



National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Notice - Votre référence

Notice - Votre référence

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

CLONING AND CHARACTERIZATION OF TWO PROTEIN TYROSINE PHOSPHATASES
(PTPases) FROM EMBRYONIC AND ADULT MURINE SOURCES

Trudy D. Marshall

Thesis submitted to
the School of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Department of Biochemistry
University of Ottawa
Ottawa, Ontario
CANADA



Trudy D. Marshall, Ottawa, Canada, 1994



National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

THE AUTHOR HAS GRANTED AN
IRREVOCABLE NON-EXCLUSIVE
LICENCE ALLOWING THE NATIONAL
LIBRARY OF CANADA TO
REPRODUCE, LOAN, DISTRIBUTE OR
SELL COPIES OF HIS/HER THESIS BY
ANY MEANS AND IN ANY FORM OR
FORMAT, MAKING THIS THESIS
AVAILABLE TO INTERESTED
PERSONS.

L'AUTEUR A ACCORDE UNE LICENCE
IRREVOCABLE ET NON EXCLUSIVE
PERMETTANT A LA BIBLIOTHEQUE
NATIONALE DU CANADA DE
REPRODUIRE, PRETER, DISTRIBUER
OU VENDRE DES COPIES DE SA
THESE DE QUELQUE MANIERE ET
SOUS QUELQUE FORME QUE CE SOIT
POUR METTRE DES EXEMPLAIRES DE
CETTE THESE A LA DISPOSITION DES
PERSONNE INTERESSEES.

THE AUTHOR RETAINS OWNERSHIP
OF THE COPYRIGHT IN HIS/HER
THESIS. NEITHER THE THESIS NOR
SUBSTANTIAL EXTRACTS FROM IT
MAY BE PRINTED OR OTHERWISE
REPRODUCED WITHOUT HIS/HER
PERMISSION.

L'AUTEUR CONSERVE LA PROPRIETE
DU DROIT D'AUTEUR QUI PROTEGE
SA THESE. NI LA THESE NI DES
EXTRAITS SUBSTANTIELS DE CELLE-
CI NE DOIVENT ETRE IMPRIMES OU
AUTREMENT REPRODUITS SANS SON
AUTORISATION.

ISBN 0-315-95992-4

Canada



UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

ABSTRACT

A PCR strategy was used to amplify novel sequences residing between two degenerate protein tyrosine phosphatase [PTPase]-specific primers. Partial Class I and II clones were isolated from a library constructed with this P19 stem cell derived-cDNA. The predicted amino acid sequence of these PCR fragments revealed sufficient identity with the PTPase consensus to indicate that a region from two individual PTPases had been cloned. Northern analysis of the Class II or PTP2 PTPase revealed a 5 Kb mRNA in all mouse tissues and cell lines examined, while the mouse CAP-1 (Class I) gene was found to be expressed as 5 and 7 Kb mRNA transcripts in a variety of mouse tissues.

CAP-1 PTPase cDNAs have been cloned from messenger RNA derived from mouse kidney and embryonal carcinoma cells. The composite cDNA contains an open reading frame which encodes 942 amino acids. The N-terminal portion of the predicted protein ($M_r = 107,000$) shares 30-37% identity with homologous regions in the cytoskeletal associated proteins, band 4.1, ezrin, moesin, radixin, and talin. This PTPase has been termed CAP-1 for cytoskeletal associated protein related PTPase. The C-terminal phosphatase domain is 33-41% identical to catalytic domains of known PTPases. The bacterially expressed GST/CAP-1 fusion protein preferentially removes phosphate moieties from tyrosine residues.

CAP-1 exhibits a specific pattern of transcription in several tissues, that is, in leg muscle as well as heart and brain. In adult leg muscle, CAP-1 is expressed as two distinct transcripts

which are approximately 500 bp shorter than that found in other murine tissues. The corresponding skeletal muscle cDNAs were cloned to elucidate the mechanism by which the different mRNAs arise. The composite leg muscle CAP-1 [LMCAP-1] cDNA, with leg muscle-specific sequence at the 5' end, predicts a truncated LMCAP-1 protein which lacks part of the protein 4.1 homology region.

This CAP-1myc fusion was transiently expressed in COS-1 cells to elucidate the localization and possible function of CAP-1. A CAP-1myc specific protein close to the predicted molecular weight (110-120 kDa) was immunoprecipitated from these metabolically labeled cells. Concurrently, indirect immunofluorescence with an α myc antibody revealed a punctate, non-nuclear pattern of exogenous CAP-1myc expression in transfected COS-1 cells. Morphological changes such as the "giant cells" observed in several CAP-1myc /pMT21- and CAP-1myc/pECE-generated NIH 3T3 clones, appear unique to CAP-1, suggesting that this phenomenon is a result of the dephosphorylation of a cytoskeleton/membrane protein found normally associated with this enzyme.

DEDICATION

To my family: I dedicate this thesis to my parents Tom and Bernice; my sisters: Paula, Claudia, Pamela, and Renée; my brothers in law- Mike, Rick, and Ron; and their children: Tracey, Sharon, Kellie, Daniel, Peter, Adam, and Ashley.

In memory of my Grandma, Mrs. B. Frampton.

And this I believe: that the free, exploring mind of the individual human is the most valuable thing in the world.

John Steinbeck

ACKNOWLEDGEMENTS

First and foremost, I would like to extend my appreciation to John Bell for his thoughtful supervision and for allowing me to do science in his laboratory. I express gratitude to the members of my graduate committee, namely Doug Gray, Mike McBurney, and Yvonne LeFebvre for their advice and direction. I would also like to acknowledge Drs. McB, D.A.G., J.B., and Vanderhyden for their guidance under the auspices of the C.R.G.

At McGill I thank: Drs. L. Bernier (and Dr.V.C.), Braun, Gros, McKensie, Ms. E. Horvath, Dr. S. Randall, and P. Icely.

I acknowledge all members of John's group (past and present) for their contribution. I thank Drs. D. Afar and B. Howell for their technical advice and for their helpful suggestions at lab meetings. I especially thank Ninan Abraham, Elizabeth Douville, Peter Duncan, and Maria Jaramillo for their help and for providing a good working environment. I also thank Jennifer, Carmen, Ellie, Mark, and Jason in this regard. I extend my gratitude to Dr. Louise Desjardine for being a part of the PTPase world. I also appreciate the help provided by animal care and I particularly thank the "Bunny Troupe" of J.B., P.D., E.D., N.A., and M.J. for injecting and bleeding the rabbits.

I am eternally grateful to Paul Wong and Sean Guenette in Dr. Tenniswood's group for providing information on the making of cDNA libraries. I also wish to thank Dr. Tuana for his advice and Elaine Petrov for donating rabbit tissue. Gratitude is also extended to Ricardo Marius and Manon Dube for their help. I appreciate Josée

Coulombe for her willingness to help and for being a good bench pal. I am grateful to Marco Difruscio for help with photography and for his humor. I thank Kalpana Gupta for her helpful suggestions.

A very special thanks to Ms. Beth Mason for her help beyond the call of duty. I also thank Julie and Joanne in Biochemistry.

I am indebted to all members of the Cancer Research group. I thank Drs. Morasuitti, Pratt, Bladdin, and Gordon. Thanks are extended to Jane Craig and Karen Jardine for always answering my questions. I appreciate the help of Dr. Ruth Slack, Leslie Sutherland, and Helen Tai. I am grateful to Dr. Paul McPherson for providing protocols and food for thought. I acknowledge Riccha Sharon for her advice and for always being able to laugh. I thank Rubina Siddiqi for her wisdom and support. I thank N. Laferriere and Dr. Staines for their advice. I especially acknowledge the contribution of Stacy Costa for her help with designing the myc tagged constructs and for her helpful suggestions throughout.

I am grateful to Maria [and to her fish and Tigger] for allowing me to clutter her computer room. I also thank Maria for her friendship and for her help during the writing of this thesis.

I appreciate the guidance and support provided by Dr. Ilona Skerjanc over the past few years and especially within the past several months during the completion of this thesis. I only wish I could express the depth of my gratitude to Ilona and her husband, Edi for their support and encouragement.

I acknowledge financial support from the Cancer Research Society (CRS) and the School of Graduate Studies.

PREFACE

An abbreviated version of the work presented in Chapters 2 and 4 has been prepared as a manuscript.

T.Marshall and J.C. Bell (1993) CAP-1: A Protein Tyrosine Phosphatase with Homology to Cytoskeletal Associated Proteins, submitted.

The data presented in this thesis are entirely my own work. Dr. Louise Desjardine sequenced the second strand of PTP2 (Figure 2.9) and provided the end sequence of several kidney cDNAs (Table 2.7). John Lui synthesized the cDNA used in the initial PCR amplification and P19 library construction. P.I. provided the "Pamoblot" Northern used in Figure 2.10, while Dr. B.H. provided the poly A⁺-selected mRNA used in Figure 2.4, and Dr. M.J. donated mRNA used in Figure 3.4. The mRNA derived from DMSO- and RA-induced cells (Figure 2.12) was obtained from G. Vidricaire and Drs. R.S. and I.S., respectively. I acknowledge the gift of mouse genomic DNA from Dr. Gray (Figure 2.13). E.D. donated bacterial *esk* extract and provided assistance with the overlay assay (Figure 4.3). R. Marius provided oligonucleotides and the MAP synthetic peptide. Oligonucleotides were also provided by P.D. and Manon Dube.

TABLE OF CONTENTS

ABSTRACT	i
DEDICATION	iii
ACKNOWLEDGEMENTS	v
PREFACE	vii
TABLE OF CONTENTS	viii
LIST OF FIGURES	xii
LIST OF TABLES	xiv
LIST OF ABBREVIATIONS	xv

CHAPTER 1

GENERAL INTRODUCTION	2
1.1 Protein Tyrosine phosphorylation	2
1.2 Protein Tyrosine Kinases	2
1.2-1 The Role for SH2 Domains in PTK function	5
1.2-2 Developmental expression of PTKs	6
1.3 Protein Tyrosine Phosphatases	7
1.3-1 Purification and Initial Characterization of PTPases	8
1.3-2 Comparison of PTPases with Other Phosphatases	9
1.4 Transmembrane Protein Tyrosine Phosphatases	11
1.4-1 CD45: The prototype receptor PTPase	16
1.4-2 PTPase catalytic domains: Receptor domain I and II	21
1.4-3 Regulation of PTPase catalytic domains	23
1.5 Intracellular Protein Tyrosine Phosphatases	24
1.5-1 Carboxyl Terminal Domain Regulation of PTP1B and TCPTP	26
1.5-2 The C-Terminal Pro-/Glu-/Ser-/Thr-Enriched or PEST Domain	28
1.5-3 N-Terminal Domains: Induction of PTPase Expression	29
1.5-4 SH2-containing PTPases	30
1.6 Cytoskeleton-Associated Proteins and CAP PTPases	33
1.7 A Role for the PTPases in Cell Cycle control	37
1.8 Protein Tyrosine Phosphatases in Development	39
1.9 PTPases: from Yeast to Human	42
1.10 PTPases and Growth Control	44
1.11 Thesis Project	47

CHAPTER 2

CLONING AND CHARACTERIZATION OF MURINE PTPase-ENCODING cDNAs	49
2.1 Introduction	49
2.2 Materials and Methods	51
2.2-1 Polymerase Chain Reaction Amplification	51
2.2-2 Characterization of PCR Amplified Fragments	51
2.2-3 Isolation of P19 cDNA Clones	56
2.2-4 Generation and Screening of a Kidney cDNA	

Library	57
2.2-5 DNA Sequencing	60
2.2-6 Synthesis and Preparation of Oligonucleotides	61
2.2-7 Hybridization of PCR clones to P19 Stem Cell-Derived mRNA	62
2.2-8 Northern Blot Analysis of mRNA Derived From Mouse Tissues	62
2.2-9 Genomic Southern Analysis	63
2.3 Results	65
2.3.1 Amplification of Two Novel PTPase-Encoding PCR Fragments	65
2.3-2. Class I and II are Expressed in P19 Stem Cells	68
2.3-3 Cloning and Characterization of Class I P19 cDNAs	68
2.3-4 Expression of Class I (CAP-1) in mRNA in Mouse Tissues	70
2.3-5 Isolation of Kidney cDNA Clones Containing 5' Sequence	74
2.3-6 Structure and Analysis of Composite Class I or CAP-1 cDNA	78
2.3-7 Isolation of a Partial Class II (PTP2) cDNA	80
2.3-8 Expression of Class II mRNA in Mouse Tissues and Cell Lines	82
2.3-9 CAP-1 is Expressed in a Range of Tissues and Cell Lines	82
2.3.10 Southern Analysis of the CAP-1 Gene	87
2.4 Discussion	87
CHAPTER 3	
ISOLATION AND ANALYSIS OF ADULT LEG MUSCLE-DERIVED cDNA CLONES	
3.1 Introduction	97
3.2 Materials and Methods	98
3.2-1 Preparation and Screening of a Leg Muscle Library	99
3.2-2 Northern Blot Analysis of Leg Muscle-Specific CAP-1	100
3.3 Results	100
3.3-1 Cloning and Characterization of Leg Muscle-Specific CAP-1	100
3.4 Discussion	108
CHAPTER 4	
DETERMINATION OF CAP-1 PTPase ACTIVITY	
4.1 Introduction	112
4.2 Materials and Methods	113
4.2-1 Construction of the GST/PTPase plasmid: pGEX-3X/CAP-1	113
4.2-2 Bacterial Expression and Solubilization of GST/CAP-1	113

4.2-3 Determination of PTPase Activity: Overlay Assay	116
4.2-4 Investigation of Phosphotyrosine-Specific PTPase Activity	117
4.2-5 Phosphoamino Acid Analysis	118
4.3 Results	119
4.3-1 The Expression and Analysis of the CAP-1 PTPase	119
4.3-2 CAP-1 is a Phosphotyrosine Specific Phosphatase	124
4.4 Discussion	126
CHAPTER 5	
GENERAL DISCUSSION	130
5.1 The PTP2 Homologue [MEG2] has a Putative Ligand Binding Domain	130
5.2 The CAP-1 PTPase is a Member of the Band 4.1 Superfamily	132
5.3 A Specific Function for the Skeletal Muscle CAP-1 Isoform?	137
5.4 Deregulation of the CAP-1 PTPase: A Piece of the Puzzle?	139
5.5 Conclusions	141
APPENDIX I	143
APPENDIX II	
Design and Generation of PTPase Minus CAP-1 Constructs	146
A ^{2.1} Introduction	146
A ^{2.2} Materials and Methods: Construction of Plasmids	146
A ^{2.2-1} Production of a CAP-1 PTPase Minus Deletion Mutant	146
A ^{2.2-2} Creation of the PTPase Minus Mutant of CAP-1	147
A ^{2.3} Summary	149
APPENDIX III	
Generation of a CAP-1/MAP Specific Polyclonal Antibody	151
A ^{3.1} Introduction	152
A ^{3.2} Materials and Methods	152
A ^{3.2-1} Synthesis of a CAP-Specific Synthetic Peptide	152
A ^{3.2-2} Immunization of the CAP-1/MAP Antigen into Animals	153
A ^{3.2-3} Specific Immunoreactivity of the α CAP-1/MAP Antibodies	154
A ^{3.2-4} CAP-1 Expression in COS-1 Cell Extracts	154
A ^{3.3} Results	154
A ^{3.3-1} Specific Immunoreactivity of the α CAP-1/MAP Antisera	154
A ^{3.4} Discussion	155

APPENDIX IV	
THE BACTERIAL EXPRESSION OF CAP-1 AND PTP2 AS TrpE FUSIONS:	
POLYCLONAL ANTIBODY PRODUCTION	158
A ^{4.1} Introduction	159
A ^{4.2} Materials and Methods	159
A ^{4.2-1} Construction of TrpE/PTPase-Encoding Plasmids	159
A ^{4.2-2} TrpE/CAP-1 Δ Fusion Protein Expression and Induction	161
A ^{4.2-3} Gel Purification of TrpE/CAP-1 Δ and Antibody Production	162
A ^{4.2-4} Construction and Analysis of FLAG/CAP-1	163
A ^{4.3} Results	166
A ^{4.3-1} Induction of TrpE/CAP-1 Δ and TrpE/PTP2 Δ Fusion Proteins	166
A ^{4.3-2} Polyclonal Antibody Production	168
A ^{4.3-3} Analysis of the FLAG/CAP-1 Δ myc Fusion	168
A ^{4.4} Discussion	170
APPENDIX V	
Immunofluorescent and Morphological Analysis of CAP-1myc-Expressing Cells	174
A ^{5.1} Introduction	175
A ^{5.2} Materials and Methods	176
A ^{5.2-1} Generation of the Composite CAP-1 cDNA Clone	176
A ^{5.2-2} Myc Epitope-Tagging of CAP-1	176
A ^{5.2-3} Insertion of CAP-1 into Mammalian Expression Vectors	178
A ^{5.2-4} Large Scale Plasmid Preparation	178
A ^{5.2-5} Transient Transfection of CAP-1 Constructs into COS-1 Cells	181
A ^{5.2-6} Immunofluorescence of CAP-1 myc	182
A ^{5.2-7} Immunoprecipitation of CAP-1myc from ³⁵ S-Met Labeled Cells	183
A ^{5.2-8} Stable Transfection of CAP-1myc Constructs	184
A ^{5.3} Results	185
A ^{5.3-1} Immunoprecipitation of the myc-tagged CAP-1 PTPase	185
A ^{5.3-2} Indirect Immunofluorescence of CAP-1myc in COS-1 cells	185
A ^{5.3-3} Stable CAP-1myc clones Exhibit Morphological Changes	188
A ^{5.4} Discussion	190
REFERENCES	194

LIST OF FIGURES

Figure 1.1 A diagrammatical representation of the intracellular and receptor protein tyrosine kinases (PTKs)	4
Figure 1.2 A schematic illustration of the transmembrane an intracellular protein tyrosine phosphatases (PTPases)	12
Figure 2.1 Oligonucleotide primers used in the polymerase chain reaction (PCR) amplification of fragments from two novel PTPases	52
Figure 2.2 Subcloning of PTPase-encoding PCR fragments into pTZ19R	54
Figure 2.3 Alignment of deduced Class I and II PCR sequence with a PTPase consensus	67
Figure 2.4 Northern analysis: Class I and II PCR fragments hybridize to P19 stem cell-selected mRNA	69
Figure 2.5 Schematic diagram illustrating the structure of the composite CAP-1 cDNA and the relatedness of four cDNA clones	72
Figure 2.6 Northern blot analysis of CAP-1 mRNA in mouse tissues	73
Figure 2.7 An autoradiogram of the fractionated second strand kidney-derived cDNA	75
Figure 2.8 The nucleotide and deduced amino acid sequence of CAP-1	79
Figure 2.9 Partial nucleotide and predicted amino acid sequence of a Class II cDNA	81
Figure 2.10 Expression of the Class I and II PTPase mRNA in several mouse tissues and cell lines	83
Figure 2.11 Expression of CAP-1 transcripts in adult and embryonic mouse tissues	85
Figure 2.12 Expression of CAP-1 mRNA in adult mouse tissues and in differentiated embryonal carcinoma cells	86
Figure 2.13 Southern blot analysis of the CAP-1 gene	88
Figure 2.14 Alignment of the amino terminal domain of the PTPases CAP-1 and MEG-1 with a homologous region in eukaryotic membrane protein 4.1	92
Figure 3.1 Size Selection of Leg Muscle-derived cDNA	101
Figure 3.2 This diagram portrays the relatedness between CAP-1 and the Leg Muscle cDNA clones	104
Figure 3.3 Partial sequence of several overlapping leg muscle-derived cDNAs	106
Figure 3.4 Analysis of Leg Muscle-Specific CAP PTPase expression	107
Figure 4.1 Generation of the GST/CAP-1 fusion protein	114
Figure 4.2 PTPase overlay assay	121
Figure 4.3 CAP-1 phosphatase activity: CAP-1 abolishes the α PY immunoreactivity of esk	122
Figure 4.4 Solubilization and affinity purification of GST/CAP-1	123
Figure 4.5 PTPase activity of the renatured GST/CAP-1 fusion protein	125
Figure 4.6 The tyrosine specific phosphatase activity of CAP-1	127

Figure 5.1 Band 4.1 superfamily and the ezrin, radixin, and moesin (ERM) family	133
Figure 5.2 Models for possible CAP-1 PTPase function at the cytoskeleton/membrane interface	136
Figure 5.3 The predicted structural design of the CAP-1 PTPases	138
Figure A ² .1 Production of a CAP-1 PTPase deletion mutant . . .	148
Figure A ² .2 Use of a PCR Strategy to Generate a PTPase Minus CAP-1	150
Figure A ³ .1 Specific immunoreactivity of the α CAP-1/MAP antisera	157
Figure A ⁴ .1 Construction of the trpE/CAP-1 Δ fusion protein . .	160
Figure A ⁴ .2 Construction of FLAG/CAP-1 Δ and FLAG/CAP-1 Δ myc fusions	165
Figure A ⁴ .3 Expression of trpE/PTPase fusion proteins	167
Figure A ⁴ .4 Induction of the trpE/CAP-1 Δ fusion protein . . .	169
Figure A ⁴ .5 Recognition of the myc epitope in context of a FLAG/CAP-1 Δ myc fusion	171
Figure A ⁵ .1 Generation of the composite CAP-1 cDNA/pTZ19R plasmid	177
Figure A ⁵ .2 Insertion of the myc epitope tag into CAP-1	179
Figure A ⁵ .3 Insertion of CAP-1 into mammalian expression vectors	180
Figure A ⁵ .4 Immunoprecipitation of CAP-1myc from COS-1 cells .	186
Figure A ⁵ .5 Indirect immunofluorescence of COS-1/CAP-1myc cells	187
Figure A ⁵ .6 Morphological changes induced in NIH 3T3/CAP-1myc clones	189

LIST OF TABLES

Table 1.1 Receptor PTPase Isoforms.	14
Table 1.2 Classification of non-transmembrane PTPases	25
Table 2.1 Northern Analysis of mouse tissues and cell lines	64
Table 2.4 Summary of P19-derived Class I or CAP-1 cDNAs	71
Table 2.6 A compendium of Class I (CAP-1) Kidney cDNA clones	77
Table 2.7 Comparison of the core catalytic domain of CAP-1 with that of several intracellular and receptor-like PTPases	91
Table 3.2 Cloning and characterization of Leg Muscle cDNAs	103

LIST OF ABBREVIATIONS

α PY	anti-phosphotyrosine antibody
ATP	adenosine triphosphate
bp	base pairs
Ca^{2+}	cytosolic free calcium
CAM	cell adhesion molecule
cAMP	adenosine 3',5' cyclic monophosphate
CAP	cytoskeletal associated protein related PTPase
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
CTP	cytidine triphosphate
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EC	embryonal carcinoma cells
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ES	embryonic stem cells
esk	embryonal carcinoma ser/thr/tyr kinase
FAK	focal adhesion kinase
FCS	fetal calf serum
FITC	fluoresceine iso-thiocyanate
Fn III	fibonectin type III repeat
GAP	GTPase activating protein
GTP	guanosine triphosphate
GST	glutathione S-transferase
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HPTP	human protein tyrosine phosphatase
Ig	immunoglobulin
IL 2R α	interleukin 2 receptor alpha
IPTG	isopropyl- β -D-thiogalactopyranosid
Kb	kilobase pairs
kD	kilodaltons
LAR	leukocyte common antigen related protein
LCA	leukocyte common antigen
MAP	multiple antigen peptide
MEG	megakaryoblastic cell line
MEM	minimal essential medium
Mg^{2+}	magnesium
Mn^{2+}	manganese
mRNA	messenger ribonucleic acid
moesin	membrane organizing extension spike protein
NCAM	neural cell adhesion molecule
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PI	phosphatidyl inositol
PI3-K	phosphatidyl inositol-3 kinase
PIP ₂	phosphatidylinositol 4,5 bis-phosphate

PKA	cAMP-dependent protein kinase
PKC	protein kinase C
PLC γ	phospholipase C- γ
PMSF	phenylmethyl-sulfonylfluoride
PNS	peripheral nervous system
PTK	protein tyrosine kinase
PTPase	protein tyrosine phosphatase
PS	phosphoserine
PT	phosphothreonine
PY	phosphotyrosine
RA	retinoic acid
RSV	Rous sarcoma virus
SDS	sodium dodecyl sulfate
SH2	src homology region 2
SH3	src homology region 3
sty	serine threonine tyrosine kinase
TCPTP	T cell PTPase
TCR	T cell receptor
TFA	trifluoroacetic acid
TLE	thin-layer electrophoresis
TRIS	tris (hydroxymethyl) aminomethane
trpE	tryptophan E

CHAPTER 1
GENERAL INTRODUCTION

1.1 Protein Tyrosine phosphorylation

Regulated changes in the phosphorylation state of tyrosyl residues in proteins have been implicated in a number of fundamental multicellular processes such as cell communication, cell growth and development. Cellular proteins are more frequently phosphorylated on serine and threonine residues (Taborsky, 1974) than on tyrosine residues (Hunter, 1982). In a normal cell, 0.01%-0.05% of the total protein phosphoamino acids is phosphotyrosine (Hunter and Sefton, 1980); whereas, this level is increased in retroviral transformed cells to 1-3% (Sefton *et al*, 1981). In fact, many of the known protein tyrosine kinases (PTKs) were discovered as products of retroviral-encoded oncogenes, indicating a role for these enzymes in the regulation of normal and aberrant cell growth (Cantley *et al*, 1991). While the biochemistry and cell biology of protein tyrosine kinases have been studied extensively, relatively little is known about the protein tyrosine phosphatases (PTPases). Clearly, in order to fully understand the role of tyrosine phosphorylation in the regulation of cellular function it is essential that these enzymes be identified and characterized.

1.2 Protein Tyrosine Kinases

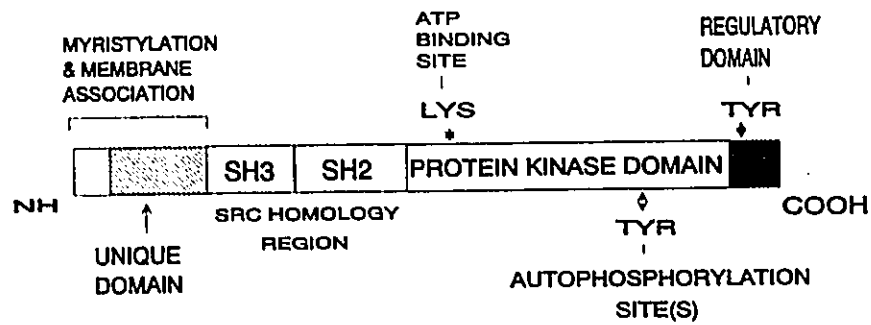
The reversible and dynamic nature of protein phosphorylation makes it ideally suited to transduce external signals across the plasma membrane and to propagate the signal throughout the cell. In the past decade it has become apparent that many of the primary signals are transmitted by activation of protein tyrosine kinases

(PTKs; Ullrich and Schlessinger, 1990). In addition, members of the serine/threonine (Hunter, 1987; Siegfried *et al*, 1990) and of the recently identified serine/threonine/tyrosine, or STY, kinases are also involved in these signalling pathways (Ben-David *et al*, 1991; Howell *et al*, 1991; Piwnica-Worms *et al*, 1991; Stern *et al*, 1991; reviewed in Linberg *et al*, 1992). Many cell growth and proliferation events are initiated via interaction of growth factor ligands with the external domain of specific receptors, followed by oligomerization and subsequent activation of the intrinsic tyrosine kinase. Other mechanisms involved in regulating receptor protein tyrosine kinases or RPTKs can be delineated as: phosphorylation at putative protein kinase C (PKC) sites; ligand-induced autophosphorylation generating kinase activation and regulation of substrate binding; and tyrosine phosphorylation of a C-terminal residue resulting in negative regulation (as illustrated in Figure 1.1; reviewed in Ullrich and Schlessinger, 1990).

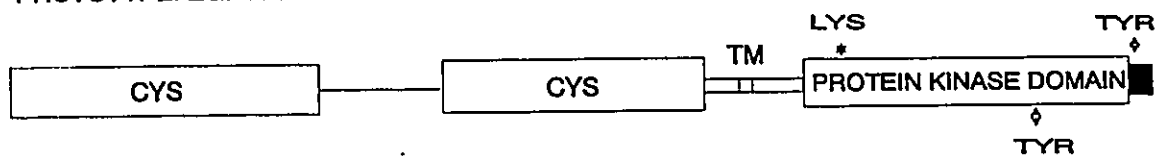
The non-receptor PTKs, with src as a prototype (see Figure 1.1), can be subdivided into four functional regions as follows: amino terminal regions necessary for myristylation and stable membrane association; the substrate interaction domain or src homology (SH) region; the catalytic domain with major activating autophosphorylation sites; and the C-terminal negative regulatory region (Pawson, 1988). Src-related PTKs are thought to be involved in regulating cellular growth control and as well, perform specialized functions in differentiated cells (Eiseman and Bolen, 1990). These membrane associated proteins may function through

Figure 1.1 A diagrammatical representation of the intracellular and receptor protein tyrosine kinases (PTKs). The generic intracellular PTK, src is divided into: the N-terminal myristylation site followed by a unique membrane association region; the src homology (SH3 and SH2) region; the catalytic domain; and negative regulatory region. The prototypic RPTKs-the EGF and PDGF receptor PTKs, have Cys-rich and Ig-like repeats within its external domain, respectively. The transmembrane spanning region (TM) is followed by putative phosphorylation sites (i.e. for protein kinase C) in its juxtamembrane region. The PDGF receptor has an insertion or kinase insert (KI) within the catalytic domain. All PTKs contain an essential lysine as the ATP binding site and have several autophosphorylation sites at tyrosine (tyr) residues throughout its kinase domain and within the KI. A negative regulatory region is also located at the C-terminal end. This illustration was modified from Veillette and Davidson (1992) and Eiseman and Bolen (1990).

SRC-RELATED PTK

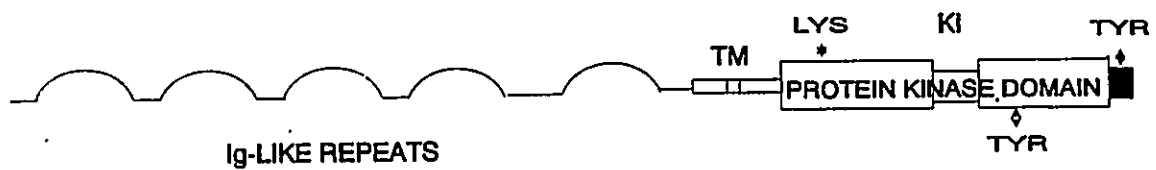


PROTOTYPE: EGF RECEPTOR



EXTERNAL DOMAIN

PROTOTYPE: PDGF RECEPTOR



interaction with integral membrane proteins. The best characterized example of this is identified in T cell activation, where glycoprotein-mediated induction activates a src-like p56^{lck} protein tyrosine kinase; the signal is transduced through a noncovalent link formed between the cytoplasmic domain of the glycoproteins CD4 or CD8 and the amino terminal domain of the intracellular p56^{lck} (Shaw *et al*, 1990; Turner *et al*, 1990; Veillette *et al*, 1989). The noncovalent association of cytokine and hemopoietic receptors with intracellular PTKs was recently identified as an important component in the transmission and reception of primary signals (Eiseman and Bolen, 1990). Integral membrane receptor and kinase combinations can function to enhance signalling possibilities; for instance, association of the interleukin or T cell antigen receptor with the src-like PTK, p59^{lck} as well as the interaction of the B-cell surface antigen receptor with p53/56^{lck} is thought to generate ligand-dependent phosphorylation of specific substrates (Gould and Hunter, 1985; Hatakeyama *et al*, 1991; Kobayashi *et al*, 1993; Murakami *et al*, 1993; Samelson *et al*, 1990; Yamanashi *et al*, 1991).

1.2-1 The Role for SH2 Domains in PTK function

Signal-mediated interaction of phosphotyrosyl residues and SH2 domains function to translocate signalling components from the cytosol to the plasma membrane where they associate with substrate and effector proteins. Upon activation and autophosphorylation, the PTK (i.e. the PDGF receptor) will form high affinity physical complexes via interaction of specific phosphotyrosines with the SH2

domain of signalling proteins (Anderson *et al*, 1990; Mayer and Hanafusa, 1990; Mayer *et al*, 1991). Stimulation of receptor or receptor-associated tyrosine kinases is known to trigger a chain of events involving multiple pathways such as phosphatidylinositol metabolism or signalling through ras (Cantley *et al*, 1991). Several of these pathways involve SH2-containing proteins such as: ras GTPase-activating protein, GAP; phospholipase C γ , or PLC γ ; and phosphatidylinositol 3-kinase (PI3-K) associated protein, p85 (Escobedo *et al*, 1991; Kaslaukas *et al*, 1990; Ostu *et al*, 1991; Skolnik *et al*, 1991; Stahl *et al*, 1988; Suh *et al*, 1988;). Interaction of these proteins with specific phosphotyrosine residues is a prerequisite for the phosphorylation of signalling molecules and subsequent stimulation of second messenger pathways which culminate in pleiotrophic cellular responses (Ullrich and Schlessinger, 1990; Williams *et al*, 1991).

1.2-2 Developmental expression of PTKs

Through the study of protein tyrosine kinases it has become apparent that tyrosine phosphorylation is an essential element in certain developmental pathways. This role is evident in the functional requirement for specific transmembrane tyrosine kinases as documented for c-kit in mouse development and for the sevenless protein in the determination of cell fate in *Drosophila* (Basler and Hafen, 1988 ;Tan *et al*, 1990). The spatial and temporal expression of the sev PTK is required for normal ommatidia development (Banerjee *et al*, 1987; Bowtell *et al*, 1988). The restriction of trk

expression to the developing neural crest and of the related trkB to specific structures in the adult brain is consistent with distinct roles for these neurotrophic receptor PTKs in the developing nervous system (Martin-Zanca et al, 1990; reviewed in Barbacid et al, 1991). However, it may be difficult to identify developmentally important enzymes if the pattern of expression is not obvious or if the differentially expressed PTK is part of a redundant pathway. For instance, mutations must be produced in both the *Drosophila* cell adhesion molecule, fasciculin and the cytoplasmic kinase, *abl*, to disrupt normal growth cone guidance in the developing CNS axon (Elkins et al, 1990). Developmental and tissue-specific processing or alternate promoter usage can generate functionally distinct isoforms of PTKs with an array of structural modifications: this has been observed in the inclusion of a neural-specific exon in *n-src* (Brugge et al, 1985; 1987), with the loss of the catalytic domain in the tissue-specific trkB (Klein et al, 1990) or by the truncation of a PDGF-binding external domain generating an embryonic isoform of the PDGF receptor kinase (Vu et al, 1989). It is clear that differentially expressed and alternative processed kinases constitute an important component in the cascade which translates external signals into developmental and proliferative decisions (Pawson and Bernstein, 1990).

1.3 Protein Tyrosine Phosphatases

The level of phosphorylation at equilibrium reflects a critical balance between the phosphorylating and dephosphorylating

enzymes, thus emphasizing a role for both PTKs and PTPases. The existence of protein tyrosine phosphatases (PTPases) was first identified in cells transformed with retroviral temperature-sensitive PTKs (Hunter et al, 1979). These cells displayed a transformed phenotype along with elevated levels of phosphotyrosine upon transformation at the permissive temperature (36°C). Cells grown at the nonpermissive temperature (41°C), at which the kinase was inactive, reverted to a normal phenotype with a corresponding decrease in the phosphotyrosine level. This effect was independent of protein turnover, suggesting the presence of a dephosphorylating enzyme (PTPase) in these cells (Radke and Mortin, 1979). Concomitantly, PTPase activity was detected in EGF-receptor-rich, A431 membrane fractions by the identification of a slow time-dependent decrease of $^{32}\text{P}_i$ from EGF-induced, in vitro [γ ^{32}P] ATP-labeled membrane proteins (Carpenter et al, 1979; Brautigan et al, 1981).

1.3-1 Purification and Initial Characterization of PTPases

The search for PTPases has since been an active area of research. PTPase activity has been partially purified from particulate and soluble fractions of many tissue and cell types using a variety of artificial substrates (Lau et al, 1989 and references therein). Many of these PTPases were identified as multiple forms due to differential separation on ion exchange (DEAE) columns and to differences in molecular weight (Lau et al, 1985). The rate-limiting step in the isolation of a highly purified

enzyme preparation was the identification of a substrate that could be made in large quantities and phosphorylated at high specific activities. In 1988, Tonks and his colleagues in Fischer's group, purified human placental PTPases to homogeneity using chemically modified lysozyme as the phosphotyrosine-labeled substrate. Most importantly, upon thiophosphorylation with $[\gamma\text{-S}]\text{ATP}$, this artificial substrate was used as a powerful affinity chromatography step (Tonks *et al*, 1988a). Comparison of this pure preparation of PTP1B with those of previously described PTPases revealed several general features. PTPase activity was shown to be: dependent on the presence of active thiol groups (i.e. requires reducing agents such as DTT); inhibited by micromolar concentrations of vanadate and zinc; independent of divalent cations; optimal at neutral pH; insensitive to fluoride; and specific for phosphotyrosine-containing substrates (Brautigan *et al*, 1981; Nelson and Branton, 1984; Tonks *et al*, 1988b).

1.3-2 Comparison of PTPases with Other Phosphatases

Analysis of these biochemical properties raised the question of whether the protein tyrosine phosphatases share similarities with other phosphatases or whether they constitute a separate class of phosphotyrosine-specific dephosphorylating enzymes. In a parallel study, a low molecular weight protein tyrosine phosphatase was purified and subsequently its cDNA was cloned. This phosphatase shares little amino acid identity with the PTPase purified by Tonks, however, it does appear to have a catalytic mechanism very

similar to PTP1B-related PTPases (Charbonneau et al, 1988; Comica et al, 1989; Waheed et al, 1988; Wo et al, 1992).

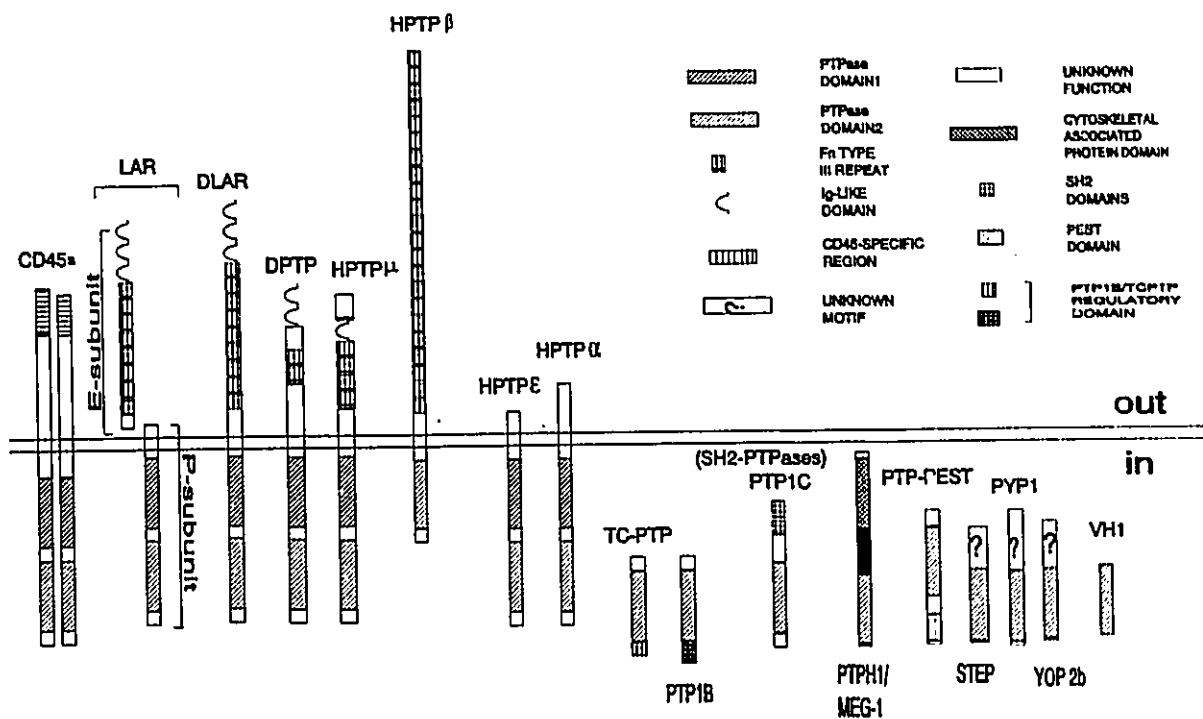
Analysis of tyrosyl-specific phosphatase structure and function has also encompassed examination of highly characterized enzymes such as the phosphoserine phosphatases. Cohen has subdivided the phosphoserine phosphatases into type 1 or type 2 based on several criteria including sensitivity (or resistance in case of type 2) towards two heat stable phosphatase inhibitors (Cohen and Cohen, 1989; Cohen, 1982; 1989). Type 2 phosphatases are further classified by virtue of their divalent cation requirement: type 2A phosphatase is Mn^{2+} -dependent; type 2B or calcineurin is regulated by Ca^{2+} and calmodulin; and the type 2C enzyme requires Mg^{2+} for activity (refer to references in the following reviews- Cohen, 1985; Lau et al, 1989). The prototypic PTPase, PTP1B and other related enzymes share no structural similarity with the deduced amino acid sequence of the phosphoseryl phosphatase catalytic domains (Charbonneau et al, 1989). However, there is preliminary data suggesting that in vitro PTPase activity is associated with zinc sensitive, Ni^{2+} /calmodulin dependent type 2B (or calcineurin) activity (Chan et al, 1986; Chernoff et al, 1984; Pallen et al, 1985). In addition, while animal and bacterial alkaline phosphatases dephosphorylate proteins in vitro, several lines of evidence argue against its physiological significance (Lau et al, 1989; Leis and Kaplan, 1982; Li et al, 1984; Swarup et al, 1981; Tamura et al, 1986). Similarly, PTPase activity attributed to the partially pure prostate-specific phosphatase is tartrate,

fluoride, and vanadate sensitive, while the osteoblastic acid phosphatase is tartrate insensitive and vanadate and fluoride sensitive (Himeno *et al*, 1989; Lau *et al*, 1985;1989; Li *et al*, 1984; Lin and Clinton, 1985; Pohlmann *et al*, 1988). It is not known if the results of these *in vitro* assays represent *in vivo* events. These studies do establish that protein tyrosine phosphatases and phosphoserine phosphatases are distinct enzymes and that PTPase activity may evolve from at least two classes of structurally different but functionally similar phosphatases.

1.4 Transmembrane Protein Tyrosine Phosphatases

The predicted protein structure of recently cloned and identified protein tyrosine phosphatases suggests that the repertoire of signalling pathways may now include transmembrane and intracellular phosphatases (see Figure 1.2; reviewed in Tonks *et al*, 1991). Initial primary sequence data obtained from the purified PTP1B had sufficient similarity with that of two tandemly-repeated cytoplasmic domains of the T and B cell-specific CD45 (T200 or leukocyte common antigen- LCA), to classify this well characterized, cell surface protein as a receptor-like PTPase (Charbonneau *et al*, 1988; 1989). Recent findings suggest that transmembrane PTPases are a family of structurally diverse, regulated enzymes involved in many facets of signal transduction pathways. The external portion of many transmembrane PTPases are classified as having receptor- or cell adhesion molecule-like domains (Table 1.1; Figure 1.2; Shaw and Thomas, 1991; Streuli *et*

Figure 1.2 A schematic illustration of the transmembrane and intracellular protein tyrosine phosphatases (PTPases). The glycosylated receptor-like domain of the prototypic transmembrane PTPase, CD45, is followed by a transmembrane spanning region and two intracellular PTPase domains (R type I). Included as an exception to this general architecture, is the single catalytic domain-containing PTPase- HPTP β (R-type III). The cell adhesion molecule CAM-like motifs: Fn type III and Ig-like repeats are found in the extracellular domain of many known receptor PTPases (R-type II). The proteolytically derived subunits of LAR interact noncovalently; the other CAM-like PTPases may have a similar structure. The HPTP α or HPTP ϵ (R-type IV) may depend on interacting integral membrane for regulation. The N-terminal domains of all intracellular enzymes have been arbitrarily positioned closest to the plasma membrane. The catalytic and non-catalytic domains of these PTPases have been illustrated by patterned boxes. The individual PTPases represented here are referenced in Tables 1.1 and 1.3 and throughout the text. This diagram is not all inclusive and has been modified from Tonks et al (1991).



al, 1990; Trowbridge, 1991). Thus it is possible that this group of PTPases plays a role in regulating cell growth and differentiation by cell adhesion- and ligand-mediated modulation of tyrosine phosphatase activity.

Transmembrane PTPases can be subdivided into several classes dependent upon the number of PTPase catalytic domains and by classification of the extracellular domain into receptor, cell adhesion molecule, or short, non-descript categories (Table 1.1; Figure 1.2). The LAR, or leukocyte common antigen-related PTPase was identified by virtue of its similarity with the receptor-like, CD45 (Streuli et al, 1988). This similarity includes the general structural architecture of two cytoplasmic catalytic domains, a transmembrane spanning region, and an extracellular domain. However, the external (E) and transmembrane/ cytoplasmic (P) regions of LAR are proteolytically cleaved and the mature protein is expressed on the cell surface as noncovalently-linked subunits (Streuli et al, 1992). This dipeptide topography has been adopted by at least another member of the transmembrane PTPases; therefore, it is not unique to LAR (Jiang et al, 1993). The external domains of LAR, of the Drosophila homologue DLAR, and of the closely related HPTP δ are composed of immunoglobulin- or Ig-like domains and fibronectin type III (Fn-III) repeats; the function of these regions is generally ascribed to cell adhesion and defines R-type IIA (Table 1.1 ;Krueger et al, 1990; Streuli et al, 1988; 1989; Saito and Streuli, 1991). DPTP and HPTP μ , derivatives of the Receptor type II subclass or R-type IIB, contain non-homologous

Table 1.1 Receptor PTPase Isoforms

This summary was modified from a Table presented in Fischer *et al*, 1991. The receptor PTPases denoted HPTP are of human origin, those referred to as RPTP were cloned from mouse, with the exception of LRP, while the letter D, identifies *Drosophila* PTPases. The CD45 PTPase was cloned from human, mouse and rat. The receptor or R-types, delineated in Figure 2 and in the text, was originally proposed in Fischer *et al*, 1991. R-type I is typically a receptor-like transmembrane molecule with two intracellular domains with similarity to the PTPase domain. Type II was subdivided into A, B, and C based on the presence of the fibronectin type III-like (Fn III) and immunoglobulin-like (Ig-like) domains (R-type IIA), the Fn III and Ig-like domains with additional (+) external domain sequence (R-type IIB) or due to the presence of fn III alone (R-type IIC). R-types are also assigned according to the number of PTPase catalytic domains present in the cytoplasmic domain (i.e. Type III has only one). Type IV PTPases have short extracellular domains with the size specified as number of amino acids (aa). Type V has a carbonic anhydrase-like region in the external domain.

Table 1.1

PTP	R-TYPE	EXTERNAL DOMAIN	PTPase #	REFERENCE
RECEPTOR				
CD45	I	Receptor-like	2	Thomas <u>et al</u> , 1985;1987 Ralph <u>et al</u> , 1987
CELL ADHESION MOLECULE				
LAR	IIA	Fn III & Ig-like	2	Streuli <u>et al</u> , 1988
DLAR	IIA	FN III & Ig-like	2	Streuli <u>et al</u> , 1989
HPTP δ	IIA	FN III & Ig-like	2	Krueger <u>et al</u> , 1990
DPTP	IIB	FN III & Ig-like +	2	Streuli <u>et al</u> , 1989
HPTP μ	IIB	FN III & Ig-like +	2	Gebbink <u>et al</u> , 1991
DPTP99A	IIC	FN type III	2	Tian <u>et al</u> , 1991; Yang <u>et al</u> , 1991; Hariharan <u>et al</u> , 1991
HPTP β	III	FN type III	1	Krueger <u>et al</u> , 1990
DPTP10D	III	FN type III	1	Tian <u>et al</u> , 1991; Yang <u>et al</u> , 1991; Hariharan <u>et al</u> , 1991
UNKNOWN FUNCTION				
RPTP α	IV	Short (123 aa)	2	Sap <u>et al</u> , 1990
HPTP α	IV	<u>ibid</u>	2	Krueger <u>et al</u> , 1990 Kaplan <u>et al</u> , 1990
HLRP	IV	<u>ibid</u>	2	Jirik <u>et al</u> , 1990
LRP	IV	<u>ibid</u>	2	Matthews <u>et al</u> , 1990
HPTP ϵ	IV	Short (27 aa)	2	Krueger <u>et al</u> , 1990
HPTP ζ	V	carbonic anhydrase	2	Wary <u>et al</u> , 1993
HPTP γ	V	like domain	2	Barnea <u>et al</u> , 1993 Krueger and Saito, 1992

sequences of unknown function in addition to the Ig-like and Fn type III repeats (Gebblink *et al.*, 1991; Streuli *et al.*, 1989). The R-type IIC Drosophila DPTP99A, meanwhile, contains only several Fn type III repeats followed by a transmembrane domain and two cytoplasmic PTPase domains (Hariharan *et al.*, 1991; Tian *et al.*, 1991; Yang *et al.*, 1991). The structure of HPTP β and DPTP10D is predicted to consist of a rod-shaped domain with 15-16 and 12 Fn type III repeats, respectively, on the outside and a single catalytic domain on the inside of the cell, placing these PTPases in the new R-type III group (Hariharan *et al.*, 1991; Kreuger *et al.*, 1990; Tian *et al.*, 1991; Yang *et al.*, 1991). These cell adhesion molecule-like motifs may employ either cell-cell, cell-extracellular matrix (ECM) or cell-ligand interactions to generate regulated changes in PTPase activity. Alternatively, signalling through the catalytic domain may in effect modify external domain-mediated cell adhesion (Cunningham *et al.*, 1987; Moos *et al.*, 1988; Norton *et al.*, 1990; Patthy, 1990).

The external domains of the R-type IV, HPTP α and HPTP ϵ , however, are relatively short, consisting of 123 and 27 residues, respectively. These regions are non-descript in that they: are not highly conserved between human and mouse homologues, lack known structural motifs, and instead encode many putative N- and O-linked glycosylation sites (Krueger *et al.*, 1990). Maintenance of the overall external domain topography rather than conservation of the primary sequence is suggestive of PTPase regulation via association with integral membrane receptors or by oligosaccharide-mediated,

lectin induction (Jirik et al, 1990; Kaplan et al, 1990; Kreuger et al, 1990; Matthews et al, 1990; Sap et al, 1990).

1.4-1 CD45: The prototype receptor PTPase

The prototypic receptor-like PTPase, CD45, has a heavily glycosylated external domain, a membrane spanning region and two imperfectly repeated catalytic domains; domain I and domain II have 40% and 33% identity with PTP1B, respectively (Table 1.1; Figure 1.2; Charbonneau et al, 1988; 1989; Thomas et al, 1985). CD45 is a heterogenous molecule with molecular weights ranging from 180 to 220 kDa. These isoforms are generated by alternative splicing of three exons (4, 5, and 6) followed by subsequent disparate O-linked glycosylation of the external domain (Barclay et al, 1987; Ralph et al, 1987; Saga et al, 1986; 1987; Streuli and Saito, 1989; Streuli et al, 1987; Thomas et al, 1985; 1987; Trowbridge, 1991). Differential expression of these isoforms allow CD45 to interact and respond to a variety of cell-linked or soluble ligands.

The recent identification of phosphatases which appear to be regulated indicates that PTPases do not simply reverse the effects of PTKs but that they are signal transducers in their own right. For instance, the cell surface expression of this hemopoietic-specific receptor PTPase, CD45, is essential for antigen-induced signal transduction in T and B cell activation and maturation (Justement et al, 1991; Koretsky et al, 1990; Ostergaard et al, 1989; Pingel and Thomas, 1989; Weaver et al, 1991). This suggests that CD45 can regulate signal transduction by specific interaction

with membrane-associated proteins, whereby antigen-induced dephosphorylation of tyrosyl residues by CD45 can effect lymphoid cell activation (reviewed in: Clark and Ledbetter, 1989; Shaw and Thomas, 1991). Antibody-mediated crosslinking of a subset of T cell differentiation antigens, such as CD2, CD3, or CD28 to form homoaggregates on the cell surface is sufficient to trigger the increase of cytoplasmic free calcium. This increase in $[Ca^{2+}]_i$ is abolished when these receptors are crosslinked to CD45 (Ledbetter *et al.*, 1987; 1988; Samelson *et al.*, 1990a). However, the CD4-mediated signalling is enhanced rather than inhibited when CD4 and CD45 are crosslinked by antibodies (Ledbetter *et al.*, 1988). In addition, when T- or B-cell associated receptors are brought into close contact with CD45, this PTPase also modulates signal transduction (i.e. increased $[Ca^{2+}]_i$) through these receptors (Justement *et al.*, 1991; Ledbetter *et al.*, 1988). Therefore, the function of CD45 in T and B cells may depend on the nature of the interacting proteins.

Isoforms of CD45 are differentially expressed at various stages of lymphocyte maturation such as resting, memory, and activated B and T cells (Powrie and Mason, 1988; Sanders *et al.*, 1988). This could account for the pleiotropic results obtained in the aforementioned crosslinking experiments. CD45 could normally undergo direct lateral contact with T and B cell surface molecules via specific ligand-mediated events, with each isoform recognizing a slightly different ligand and evoking a particular response (Clark and Ledbetter, 1989).

Phosphotyrosyl phosphorylation is known to be one of the earliest events to occur upon antigen-induced T-cell activation (Samelson et al, 1985). Mutant T cells lacking cell surface expression of CD45 (CD45⁻/CD4⁺ or CD45⁻/CD8⁺) have impaired responses to antigen-induced stimulation through the T cell receptor or TCR/CD3 complex (Pingel and Thomas, 1989; Weaver et al, 1991). These cells fail to show increased tyrosine phosphorylation, phosphatidylinositol 4,5-bisphosphate (PIP₂) metabolism and PIP₂ turnover-dependent elevation of [Ca²⁺]_i levels upon antigen induction (Koretzky et al, 1990; 1991). CD45 "deficient" cells have, in effect, uncoupled both the T cell antigen and CD2 receptors from PIP₂ metabolism. Since phosphotyrosine phosphorylation is found to precede and is a prerequisite for activation of the PIP₂ hydrolysing enzyme phospholipase Cγ (PLCγ), it follows that the CD45 PTPase is involved in activating a PTK (June et al, 1990; Koretzky et al, 1991; Ledbetter et al, 1991; Mustelin et al, 1990; Nishibe et al, 1990). This kinase can then bring about the events which immediately follow antigen-induced stimulation such as tyrosine phosphorylation of PLCγ and of the zeta subunit of the TCR/CD3 complex (Baniyash et al, 1988; June et al, 1990; Mustelin et al, 1990).

During T cell activation, the accessory protein CD4 (or CD8) interacts intercellularly with antigen/major histocompatibility complex (MHC) and is found to colocalize with the TCR/CD3 complex at the antigen presenting cell, APC- T cell interface (Bolen and Veillette, 1989; Emrich, 1988; Kupfer et al, 1987). Transmission

of signals resulting in antigen-induced proliferation of T cells is coincident with interaction of the membrane-associated PTKs, p59^{fyn} and p56^{lck}, with TCR/CD3 and CD4 (or CD8), respectively (Rudd *et al*, 1988; Samelson *et al*, 1990b; Veillette *et al*, 1988; 1989). Kinase activity of the src-like PTKs is suppressed by phosphorylation of a tyrosine located in the C-terminal regulatory domain (reviewed in Cantley *et al*, 1991). In unstimulated T cells, phosphate occupancy is high at tyrosine 505 of p56^{lck}, this is coincident with low kinase activity (Veillette *et al*, 1988; 1989). Activation of T cells upon overexpression of the F505 (Tyr to Phe) mutant of p56^{lck} confirms this regulatory mechanism (Abraham *et al*, 1991). CD45 may exert its effect at this critical residue on the CD4- or CD8-linked PTK, p56^{lck} or on the TCR/CD3-associated p59^{fyn} (Mustelin and Altman, 1990; Mustelin *et al*, 1989; 1992; Samelson *et al*, 1990b). In fact, analysis of parental, CD45-deficient, and revertant cell lines have revealed a correlation between the presence of cell surface expression of CD45 and dephosphorylation of tyrosine 505 on p56^{lck} (Ostergaard *et al*, 1989; Ostergaard and Trowbridge, 1990). Similarly by employing immunofluorescence labeling techniques, p59^{fyn} was found to co-cap or co-distribute with CD45 under physiological conditions (Mustelin *et al*, 1992). This data is consistent with CD45-mediated dephosphorylation and subsequent activation of receptor-associated PTKs in T and B cell stimulation and maturation.

Ligand-induced oligomerization and cell surface relocalization as well as putative Ser/Thr phosphorylation and

auto-dephosphorylation are events proposed to broker changes in CD45 catalytic activity and substrate selection. Antigen- or anti-CD3-induced tyrosine phosphorylation on CD45 was found to be transient, indicating the ability of CD45 to undergo autodephosphorylation. This is consistent with a bilateral feedback loop involving the associated PTK and the CD45 PTPase (Stover *et al.*, 1991). The CD45 primary sequence predicts several protein kinase C (PKC) phosphorylation sites, similar to those found in the juxtamembrane region of receptor PTKs, indicating a role for serine phosphorylation in the regulation of CD45 activity (Charbonneau *et al.*, 1988). Treatment of T cells with the Ca^{2+} ionophore, ionomycin, induced intracellular Ca^{2+} levels which in time generated a large decrease in CD45 PTPase activity. This was coincident with a decrease in phosphoserine phosphorylation on CD45, presumably catalyzed via the Ca^{2+} / Calmodulin protein phosphatase (Ostergaard and Trowbridge, 1991). This regulatory event may reflect the actual feedback mechanism in place upon antigen-induced increase in intracellular Ca^{2+} levels. However, phosphorylation of CD45 with various serine/threonine kinases *in vitro* does not appear to alter the catalytic activity of this PTPase (Tonks *et al.*, 1990b).

Stimulation of lymphocytes result in an increase in the co-distribution of CD45 with the cytoskeletal protein fodrin found underneath the patched or capped lymphocyte receptor structure (Bourguignon *et al.*, 1985). This spectrin-like protein, fodrin preferentially co-isolates with CD45 and binds in a saturable manner to an *in vivo*-labeled 48 kDa phosphopeptide of CD45. Most

importantly, in vitro complex formation of CD45 with fodrin and with spectrin (to a lesser extent), is found to increase CD45 PTPase activity (Lokeshwar and Bourguignon, 1992). Accordingly, phosphorylation of CD45 on Ser/Thr residues by PKC in vivo, in response to phorbol ester, is concomitant with down-regulation of CD45 PTPase activity as well as a change in lateral mobility on the cell surface; this appears to involve an induced binding to the cytoskeleton and may alter substrate accessibility to this PTPase (Autero and Gahmberg, 1987; Patarroyo and Gahmberg, 1984). The covalent attachment of a particular lipid to CD45, may contribute to changes in the lateral membrane localization or regulation of CD45 function (Takeda and Maizel, 1990). The functional similarity of CD45 to the EGF receptor was recently found to extend to the receptor-like oligomerization of CD45. However, homodimer formation was not sufficient to induce a functional change in PTPase activity in vitro and instead observed differences in activity were clearly dependent upon other factors (Takeda et al, 1992). This is consistent with a complex mechanism of ligand-mediated regulatory control of CD45 function.

1.4-2 PTPase catalytic domains: Receptor domain I and II

A unique feature of the transmembrane PTPase is the presence of two catalytic domains; the exception to this rule is the discovery of a single PTPase domain in HPTP β and DPTP10D (Krueger et al, 1990). Initial studies revealed that these catalytic domains are sufficient to confer phosphotyrosine-specific activity to both

the purified and recombinantly expressed receptor PTPases, CD45 and LAR (Charbonneau *et al*, 1988; Streuli *et al*, 1989; 1990; Tonks *et al*, 1990). The bacterially expressed domain I of these PTPases was shown to have catalytic activity towards several substrates, whereas domain II did not have activity towards these artificial substrates (Streuli *et al*, 1990). However, complete or partial removal of the second domain appeared to influence the substrate specificity of domain I (Streuli *et al*, 1989; 1990). A subsequent study was able to demonstrate PTPase activity in domain 2 of CD45 (Tan *et al*, 1993). Wang and Pallen (1991) have also characterized domain II of HPTP α as having activity, however, its artificial substrate specificity is distinct from that of domain I. This is suggestive of an important function for domain II in context of ligand-mediated activation. In this scenario, each domain may dephosphorylate a specific set of substrates upon differential activation by a particular ligand. Alternatively, the second domain may serve as a docking area or regulatory region such that the binding of a particular substrate or modulatory protein will alter the activity of the first domain. This is consistent with the lack of an essential cysteine in the second domain of several receptor PTPases (Krueger *et al*, 1990; Guan and Dixon, 1990; Tian *et al*, 1991; Yang *et al*, 1991). This essential cysteine is present in and necessary for active PTPases; it is known to be involved in the catalytic process due to the recent isolation of covalent phosphocysteine intermediates (Guan and Dixon, 1991a; Itoh *et al*, 1992; Pot *et al*, 1991; Streuli *et al*, 1989; 1990). This is

suggestive of a regulatory rather than a catalytic role for the distal PTPase domain in at least some RPTPases. Elucidation of the actual cell-associated or soluble ligand(s) and physiological substrates of PTPases are essential to determine the regulatory significance of domain I and II and ultimately to understand the function of these transmembrane phosphatases.

1.4-3 Regulation of PTPase catalytic domains

Phosphorylation on tyrosine residues in proteins is generally substoichiometric and transient. This is demonstrated by the relatively higher specific activity of PTPases (by 10-1000 fold) relative to PTKs (Fischer *et al*, 1991). Therefore, signal transmission via phosphotyrosine-mediated events must correspond with strict regulatory control of the dephosphorylating enzymes (Tonks *et al*, 1991). Modulation of these PTPases may include regulation via enzyme-specific binding or by post-translational modification such as phosphorylation. Evidence for the former lies in the identification of two heat-stable PTPase-specific protein inhibitors from bovine brain and rabbit tissues which were found to inhibit the activities of specific PTPase isoenzymes (Ingebritsen *et al*, 1988; Ingebritsen, 1989). There is no *in vitro* evidence for the involvement of phosphorylation in the modulation of PTPase activity, however, the regulation of CD45 function as discussed earlier sets a precedent for such a role.

Core catalytic domains of purified or bacterially expressed PTPases have been shown to differ in their preference for

artificial substrates and in their stimulation versus inhibition by polyionic compounds (Cho *et al*, 1991; 1992; Daum *et al.*, 1991; Itoh *et al*, 1992; Pot *et al*, 1991; Tonks *et al*, 1988b; Wang and Pallen, 1991; 1992). The relatively high similarity of subgroup catalytic domain sequence- TCPTP and PTP1B (72%) or LAR and HPTP δ (87%), compared to that of the general PTPase domain amino acid identity (29-42%) may extend into similarities in substrate or effector preference (Charbonneau *et al*, 1989; Cool *et al*, 1989; Kreuger *et al*, 1990; Saito and Streuli, 1991). It is not yet known whether the difference in core domain sequence and in *in vitro* catalytic parameters corresponds with a difference *in vivo*.

1.5 Intracellular Protein Tyrosine Phosphatases

Intracellular phosphatases, such as the prototypic PTPase, PTP1B appear to be modulated via regulatory domains (Cool *et al*, 1990; Tonks *et al*, 1988a). The non-receptor enzymes are subdivided into groups by the nature and position of the noncatalytic domain (Figure 1.2; Table 1.2). These include the PTP1B-/TCPTP-like or alternatively PEST C-terminal domain and the N-terminal: putative hydrophobic ligand-binding motif, SH2 domain, or membrane/cytoskeleton-associated protein region (see Table 1.2; Brown-Shimer *et al*, 1990; Chernoff *et al*, 1990; Gu *et al*, 1991; 1992; Matthews *et al*, 1992; Plutzky *et al*, 1992; Shen *et al*, 1991; Yang and Tonks, 1991).

Table 1.2 Classification of non-transmembrane PTPases

The non-receptor PTPases are classified by the nature and position (N-terminal or C-terminal; N or C) of its non-catalytic domain. These domains appear to confer subcellular localization, protein degradation, or protein association to the intracellular PTPases. Several PTPases have amino terminal domains with no known sequence homology (no known motifs).

Table 1.2

NONCATALYTIC			REFERENCE
PTP	N or C	DOMAIN TYPE/FUNCTION	
SUBCELLULAR LOCALIZATION			
PTP1B	C	Charged but last 35 aa (Hydrophobic) targets PTP1B to ER membrane	Charbonneau <u>et al</u> , 1989 Chernoff <u>et al</u> , 1990 Brown-Shimer <u>et al</u> , 1990 Frangioni <u>et al</u> , 1992
PTP1	C	<u>ibid</u>	Guan <u>et al</u> , 1990
TCPTP	C	Charged but Hydrophobic C-terminal 19 aa may predict particulate TCPTP isoform	Cool <u>et al</u> , 1989 Mosinger <u>et al</u> , 1992 Swarup <u>et al</u> , 1991
PROTEIN DEGRADATION			
PEP	C	PEST or Pro-, Glu-, Ser-, and Thr-rich domain predicts rapid protein turnover	Matthews <u>et al</u> , 1992 Yang <u>et al</u> , 1993
PROTEIN ASSOCIATION			
PTP1C, SHP	N	Two SH2 domains that	Shen <u>et al</u> , 1992
SHPTP1, HCP		bind to phosphotyrosine-	Matthews <u>et al</u> , 1992
SHPTP2, SypN		containing proteins	Plutzky <u>et al</u> , 1992
PTP1D	N		Vogel <u>et al</u> , 1993
PTP2C	N		Ahmad <u>et al</u> , 1993
csW	N	Required for normal Drosophila termini	Freeman <u>et al</u> , 1992 Perkins <u>et al</u> , 1992 Feng <u>et al</u> , 1993
PTPH1	N	Protein 4.1 family member: with identity to cytoskeleton associated protein CAP domain	Yang and Tonks, 1991
MEG	N	Protein 4.1 CAP domain	Gu <u>et al</u> , 1991
UNKNOWN FUNCTION/mRNA INDUCED			
STEP	N	No known motif; Striatum- enriched PTPase (3 Kb mRNA)	Lombroso <u>et al</u> , 1991
HePTP	N	No known motif; mRNA found and induced in T cells	Zanke <u>et al</u> , 1992
CL100	N	No known motif; induction	Keyse and Emslie, 1992
3Ch134		of mRNA with heat shock	Charles <u>et al</u> , 1992
CELL CYCLE REGULATED			
cdc25	N	CD45 isoforms posted at cell cycle checkpoints (i.e. G2/M); Dual-specificity Phosphatase	Moreno and Nurse, 1991
"VIRAL AND PATHOGENIC BACTERIAL" FUNCTION			
VH1	-	"Core" PTPase is late stage Vaccinia virus protein with Ser/Tyr phosphatase activity	Guan <u>et al</u> , 1991
YOP2b		No known motif; essential for Yersinia virulence	Guan and Dixon, 1990 Bliska <u>et al</u> , 1991

1.5-1 Carboxyl Terminal Domain Regulation of PTP1B and TCPTP

Data obtained from extensive microsequencing of the first homogenously purified PTPase, the non-transmembrane PTP1B, was corroborated by the predicted primary structure of the first 321 amino acids of the cloned PTP1B, however this cDNA also predicted another 114 C-terminal amino acids (Brown-Shimer *et al.*, 1990; Chernoff *et al.*, 1990). The fairly high activity of the truncated PTP1B may be similar to results found in other studies where truncation in size by limited proteolysis during purification generated an increase in enzymatic activity; this suggests the presence of a regulatory region in these PTPases (Pallen *et al.*, 1991; Rotenberg and Brautigan, 1987; Swarup and Subrahmanyam, 1989). Similarly, the enzyme activity of TCPTP is enhanced upon limited proteolytic cleavage of the C-terminal region (Cool *et al.*, 1990; Zander *et al.*, 1991). In addition, overexpression of the truncated isoform of TCPTP resulted in a soluble protein with higher activity than that of the intact TCPTP (Cool *et al.*, 1990). Relatively high K_{cat} or turnover numbers were observed when the truncated PTP1B or bacterially expressed catalytic "core" domains of LAR and the transmembrane CD45 were purified and used to dephosphorylate phosphotyrosyl substrates (Cho *et al.*, 1991; Pot *et al.*, 1991; Tonks *et al.*, 1988c; 1990). This indicates that domains extraneous to those found in the enzymatic core may function to regulate activity and substrate selection.

Subcellular localization of the PTPase may in effect restrict phosphatase function by limiting the range of putative *in vivo*

substrates. The full length intracellular PTPases, PTP1B and TCPTP, are localized to a specific subcellular compartment(s) or fraction by virtue of their C-terminal domain. The final 34 hydrophobic-rich residues of PTP1B are both necessary and sufficient to localize this enzyme or a heterologous protein to the cytoplasmic face of the endoplasmic reticulum, or ER, membrane (Frangioni *et al*, 1992). Membrane attachment may occur by insertion of these hydrophobic sequences into the lipid bilayer directly, by association with integral membrane proteins, or alternatively by specific attachment of the polyisoprenylated and/or palmitoylated moieties predicted to modify the C-terminal end (Frangioni *et al*, 1992; Guan *et al*, 1990). This is consistent with the identification of PTP1B in both the particulate and soluble fractions of human placenta and with the subsequent purification of the soluble truncated form lacking C-terminal sequences (Brown-Shimer *et al*, 1990; Charbonneau *et al*, 1989; Chernoff *et al*, 1990; Guan *et al*, 1990; Miyasaka and Li, 1992; Tonks *et al*, 1988a). In concordance, the final 98 amino acids of the full length TCPTP are required for localization to a high molecular weight/particulate fraction (Cool *et al*, 1990). Two cDNAs that differ markedly at their 3'-termini, predict intracellular TCPTPs that retain or lose the hydrophobic C-terminal sequences (Cool *et al*, 1989; Mosinger, Jr. *et al*, 1992; Swarup *et al*, 1991). The identification of alternatively spliced PTP1B transcripts encoding a different C-terminus may translate into differential localization and function for the PTP1B isoforms (V. Shifrin and Neel, B.G as cited in Frangioni *et al*, 1992). The alternative usage

of these hydrophobic domains may provide a mechanism whereby differential targeting could direct dephosphorylation of distinct substrates leading to specific physiological responses.

1.5-2 The C-Terminal Pro-/Glu-/Ser-/Thr-Enriched or PEST Domain

The modulation or subcellular localization of PTPases appear to determine the preferred tyrosyl phosphorylated substrate(s). The identification of a putative nuclear localization signal in the hematopoietic PTPase, PEP, is suggestive of a nuclear PTPase (Dang and Lee, 1988; Garcia-Bustos *et al*, 1991; Matthews *et al*, 1992). In addition, the timing of PTPase expression and degradation may serve to determine the functional characteristic of these enzymes. For instance, the C-terminal 300 amino acids of the PTPases- PEP and PTP-PEST feature a PEST or Pro-, Glu-, Ser-, and Thr-enriched domain that is generally coincident with rapid protein turnover (Matthews *et al*, 1992; Rogers *et al*, 1986; Yang *et al*, 1993). PEST sequences have been reported in a number of nuclear proteins, such as c-fos, c-myc, and p53 (Rogers *et al*, 1986; van Lohuizen *et al*, 1991; McCarty *et al*, 1991). The identification of three potential p34^{cdc2} phosphorylation sites in PEP and PTP-PEST is sufficient to propose a cell-cycle dependent regulation for these PTPases (Matthews *et al*, 1992; Yang *et al*, 1993). A corollary is the cell cycle regulated Cln3 cyclins which undergo PEST-dependent, relatively rapid turnover ($t_{1/2}$ = 10 minutes; Matthews *et al*, 1992; Tyler *et al*, 1991; Peter *et al*, 1990). There may be a role for these PTPases in cell cycle control by the proposed use of cell-cycle

regulation and rapid turnover in creating a narrow window through which PEP or PTP-PEST may function.

1.5-3 N-Terminal Domains: Induction of PTPase Expression

Tissue specific expression or induction-mediated increase in transcript levels may translate into the spatial and temporal expression of PTKs, PTPases, and/or their substrates; this regulated expression may function to define signal transduction networks. The N-terminal catalytic domain of STEP and HePTP share little similarity with PTP1B, TCPTP or with other known functional motifs; it is possible that these small regions encode targeting domains or alternatively promote binding to modulatory proteins (Lombroso *et al*, 1991; Zanke *et al*, 1992). Alternative processing of the neural-specific STEP (striatum-enriched phosphatase) transcripts generates a 3 Kb form differentially enriched in the striatum, while the 4.4 Kb message is most abundant in the cerebral cortex and is rare in the striatum (Lombroso *et al*, 1991). Whereas, the pattern of lectin-, T cell receptor antibody (Ab)-, or anti-CD3 Ab-dependent induction of HePTP encoding mRNA levels is similar to that of a variety of the lymphocyte-induced proteins. Interleukin 2 receptor α (IL 2R α), IL2, IL6, p56^{lck}, and HePTP mRNA transcripts receive maximal induction at 24-48 hours; this is indicative of a role for this phosphatase in maintaining the activated lymphocyte (Ullman *et al*, 1990; Zanke *et al*, 1992). Similarly, oxidative stress and heat shock were recently shown to induce mRNA transcripts corresponding to a human PTPase (CL100). The predicted

phosphatase domain has similarity to the "catalytic (HGXAG) domain" of the (Ser/Tyr) dual-specificity, vaccinia virus (VH1) phosphatase; while the N-terminal region of CL100 has no similarity to known regulatory motifs (Charles *et al*, 1992; Keyse and Emsile, 1992). The level of PTPase activity may also be regulated as proposed with the MEG PTPase in which the N-terminal domain is similar to known hydrophobic ligand binding domains (Gu *et al*, 1992).

1.5-4 SH2-containing PTPases

The identification of SH2 domain-containing protein tyrosine phosphatases provides another mechanism by which PTPases may regulate signal transduction (Adachi *et al*, 1992; Ahmad *et al*, 1993; Freeman *et al*, 1992; Feng *et al*, 1993; Matthews *et al*, 1992; Pei *et al*, 1993; Perkins *et al*, 1992; Plutzky *et al*, 1992; Shen *et al*, 1991; Vogel *et al*, 1993; Yi *et al*, 1992; Zhao *et al*, 1993). The src homology (SH) regions 2 and 3, initially described in src-like tyrosine kinases, were recently identified in a variety of signalling molecules. The noncatalytic adaptor molecules, such as crk and nck, are also members of the known SH2 family, which include: PLC γ , ras GAP, PI3-K associated p85, and the src-, fps-, abl-, and syk-like intracellular PTKs (Pawson and Gish, 1992). These SH2- and SH3-containing proponents also include two polypeptides from the Interferon-stimulated gene factor 3 α (ISGF3 α) complex (reviewed by Koch *et al*, 1991; Pawson and Gish, 1992; Fu, 1992). In the latter case, interferon-induced translocation of the

SH2-containing ISGF3 α complex to the nucleus promotes interaction with the DNA-binding protein ISGF3 γ and induces a coincident increase in DNA binding affinity. The interferon response-essential PTK, tyk2 appears to link interferon α/β receptor stimulation to transcription of interferon-responsive genes via SH2-dependent ISGF3 α complex assembly or targeting of tyk2 to nuclear substrates such as ISGF3 γ (Valazquez *et al*, 1992; Fu, 1992; Pawson and Gish, 1992).

Src homology region-containing proteins function as signalling molecules. SH2/SH3-dependent association mediates the translocation of cytosolic signal transducers to plasma membrane or cytoskeletal locations where they can interact with specific substrates (Koch *et al*, 1991). Cytoskeleton localized proteins include the actin-binding, SH3 domain-containing α spectrin subunit and tensin, an SH2 domain-containing and tyrosine phosphorylated, focal contact constituent (Davis *et al*, 1991; Lehto *et al*, 1988). These SH3 domains were recently postulated to associate with a proline-rich region found in specific polypeptides (Cicchetti *et al*, 1992). Specific phosphotyrosine (PY) residues found in the autophosphorylated RPTK/PTK act as discrete SH2 docking sites to support regulatory complex formation at or near the plasma membrane. This provides a mechanism whereby a molecule, such as v-crk, with SH2 and SH3 domains but lacking catalytic domains can increase intrinsic phosphotyrosine levels and thereby transform cells. The SH2 and SH3 domains of p47^{gag-crk} may in fact function as a nucleus for complex formation between PTKs and their substrates,

and in doing so, may enhance kinase activity, block dephosphorylation, or protect tyrosine phosphorylated proteins from proteolysis (Koch *et al.*, 1991 and references therein). This model is supported by the report of several adapter molecules (SH2/SH3) which were found to recruit a ras activator to an autophosphorylated RPTK and thus form a stable complex (reviewed by, McCormick, 1993)

The SH2-PTPases may modulate the PY/SH2-mediated complex by interacting with and dephosphorylating phosphotyrosine residues present on associated PTK, substrate, or regulatory protein molecules. The SH2-PTPase may negatively regulate a PTK by binding to a complex component and upon dephosphorylation of a specific phosphotyrosine it can inactivate the kinase and/or decrease its ability to interact with SH2-containing proteins. Alternatively, the bound PTPase may dephosphorylate proteins previously modified by the PTK. In support, the human and mouse SH2 PTPase homologues, PTP1C and SHP, were recently shown to interact with cellular proteins (Matthews *et al.*, 1992; Shen *et al.*, 1991). In another scenario, the SH2-containing phosphatase may amplify the initial signal via removal of the negative regulatory C-terminal phosphate from PTKs. This would prevent SH2 domain/C-terminal PY intramolecular association and alleviate the negative regulation of SH2-containing PTKs. High affinity association of SH2-PTPases with regulatory molecules such as receptor or intracellular PTKs may modify the activity of the PTPase, through phosphorylation or by conformational change. For instance, putative phosphotyrosine sites

located in the homologous C-terminal domain could mediate an intramolecular interaction with the N-terminal SH2 domain; this allosteric model of SH2-PTPase inactivation may be similar to the mechanism proposed above for the negative regulation of src-like PTKs (Cantley *et al*, 1991). Alternatively, recent studies report the EGF- and PDGF-stimulated phosphorylation of RPTK-associated SH2-PTPases (Feng *et al*, 1993; Vogel *et al*, 1993). This increase in phosphate residency on these tyrosine moieties was found to induce the PTPase activity, however, the SH2-PTPase did not dephosphorylate the RPTKs, indicating a coordinated function for both enzymes (Vogel *et al*, 1993).

1.6 Cytoskeleton-Associated Proteins and CAP PTPases

Several recently cloned PTPases have N-terminal homology to a family of cytoskeletal associated proteins (Marshall and Bell, submitted; Chapters 2 and 4; Gu *et al*, 1991; Yang and Tonks *et al*, 1991); this band 4.1 superfamily is found to include; protein [band] 4.1, talin, ezrin, radixin, and moesin (Conboy *et al*, 1986; Funayama *et al*, 1991; Gould *et al*, 1989; Gu *et al*, 1991; Lankes and Furthmayr, 1991; Rees *et al*, 1990; Turunen *et al*, 1989; Yang and Tonks, 1991). These protein 4.1 family members appear to bind via their N-terminal region to the cytoplasmic domain of integral membrane proteins while the diverse C-terminal domains seem responsible for interaction with cytoskeletal proteins (Luna, 1991). Protein 4.1 was shown to play a pivotal role in cell shape and membrane flexibility by its promotion and stabilization of the

spectrin-actin complex and via interaction with the inner face of the plasma membrane (Cohen et al, 1980; Cohen and Foley, 1980; Fowler and Taylor, 1980; Ungewickell et al, 1979). Glycophorin A, B, and C are the main candidate integral membrane proteins proposed to anchor the N-terminal domain of protein 4.1 in a phosphatidylinositol-dependent manner to the erythrocyte skeleton-lipid bilayer (Anderson and Marchesi, 1985; Anderson and Lovrien, 1984; Mueller and Morrison, 1981; Pasternak et al, 1985; Wolfe et al, 1980). Likewise, co-distribution of vinculin-binding talin with the fibronectin receptor (integrin) at focal adhesion plaques is consistent with talin providing a link between the extracellular matrix and the cytoskeleton at regions of closest cell-substratum contact (Horwitz et al, 1986; Rees et al, 1990; reviewed in Burridge and Fath, 1989). Ezrin is found at the submembranous region of microvilli in intestinal epithelial cells and in other cell types (Bretscher et al, 1983). The amino terminal region of ezrin is seen to restrict subcellular localization to cortical cytoskeleton and plasma membrane linkage sites; while the overexpressed carboxy terminal region of ezrin co-distributes with the stress fibers or actin patches (Algrain et al, 1993). Whereas, ezrin-related but distinct, moesin is a cytoskeletal component of filopodia (Furthmayr et al, 1992; Lankes and Furthmayr, 1991), radixin is highly concentrated in the undercoat of cell-cell adherens junctions during interphase and at the cleavage furrow during cytokinesis (Funayama et al, 1991; Itoh et al, 1991; Sato et al, 1991; Tsukita et al, 1989).

Many proteins found in these cytoskeleton-membrane linkage sites are phosphorylated on tyrosine residues in normal and RSV-transformed cells; this list includes ezrin, talin, the β subunit of integrin, vinculin, tensin, the focal adhesion plaque, PTK p125^{PAK} and paxillin (Bretscher, 1989; Davis *et al.*, 1991; Gould *et al.*, 1986; Hirst *et al.*, 1986; Horvath *et al.*, 1990; Igarashi *et al.*, 1990; Pasquale *et al.*, 1986; Tapley *et al.*, 1989; Turner *et al.*, 1990; Turner, 1991). Redistribution of ezrin into EGF-stimulated microvilli and membrane ruffles of A431 cells is concomitant with rapid phosphorylation on ezrin tyrosine residues (Bretscher, 1989). In a related study, phosphorylation on an erythroid-maturation-specific tyrosine residue was found to reduce protein 4.1/spectrin/actin ternary complex formation *in vitro*; the *in vivo* tyrosine phosphorylation of protein 4.1 may effect transient changes in cytoskeleton/membrane plasticity (Anderson and Marchesi, 1985; Subrahmanyam *et al.*, 1991; Tang *et al.*, 1988). PTK-modulated proteins are concentrated at gap junctions, growth cones, focal adhesion plaques, and other membrane formations indicating a regulatory role for cytoskeleton/ membrane associated PTPases in the assembly and function of these specialized structures (Bishop, 1991; Matten *et al.*, 1990).

Phosphotyrosine-containing proteins found at focal adhesions and intercellular junctions appear to be modified by colocalizing enzymes (Maher *et al.*, 1985; reviewed in Burridge and Fath, 1989). This indicates that regulation of the cytoskeleton-membrane bridge by specific PTKs and PTPases is important in normal growth and

development and that deregulation of these enzymes may play a role in transformation. Translocation of p60^{c-*src*} to the cytoskeleton during platelet aggregation is correlated with GPIIb/IIIa receptor-fibronectin binding, while cytoskeletal association of p60^{v-*src*} is required for RSV-mediated transformation (Horvath *et al*, 1992). The focal adhesion plaque-localized kinase, p125^{PAK} exhibits a low level of tyrosine-specific phosphorylation in normal fibroblasts and this level is greatly enhanced in p60^{v-*src*} transformed cells (Kanner *et al*, 1990; Schaller *et al*, 1992). This kinase (p125^{PAK}) is activated and may be a common factor in integrin-mediated cell anchorage, platelet aggregation and in signal transduction via the neuropeptide growth factors- bombesin, vasopressin, and endothelin (Burridge *et al*, 1992; Lipfert *et al*, 1992; reviewed in Zachary and Rozengurt, 1992).

The level of tyrosine phosphorylation on membrane-cytoskeleton proteins may influence the relative affinities of interacting components such as cell adhesion molecule (CAM) association with cytoskeleton. In fact, cadherin-mediated multicellular organization requires interaction of the CAM's cytoplasmic domain with the cytoskeletal-linking catenins (Hirano *et al*, 1992; Takeichi, 1988; 1991). Disruption of this cell adhesion by p60^{v-*src*} transformation is possibly due to deregulated tyrosine phosphorylation on the 98-kD catenin (Matasuyoshi *et al*, 1992). This would imply a role for submembranous tyrosine kinases and phosphatases in the regulation of vertebrate morphogenesis by modification of components pivotal in promotion and maintenance of the cytoskeleton/membrane scaffold.

1.7 A Role for the PTPases in Cell Cycle control

The cell cycle is defined as the ordered progression from growth (G1) and DNA synthesis (S) into preparation and completion of nuclear division; contiguous with mitosis is cell division or cytokinesis which culminates in the creation of daughter cells. Transition control points such as those at G1 and at the G2/M demarcation are governed by regulatory feedback as the cell checks its readiness "in a rearview mirror" before proceeding to the next phase (Hunt, 1991; Lewin, 1990). Initiation of mitosis is triggered by activation of the MPF (M phase promoting factor) kinase at the G2/M boundary (reviewed in Lewin and Nurse, 1990). The MPF-serine/ threonine kinase (cdc2 in fission yeast, cdc28 in budding yeast and p34^{cdc2} in mammals) is modulated by interaction with the regulatory protein, cyclin B. Cyclin B is accumulated during interphase and rapidly degraded by proteolysis as cells exit mitosis (Beach et al, 1982; Lee and Nurse, 1987). Upon binding to cyclin B, the yeast cdc2 kinase is negatively regulated by phosphorylation at a tyrosine residue (Tyr 15) found within the ATP binding site; the human p34^{cdc2} faces a double block with phosphate moieties stationed at both Thr 14 and Tyr 15 (Gould and Nurse, 1987; Krek and Nigg, 1991a). Site-directed mutagenesis of the yeast cdc2 with the substitution of tyr 15 by a nonphosphorylatable residue or by a double mutation on p34^{cdc2} was sufficient to trigger premature entry into mitosis (Gould and Nurse, 1989; Krek and Nigg, 1991a; 1991b; Nigg and Krek, 1991). This phenotype is similar to that observed upon mutation of the weel kinase (Nurse, 1975;

Russell and Nurse, 1987; Thuriaux et al., 1978) This weel Ser/Thr/Tyr (STY) kinase along with the redundant mik1 kinase, serve as negative regulators by phosphorylating Tyr 15 (and Thr 14) on the MPF kinase (Featherstone and Russell, 1991; Lundgren et al., 1991; Parker et al., 1991; Piwnicka-Worms et al., 1991).

Overexpression of cdc25 was also found to promote mitotic advancement, consistent with a role for cdc25 as a dose-dependent mitotic inducer (Edgar and O'Farrell, 1990; Millar et al., 1991a; Russell and Nurse, 1986). This predicts a mitotic timer equipped with dosage sensors armed to detect threshold levels and/or activities of cdc25 (Millar et al., 1991a; Millar and Russell, 1992). Activation of the M-phase kinase is coincident with dephosphorylation at Tyr 15 and perhaps Thr 14 in the cyclin B/p34^{cdc2} context, indicating that this cdc25-mediated decline in phosphate occupancy, acts as a rate-determining switch at G2/M (Dunphy and Newport, 1989; Gautier et al., 1989; 1991; Gould et al., 1990; Moreno and Nurse, 1991; Morla et al., 1989; Solomon et al., 1991). Recombinantly purified cdc25 can dephosphorylate and activate the M-phase kinase within a cyclin B/p34^{cdc2} milieu and as well, dephosphorylates monomeric p34^{cdc2} and other Ser/Thr and Tyr phosphorylated substrates (Devault et al., 1991; Dunphy and Kumagai, 1991; Kumagai and Dunphy, 1991a; 1991b; Millar et al., 1991b; Strausfeld et al., 1991). In addition, wild-type human and *Drosophila* cdc25 PTPase activity is sufficient to rescue cdc25 mutants of *S. pombe* in vivo; substitution of the essential Cys or Arg residues of cdc25, both necessary for in vitro PTPase activity,

is sufficient to abrogate this in vivo function (Edgar and O'Farrell, 1990; Galaktionov and Beach, 1991; Nagata et al, 1991; Sadhu et al, 1990; Solomon et al, 1991).

Combination of various regulatory molecules create the redundant and fine-tuned checkpoints required to control the cell cycle. This model was exemplified by the recent identification of multiple phase-specific cyclins: Cyclin A (S phase), cyclin B (G2/M), and the G1 cyclins- or CLNs; cyclin-dependent kinases (cdks); several cdc25 PTPase isoforms (A, B, and C) from a single species (Beach et al, 1988; Galaktionov and Beach, 1991; Girard et al, 1991; 1992; Hunt, 1991; Matsushime et al, 1991; Meyerson et al, 1991; Millar et al, 1991b; Nagata et al, 1991; Pines and Hunter, 1991a; 1991b; Reed et al, 1991; Sadhu et al, 1990). Several structurally-related human cdc25 genes express functionally different PTPases such that microinjection of the type-specific anti-cdc25C antibody generated a flattened interphase morphology consistent with a late G2 function while formation of a rounded mitotic-state was obtained using the anti-cdc25A antibody (Galaktionov and Beach, 1991; Millar et al, 1991c). Differential expression and activation of the cdc25 family of PTPases may help provide the intricate timing required in cell cycle control.

1.8 Protein Tyrosine Phosphatases in Development

While protein tyrosine kinases have been shown to play major roles in Drosophila and mouse development, the dephosphorylating enzymes have only been recently identified in these multicellular

processes (Howard et al, 1992; Hariharan et al, 1991; Pawson and Bernstein, 1990; Perkins et al, 1992; Tian et al, 1991; Yang et al, 1991). For instance, the regulation of cell fate at the termini of the Drosophila embryo requires the maternal and embryonic expression of several terminal genes (Pawson and Bernstein, 1990). Of these, the SH2-containing PTPase corkscrew, or csw is known to play an important role in terminal structure development. In addition, other terminal genes include: torso, a receptor PTK and D-raf, a Ser/Thr kinase homologous to the human c-raf (Perkins et al, 1992). Through loss of function mutations, D-raf and csw were shown to act in concert to synergistically translate the torso ligand-stimulated signal into transcription of other terminal genes. Although, the csw protein is essential for normal embryonic development of Drosophila termini, its alternative transcripts are not exclusively localized to the early embryo, with csw expression observed in larval, pupal and adult stages as well (Perkins et al, 1992; reviewed in Pawson and Bernstein, 1990).

Multicellular processes such as development of the CNS and PNS are tightly linked to the expression and action of cell adhesion molecules (CAM) in governing neuronal-specific differentiation, migration, and guidance. CAM-like fibronectin type III (Fn III) and immunoglobulin (Ig) repeats were recently found in the external domain of several PTPases, such as the mouse LAR and Drosophila DLAR and DPTP, while the neural-specific DPTP10D and DPTP99A has only Fn III repeats (Hariharan et al, 1991; Streuli et al, 1988; 1989; Tian et al, 1991; Yang et al, 1991). The structure of these

CAM-PTPases and of the Drosophila N-CAM-like receptor PTK, Dtrk is suggestive of regulated changes in the phosphorylation state in response to cell surface, extracellular matrix- and ligand-mediated stimuli (Barbacid et al, 1991; Rutishauser et al, 1988). Alternatively, the cytoplasmic catalytic activity may affect the cell adhesion properties of the molecule through an "inside out" signalling event. One intriguing possibility may involve PTPase-mediated regulation of the developmentally important PTKs; for instance, the intracellular PTK, D-abl is required for correct formation and fasciculation of the CNS axon array (Elkins et al, 1990; Henkemeyer et al, 1990). Similarly, regulation of the embryonically expressed Drosophila src isoforms may affect membrane/cytoskeleton plasticity and as such promote growth cone guidance in the developing CNS axon (Maness et al, 1988; Maness and Matten, 1990). Complex patterns of spatially and temporally expressed isoforms of the Drosophila CAM-PTPases, DPTP10D and DPTP99A, appear to program the embryonic establishment and maintenance of subsets of axonic bundles and of several pioneer cell bodies (Hariharan et al, 1991; Tian et al, 1991; Yang et al, 1991).

Dictyostelium discoideum is the quintessential model for multicellular development, in that independent amoebae, when disrupted by starvation, can stop dividing and become attracted to aggregation centers via pulsed secretion of chemical signals (i.e. cAMP). This continuous aggregation will produce compact, multicellular slugs which ultimately form attached, spore-

containing fruiting bodies (Firtel, 1991). The Dictyostelium protein tyrosine phosphatase, PTP1, exhibits developmentally regulated expression with the lowest transcript levels observed in the vegetative state while maximal levels of PTP1 mRNA are found in the tightly aggregated or multicellular stages (Howard et al, 1992). Similarly, PTP1 promoter-driven expression of β -galactosidase activity is predominant in the prestalk and in the prefruiting body or anterior-like cells (ALCs) of the slug. In later stages, the top and bottom caps surrounding the fruiting body spore mass are also stained. PTP1 null mutants generate normal fruiting bodies, however the timing of the aggregation process is accelerated. This is reminiscent of the role for the CDC25 PTPase in timing the yeast and mammalian entry into mitosis (Howard et al, 1991; Millar and Russell, 1992). The highest PTP1 over-expressing clones experienced a 48 hour delay in aggregation and few fruiting bodies were formed, whereas moderate levels of the exogenous PTP1 produced severe morphological defects such as multitipped aggregates. The developmental pattern of phosphotyrosyl proteins is changed upon disruption or overexpression of this putative myristylated, intracellular PTPase indicating a role for dephosphorylation in multicellular aggregation and migration (Howard et al, 1992).

1.9 PTPases: from Yeast to Human

The importance of tyrosine phosphorylation is exemplified by the presence of dephosphorylating enzymes (PTPases) throughout

evolution. This was illustrated by the recent identification of PTPase isoforms from a variety of species which include: the fission and budding yeast (Guan et al, 1991; Ottlie et al, 1991); the arthropod or more specifically Drosophila melanogaster (Hariharan et al, 1991; Perkins et al, 1992; Streuli et al, 1988; 1989; Tian et al, 1991; Yang et al, 1991); the nematode, C elegans (Matthews et al, 1991); the species Iguana iguana (Matthews et al, 1989); the slime mold, Dictyostelium discoideum (Howard et al, 1992). In keeping with this tenet, the PTPase-related CDC25, is known to regulate cell cycle control in the human, mouse, Drosophila, and yeast and in addition, a similar role is predicted in Xenopus and the starfish (Millar and Russell, 1992 and references therein). Computer search analysis and biochemical data distinguished the YOPH PTPase as the essential virulence determinant of the pathogenic bacteria, Yersinia (Bliska et al, 1991; Guan and Dixon, 1990). Similarly, the identification of the late stage vaccinia virus-expressed protein, VH1, as a Tyr and Ser/Thr-specific phosphatase (Guan et al, 1991a), is reminiscent of the proposed horizontal transmission and modification of proto-oncogene DNA by the retroviral family of viruses. These dual-specificity phosphatases were also identified in the baculovirus and orthopoxvirus (Hakes et al, 1993; Sheng and Charbonneau, 1993). The recent PCR amplification of twenty-seven distinct [PTPase-like] sequences from the sea squirt, Styela, also promotes the notion that the PTPase family is as large, diverse, and evolutionally conserved as the PTKs (Matthews et al, 1991a).

1.10 PTPases and Growth Control

Receptor and receptor-linked PTKs are responsible for the transmission of many growth factor and proliferation signals. PTPases may counteract this kinase-mediated activity and transduce cell-cell contact inhibition into cellular growth arrest. This is consistent with the observed stimulation of membrane-associated PTPase activity in high density, growth arrested cells; a reduced level of enzyme activity is found in a lower density NIH 3T3 monolayer (Pallen and Tong, 1991; Ramponi et al, 1992; Rijksen et al, 1993). In addition, cells develop an increased ability to overcome contact inhibition and acquire a transformed phenotype, upon treatment with the PTPase inhibitor vanadate (Klarlund, 1985). A putative candidate for this growth arrest-mediated control of PTPase may be the CAM-like transmembrane PTPase, LAR. The non-covalently linked external or E-subunit of LAR is shed during cell growth, suggesting that a cellular CAM- or ligand-mediated subunit dissociation may provide a mechanism for regulating PTPase activity (Streuli et al, 1992; Yu et al, 1992).

In keeping with this notion, putative tumor suppressor genes have been mapped to the LAR gene on chromosome 1 (Streuli et al, 1992). In addition, other PTPases may function as antioncogenes or tumor suppressors and mapping of RPTPy to a genomic locus frequently deleted in human lung and renal tumors serves as an example of this phenomenon (LaForgia et al, 1991). Loss of function of such a PTPase could result in deregulated cell growth leading to transformation.

The proposed role for certain PTPases as 'anti-oncogenes' is supported by experimental suppression of cellular transformation via overexpressed PTPases. For example, the expression of the intracellular PTPase, PTP1B in the NIH 3T3 cells, preclude activated RPTK/ neu oncogene-dependent transformation of these cells (Brown-Shimer et al, 1992). The transformed phenotype of v-src/NIH 3T3 cells was partially reversed in clones expressing PTP1, the rat homologue of PTP1B or upon constitutive overexpression of the putative membrane-cytoskeleton localized PTPase, PTPH1 (Tonks et al, 1991; Woodford et al, 1991). Consistent with this hypothesis, transfection of v-erbB-transformed NIH 3T3 cells with a structurally unrelated, low molecular weight PTPase, counteracts transformation by the oncogene; this was exhibited as a decrease in growth rate, thymidine uptake, and growth in soft agar (Ramponi et al, 1992). v-fms transformed cells are also affected in a similar manner by the introduction of a truncated and more active form of the T cell PTPase (Zander et al, 1993). Growth inhibition was also detected in the NIH 3T3/PTPase overexpressing clones, indicating that this response was not only due to dephosphorylation of the transforming PTK; this is also suggestive of a role for these PTPases in downstream signalling mechanisms (Brown-Shimer et al, 1992; Ramponi et al, 1992). Likewise, microinjection of the truncated and deregulated PTP1B phosphatase into Xenopus oocytes inhibited insulin-stimulated maturation via downstream signalling events (Cicerelli et al, 1990; Tonks et al, 1990a).

PTPases may amplify rather than antagonize PTK-dependent

growth control and differentiation events. Overexpression of the transmembrane PTPase, RPTP α in rat embryo fibroblasts results in cellular transformation. This PTPase, appears to deregulate the mitotic control of pp60^{c-src} by persistent activation of the PTK. (Zheng et al, 1992). This is consistent with the normal role proposed for the regulated PTPases: CD45 and cdc25 in antigen-dependent T cell proliferation and mitotic induction of the cell cycle, respectively, in that both are predicted to dephosphorylate and activate a kinase (Millar and Russell, 1992).

Overexpression of the full length rat PTP1 in NIH 3T3 cells generated a substantial increase in the proportion of bi- and multinucleated cells (Woodford-Thomas et al, 1992). Similarly, introduction of the C-terminal truncated/constitutively activated TCPTP into BHK cells produced a 50% decline in growth rate and an increase in the relative number of cells with two or more nuclei. This formation of syncytial cells appears to occur through an obstruction in cytokinesis; the nuclei in these cells display an interesting asynchronous entry into mitosis (Cool et al, 1992). The particulate fraction-localized full length TCPTP proteins could not generate these results, indicating a requirement for deregulation of normal PTPase function. It would appear that, cycling cells have completion clauses, whereby the cell must complete an event (i.e. DNA replication) before the next event (or mitosis) can occur (Murray, 1992). These data describe PTPase-mediated uncoupling of normal control parameters set in place to order and synchronize the correct mitotic sequence of events.

1.11 Thesis Project

The purpose of this study was to clone and characterize tyrosine-specific phosphatases (PTPases) cDNAs from mammalian cells. Chapter 2 describes how a PCR strategy was used to amplify a region from two distinct PTPase cDNAs. This section deals with the cloning of the complete coding sequence for one (CAP-1) and partial sequence for the other (PTP2) PTPase from two separate cDNA libraries. The remainder of this Chapter studies the expression pattern of these phosphatases in mouse tissues and cell lines.

The Northern analyses presented in Chapter 2 identified a distinct CAP-1 pattern of transcription in adult leg muscle. Chapter 3 introduces an attempt to further characterize these mRNAs; cDNAs were cloned from a leg muscle library and the unique sequence obtained appears to be leg muscle specific.

Chapter 4 describes the bacterial expression and solubilization of a GST/truncated CAP-1 fusion for use in subsequent studies. The resulting enzyme was found to preferentially remove phosphates from the tyrosine residues of the sty kinase indicating that it is indeed a tyrosine-specific phosphatase (PTPase).

The majority of this thesis deals with the CAP-1 PTPase which has homology in its N-terminal region with the protein 4.1 family of proteins. These proteins are found to link proteins located in plasma membrane with those in the cytoskeleton. Appendix V describes initial experiments designed to analyze the subcellular localization and putative function of the CAP-1 PTPase.

CHAPTER 2

CLONING AND CHARACTERIZATION OF MURINE PTPase-ENCODING cDNAs

2.1 Introduction

Tyrosine phosphorylation appears to play an important role in the regulation of early mouse development. Phosphotyrosine-containing proteins have been found in early mouse inner cell mass (ICM) and embryonal carcinoma (EC) cells of teratoma or teratocarcinoma cell origin (Carlin *et al.*, 1983; Kondo *et al.*, 1989). These EC cells appear to be morphologically, immunologically, and biologically equivalent to embryonal stem (ES) cells, a pluripotent stem cell line derived from the ICM of the early mouse blastocyst (Martin, 1980; 1981; Evans and Kaufman, 1981).

The EC cell appears to serve as a model for both neoplasia and embryogenesis (Strickland, 1981). The role for phosphotyrosine phosphorylation in the latter process was substantiated by the ability of the PTK inhibitor, herbimycin A, to inhibit the differentiation of embryonal carcinoma (F9) and erythroleukemia (MEL) cells (Kondo *et al.*, 1989). Many of the known PTKs are found in low (c-abl or c-src) or non detectable levels in EC stem cells, however, their expression is enhanced soon after differentiation (Adamson, 1987; Sejersen *et al.*, 1985). In addition, the developmental use of an alternative promoter was shown to create a truncated form of the PDGF receptor (a RPTK) which is only expressed in the undifferentiated EC and ES cell (Vu *et al.*, 1989). These findings suggest that stem cell-specific or developmentally expressed forms of both PTKs and PTPases may be essential components of early embryonic cells.

P19 is a particularly useful pluripotent EC cell line in that these cells can be induced to differentiate by dimethyl sulfoxide (DMSO) to generate skeletal and cardiac muscle and by retinoic acid (RA) to obtain neurons and astroglia (McBurney *et al.*, 1982; Rudnicki *et al.*, 1987). This cell line was recently chosen to identify and clone novel Ser/Thr/Tyr kinases, such as *esk* and *sty* which may play an important role in early mouse development (Douville *et al.*, 1992; Howell *et al.*, 1991). Over the past four years a great number of PTPases have been identified using cloning strategies such as low stringency hybridization with known catalytic (PTPase) domains or PCR amplification of DNA fragments encoding novel PTPase sequence. Preliminary evidence indicated the P19 stem cell line to be an ideal system from which to amplify and clone putative developmentally important PTPases.

Two individual classes of DNA with PTPase-related sequence were amplified from P19-derived cDNA. Partial class I and II clones were isolated from a library constructed with this cDNA. Complete coding sequence for the Class I or CAP-1 PTPase was obtained by combining a 5'-extended kidney cDNA with an overlapping P19 clone. Northern analysis of the Class II PTPase revealed a 5 Kb mRNA in all mouse tissues and cell lines examined. The single CAP-1 gene was found to express two major mRNA transcripts of 5 and 7 Kb in a variety of mouse tissues. Several tissues, however, namely leg muscle as well as heart and brain exhibit slightly smaller or additional mRNA species, respectively.

2.2 Materials and Methods

2.2-1 Polymerase Chain Reaction Amplification

A pair of degenerate oligonucleotides encoding the PTPase domain-specific peptides YINAS and HCSAG were used to amplify 450 bp fragments from novel PTPase clones (primers 1 and 2, respectively, as shown in Figure 2.1). A total of 10 ng of template cDNA, prepared from P19 embryonal carcinoma cell poly A⁺ mRNA (Howell *et al.*, 1991), was added along with 1 μ g of each of the two primers to reaction buffer and nucleotides (final concentration 200 μ M dNTPs) and 2.5 units of Amplitaq DNA polymerase obtained from the AmpliTag Perkin-Elmer-Cetus kit. Prior to the addition of the DNA polymerase, each 100 μ l sample was placed in the Thermocycler for 1 minute at 94°C; this was performed to inactivate possible proteases. After addition of the enzyme, the reaction mix was overlaid with about 100 μ l of mineral oil to prevent evaporation. Thirty to forty cycles of amplification were performed with 1 minute of denaturation at 94°C, 2 minutes of annealing at 50°C, and 3 minutes of extension at 72°C. The 450 bp PCR amplified DNA was excised from agarose by GENECLEAN (Bio 101, Lajolla, California) and these fragments were used as template in further PCR reactions to acquire more material for subsequent steps.

2.2-2 Characterization of PCR Amplified Fragments

This PCR amplified DNA could represent a population of PTPase-encoding fragments. Therefore, to obtain specific probes, the DNA was subcloned into a vector (pTZ19R) by blunt-end ligation and

Figure 2.1 Oligonucleotide primers used in the polymerase chain reaction (PCR) amplification of fragments from two novel PTPases. These degenerate primers correspond to highly conserved residues found in the catalytic domains of several known PTPases (YINAS and HCSAG; Charbonneau *et al*, 1989; Cool *et al*, 1989). The oligonucleotides, written 5' to 3', are designed to prime synthesis by binding to opposing ends of complementary DNA strands. The restriction enzyme site of HindIII and KpnI are synthesized at the 5' end of primer #1 and #2, respectively.

PRIMER #1

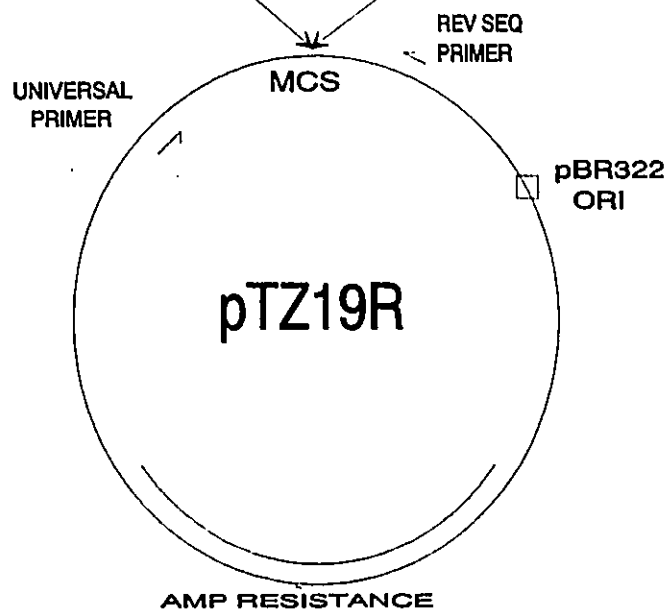
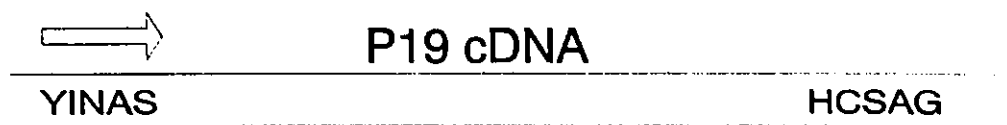
		Y	I	N	A	S	
5'	TAAGCT	TAT	ATA	AAT	GCG	AG	3'
		C	T		A		
HindIII			C		T		
					C		

PRIMER #2

H C S A G
 3' GTA ACA TCA CGT CCA TGGC 5'
 G G A
 G KpnI

individual clones were characterized (Figure 2.2). To this end, the PCR product was purified by the GENECLAN protocol and then incubated with the Klenow fragment in extension buffer (60 mM Tris-HCl, 60 mM NaCl, 6 mM MgCl₂, 2 mM DTT, pH 7.5) and 250 μ M final of each dNTP for 30 minutes at 37°C; the reaction was halted by heat treatment at 65°C for 15 minutes. This DNA was again purified by electrophoresis and standard GENECLAN procedures. Since the PCR primers were devoid of 5'-end phosphates, the PCR fragments were treated with T4 polynucleotide kinase (Pharmacia) in an Invitrogen kinase buffer and 1 mM ATP at 37°C for 30 minutes, followed by incubation at 65°C for 10 minutes. The purified PCR fragment (GENECLAN) was subcloned into pTZ19R (Pharmacia) following treatment with SmaI and calf intestinal alkaline phosphatase (CIP, Boehringer Mannheim; refer to Maniatis *et al*, 1982). The ligations were performed using BRL ligase and ligase buffer in a 20 μ l assay at 25°C for 12-16 hours. The ligation reactions were ethanol precipitated using 10 μ g of tRNA as a carrier and the reconstituted DNA (about 10 μ l) was electroporated into 40 μ l of JM103 electroporation competent bacteria using standard procedures. For instance, the Gene Pulser apparatus (BioRad) was set at 2.5 kV and 25 [μ F] while the Pulse controller was dialed to 200 ohms. The electroporated bacteria and DNA solution were immediately diluted into 1 ml of SOC and grown at 37°C for 1 hour (see Maniatis *et al*, 1982). Approximately 100-200 μ l were plated onto Ampicillin-containing LB plates and colonies were allowed to form overnight at 37°C.

Figure 2.2 Subcloning of PTPase-encoding PCR fragments into pTZ19R. Regions of P19 cDNA were amplified using degenerate primers designed from PTPase consensus sequence. The modified 450 bp fragments were then subcloned into the SmaI site of the sequencing vector, pTZ19R. Several salient features of this cloning vector are the: multicloning site (MCS), origin of replication (ORI), ampicillin resistance gene, and the binding sites for the universal and reverse sequencing (Rev Seq) primers.



➡ **DEGENERATE
OLIGONUCLEOTIDE**

Individual colonies were selected, grown in 5 ml of LB plus Ampicillin (50-100 μ g/ml final), and minipreparations of plasmid DNA (minipreps) were performed by alkaline lysis (as in Maniatis et al, 1982; Birnboim and Doly, 1979). The presence or absence of the PCR fragment was determined by excision of the potential 450 bp fragment with KpnI/HindIII. The digested plasmids were examined by electrophoresis and several insert positive clones, chosen at random, were analyzed by single-stranded DNA sequencing using the Taquence (United States Biochemical Company) and T7 polymerase (Pharmacia) kits (see Section 2.2-5). Agarose blots containing all digested/ insert positive clones were transferred to Hybond N according to company specifications and as described in Maniatis, while hybridization was executed as given below in Section 2.2-3 (Maniatis et al, 1982). These blots were washed 2X in 0.2X SSC/0.1% SDS for 30 minutes at 60°C and exposed for 2 hours to kodak XAR-5 x-ray film. The probes employed in these experiments were prepared by random-primed oligolabeling of single-stranded pTZ19R-PCR template DNA using 50 μ Ci [α^{32} P] dCTP and components from a Pharmacia labeling kit. These miniprep Southern blots were reprobbed after treatment in 50% formamide/2X SSPE for 1 hour at 65°C. Several individual PCR inserts were liberated from large scale DNA preparations; this plasmid DNA was isolated and then selected on a CsCl gradient (50VTi rotor at 48 K for 24 hours or TLV100K rotor at 100 K for 4 hours) as described by Maniatis et al, (1982).

2.2-3 Isolation of P19 cDNA Clones

The oligo (dT)-primed cDNA (20 or 50 ng), used to PCR amplify PTPase-related fragments, was ligated together with 1 μ g of λ gt11 arms (Amersham) and 6 units of the Pharmacia ligase in Amersham ligase (L) buffer overnight at 16°C. The ligated insert/phage DNA was subsequently packaged with the Gigapack Gold packaging extract (Stratagene) to generate a cDNA library derived from mouse EC stem cells. Procedures required to titre, plate, and screen the library were derived from the Amersham cDNA cloning system booklet and from Maniatis *et al* (1982). To estimate the cloning efficiency or percentage of recombinant phage in the libraries, 2% X-gal (5-Brom-4-Chlor-3-indoyl- β -D-galactopyranosid; Boehringer Mannheim) and 100 mM IPTG (isopropyl- β -D-thiogalactopyranosid; Boehringer Mannheim) were added per ml of top agarose to each plate and the blue(wt)/white(recombinant) ratio was determined for several dilutions. The plaque DNA was transferred to Hybond N filters and denatured by autoclaving at 100°C in the liquid cycle for about 3 minutes (Sambrook *et al*, 1989).

Approximately 800,000 recombinants or 40,000/filter were screened consecutively with random-primed ^{32} P-labeled probes (Gibco-BRL kit) representing the PCR amplified fragments (probe #1, Figure 2.5; 2435 to 2888 [bp] in CAP-1, Figure 2.8 and then probe PTP2, 156 to 641 [bp] in PTP2, Figure 2.9). Prehybridization and hybridization was done in 50% formamide, 5x SSC, 2.5x Denhardt's solution (1% [wt/vol] Ficoll, 1% [wt/vol] polyvinylpyrrolidone, 1% bovine serum albumin [BSA; fraction V), 0.5% SDS, and 100 μ g/ml

denatured herring sperm DNA for 16-18 hours at 42°C. The probes were added at 43×10^6 cpm/20ml of hybridization buffer by soaking each filter in the solution and stacking the filters into a single bag. These filters (Hybond N) were washed in 0.1x SSC/0.1% SDS at 65°C for 2 x 30 minutes and positive plaques, identified as hybridizing spots on duplicate filters, were observed after exposure to Kodak XAR-5 x-ray film at -70°C for 24 to 48 hours. These positives were purified to a single phage-type using several rounds (secondary and tertiary) of screening with the corresponding SM buffer-eluted plugs acting as phage for the next step (Maniatis *et al*, 1982). A medium scale DNA phage preparation, generated from phage-infected Y1090 bacteria, was digested with EcoRI and the released insert was subsequently ligated into the multicloning site (MCS) of pTZ19R (EcoRI). These subcloned cDNAs were appraised by miniprep Southern blot (see Section 2.2-1) and sequencing (Section 2.2-5) analysis.

2.2-4 Generation and Screening of a Kidney cDNA Library

The P19-derived cDNA clones did not contain the complete coding sequence. Further cDNA clones were isolated by generating and screening a random-primed cDNA library prepared from mouse kidney. Random hexamers (100 ng; Pharmacia) were used to prime first strand synthesis with approximately 5 µg of Kidney-derived poly A⁺ mRNA. cDNA was prepared using the BRL Synthesis kit supplemented with 1 µCi [α^{32} P] dCTP and BRL superscript reverse transcriptase. The yield of the single- (tracer reaction product)

and double-stranded cDNA was determined using a cDNA synthesis protocol. Following heat (15 minutes at 65°C) and klenow (30 minutes at 37°C) treatment, the blunt-ended cDNA was cleaned by phenol/chloroform extraction and ethanol precipitation. The resuspended cDNA (in TNE; 10 mM Tris-HCl, pH 8.0; 100 mM NaCl ; 1 mM EDTA, pH 8.0) was further separated from the free [α^{32} P] dCTP, salts, and cDNA < 500 bp by fractionation through a 1 ml Sepharose 4B sizing column. An autoradiogram of the dried agarose gel containing aliquots of the various fractions as well as Cerenkov counting of the entire sample, identified the fractions to be used in subsequent steps. EcoRI/NotI linker/adaptors (Pharmacia) were ligated to a pooled fraction of flush-ended cDNA using T4 DNA ligase in ligase buffer (50 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 1mM DTT) and 1 μ l of an ATP solution from the "You Prime Synthesis Kit" (Pharmacia) in an overnight reaction at 16 °C. The cDNA was purified using standard procedures (GENECLEAN) to remove excess linker/adaptors which may interfere with the subsequent steps. The EcoRI-ended cDNA underwent phosphorylation by incubating with T4 polynucleotide kinase (Pharmacia) in 50 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 5mM DTT, and 1 mM ATP for 30 minutes at 37°C. The revised cDNA was purified (GENECLEAN) and ligated to dephosphorylated λ gt10 arms (Amersham) using the ligase, ligase buffer, and conditions given above for the linker/adaptor modification. The ligated λ gt10 DNA was packaged (Stratagene), plated, titered, and transferred to Hybond N filters as described in Section 2.2-3 and Maniatis *et al* (1982). Recombinant phage were selected for by plating with

maltose-induced C600 HflA150 in SM (20 mM Tris, pH 7.5; 100 mM NaCl; 8 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), in bacteria with the hfl (high frequency of lysogeny) mutation, the λ vector promotes lysogen formation unless the lambda cI repressor gene is disrupted by an insertion (of a cDNA).

Screening of about 500,000 recombinants using the hybridization and washing stringency shown above revealed several positives. Supplemental to the procedures outlined in Section 2.2-3, 5X Denhardt's solution was substituted for 2.5X in the hybridization buffer and 45×10^6 cpm of the heat-denatured 1.3 Kb P19 cDNA probe (probe #2; Figure 2.5; 2453 to 3776 in CAP-1, Figure 2.8) was added to 20 ml of this buffer. A subsequent round of screening employed a ^{32}P -labeled 275 bp PCR-derived probe (probe #3; Figure 2.5). This PCR amplification utilized the P19/Kidney 2.0 Kb cDNA (10 ng) as template, 20 pmoles of the #13 and #15 oligonucleotides (Appendix I) as primer, and was performed using Cetus kit components directed by the "Bum2" program ([94°C, 40 seconds; 50°C, 60 sec; 72°C, 120 sec] X 30 cycles; 70°C, 10 minutes). This PCR fragment (probe #3, Figure 2.5; 1848 to 2125 in CAP-1, Figure 2.8) was random-labeled and 30×10^6 cpm of the heat-denatured probe was added to 20 mls of hybridization buffer (see above). Ultimately, these filters were probed with 28×10^6 cpm/40 filters of a 145 bp PCR fragment (F25; 1195 to 1339 in CAP-1, Figure 2.8) amplified from the 12-1b phage clone utilizing the $\lambda\text{gt}10$ forward and #25 as primers. Both probes [#3 and F25] were excised from low melt agarose, melted, added to 1 volume of dH_2O ,

and 23 μ l of this DNA mixture was used in a standard random labeling reaction (Section 2.2-3). Once positive phage were purified to homogeneity, the EcoRI-digested or NotI/blunt-end cDNA clones were isolated, subcloned into pTZ19R at the EcoRI or SmaI site, respectively. These cDNAs were related to each other and to the P19 clones by restriction enzyme mapping and by sequencing.

2.2-5 DNA Sequencing

The nucleotide sequence of specific CAP-1 inserts were obtained using the dideoxy-chain termination procedure and kit provided by Pharmacia (Sanger *et al*, 1977). Single stranded DNA was obtained from the pTZ19R-PTPase clones using the M13K07 helper phage (Promega). Double-stranded DNA was prepared according to a technique provided in Biotechniques (Kraft *et al*, 1988). DNA sequencing was carried out using either the universal or reverse sequencing primers (Pharmacia). These primers bind to a complementary sequence found on either side of the pTZ19R/pTZ18R vector multicloning site and can initiate sequencing of opposing strands or sides (Figure 2.2). Full length cDNA sequence using these primers was obtained by the construction of nested deletions with the Exo III-Mung Bean deletion kit from Stratagene. The Exo III-Mung Bean deleted clones were related to the original clone by digestion with restriction enzymes that generate both simple (single site) or complicated (multi-site) patterns. Alternatively, synthetic oligonucleotides designed from previous sequence of the cDNA were used to verify or obtain new coding or noncoding strand

sequence. Sequencing gels were prepared, run, and manipulated according to standard protocol (Pharmacia Sequencing Booklet; Sambrook *et al*, 1989). The various sequence files were organized into overlapping "contigs" automatically using the SeqFit computer program (DNASTar). Final primary structure was obtained by sequencing both cDNA strands. Amino acid sequences were predicted (MapSeq) and compared with the protein sequence in the NBRF-PIR data bank using Proscan, while protein alignments were optimized using the DNASTar program of Needleman-Wunsch.

2.2-6 Synthesis and Preparation of Oligonucleotides

The oligonucleotides used in the initial PCR amplification experiments (Section 2.2-1) were prepared using cyanoethyl chemistry and full length products were purified on HPLC by Ms. N. Delcellier (Applied Biosystems). The majority of remaining oligonucleotides were synthesized by Mr. R. Marius on a 391 DNA Synthesizer (Applied Biosystems). The oligonucleotides were synthesized 3' to 5' on dC, dA, dT, or dG synthesis CPG columns; these columns were then rinsed and incubated with ammonium hydroxide overnight at 55°C. The deprotected oligonucleotides were purified on an OPC column (oligonucleotide purification cartridges; Applied Biosystems) and the eluted material was dried down in a speed vacuum apparatus. The primers were resuspended in dH₂O and the concentration was determined by absorbance spectrometry. These primers, summarized in Appendix I, were used in sequencing, PCR, and ligation reactions as outlined in the materials and methods

Sections.

2.2-7 Hybridization of PCR clones to P19 Stem Cell-Derived mRNA

Poly A⁺ selected mRNA (3.5 µg), prepared as reported in Howell *et al* (1991) was electrophoresed on a formaldehyde-containing agarose gel, and then transferred to Hybond N using 10X SSC and specifications given in Maniatis *et al* (1982). These uv (ultraviolet) crosslinked filters were hybridized via conditions described in Section 2.2-4 using about 1×10^6 cpm/ml of the 450 bp random-labeled PCR probes (#1, Figure 2.5 and PTP2, Figure 2.9). The two strips were initially washed twice in 2X SSC; 0.1% SDS at room temperature, exposed overnight to film, then washed with 0.2X SSC; 0.1% SDS at 42°C for 30 minutes, and finally subjected to a second round of exposure to Kodak XAR-5 x-ray film. The position of the 18 and 28S ribosomal RNA (rRNA) markers were visualized by acridine orange staining of total RNA (Howell *et al*, 1991).

2.2-8 Northern Blot Analysis of mRNA Derived From Mouse Tissues

mRNA was extracted and selected from various mouse tissues (Auffray and Rougneon, 1980; Maniatis *et al*, 1982); poly A⁺ mRNA was prepared from P19 cells that were aggregated according to the procedures outlined previously (McBurney *et al*, 1982; Rudnicki *et al*, 1987). Freshly dissected tissues were immediately placed into liquid nitrogen and stored at -70°C or manipulated by homogenizing (Polytron) in a LiCl/Urea solution. After SDS/Proteinase K treatment and multiple phenol/chloroform extractions the

deproteinized/ ethanol precipitated RNA was poly A⁺ selected as outlined in Maniatis with a few minor modifications; this mRNA was then slowly fractionated at 46 volts overnight on a 1% agarose gel containing formaldehyde (Maniatis *et al*, 1982 and references therein). The location of 28S and 18S rRNA could be visualized by ultraviolet illumination of the gel due to the addition of ethidium bromide to the RNA loading buffer. This electrophoretically separated RNA was transferred to nitrocellulose membranes (MSI) using standard procedures which included baking the membranes under vacuum for 2 hours at 80°C. Blots were prehybridized at high stringency in 50mM Tris-HCl, pH 7, 750 mM NaCl, 2 mM EDTA, 50% formamide, 10x Denhardt's, 0.5% SDS, and 100 µg/ml denatured salmon sperm DNA at 42°C for 16-18 hours. These tissue blots were hybridized with approximately 1×10^5 cpm/ml of ³²P-labeled cDNA (i.e. the 1.3 Kb P19 cDNA or probe #2; Figure 2.5) at 42°C under similar conditions and washed in 0.1x SSC; 0.1% SDS at 65°C for 20-30 minutes. The specific parameters of several Northern analyses are outlined in Table 2.1.

2.2-9 Genomic Southern Analysis

BALB/c mouse DNA was isolated using the modified procedure of Marmur (1962). The genomic DNA (25 µg) was digested to completion with restriction enzymes, slowly separated on a preparative 1% agarose gel by running overnight at 25-34 volts, denatured, and transferred to Hybond N (Maniatis *et al*, 1982). Hybridization of the uv crosslinked membrane was performed at high stringency

Table 2.1 Northern Analysis of mouse tissues and cell lines

This is a summary of the specific conditions and other parameters involved in the preparation and hybridization of Northern blots.

(+) Standard hybridization (hyb) buffer and washing stringency (wash) are as outlined in Section 2.2-8.

(*) These blots were washed at 42°C for 30 minutes.

Probe Type-probe #2 is delineated in Figure 2.5 and PTP2.5 is shown in Table 2.6; Figure 2.9, while the 5' CAP-1 probe represents 1-1805 bp of CAP-1 (Figure 2.8)

(**) The hybridization buffer and conditions are as given in Section 2.2-4.

Table 2.1

Figure #	Filter	Standard		Probe Type	cpm/ml	μ g mRNA/lane
		Hyb	Wash			
2.6	MSI	+	+	probe #2	10^5	10
2.10 A)	HybondN	+	*	PTP2.5	10^6	5
2.10 B)	HybondN	+	+	probe #2	10^6	5
2.11 A)	MSI	+	*	probe #2	10^6	10
2.11 B)	MSI	+	+	probe #2	10^6	4
2.12	HybondN	**	+	5' CAP-1	10^6	5

(Section 2.2-4) using the 1.3 Kb P19 (probe #2) described above. Filters were washed with 2X SSC; 0.1% SDS for 1-2 hours at room temperature and to a final stringency of 0.2x SSC; 0.1% SDS at 65°C for 15 minutes.

2.3 Results

2.3.1 Amplification of Two Novel PTPase-Encoding PCR Fragments

A Polymerase Chain Reaction (PCR) strategy was used to amplify novel sequences residing between two degenerate PTPase-specific primers using P19 cell-derived cDNA as template (Figure 2.2). The primers were designed to create a HindIII and KpnI site at the 5' and 3' end, respectively (Figure 2.1). Attempts made to subclone the KpnI/HindIII digested 450 bp fragment(s) into the corresponding site of pTZ19R, were not successful. Blunt-ended ligation of the modified PCR fragment(s) into the SmaI site of pTZ19R generated many colonies. Approximately 1/3 of these KpnI/HindIII digested clones were initially identified as positive by separation on ethidium bromide stained agarose gels. Roughly 65% of these insert positive clones were assigned to Class I by analysis on Southern blots using the arbitrarily chosen PCR/pTZ19R clone- 2.1 (analogous to probe #1; Figure 2.5). When these blots were stripped and reprobed with a ³²P-labeled plasmid not detected in the previous round of screening (2-64; same insert as probe PTP2; Figure 2.9), a second Class was identified; the third cycle revealed members of Class III (Table 2.2).

Table 2.2 Sorting PCR/pTZ19R Clones by Southern Blot Analysis

Class	Probe	Positive PCR/pTZ19R Clones
I	2-1	2-6, 2-7, 2-20, 2-22, 2-23, 2-28, 2-33, 2-40, 2-44, 2-48, 2-50, 2-62, 2-70, 2-71, 2-72, 2-76, 2-85
II	2-64	1-13
III	2-19	2-14, 2-25, 2-43, 2-45, 2-52, 2-56, 2-57

Positive PCR/pTZ19R clones were categorized into three separate classes by several rounds of Southern miniprep analysis (Table 2.2). These data were substantiated by the identification of representative Class I (2-1); Class II (2-64); and Class III (2-19) nucleotide sequence. The Partial sequence of 2.1-like: 2-6, 2-7, 2-28, 2-71, and 2-76 was also obtained along with that of the class II family member, 1-13. The predicted amino acid sequence of these Class I and II PCR fragments revealed sufficient identity with the CD45/ TCPTP/ PTP1B-based PTPase consensus to indicate that a region from two unique PTPases had been cloned (Figure 2.3; Charbonneau et al, 1989; Cool et al, 1989). The deduced protein sequence of Class III, however, was identical to c-fer, which is a P19 cell-expressed PTK recently cloned in our laboratory and by others (B. Howell and J.C. Bell, unpublished data; Hao et al, 1989). These 2-19-like PCR fragments appear to have been amplified by virtue of non-specific primer binding. PTPase-encoding PCR fragments representative of Class I and II were isolated by KpnI/HindIII digestion and used as probes to clone relevant cDNAs and to characterize the expression of these unique PTPases.

Figure 2.3 Alignment of deduced Class I and II PCR sequence with a PTPase consensus. The partial sequence of Class I and II PCR fragments was deduced and aligned to a consensus derived from the intracellular PTPases-PTP1B and TCPTP (Charbonneau *et al*, 1989; Cool *et al*, 1989). Sequences were compared for optimal alignment and gaps are indicated by a dashed line; boxed residues delineate areas of identity.

CLASS I	YINAS	YVN	MEM	PAAN	LVNK	YI	AT	QG	PL	PN	T	CAQ	FW	QV	VW
CLASS II	INAS	FMD	GS	NEK	- - - -	NR	YI	GT	QG	PL	EN	YRD	FW	LM	VW
PTPase CONSENSUS	YINAS						YI		QG	PL	T		FW		VW

2.3-2. Class I and II are Expressed in P19 Stem Cells

Class I and II PCR-amplified fragments were used to probe for PTPase-encoding transcripts in P19 stem cells. Northern analysis identified undifferentiated P19 cell expressed transcripts of 5 and 7 Kb which hybridize to Class I (probe #1; Figure 2.5), while the Class II probe (probe PTP2; Figure 2.9) generated bands at 5 Kb as well as another light band at about 8 Kb (Figure 2.4). This preliminary Northern data was necessary to ensure success in subsequent experiments which include screening a P19 stem cell-derived cDNA library for Class I and Class II clones.

2.3-3 Cloning and Characterization of Class I P19 cDNAs

A P19 stem cell cDNA library was generated and screened to identify PTPase-specific cDNAs. Several insert/P19 cDNA to vector/ λ gt11 ratios were used in ligation reactions and as indicated in Table 2.3, a relatively high titer of 1.15×10^6 pfu/ml was observed upon ligation of 50 ng of the P19 cDNA to λ gt11 arms. In addition, this library (B) was shown to have a high cloning efficiency; in fact, the percentage of recombinant phage were calculated to be 96.5% (Table 2.3). Therefore, this P19 cDNA library (B) was chosen to screen for PTPase-encoding cDNAs.

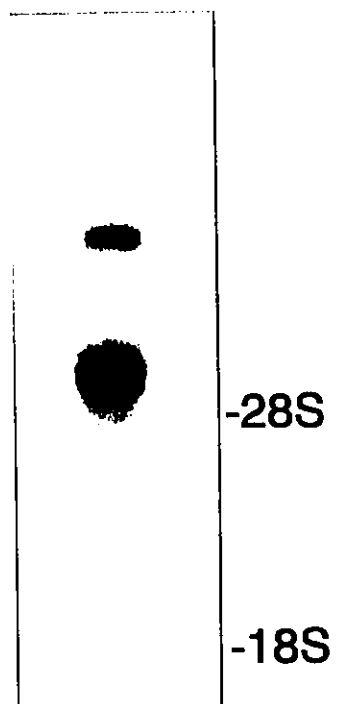
Table 2.3 Titer and Cloning Efficiency of P19 cDNA

P19 cDNA(amt)	Dilutions	# Plaques	Titer*	% Recombinants
A) 20 ng	$10^{-3}/10^{-4}/10^{-5}$	275/30/4	3×10^5	94%
B) 50 ng	$10^{-3}/10^{-4}/10^{-5}$	TMTC/115/10	1.15×10^6	96.5%

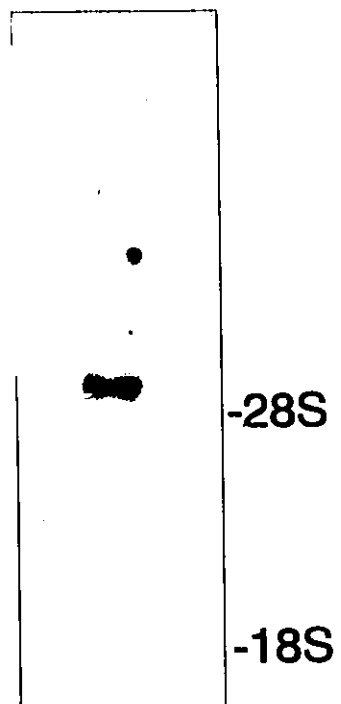
TMTC, too many to count; [*] pfu/ml, plaque forming units per ml

Figure 2.4 Northern analysis: Class I and II PCR fragments hybridize to P19 stem cell-selected mRNA. PCR-amplified probes were used to detect expression levels of Class I and II PTPases. Approximately 3.5 μ g of P19-derived poly A⁺ mRNA were loaded per lane. Transcript sizes were estimated by comparison with the position of 28S (4712 bp) and 18S rRNAs.

CLASS I



CLASS II



Initial screening of the oligo (dT)-primed P19 cDNA library (B) with a class I PCR-amplified insert (probe #1; Figure 2.5) generated 6 true positives (Table 2.4). Four of the Class I positives were isolated and subcloned into pTZ19R. The 5' end sequence of 1.3 Kb cDNA (in plasmid 4-1), revealed virtual identity to the Class I PCR insert (Figure 2.5; Table 2.4). This sequence was also found to overlap with that of another cDNA clone (plasmid 2-4; Table 2.4). Consequent investigation of the largest cDNA clones revealed that the P19 2.0 Kb cDNA sequence, in plasmid 5-4, predicts a single PTPase domain in addition to in frame coding sequence upstream, whereas the 1.3 Kb clone (4-1) encodes only a portion of the putative catalytic domain (Figure 2.5; Table 2.4). The Northern expression analysis described above, using the Class I PCR fragment as probe, revealed hybridizing transcripts of greater size than that of the largest P19-derived cDNAs (Figure 2.4). These data directed subsequent efforts toward obtaining full length coding sequence for this clone.

2.3-4 Expression of Class I (CAP-1) in mRNA in Mouse Tissues

Class I, or CAP-1, expression was examined at the RNA level to determine the abundance and presence of alternative transcripts in specific adult mouse tissues. Northern tissue blot analysis revealed a host of transcripts that hybridize to the 1.3 Kb P19 cDNA (probe #2; Figure 2.5) at high stringency (Figure 2.6). Two major transcripts, with sizes of 5 and 7 Kb were identified in brain, heart, lungs, liver, and kidney, while putative minor bands

Table 2.4 Summary of P19-derived Class I or CAP-1 cDNAs. Isolation of Class I positive phage cDNA was followed by determination of insert size (bp). EcoRI-digested inserts were verified by Southern miniprep analysis using probe #2 (Figure 2.5). The hybridizing inserts are denoted by (+), with the exception of phage 193, which was not released by EcoRI (*). These inserts were subcloned into pTZ19R in both the sense (+) and antisense (-) orientation. ND, represents not determined. The 5' border of the sequenced cDNA is indicated as position of nucleotide sequence (bp) in CAP-1 (Figure 2.8).

Table 2.4

Phage #	Insert Size	Probe #2	Plasmids	CAP-1
				5' Sequence
192	1125	+	2-4 (+) 2-9 (-)	2579
193	ND	*	ND	ND
194	1323	+	4-1 (+) 4-10 (-)	2453
195	1990	+	5-4 (+)	1787
197	2700	+	7-2 (+)	2463
198	900	ND	ND	ND

Figure 2.5 Schematic diagram illustrating the structure of the composite CAP-1 cDNA and the relatedness of four cDNA clones. The thin lines represent cDNA sequence whereas the open boxes denote protein structure. The probes depicted as narrow boxes were designed for screening cDNA libraries and for hybridizing to poly A⁺ mRNA and genomic DNA. These probes represent the following sequences in CAP-1: probe #1- 2435 to 2888; probe #2- 2453 to 3776; probe #3- 1848 to 2125 (Figure 2.8). Sequence of all four clones were combined to generate the composite cDNA. The 5' and 3' UTR indicates the position of untranslated regions containing in frame stop codons.

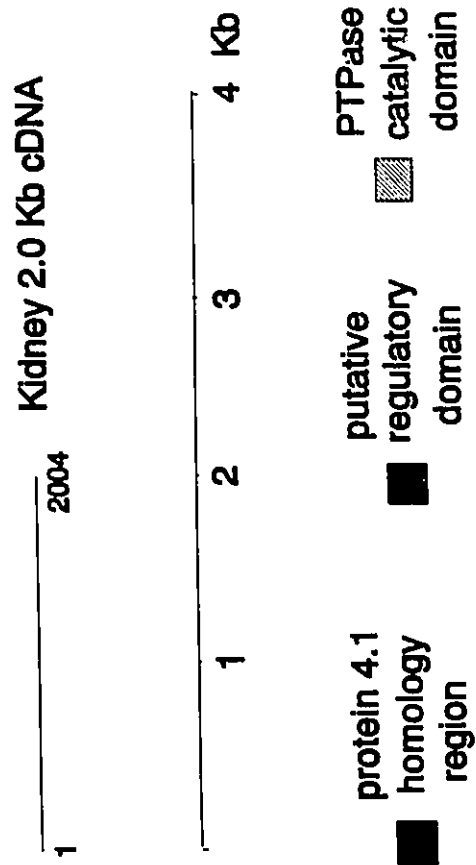
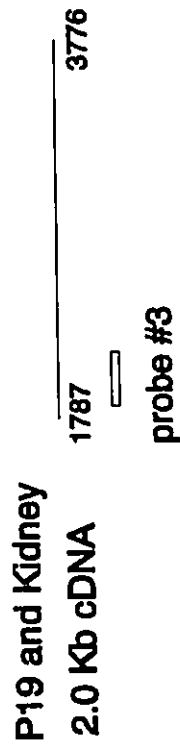
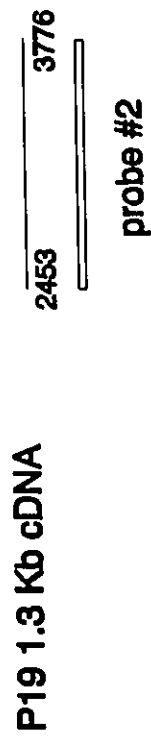
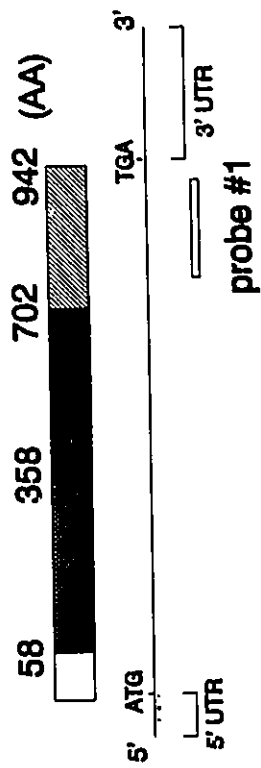


Figure 2.6 Northern blot analysis of CAP-1 mRNA in mouse tissues. Each lane contains 10 μ g of poly A⁺ RNA isolated from adult mouse tissues which are represented as: B, brain; H, heart; Lu, lungs; Li, liver; K, kidney; and M, leg muscle. The resulting tissue blot was probed with a partial CAP-1 cDNA (probe #2; Figure 2.5). The position of the 28s and 18s rRNA (see arrows) are determined by ultraviolet visualization of ethidium bromide staining of the gel.

B H Lu Li K M

28S ➤

18S ➤



are seen in brain and heart. These two constitutively expressed transcripts are also detected in undifferentiated P19 cells (Figure 2.4). In adult leg muscle, however, the constitutively expressed transcripts appear as two reproducibly smaller mRNAs (Figure 2.6). The smallest transcript in most tissues is about 5 Kb, 4.5 Kb in leg muscle, indicating the existence of additional CAP-1 sequence.

2.3-5 Isolation of Kidney cDNA Clones Containing 5' Sequence

A random-primed cDNA library was constructed to acquire the complete coding sequence for the Class I PTPase. This library was generated from mouse kidney-derived poly A⁺ mRNA since this appeared to be a rich source of CAP-1 mRNA (Figure 2.6). This cDNA, prepared as described in Section 2.2-4, was subjected to fractionation on a sizing column. An autoradiograph of the dried agarose gel containing aliquots of these fractions determined the cDNA to be pooled (fractions 10-18) and used in subsequent steps (Figure 2.7). The titer of one of the subsequently generated libraries, K1 was identified as 1.4×10^6 pfu/ml; therefore this library was used in subsequent screening strategies (Table 2.5).

Table 2.5 Titration of Kidney-Derived cDNA\lambdagt10 Libraries

Amt of cDNA	Dilution	#Plaques	Titer(pfu/ml)	Total Titer
K1) 15ng	10^4	306		
	10^5	28*	2.8×10^6	1.4×10^6
K2) 6ng	10^4	133		
	10^5	7*	7.0×10^5	3.5×10^5

* These calculations were based on duplicate results

Figure 2.7 An autoradiogram of fractionated second strand kidney-derived cDNA. Each lane (4-28) represents aliquots of individual fractions that were collected from the Sepharose 4B sizing column. The ethidium stained BRL 1.0 Kb ladder, was used as a marker (M) for size determination. The marks indicated here represent the position of approximately 7.0, 6.0, 5.0, 4.0, 3.0, 2.0, 1.6, 1.0, and 0.5 Kb. While, T, indicates the total input cDNA loaded on the Sepharose 4B gravity column.

T 4 5 6 7 8 9 10 11 12 13 14 15 M 16 17 18 19 20 21 22 23 24 25 26 27 28

To obtain complete coding sequence for this enzyme, 500,000 recombinants from the K1 library were subjected to several rounds of screening. Initially, the 1.3 Kb cDNA (probe #2; Figure 2.5) was employed to identify cDNA sequence similar to that isolated from the P19 cDNA library. All 8 positives contain inserts which hybridize to probe #2 (Figure 2.5); a notable exception is the 9-1 phage whose insert is not liberated by EcoRI nor by NotI digestion. In addition, only one of the two EcoRI fragments of 5-2 and 19-1 is detected by this P19 cDNA probe (Table 2.6).

Analysis of 52-T7 and 53-1 reveals cDNAs with nucleotide sequences very similar to those of the P19 cDNAs. These clones all have a putative termination codon in the same position (3093 in CAP-1; Figure 2.8). Interestingly, the 3' borders of all four cDNA clones are essentially equivalent; this sequence does not harbor a poly A tail, however, it does contain a repeated sequence which predicts a potentially complex mRNA structure. This $(CCCT)_n$ repeat, where $n=12$ for P19 clones and $n=9$ for kidney cDNAs, in addition to three single nucleotide substitutions are the only differences observed between these two sets of cDNAs.

Several of these kidney cDNAs have unusual features which include a 990 bp extension at the 5' end of 5-1 (51-4) with a NotI site at the junction sequence. Similarly, when the PTPase-encoding 2000 bp EcoRI fragment of the 5-2 phage (52-T7; Table 2.6) is used as a probe, it generates a Class I Northern expression pattern, whereas the 1600 cDNA insert (52-B2; Table 2.6) does not; instead, this cDNA hybridizes to many transcripts present in several mouse

Table 2.6 A compendium of Class I (CAP-1) Kidney cDNA clones. Round I positive phage clones were analysed for size estimation (bp) and hybridization to probe #2 (Figure 2.5). Sequence of the subcloned inserts (+/-; sense/antisense) were determined; 5' and 3' borders are represented as nucleotide position in CAP-1 (Figure 2.8). Second and third round positives were characterized in a similar manner with the following exceptions. The 12-1b phage insert was liberated with NotI and cDNA inserts were validated by hybridizing with the round II (probe #3; Figure 2.5) and round III (F25; Section 2.2-4) probes. The presence of nonCAP-1 sequence in the kidney cDNAs has been noted.

Table 2.6

Phage # Round I	Insert size		Plasmids	CAP-1 Sequence		nonCAP-1
				5' Border	3' Border	
2-1	600	+	ND	ND	ND	yes
5-1	2700	+	51-4 (+) 51-5 (-)	2350	ND	
5-2	2000	+	52-T7	1787	3776	yes
	1600	-	52-B2	-	-	
5-3	1800	+	53-1 (?)	1979	3776	
9-1	ND		ND	ND	ND	
12-1	500	+	ND	ND	ND	
15-1	750	+	ND	ND	ND	
19-1	900	-	ND	ND	ND	
	700	+	ND	ND	ND	
Round II						
12-1b	900	+	ND	1194	2010	
20-2	1000	+	20-2 (+) 20-4 (-)	1461	1998	yes
Round III						
11	2000	+	111-14 (+) 111-8 (-)	1	2004	
41	1600	?	T41-2 (+)	528	ND	
81	900	+	811-1 (+) 811-3 (-)	ND	1790	
181	750	?	181-3 (-) 181-4 (+)	1149	1886	

tissues (data not shown). The pattern is indicative of the presence of repetitive sequences, which are found in untranslated and intronic regions.

Kidney cDNA clones identified in the second and third round of screening were all 5' extensions of the P19 and round I Kidney clones (Table 2.6). The PCR-amplified probe #3 (Figure 2.5), representing the 5' most cDNA sequence of 5-4 or 52-T7 (Tables 2.4 and 2.6), revealed two definite positives: 12-1b and 20-2. However, the positive observed on plate #1 was very light and difficult to isolate. A PCR generated probe (F25: 1195 to 1339 in CAP-1; Figure 2.8) amplified from round II phage DNA, clearly recognized this (referred to as 11) as well as several other positives (Table 2.6).

2.3-6 Structure and Analysis of Composite Class I or CAP-1 cDNA

Complete coding sequence was obtained by combining the 2.0 Kb/5'-extended kidney cDNA (phage #11) with the overlapping 2.0 Kb P19 clone (Figure 2.5). The composite CAP-1 cDNA predicts a continuous open reading frame of 942 amino acids (Figure 2.8). The putative initiating methionine, at position 267, with sequence close to that of the Kozak consensus [(A/G)CCAUG(G); Kozak 1986] is preceded by four in frame stop codons, while the termination codon is located at 3093 (Figure 2.8). The CAP-1 protein deduced from this cDNA has a predicted M_r of about 107,000.

Comparison of the deduced CAP-1 protein sequence with that of the human PTPH1 reveals 91.6% shared amino acid identity starting at a methionine residue located 30 amino acids downstream from the

Figure 2.8 The nucleotide and deduced amino acid sequence of CAP-1. The N-terminal domain homologous to band 4.1 family members is enclosed in a box by a solid line. The putative catalytic domain of CAP-1 is denoted by an open box (dotted lines) with the arrows representing the PCR primer region. The region between the N-terminal and C-terminal region represents a putative regulatory domain. Deduced amino acid residues are denoted as single letters above the nucleotide sequence.

[illegible]

predicted initial methionine (Yang and Tonks, 1991). The composite cDNA encodes a putative PTPase domain in its C-terminal 702-929 amino acids; this phosphatase domain of CAP-1 has 33-41% identity to core catalytic domains found within PTPases having transmembrane and intracellular structure (Charbonneau *et al*, 1989; Cool *et al*, 1989; Krueger *et al*, 1990; Ralph *et al*, 1987; Thomas *et al*, 1985; 1987; Streuli *et al*, 1988; 1989; Table 2.7). The N-terminal portion of this PTPase (residues 58-358 in CAP-1; Figure 2.8) was shown by Proscan analysis to share marked similarity with the cytoskeleton/membrane localized proteins: band 4.1, ezrin, radixin, moesin, and talin (Conboy *et al*, 1986; Funayama *et al*, 1991; Gould *et al*, 1989; Gu *et al*, 1991; Lankes and Furthmayr, 1991; Rees *et al*, 1990; Turunen *et al*, 1989; Yang and Tonks, 1991; Figure 2.14). This PTPase is, therefore called CAP-1 for Cytoskeletal Associating Protein related PTPase. The potential relevance of this homology will be discussed later on.

2.3-7 Isolation of a Partial Class II (PTP2) cDNA

Several positives were obtained upon screening of the P19 cDNA library with the Class II PCR fragment as probe (2-64; Table 2.2 or PTP2; 156 to 641, Figure 2.9). One of these PTP2 phage inserts were isolated and ligated into pTZ19R to generate the plasmids, 8-1 (+) or 8-2 (-). Nested or ExoIII/Mung Bean deletions were constructed in this 2.5 Kb cDNA. Partial sequence analysis of the 8-1 insert, as outlined in Figure 2.9, would appear to predict a continuous open reading frame from the 5' border through the putative PTPase

Figure 2.9 Partial nucleotide and predicted amino acid sequence of a Class II cDNA. The putative PTPase domain is found within parenthesis [] with PCR primers denoted by underlined sequence and by arrows. This is the partial sequence of a PTP2 cDNA and represents only a portion of probe PTP2.5. Predicted amino acid residues are delineated as single letters above the nucleotide sequence.

E	Y	E	D	I	R	R	E	N	P	V	G	T	F	v14
cggag	tat	gaa	gac	att	cgt	cgt	gag	aac	cct	ggt	ggc	act	ttc	44
H	C	S	M	S	P	G	N	L	E	K	I	N	R	v28
cac	tgt	tcc	atg	tct	cca	gga	aac	cta	gaa	aaa	aac	aga	tat	86
G	D	V	P	C	L	D	Q	T	R	V	K	L	T	v42
gga	gat	gta	ccc	tgc	cta	gac	caa	acc	cga	gtg	aag	ctg	aca	128
K	R	S	G	H	T	Q	T	D	Y	I	N	A	S	v56
aaa	cgg	agt	ggc	cac	act	cag	aca	gat	<u>tac</u>	<u>atc</u>	<u>aat</u>	<u>gcc</u>	<u>agt</u>	170
F	M	D	G	Y	K	Q	K	N	A	Y	I	G	T	v70
ttc	atg	gat	ggt	tac	aag	cag	aag	aat	gct	tat	att	ggt	aca	212
Q	G	P	L	E	N	T	Y	R	D	F	W	L	M	v84
caa	ggt	cct	ttg	gag	aac	acc	tat	cgt	gac	ttc	tgg	ctt	atg	254
V	W	E	Q	K	V	L	V	I	V	M	T	T	R	v98
ggt	tgg	gag	caa	aaa	ggt	ttg	gtg	att	gtc	atg	act	acc	cgc	296
F	E	E	G	G	R	R	K	C	G	Q	Y	W	P	v112
ttt	gag	gaa	ggc	ggc	agg	aga	aaa	tgt	ggc	cag	tac	tgg	cct	338
L	E	K	D	S	R	I	R	F	G	F	L	T	V	v126
tta	gaa	aaa	gac	tcc	cgg	atc	cga	ttt	ggc	ttc	ctc	acg	gtg	380
T	N	L	G	V	E	N	M	N	H	Y	K	K	T	v140
act	aat	cta	gga	gtg	gaa	aac	atg	aac	cat	tat	aag	aaa	aca	422
T	L	E	I	H	N	T	E	E	R	E	K	R	Q	v154
act	cta	gaa	att	cac	aac	aca	gag	gag	cgg	gag	aaa	cgc	cag	464
V	T	H	F	Q	F	L	S	W	P	D	Y	G	V	v168
ggt	acc	cac	ttc	cag	ttc	cta	agc	tgg	cca	gat	tat	ggt	gtc	506
P	S	S	A	A	S	L	I	D	F	L	R	V	V	v182
cct	tcc	tca	gct	gct	tcc	ctc	att	gac	ttc	ctg	aga	gtg	gtc	548
R	N	Q	Q	S	M	A	V	G	N	L	G	A	R	v196
aga	aac	cag	cag	agt	atg	gct	gta	ggc	aac	ctg	gga	gct	cgc	590
S	K	G	Q	C	P	E	P	P	I	V	V	H	C	v210
tcc	aaa	ggg	cag	tgc	cct	gag	cca	ccc	att	gtg	<u>gtc</u>	<u>cac</u>	<u>tgc</u>	632
S	A	G	I	G	R	T	G	T	F	C	S	L	D	v224
<u>agt</u>	<u>gct</u>	<u>ggc</u>	atc	ggc	agg	aca	ggt	acc	ttc	tgc	tca	ctg	gac	674
I	C	L	A	Q	L	E	E	L	G	T	L	N	V	v238
atc	tgc	ctg	gca	cag	ctg	gag	gag	ctt	ggc	acc	ctt	aat	gtg	716
F	Q	T	V	S	R	M	R	T	Q	R	A	F	S	v252
ttc	cag	acg	gtg	tca	cgc	atg	agg	acc	caa	agg	gcc	ttc	agt	758
I	Q	T	P	E	Q	Y	Y	F	C	Y	K	A	I	v266
atc	cag	acc	cct	gag	cag	tac	tac	ttt	tgc	tac	aag	gca	att	800
L	J	E	F	A	E	R	E	G	M	V	P	S	G	v280
ctg	gag	ttt	gca	gag	agg	gaa	ggc	atg	gtt	ccc	tct	ggt	cac	842
S	L	L	A	M	D	G	Q	.						v288
agc	ctg	ttg	gcc	atg	gac	ggt	caa	taa						869

domain.

In an effort to clone further upstream sequences, the random-primed kidney cDNA library was probed with this 2.5 Kb insert (PTP2-5) and 6 positives were realized. The 5' and 3' borders of these cDNAs appear to lie within 2.5 Kb (not shown). The smallest class II hybridizing transcript is about 5 Kb (Figure 2.4) indicating the existence of additional cDNA sequence. Subsequent comparison of translated PTP2 sequence by DNASTAR revealed virtual identity with the putative retinaldehyde-binding, human PTPase, MEG2 (Gu *et al.*, 1992). Further studies should focus on the isolation of the full length coding sequence, estimated from MEG2 sequence, to be another 930 nucleotides.

2.3-8 Expression of Class II mRNA in Mouse Tissues and Cell Lines

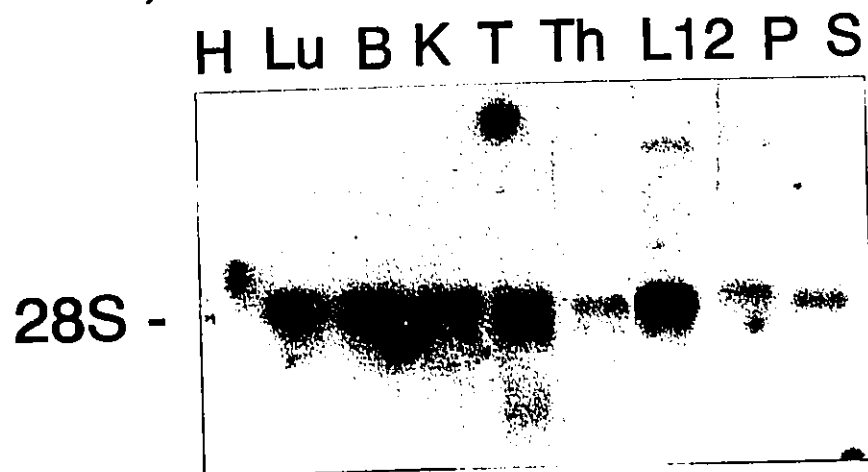
Several mouse tissues and cell lines were found to express a Class II-specific transcript of 5 Kb (Figure 2.10A). The expression pattern observed in mouse testis was a little more complex, with the 2.5 Kb cDNA probe recognizing the 5 Kb as well as a 4.5 Kb form. This tissue may serve as a good source from which to clone further PTP2 cDNAs.

2.3-9 CAP-1 is Expressed in a Range of Tissues and Cell Lines

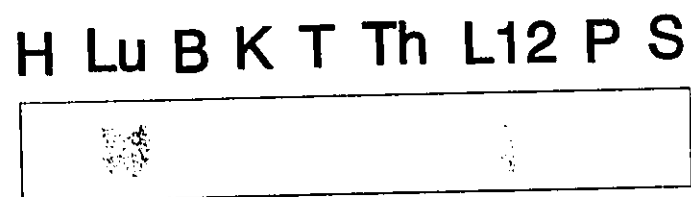
Class I (CAP-1) probes were used to reaffirm and extend the expression information exhibited previously in Figures 2.4 and 2.6. Hybridization of the Northern blot used above (Figure 2.10A) to the CAP-1 probe #2, revealed a putative multi-transcript expression

Figure 2.10 Expression of the Class I and II PTPase mRNA in several mouse tissues and cell lines. A) The PTP2.5 probe was employed to identify Class II or PTP2-hybridizing transcripts in mouse tissues such as: H, heart, Lu, lung; B, brain; K, kidney; T, testis; Th, thymus and in the cell lines P, P388; S, SP10; and L12, L1210. B) This Northern blot was standardized with a β_2 -microglobulin probe. C) The Northern blot, used above, was reprobed with a partial CAP-1 probe (probe #2; Figure 2.5). The 28S RNA marker represents 4712 bp.

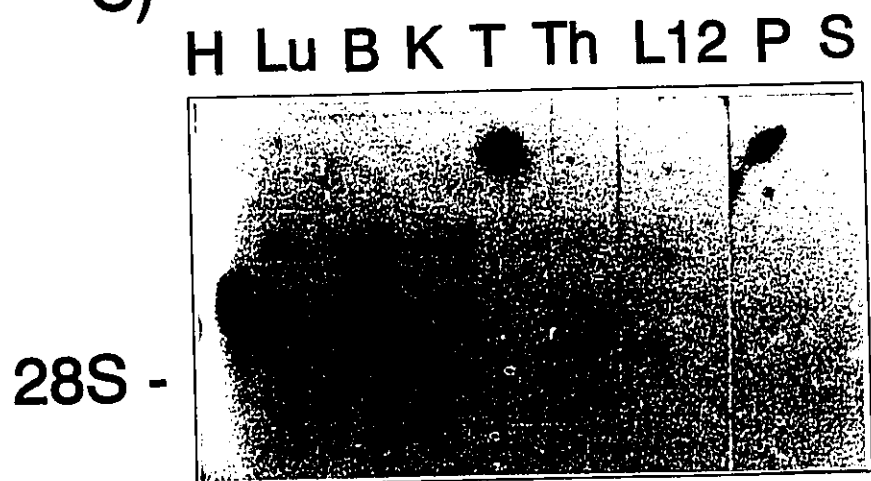
A)



B)



C)



pattern in heart and brain, while Class I-like transcripts of 5 and 7 Kb were observed in kidney and lung (Figure 2.10C). This CAP-1 probe barely detected thymus-derived mRNAs in this Northern analysis (Figure 2.10C), however, the standard Class I pattern was identified when higher levels of thymus mRNA were used (Figure 2.11A). CAP-hybridizing mRNAs were also non detectable in all cell lines examined, with the exception of the L1210 cell line which has two smaller mRNAs (Figure 2.10C); these transcripts differ in size by about 0.5 Kb which is similar to the pattern found in leg muscle (Figure 2.11B). When the β_2 -microglobulin standardization (Figure 2.10B) was considered, mouse brain, kidney, and heart were found to express relatively high levels of CAP-1, while lung and thymus contained much less (Figure 2.10C). Similarly, screening a 5' CAP-1 probe-hybridized (1 to 1805; Figure 2.8) tissue blot with β -tubulin, reaffirmed the lower level of expression in liver (previously seen in Figure 2.6) relative to the higher levels of CAP-1 transcripts observed in mouse kidney and brain (Figure 2.12).

The CAP-1 PTPase was originally amplified and cloned from the embryonal carcinoma cell line, P19. A Class I-like pattern of expression was previously observed in the P19 stem cell (Figure 2.4). Preliminary evidence indicate that differentiation of P19 cells with dimethyl sulfoxide (DMSO) or retinoic acid (RA) does not change CAP-1 expression qualitatively, while the transcripts levels are decreased slightly in the DMSO-induced cells and to a considerable degree in RA-differentiated cells once the results are corrected for loading (Figure 2.12). Poly A⁺ mRNA was selected from

Figure 2.11 Expression of CAP-1 transcripts in adult and embryonic mouse tissues. A) Poly A⁺ mRNA (10 µg) was selected from day 14 whole mouse embryo (E) and from adult mouse tissues: K, kidney; Li, liver; and Th, thymus. B) Approximately 4 µg of poly A⁺ mRNA from B, brain; K, kidney; and M, leg muscle were loaded per lane. These tissue blots were probed with a partial CAP-1 cDNA (probe #2; Figure 2.5). Ethidium bromide stained 28S and 18S (ribosomal) rRNAs served as molecular weight markers.

A)
K Li E Th

B)
B K M

28S -

18S -

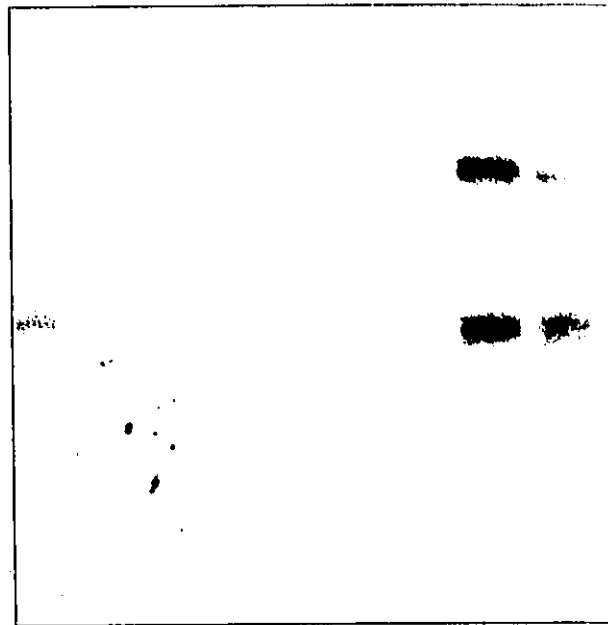
28S -



Figure 2.12 Expression of CAP-1 mRNA in adult mouse tissues and in differentiated embryonal carcinoma cells. The predicted non-catalytic or 5' region of CAP-1 was used to probe CAP expression during P19 cell differentiation and in the adult mouse. A) Poly A⁺ mRNA was selected from DMSO-treated EC cells at day 1 (1); day 2 (2); and day 7 (7), while day 6 (6) RA-differentiated P19 cells were also analyzed. In addition, whole mouse embryo, day 14 of gestation (E) and adult mouse tissues: (Li) Liver; (K) Kidney; and (B) Brain were examined. B) RNA loading was standardized with β -tubulin hybridization.

A

DMSO			RA	TISSUES			
1	2	7	6	E	Li	K	B



B



day 0 or P19 stem cells and run on the same gel; CAP-1 expression is similar to that observed in the day 1, DMSO-treated lane (data not shown; Figure 2.12). These Class I-specific CAP-1 transcripts are also found in early mouse development, namely at day 14 of gestation; however, the high levels observed in Figure 2.11A appears to result from overloading (see Figure 2.12).

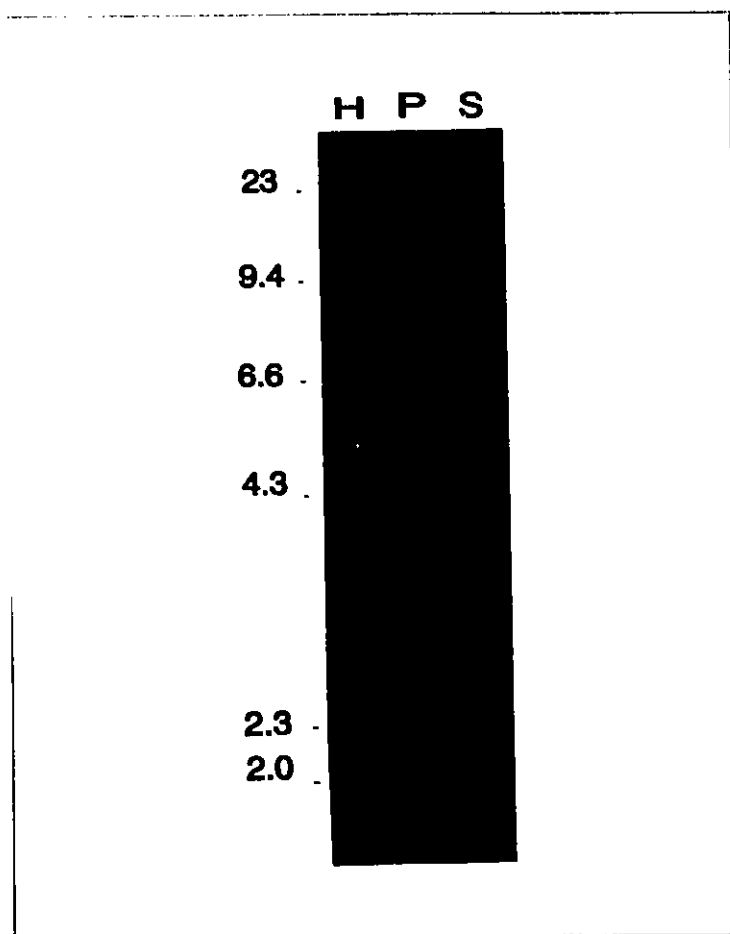
2.3.10 Southern Analysis of the CAP-1 Gene

These rather complex Northern results raise the issue of whether the CAP-1 probes may be recognizing transcripts from a different, but related gene. Hybridization with the 1.3 Kb partial cDNA (i.e. probe #2, Figure 2.5) under similar high stringency conditions generated a simple pattern with SacI-digested genomic DNA, while the HindIII and PstI lanes have a single reacting band in each (Figure 2.13). This genomic Southern result is consistent with that of multiple transcripts arising from a single CAP-1 gene.

2.4 Discussion

Many new PTPases have been identified in the past few years; this may now illuminate signal transduction pathways by shedding light on the structure and function of the relatively unknown dephosphorylating enzymes. This study reports the use of PCR amplification to identify and clone two unique PTPase cDNAs from a embryonal carcinoma (P19)-derived cDNA library. The degenerate primers used in this amplification reaction, were designed based on two short stretches or conserved motifs found in several human

Figure 2.13 Southern blot analysis of the CAP-1 gene. Mouse genomic DNA was digested to completion with HindIII (H), PstI (P) and SacI (S). Size of the molecular weight markers (Kb), lambda DNA digested with HindIII, are provided.



PTPases, such as: PTP1B, TCPTP, CD45, and LAR (Charbonneau *et al.*, 1989; Cool *et al.*, 1989; Ralph *et al.*, 1987; Streuli *et al.*, 1988). This has probably restricted the outcome somewhat, since with the isolation of further PTPases, Y(I/V)NA(S/N), instead of YINAS, is seen to be conserved among many intracellular and receptor-like PTPases; the YINAS motif is not present at all in others (Lambroso *et al.*, 1991; Krueger *et al.*, 1990; Streuli *et al.*, 1989; Zanke *et al.*, 1992; Keyse and Emslie, 1992). The catalytic site of PTPases is generally represented by the HCSAG sequence in most PTPases. However, this sequence is not as tightly conserved in several distantly related subgroups, such as the dual-specificity vaccinia virus, VH1, phosphatase which harbors the motif, HCAAG, or the cell-cycle cdc25 enzymes with the HCXXG consensus (Guan *et al.*, 1991a; Moreno and Nurse, 1991).

These degenerate oligonucleotides were designed by the analysis of mammalian codon frequency charts (Grantham *et al.*, 1981) or by the comparison of rat, mouse, and human CD45 codon usage to that observed in TCPTP (Cool *et al.*, 1989; Ralph *et al.*, 1987; Thomas *et al.*, 1985; 1987). The low level of degeneracy (Figure 2.1) along with the PCR conditions (i.e. annealing temperature- 50°C) were probably most influential in limiting the gamut of possible amplified PTPase regions to a smaller field of two novel Class I (CAP-1) and Class II (PTP2) clones.

The CAP-1 phosphatase was identified by virtue of its homology to conserved regions found in several PTPases (Charbonneau *et al.*, 1989; Cool *et al.*, 1989). Consistent with this, the putative PTPase

domain of CAP-1 is 33-41% identical to the catalytic domains found within PTPases having transmembrane and intracellular structure (Table 2.7). Comparable levels of catalytic domain identity are shared between most PTPase groups (36-46%; Krueger *et al*, 1990). However similarity within the same subgroup is much higher; PTP1B and TCPTP are highly homologous to each other, having 71% identity within the catalytic domain (Charbonneau *et al*, 1989; Cool *et al*, 1989). The PTPase-specific 702-929 amino acid region of CAP-1 has 64% identity with the catalytic domain of the related PTPase, MEG1 (Table 2.7; Gu *et al*, 1991). This level of similarity is indicative of a shared general function for members of this new subgroup of PTPases.

CAP-1 is the murine homologue of a human PTPase, PTPH1 (Yang and Tonks, 1991). The alignments generated by comparison of CAP-1 with homologous regions in various cytoskeletal-associated proteins are similar to those displayed by PTPH1 (Yang and Tonks, 1991). Residues 58-358 of CAP-1 share 33-36% identity with the N-terminal domain of the cytoskeletal membrane protein 4.1 isoforms and 29% identity with the human ezrin domain. In addition, the N-terminal portion of CAP-1 shares 55% identity within the protein 4.1 homology domain of the related human PTPase, MEG1 (Gu *et al*, 1991). Comparison of the N-terminal region of CAP-1 (58-358) and MEG1 (29-329) with the homologous domain in protein 4.1 (1-296) reveals a shared consensus (Figure 2.14). This identifies CAP-1/PTPH1 and MEG1 as members of a new family of cytoskeletal associated protein-like or CAP PTPases (Gu *et al*, 1991; Yang and Tonks, 1991). This

Table 2.7 Comparison of the core catalytic domain of CAP-1 with that of several intracellular and receptor-like PTPases.

The predicted PTPase domain sequence of CAP-1 was aligned with the core domain of other PTPases using DNASTAR programs. Alignments were made between the CAP-1 PTPase sequence (698-942) and the core PTPase regions of PTP1B (Brown-Shimer et al, 1990; Charbonneau et al, 1989; Chernoff et al, 1990; Guan et al, 1990); TCPTP (Cool et al, 1989); CD45 Domain 1 (D1; Thomas et al, 1985; 1987; Ralph et al, 1987); LAR D1 (Streuli et al, 1988); MEG1 (Gu et al, 1991).

Table 2.7

	CAP-1	PTP1B	TCPTP	CD45 D1
CAP-1				
PTP1B	39			
TCPTP	41	71		
CD45 D1	33	37	36	
LAR D1	ND	39	42	50
MEG1	64	39	39	ND
PTP2	37	39	40	ND

Figure 2.14 Alignment of the amino terminal domain of the PTPases CAP-1 and MEG-1 with a homologous region in eukaryotic membrane protein 4.1. Identity between CAP (58-358), MEG (29-329), and those of the cytoskeletal-associating protein, band 4.1 (1-296) is represented by the presence of single letter amino acids, which are boxed. The colon indicates the presence of two highly conserved residues while the period delineates moderate conservation.

V:Q:I:LD:ND:FKVNR:D:EQ:LLD:HL:TR:YFGLQ:DD:D:PRWL:KH:RKQLK:MEG
VICIRFLDGLVDTFKVNRQDIEQS:LD:MAYGHGVTEKVFGLQ:HGDDPVDSPRWLEASKH:RKQLK:CAP
:C:LD:LD:V:V:K:EQ:LLD:HL:HL:YFGL:D:....WL:K:KO:4.1

G:E:L:FRV:F:DPM:LD:E:TR:YFLO:K:D:GRU:CF:N:A:LAS:AVQS:GD:S:MEG
GGFECTLHFRVRFIPDPMTLQEQTRHLVFLQKMDVSQGRU:CF:LSAVLASAVQSHFGDNSS:CAP
G:D:E:M:RDE:U:VR:L:LOL:D:GRU:C:LSY:OS:GD:4.1

:CWL:D:FIR:Q:DF:....LH:OH:GL:RAE:Y:N:ARTL:YGV:E:H:RD:M:MEG
IHHFGYLAQSQFIPDQNDLFLSKVESLHROHSLKQSEAESCYINIARTL:DFYGV:HLH:GD:HLNLDL:CAP
:H:Y:N:D:E:Q:KV:LLH:.....LA:U:LVGY:LE:DU:D:4.1

MIG:S:GI:Y:PW:I:KISFN:K:FPI:R:ESRE:FM:NYR:CKNLK:MEG
MIGLASAGIAYRKVICTSFYPMVNLKISFGRKFFIHQROKQAESREHIVAFNMLNYS:CKNLK:CAP
:RG:S:G:VY:PE:PKIS:KB:FPI:R:E:E:YR:K:LNK:4.1

CVEHH:FF:IP:KN:Y:TLGS:MEG
SCVEHHSFFQAKLLPQEKVLSQYWLGS:CAP
CVEHH:FE:L:S:PLGS:4.1

N-terminal domain localizes or restricts, 4.1 family members to the underside of various membrane structures (Algrain *et al*, 1993; Anderson and Lovrien, 1984; Bretscher *et al*, 1983; Furthmayr *et al*, 1992; Gould *et al*, 1986; Lankes and Furthmayr, 1991; Sato *et al*, 1991; Tsukita *et al*, 1989). Therefore, the CAP domain may direct the association of these PTPases with a transmembrane molecule and thereby serve to effect reversible changes in the cytoskeleton-plasma membrane scaffold.

The predicted amino acid sequence of CAP-1/PTPH1 has the same structural design as the PTPase, MEG1, with a C-terminal localized PTPase domain, an N-terminal domain with homology to cytoskeletal-associated proteins, and a central putative regulatory domain (Gu *et al*, 1991; Yang and Tonks, 1991). Analysis of the amino acid composition of the central region of CAP-1 reveals a relatively high level of the hydroxy amino acid serine (a striking 12%) and the conservation of many of the putative casein kinase II and p34^{cdc2} phosphorylation sites indicated in the human, PTPH1 (Yang and Tonks, 1991). This indicates that phosphate occupancy at these sites may modulate the PTPase activity or substrate specificity of this enzyme, as seen with the modulation of the lateral mobility and tyrosine-specific phosphatase activity of CD45 (Autero and Gahmberg, 1987; Patarroyo and Gahmberg, 1984; Yamada *et al*, 1990). Alternatively, the central domain may act as a "hinge" to bring the C-terminal PTPase domain in contact with codistributing CAP domain-associating substrates.

CAP-1 was cloned out of an undifferentiated embryonal

carcinoma cell line, however, it appears to be expressed in a wide variety of adult mouse tissues. When the results are standardized for β -tubulin, the expression level of CAP-1 transcripts appears much lower in the RA and DMSO differentiated P19 cells and day 14 whole embryo, than in the highest adult tissue expressers, kidney and brain (Figure 2.12). The low level of CAP-1 expression in these differentiated cells does not preclude a developmental role for this PTPase. The transcript level of another unrelated P19-PTPase was recently shown to be greatly enhanced upon aggregation of P19 cells; the replating of RA- and DMSO-induced or untreated P19 cells in monolayers abolished this response (den Hertog *et al*, 1992). The predicted subcellular localization of the CAP-1 PTPase to the cytoskeleton-membrane interface is also consistent with a role for this enzyme in the development and/or maintenance of multicellular structure. The expression of CAP-1 mRNAs in the whole embryo may reflect the adult tissue preference for this enzyme, that is CAP-1 may play a role in embryonic kidney, brain, or heart development and function. The generation of a CAP-specific antibody should reveal whether transcript levels correspond with protein or enzyme amounts.

The pattern and tissue distribution of CAP-1 mRNA reveals a variety of widely expressed transcripts. There are multiple transcripts present in brain and heart, while the majority of mouse tissues express two main mRNAs (Figure 2.6; Figure 2.10). It is not yet known whether the entire composite cDNA sequence is represented in all transcripts; the larger mRNAs may be partially processed

transcripts or alternatively, they may contain additional untranslated sequences as observed by Guan *et al* (1990). The identification of putative untranslated non-CAP-1 sequence in the subcloned cDNAs of 51-4, 20-2 and 52-B2 is consistent with one or both of these hypotheses (Table 2.6). While it is not yet clear as to the mechanism responsible for generating these various transcripts, the distinctive pattern of leg muscle expression is suggestive of tissue-specific splicing or alternative promoter usage by the CAP-1 gene (Figure 2.6; Figure 2.11B). The existence of specific leg muscle cDNAs has been investigated and is presented in Chapter 3. Translation of these transcripts into differential CAP-1 protein structure could affect subcellular localization or substrate preference, thereby defining the functional characteristics of CAP-1 isoforms.

The partial Class II clone predicts a PTPase (PTP2) with almost identical amino acid sequence to that of the MEG2 PTPase indicating it to be the murine homologue of this human enzyme (Gu *et al*, 1992; Figure 2.9). The deduced N-terminal region of MEG2 has 28% identity with the cellular 11-cis retinol or 11-cis retinaldehyde-binding protein, CRALBP and 24% identity with the phosphatidylinositol (PI) binding yeast SEC14p (Aitken *et al*, 1990; Bankaitis *et al*, 1989; 1990; Crabb *et al*, 1988; 1991; Salama *et al*, 1990). The yeast protein, SEC14p, has PI transfer activity and is required for secretion through the Golgi apparatus (Aitken *et al*, 1990; Bankaitis *et al*, 1990). This amino terminal domain homology is sufficient to suggest that MEG2 binds to a hydrophobic ligand

(Gu et al, 1992). Preliminary evidence, not shown here, indicates that the PTP2 transcript levels are upregulated during RA-induced differentiation [of P19 cells]; it would be interesting to determine whether this enzyme plays a role in retinoic acid function. PTP2 is expressed, at the mRNA level, in the embryonal carcinoma (P19), myeloma, pre B, and lymphocytic leukemia cell lines and in several mouse tissues (Figures 2.4; 2.10A). This would predict a more universal function for PTP2 such as transport of or regulation by a lipid moiety (i.e. phosphatidylinositol).

CHAPTER 3

ISOLATION AND ANALYSIS OF ADULT LEG MUSCLE-DERIVED cDNA CLONES

3.1 Introduction

The scope of eukaryotic gene function is often extended by the expression of alternative transcripts from a single gene. The cell-type specific splicing of CD45 was recently shown to depend upon the presence of distinct cis-elements in the preprocessed RNA; this process also appears to be mediated by thymocyte and/or B-cell specific trans-acting factors (Streuli and Saito, 1989). Translation of these differentially expressed mRNAs produce CD45 PTPase isoforms, ranging in size from 180 to 220 kDa, which differ in their glycosylation pattern (Johnson *et al*, 1989; Barclay *et al*, 1987; Streuli *et al*, 1987). Tissue-specific external or "ligand binding" domains may in effect modulate CD45-dependent PTPase activation (Trowbridge, 1991).

Alternatively, post-transcriptional processing may produce changes in untranslated regions. Differential usage of polyadenylation sites has been proposed to account for several PTP1-hybridizing mRNAs (Guan *et al*, 1990). Similarly, two of the many developing axon- and pioneer neuron- expressed DPTP10D transcripts are found to differ at their 3' untranslated region due to alternative polyadenylation (Yang *et al*, 1991).

In adult leg muscle, CAP-1 is expressed as two transcripts which are reproducibly about 500 bp shorter than that found in other murine tissues (Figure 2.6; Figure 2.11). The corresponding skeletal muscle cDNAs were cloned and characterized in an effort to elucidate the mechanism by which these different mRNAs arise. Construction of a composite leg muscle cDNA, from several

overlapping clones, revealed leg muscle-specific sequence at the 5' end. This cDNA predicts a truncated leg muscle protein which lacks part of the protein 4.1 homology region.

3.2 Materials and Methods

3.2-1 Preparation and Screening of a Leg Muscle Library

A cDNA library was made from adult mouse leg muscle poly A⁺ mRNA (3 μ g), in an effort to clone and characterize tissue-specific CAP-1 transcripts. The cDNA was synthesized, modified, and fractionated using the methods outlined in Section 2.2-4. The phosphorylated, linker/adaptor modified cDNA was ligated overnight at 16 °C using T4 DNA ligase, ligase buffer, and λ gt10 arms (Amersham cDNA synthesis and cloning kit). The packaged leg muscle library was screened using random-labeled probes corresponding to the entire CAP-1 cDNA or the 5' (1 to 1802 bp in CAP-1, Figure 2.8; probe A, Figure 3.2) and 3' (1787 to 3776 bp in CAP-1, Figure 2.8; probe B, Figure 3.2) halves of the composite kidney CAP-1 clone. The subcloned leg muscle-derived cDNAs were characterized by comparing restriction enzyme patterns and primer-derived sequence (as described in Section 2.2-5) to that of the composite CAP-1 clone. Southern analysis of digested phage and plasmid DNA was carried out as in Section 2.2-2. Most probes used in these experiments and in the Northern analysis (below), were prepared according to standard procedures (described in Section 2.2-3). The 200 bp, 5' EcoRI/HaeIII fragment (probe 61; Figure 3.2) of 611-12 was purified from an acrylamide gel by a "Crush and Soak" method (Sambrook *et*

al, 1989) and used in a random-labeling reaction as delineated previously (Section 2.2-3).

3.2-2 Northern Blot Analysis of Leg Muscle-Specific CAP-1

Poly A⁺ mRNA was selected from various mouse tissues, fractionated on gels, and blotted to a filter in a manner similar to procedures given in Sections 2.2-4 and 2.2-8. The specific details of leg muscle-specific Northern blot analysis are outlined below in Table 3.1.

Table 3.1 Northern Analysis of Murine Tissues

Figure	Filter	Hyb.Buffer	Wash	Probe/cpm/ml	µg mRNA/Lane
3-4A	Hybond N	+	*	Probe 61/10 ⁶	2.5
3-4B	Hybond N	+	*	Probe 51/10 ⁶	2.5

+ Hybridization (Hyb.) buffer and conditions as in Section 2.2-4

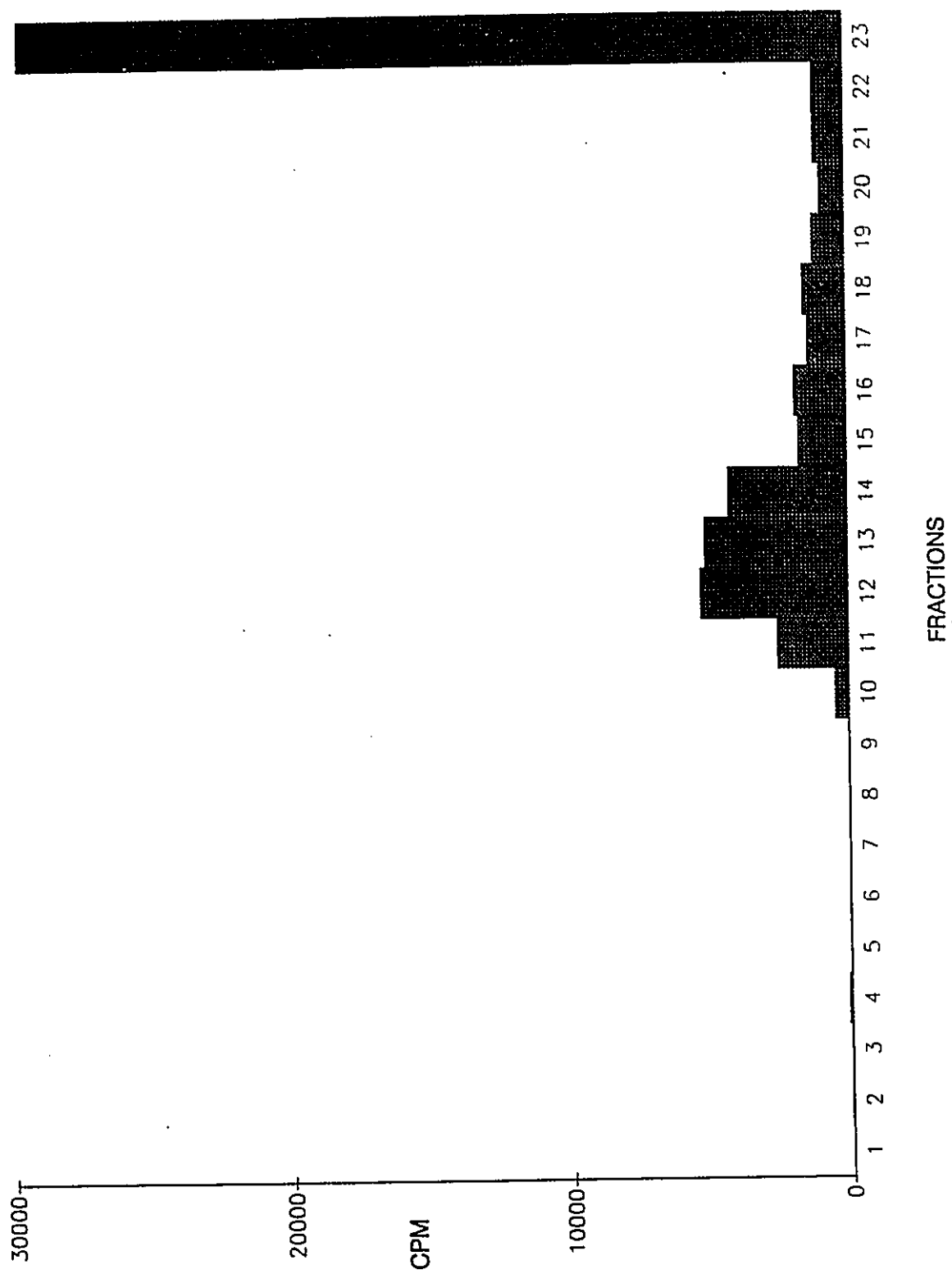
* Washing stringency is 2xSSC;0.1%SDS at 42°C for 1 hour

3.3 Results

3.3-1 Cloning and Characterization of Leg Muscle-Specific CAP-1

A cDNA library was generated and screened to identify leg muscle-specific CAP-1 clones. These isoforms reflect the tissue-specific patterns revealed upon Northern analysis of adult leg muscle transcripts (Figures 2.6 and 2.11). Murine leg muscle-derived cDNA was fractionated on a sizing column; fractions 10-16 were chosen, based on the Cerenkov count profile (Figure 3.1), and pooled for subsequent λgt10 library production. The resulting

Figure 3.1 Size Selection of Leg Muscle-derived cDNA. Radiolabeled second strand leg muscle cDNA was fractionated on a Sepharose 4B sizing column. The Cerenkov counts of each individual fraction are presented as cpm (counts per minute)/fraction.



library had a titer of 1.9×10^6 pfu/ml. Approximately 300,000 recombinants were subjected to primary and secondary screening with the composite CAP-1 clone (probe CAP-1, Figure 3.2). Ultimately, in the third round, one lot of nitrocellulose lifts was probed with the 5' half of CAP-1 (probe A, Figure 3.2), while the other two sets of filters were screened with the 3' portion (probe B, Figure 3.2). The intensity of these positives were graded relative to each other (vertically), however, the hybridization levels of the A and B probes are not comparable (Table 3.2).

Three of the thirteen tertiary positives were isolated and characterized by sequencing, restriction mapping, and miniprep Southern blotting (Table 3.2). Partial analysis of the 321-1 insert revealed about 2000 nucleotides (1300-3300 bp in CAP-1, Figure 2.8) of sequence identical to CAP-1 (Table 3.2; Figure 3.2). This cDNA had additional noncoding sequence at its 3' end. However, the kidney- and P19 cell-derived CAP-1 transcripts may also include this and additional 3' untranslated sequence considering that all CAP-1 cDNAs cloned thus far lack a poly A tract.

The search for leg muscle specific sequences led to a more diligent characterization of the 5'-extended clones. The central portion of 611-12 was also identical to CAP-1 (938-2000; Figure 2.8), while, the 5' non-CAP region of this insert was found to share short stretches of identity (59.7%-85.7%) with a variety of genomic and untranslated cDNA sequences. Additional cDNAs appear to corroborate the 611-12 clone; the 411 and 711 inserts hybridized to the 5'-most 200 bp fragment of 611-12 (probe 61; Figure 3.2; data

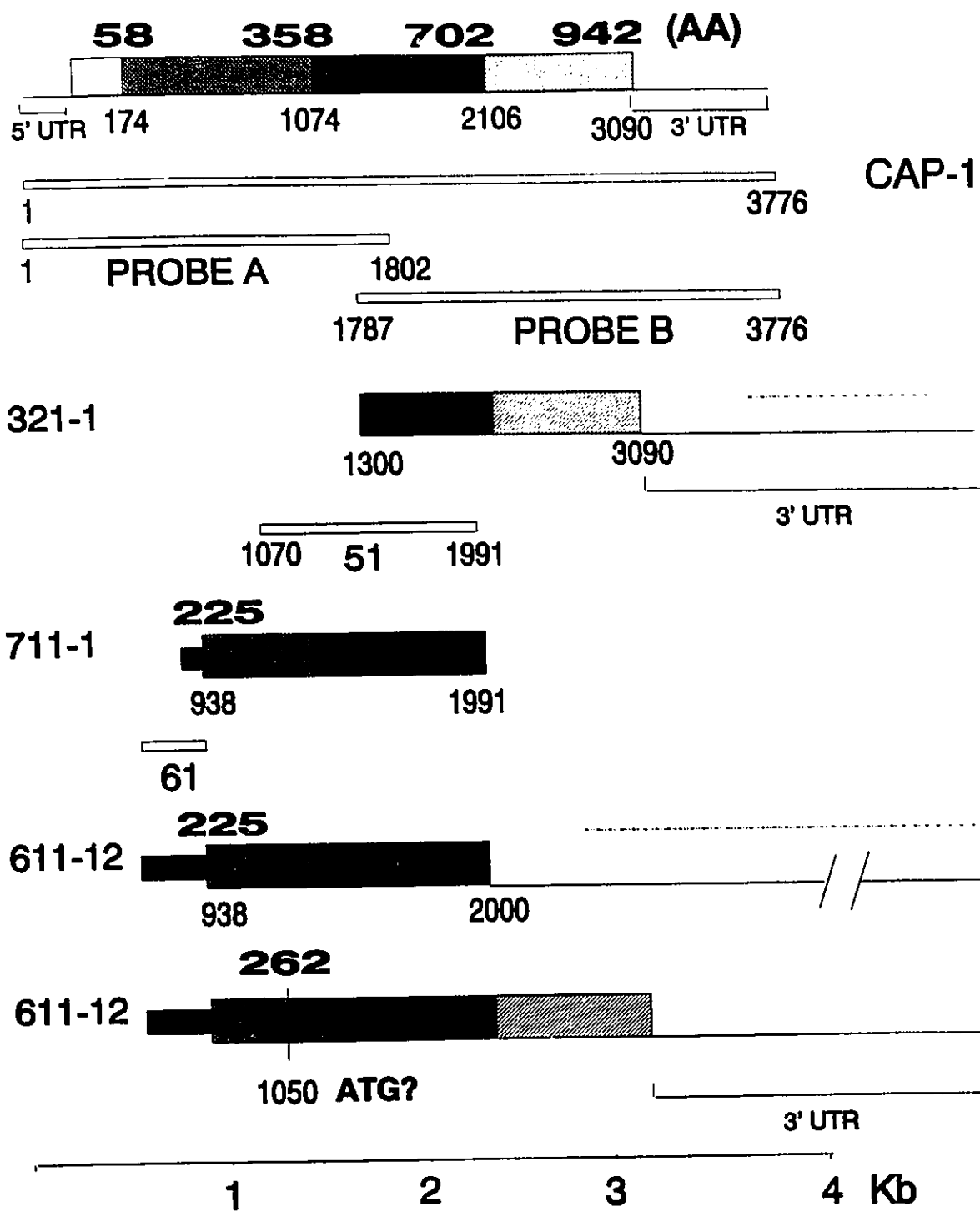
Table 3.2 Cloning and characterization of Leg Muscle cDNAs

Tertiary positive clones were identified by hybridization to the 5' or 3' half of CAP-1 (probe A or B, respectively; Section 3.2.1; Figure 3.2). These results were graded within each probe as non detectable (-), low (+), medium (++), or high (+++). Phage insert size (bp) was determined upon EcoRI digestion. The subcloned leg muscle cDNAs were related to CAP-1 by sequence comparison; 5' and 3' border sequence is delineated in terms of its location in CAP-1 sequence (Figure 2.8). All four characterized phage inserts have NONCAP sequences (Figure 3.2). Several phage inserts were not released, however, the phage did hybridize to CAP-1 probes (*).

Table 3.2

Phage #	Probes		Insert Size	NON CAP-1	CAP-1 Sequence		Plasmid
	A	B			5' Border	3' Border	
3-11	+	++	*		ND	ND	ND
3-21	+++	++	2800	yes	1300	3300(?)	321-1
3-31	++	++	*		ND	ND	ND
3-51	-	++	ND		ND	ND	ND
4-11	+++	-	600		ND	ND	ND
4-21	-	+++	ND		ND	ND	ND
5-11	+++	+++	1500	yes	1050	2150	511-9
5-21	+	++	500		ND	ND	ND
6-11	++	++	3500	yes	938	2000	611-12
7-11	+++	++	1200	yes	938	1986	711-1
7-21	-	++	ND		ND	ND	ND

Figure 3.2 This diagram portrays the relatedness between CAP-1 and the Leg Muscle cDNA clones. The thin lines symbolise nucleotide sequence, while the open boxes depict deduced protein structure. A key to the protein homology regions and 5' leg muscle cDNA character is provided in the Figure. The 5' and 3' UTR represent untranslated regions. The probes, denoted as narrow boxes, were designed for screening cDNA libraries and for Northern analysis. The characteristics of these probes are as follows: probe CAP-1 represents 1 to 3776 or the entire composite cDNA clone; probe A encompasses 1 to 1802; probe B has sequences 1787 to 3776; while probe 51 was derived from a PstI fragment or 1070 to 1991 in CAP-1 (kidney/P19; Figure 2.8). The remaining probe contains leg muscle-specific sequence (61; Figure 3.3). The nonsequenced regions of each subcloned leg muscle cDNA is indicated as dotted lines.



not shown). In addition further characterization of the 711 phage insert revealed identity [to 611-12] at the junction sequence and for another 150 bp upstream (Figure 3.3). The junction sequence of T,CCAGT shares identity with the 3' splice site consensus of [Y_nNCAG/G], where Y=polypyrimidine tract, however, unlike the consensus the underlined base pairs of CAT represent coding sequences in CAP-1 (Figure 3.3). This region appears to be leg muscle-specific since it hybridizes to the 4.5 and 6.5 Kb mRNAs in leg muscle (lane 2, Figure 3.4A). It also recognizes an upper [partially processed] transcript in brain, leg muscle and kidney (Figure 3.4A). This band was not observed in low CAP-1 expressing mouse liver or P19 cells, however, it may be a probe- rather than a CAP-1-specific hybridization event (data not shown). When the identical blot was probed with a sequence found in both leg muscle and kidney cDNAs (probe 51, Figure 3.2), the general CAP-1 expression pattern was generated (Figure 3.4B; refer to Figure 2.6). While it is not yet known how this skeletal muscle-type of expression occurs, a preliminary result implies that the phenomenon is conserved, at least from mouse to rabbit. Northern analysis revealed that the typical murine "leg muscle/LMCAP-1-specific" pattern is present in rabbit skeletal muscle (data not shown).

The 3' sequence of the 611-12 cDNA, downstream of the 2000 bp mark, is also nonCAP-1 and appears to be noncoding due to the presence of in frame termination codons (Figure 3.2; data not shown). This region has no homology with sequences present in the GENE BANK and EMBL databank, and appears from its sequence to be

Figure 3.3 Partial sequence of several overlapping leg muscle-derived cDNAs Partial or 5' end nucleotide sequence of the 6-11 and 7-11 derived cDNA clones are provided here. The starting position of the 7-11 insert begins has been indicated [Δ] in addition to the location of three in frame stop codons (***). The junction sequence including the nucleotide () and the predicted amino sequence (above) of CAP-1 is given. Probe 61 was prepared from a fragment spanning the initial bp to a HaeIII site.

AACATGGTACGATGAGAGGGCAGGGCATCTTGAAAGGTGATAGACAGTTT 50

TCACTGTCGGGAGACCTCCGGAAGAGCCCACCAAGTGGGAAGATGTCTTA 100

711

Δ
GTAAAAACCTAGGGGTCCAGAGGAGGTCAGGTTCCATGGACAAGAAGAAA 150

AACCTGCTTCATTAGCATTTAAAGCTAGAGTGCTTACGGGGGACTGGCCC 200

HaeIII

AAGGCACTTAGTAACTGCTAATAACTTACAGAGGACTATTACAACATTTT 250

S G L K Q S E A E S C Y I N I
TTTCCAGTGGGCTAAAGCAGTCGGAAGCCGAATCTTGCTACATCAACATA

Figure 3.4 Analysis of Leg Muscle-Specific CAP-1 PTPase expression
Murine tissue-derived poly A⁺ mRNA was analysed by Northern blot analysis. A) The expression of leg muscle-specific transcript(s) was revealed by screening with probe 61; Figure 3.2. B) The CAP-specific probe 51 (Figure 3.2) was used to probe the same blot to determine CAP-1 mRNA transcript levels. The lanes represent mRNA from the mouse tissues: B, brain; M, leg muscle; K, kidney. The β -tubulin standardization for each lane is shown in the lower panel. Ethidium bromide stained 28S and 18S rRNAs served as molecular weight markers.

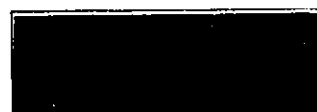
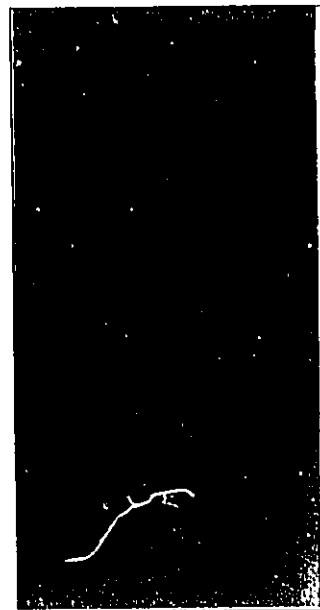
A) B M K

28S-



B) B M K

28S-



untranslated and may represent an unprocessed intron. A "single copy" probe from this region may, in this scenario, hybridize to the upper band which runs at about 7.0 Kb (6.5 in the leg muscle); this is assuming that the higher transcript is actually a relatively stable unprocessed or partially processed mRNA. Pulse field gel experiments should more clearly substantiate whether the 3' sequence of 6-11 is from a separate genomic locus or if it is part of the CAP-1 gene.

3.4 Discussion

I have cloned CAP-1 hybridizing cDNAs from a murine leg muscle library in an attempt to characterize the specific CAP-1 transcription pattern observed in this tissue. All three characterized leg muscle cDNAs were found to share internal sequence identity with that observed in the CAP-1 P19 and/or kidney derived clones (Figure 3.2). However, the end sequence of several cDNAs (6-11 and 7-11) were found to differ and are possible candidates for the observed tissue-specific variation. These cDNA inserts share sequence identity with CAP-1 up to the 938 bp demarcation (refer to CAP-1, Figure 2.8; Figure 3.3). That both cDNAs have similar junction and upstream sequence, would indicate that these are not artefacts [joined clones]. This leg muscle-specific sequence may have arisen from alternative promoter usage or from differential splicing of a noncoding exon. Alternatively, this sequence may represent the presence of an unspliced intron. There is no direct evidence for this; however, intronic regions

have been cloned from reverse transcribed premRNAs (Duncan, Howell, and Bell, unpublished; Scott *et al*, 1990; Haars *et al*, 1986; Qian *et al*, 1993). Fractionation of CAP-1 mRNA into nuclear versus cytoplasmic portions (with only the mature form present in the cytoplasmic fraction) should indicate whether this is the case here.

Recognition of the lower leg muscle CAP-1 transcript by a leg muscle-specific probe (5' region of 6-11 and 7-11), implies that this sequence is also part of a mature, fully processed CAP-1 mRNA. This domain appears to be noncoding because of its identity with short stretches of several genomic and untranslated cDNAs and due to the presence of termination codons in all frames. There are no putative initiation codons upstream of the junction sequence in the 6-11 and 7-11 cDNA inserts; however, the ATG present at position 1050 [bp] in CAP-1 (Figure 2.8) may serve to initiate a truncated message. As predicted, hybridization of a Northern blot (Figure 3.4) with a 5' CAP-1 specific probe (i.e. fragment 267-635 bp in CAP-1) generates both major 5 and 7 Kb bands in kidney and brain but not in the leg muscle lane (data not shown). Production of polyclonal antibodies against epitopes located in the N-terminus as well as C-terminus (Appendix III) of CAP-1 should provide the necessary tools to definitively prove the existence of this truncated isoform in leg muscle.

This leg muscle specific transcript is generated by an as yet unknown mechanism. The cloning of the CAP-1 genomic DNA and analysis of its exon/intron boundaries would determine if

alternative splicing is occurring. Examination of the junction sequence for a 3' splice site revealed a region having identity to the known consensus sequence. Alternatively, primer extension experiments may elucidate whether the leg muscle and kidney mRNAs initiate at different transcription sites.

As expected, the leg muscle specific pattern is not observed in days 1-7 DMSO induced P19 cells since there is little skeletal muscle present in these preparations (Figure 2.12; McBurney *et al*, 1982). Induction of a skeletal muscle population in P19 cells and the differentiation of L6 cells in culture may provide a system by which to initiate study of these tissue specific CAP-1 transcripts.

The unique cytoskeletal and intracellular membrane structure of skeletal muscle has been well documented (DeRobertis and DeRobertis, 1979). A great number of skeletal muscle proteins arise from differentially spliced transcripts; several of these include the cytoskeletal proteins: α -tropomyosin, myosin light chain, and troponin-T (Smith *et al*, 1989 and references therein). The cloning of a leg muscle specific cDNA which predicts a truncated CAP-1 PTPase is consistent with a distinct role for this LMCAP-1 isoform in the regulation of cytoskeleton/membrane interaction in skeletal muscle.

CHAPTER 4

DETERMINATION OF CAP-1 PTPase ACTIVITY

4.1 Introduction

Hybrid fusions engineered between prokaryotic and eukaryotic specified proteins are generally used to characterize the function of eukaryotic genes. The protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPases) are no exception to this rule. The validity of experiments conducted with this catalytic material is consolidated by the absence of tyrosine-specific phosphorylating or dephosphorylating enzymes in nonpathogenic bacteria (Douville et al, 1992; Howell et al, 1991; Icely et al, 1991; Letwin et al, 1988; and refer to Section 1.9).

The similarity of the CAP-1 catalytic domain to the PTPase consensus would imply a catalytic role for this protein (Table 2.7). The core catalytic domains of several PTPases have been expressed in a variety of bacterial systems (Section 1.4-3 and references therein). The majority of these enzymes are soluble, have high specific activity, and are phosphotyrosine specific. In fact, although these phosphatases differ in their artificial substrate preference, they do share biochemical properties similar to those documented earlier for the purified human placental PTP1B (Tonks et al, 1988a; Section 1.3-1). In a recent report, a soluble GST/PTPase (rat PTP1) fusion was shown to be readily purified on a glutathione affinity column; following proteolytic removal of the GST portion, the "catalytic core" domain functioned as a PTPase in an in vitro assay (Guan and Dixon, 1991).

The CAP-1 PTPase domain was also expressed in a similar system to help elucidate the functional parameters of this enzyme. This

bacterially expressed GST/CAP-1 fusion was found to preferentially remove radiolabeled-phosphate moieties from tyrosine residues of the autophosphorylated sty kinase.

4.2 Materials and Methods

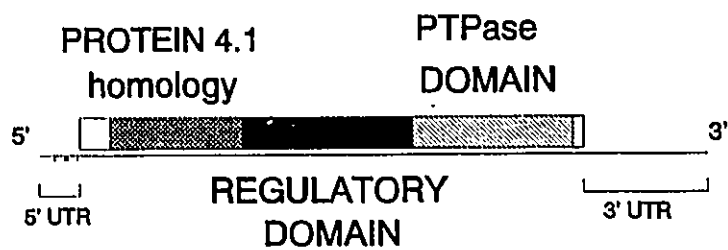
4.2-1 Construction of the GST/PTPase plasmid: pGEX-3X/CAP-1

A partial gene product of CAP-1 was expressed in bacteria using the pGEX-3X vector system (Smith and Johnson, 1988; Pharmacia). The C-terminal 2.0 Kb cDNA (1781 to 3776 in CAP-1; Figure 2.8) was inserted in frame with GST (glutathione-S-transferase) to create a GST/truncated CAP-1 PTPase (Figure 4.1). The CAP-1 cDNA/pTZ19R plasmid (52-T7; Table 2.6) was linearized by partial digestion at the Linker/Adaptor with NotI. This site was blunt-ended with Mung Bean nuclease (BRL) and the CAP-1 cDNA was subsequently liberated by cutting with EcoRI. Digestion of the vector with SmaI/EcoRI was followed by treatment with Calf Intestinal Phosphatase (Boehringer Mannheim). The blunt-end/cohesive-end insert and vector were ligated and electroporated into Tg1 bacteria. Positive clones were selected by restriction enzyme mapping and by sequencing of the GST-PTPase insertion site as described in materials and methods, Chapter 2.

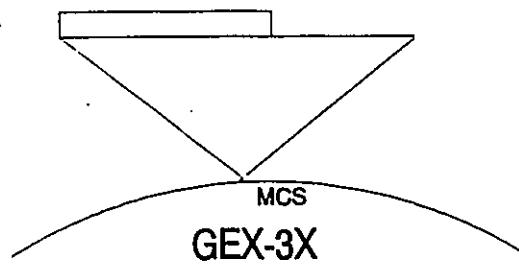
4.2-2 Bacterial Expression and Solubilization of GST/CAP-1

The GST fusion protein was induced according to procedures reported earlier (Guan and Dixon, 1991; Smith and Johnson, 1988). An overnight culture (10 ml) of GST/CAP-1-expressing Tg1 bacteria was inoculated into 250 mls of LB with 50 μ g/ml ampicillin and grown

Figure 4.1 Generation of the GST/CAP-1 fusion protein. A partial 2.0 Kb kidney cDNA (1787 to 3776 in CAP-1; Figure 2.8) was inserted into the MCS (multicloning sequence) pGEX-3X in frame with GST to create a GST/truncated CAP-1 PTPase. The protein homology regions of CAP-1 are delineated as patterned boxes. The 5' and 3' UTRs, are represented as thin lines and denote the untranslated regions of CAP-1.



2.0 Kb cDNA



to an OD₆₀₀ of about 0.4-0.5. This culture was induced to express the GST-CAP fusion protein upon the addition of 0.4-1.0 mM IPTG and grown for three hours. Purification of the insoluble fusion protein from inclusion bodies was carried out according to the procedures of Kumagai and Dunphy (1991b) with the following modifications. Bacteria were harvested by a brief centrifugation and sonicated three times for 20 seconds in lysis buffer containing 10 mM Tris-HCl, pH 7.4; 100 mM NaCl; 1 mM MgCl₂; 1mM DTT; 1 mM EDTA; and 200 µg/ml PMSF (Boehringer Mannheim). The inclusion body fraction, obtained by centrifugation, was washed for 10 minutes at room temperature with a high salt solution containing 0.2 M Tris-HCl, pH 8.2, 0.5 M NaCl, 1 mM DTT, 1 mM EDTA, and 200 µg/ml PMSF. The washed pellet was denatured and solubilized by rotating a 7 M urea/high salt solution for 30 minutes at room temperature. The fusion protein was slowly refolded by successive dialysis in 3.5 M urea/high salt for 12 hours, in high salt lacking urea for 12 hours and in a low salt solution containing 100 mM Tris-HCl, pH 7.4; 100 mM NaCl; 1 mM DTT; 1 mM EDTA; and 200 µg/ml PMSF for 12 hours at 4°C. The solubilized protein fraction was cleared by centrifugation at 8K (10,000 xg) for 30 minutes at 4°C.

The refolded protein was rotated in the low salt solution plus 1% triton X-100 for 30 minutes at 4°C with 1 ml of equilibrated 50% glutathione-coupled Sepharose beads (Douvillie *et al.*, 1992; Pharmacia). The beads, collected by a brief centrifugation, were loaded on a 1 ml column (poly prep chromatography column; BIO-RAD) and washed with 10 bed volumes of the low salt loading buffer. The

GST/CAP-1 fusion protein was eluted with 5 bed volumes of 5-10 mM reduced glutathione (Boehringer Mannheim). The eluted enzyme was stored aliquoted in a final glycerol concentration of 33% at -20°C. The induced, solubilized, and affinity purified GST/CAP-1 fractions were boiled in sample buffer (5% SDS; 20% glycerol; 50 mM Tris-HCl, pH 6.8; 0.01% bromophenol blue; 0.1% β -mercaptoethanol or 100 mM DTT) and analyzed by SDS polyacrylamide gel electrophoresis or SDS-PAGE. These 10% gels were run in 25 mM tris base; 192 mM glycine, pH 8.3; 0.1% SDS and stained with Coomassie Blue and/or silver stain (Sambrook *et al*, 1989; Biorad Silver stain kit and protocol, respectively).

4.2-3 Determination of PTPase Activity: Overlay Assay

The ability of the truncated CAP protein to act as a phosphatase was measured using an overlay assay as described previously (Douville *et al*, 1992). This assay operates on the premise that PTPase-mediated removal of phosphates from tyrosine residues, represented as autophosphorylated kinases immobilized on nitrocellulose, can be visualized by probing with a mouse anti-phosphotyrosine antibody (IgG2bk α PY Ab; UBI). The bacterial *lysate* (30-40 μ g/lane) was prepared as recounted by Douville *et al* (1992). The *trpE*/STY fusion was expressed as described by Howell *et al* (1991) with minor modifications. *TrpE*/STY containing-bacteria, grown in tryptophan-free M9 media to an OD₆₀₀ of 0.6, were induced for sty production by growing in IAA for 4 hours (Sadowski *et al*, 1986). Bacteria were pelleted by centrifugation and frozen at -70°C

or were lysed directly in sample buffer (Section 4.2-2) by sonicating 2 to 3 times with a probe (Fisher Sonic Dismembrator, 300) at 30 second intervals. These *esk* and *sty* kinase-expressing extracts were resolved on SDS-PAGE and electrophoretically transferred for 1-1.5 hours at 100 volts to nitrocellulose (Schleicher and Schuell Inc.) in Western transfer buffer (25 mM Tris base; 192 mM glycine, pH 8.3; 20% Methanol). These filters were blocked with 3% bovine serum albumin, or BSA/Fraction V (Boehringer Mannheim) for 30 minutes at 20°C or overnight at 4°C. These kinase-embedded nitrocellulose strips were then incubated with the CAP-1 PTPase or control solution for 1 hour shaking at 37°C. The solubilized PTPase was diluted into a low salt buffer (Section 4.2-2) and pretreated with or without 1 mM vanadate. These filters were blocked in 20% FCS/TBST and treated with the α PY Ab (ICN Biochemicals) at a 1:1000 dilution for 1 hour at room temperature. The phosphotyrosine reactivity (absence of PTPase activity) was detected by a 1 hour incubation with the ^{125} I-labeled anti-mouse secondary antibody (Amersham). Non-specific antibody (1° and 2°) binding was prevented by washing 3 times for 5 minutes with TBST. The results were visualized by exposure to x-ray film. Please refer to Maniatis *et al* (1982); Sambrook *et al* (1989); and Harlow and Lane (1991) for a more extensive Western blotting protocol.

4.2-4 Investigation of Phosphotyrosine-Specific PTPase Activity

The phosphoamino acid specificity of the truncated GST/CAP-1 Δ fusion protein was ascertained by analyzing its ability to

dephosphorylate the immunoprecipitated trpE/STY kinase. About 50 mls of induced culture was pelleted and sonicated (3 x 20 seconds) in lysis buffer (40 mM Tris-HCl, pH 7.5; 150 mM NaCl; 2 mM EDTA; 1% Triton X-100; 2 μ g/ml aprotinin; and 5 μ g/ml leupeptin). Insoluble materials were removed by a 15 minute eppendorf centrifugation (13,000 xg) at 4°C. Cleared lysates containing trpE/STY kinase were immunoprecipitated with the α -trpE antibody for 2 hours at 4°C in lysis buffer without vanadate; approximately 50 mls of induced culture was used per immunoprecipitation. Later, the supernatant of a 10 minute centrifugation at 13,000 Xg was added to 50 μ l of pre-equilibrated protein A beads. After rotating at 4°C for 30 minutes, the beads were washed with 3 bed volumes of lysis buffer and with 2 bed volumes of the kinase buffer (see below). The kinase-attached beads were allowed to autophosphorylate in 40 mM HEPES, pH 7.0; 10 mM MgCl₂; 2 mM MnCl₂; along with [γ -³²P] ATP for 30 minutes at 37°C (Howell *et al.*, 1991). Following several washes in lysis buffer, the beads were equilibrated with 2 bed volumes of the low salt buffer (Section 4.2-2), these samples were treated with an aliquot of solubilized phosphatase (pretreated with or without vanadate). The immunoprecipitated kinases were eluted from lysis buffer rinsed beads with 50 μ l of sample buffer. These ³²P-labeled proteins were then subjected to SDS-PAGE.

4.2-5 Phosphoamino Acid Analysis

SDS-PAGE separated reaction products were transferred to methanol pretreated immobilon membranes and upon a relatively short

exposure to film the phosphorylated protein bands were localized and excised. The samples were eluted from the immobilon and acid hydrolysed in double-distilled 6N HCl (Pierce) at 110°C for 75 minutes. The radioactive supernatant was dried down, washed several times with dH₂O, and resuspended in 5 µl of TLE buffer (0.5% pyridine; 5% acetic acid) + 5 µl of phosphoamino acid standards. The phosphoamino acids were carefully spotted onto thin-layer cellulose plates (Sigma) and resolved by one-dimensional thin-layer electrophoresis using previously discussed procedures (Cooper *et al*, 1983; Edwards *et al*, 1988; Kamps and Sefton, 1989). These known phosphoamino acid standards were detected by ninhydrin staining and the radiolabeled phosphoamino acids were observed by autoradiography.

4.3 Results

4.3-1 The Expression and Analysis of the CAP-1 PTPase

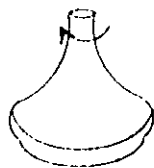
Bacterial expression of CAP-1 was utilized to ascertain whether this enzyme has the tyrosine-specific phosphatase activity intimated by the PTPase catalytic domain homology (Table 2.7). The entire catalytic domain was subcloned into the vector pGEX-3X and expressed as a GST/CAP-1 fusion protein (Figure 4.1). CAP PTPase activity was determined by the ability of the GST/CAP-1 fusion to dephosphorylate a Ser/Thr/Tyr-autophosphorylated kinase (Douville *et al*, 1991; Howell *et al*, 1991). This PTPase overlay assay involved resolving the kinase-containing bacterial extracts on an SDS PAGE gel. The *esk* or sty kinase-embedded nitrocellulose strips

were then treated with or without active PTPase. These phosphatase assay results were detected by antiphosphotyrosine (α PY) antibody staining with the absence of α PY-immunoreactivity denoting the presence of PTPase activity (Figure 4.2).

The dephosphorylation of the *esk* kinase in this overlay assay attributes vanadate-sensitive phosphatase activity to the GST/CAP-1 fusion (lanes 1 and 2; Figure 4.3A; Douville *et al*, 1992). However, only a slight loss in antiphosphotyrosine (α PY)-immunoreactivity was observed upon treatment of *sty* and *aki* kinase-embedded filters with this enzyme preparation (data not shown). This soluble GST/PTPase fusion could not be concentrated by glutathione affinity chromatography (lanes 4 and 5; Figure 4.3A). The enzyme activity was recovered, however, in the unbound or flow through fraction (lane 3; Figure 4.3A); the comparative level of initial (loaded) or unbound phosphatase activity is difficult to quantify due to the nature of the assay.

Virtually all of the recombinant fusion was localized to the insoluble pellet (lane 3; Figure 4.4); this potentiated the urea-mediated solubilization of GST/CAP-1 from the inclusion body fraction followed by slow renaturation with successive dialysis (Section 4.2-2; lane 6, Figure 4.4). A fraction of this solubilized GST/CAP-1 activity was affinity purified (lane 3 and 4, Figure 4.3B; lane 10, Figure 4.4), however, the phosphatase activity (lane 2, Figure 4.3B) and the majority of 72-74 kDa fusion protein flowed through the column (lane 7, Figure 4.4). This renatured CAP-1 enzyme catalyzed the dephosphorylation of both the *esk* (lane 2,

Figure 4.2 PTPase overlay assay. This illustration describes an assay which is based on the correlated loss of α phosphotyrosine-immunoreactivity with the presence of an active PTPase. The protocol outlined in Section 4.2-6 involves treatment of kinase-embedded nitrocellulose strips in a buffer +/- active PTPase. The results were detected by the specific binding of primary (α PY) and secondary ^{125}I -conjugated antibodies to these filters.



Bacterial
Induction
of kinase

(Autophosphorylation)



Lysate
preparation



10% SDS-PAGE



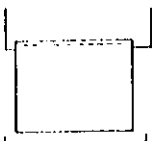
Western Transfer



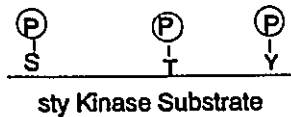
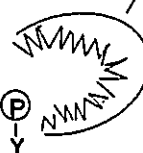
Treatment
with PTPase



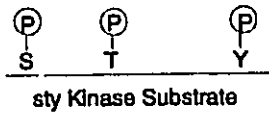
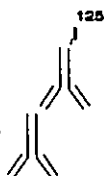
anti-PY Antibody
Immunoreactivity



Tyrosine-
Specific
Phosphatase



anti-PY



or



+PTPase

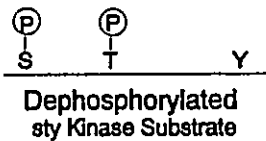
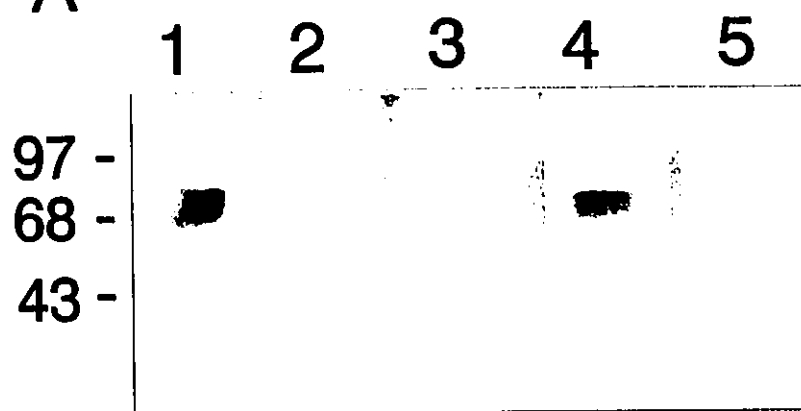


Figure 4.3 CAP-1 phosphatase activity: CAP-1 abolishes the α PY immunoreactivity of esk. Lysates of esk-expressing bacteria were run on 10% SDS PAGE, electroblotted to nitrocellulose and treated with PTPase buffer or GST/CAP-1. A) These esk-embedded strips were treated with aliquots of GST/CAP-1 containing sonicate supernatant plus vanadate, lane 1; GST/CAP-1 supernatant alone (lane 2); or with glutathione affinity column unbound, lane 3; and eluate (1 volume, lane 4; 2 volumes, lane 5) fractions. The first three mixtures contained the same amount of total protein. B) Similarly, nitrocellulose filters were incubated with PTPase buffer, lane 1; or with the affinity purified GST/CAP-1 fractions: unbound, lane 2; eluate (1 volume, lane 3; 2 volumes, lane 4); and vanadate pretreated eluate (2 volumes, lane 5). The assay results were detected by probing with the α PY and ^{125} I-conjugated secondary antibodies. The protein sizes were estimated by comparison with the electrophoretic migration of BRL protein standards.

A



B

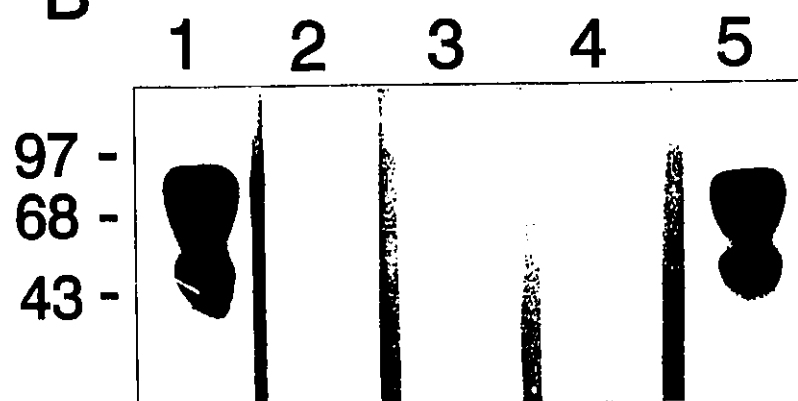


Figure 4.4 Solubilization and affinity purification of GST/CAP-1. Aliquots of the GST/CAP-1 fusion were resolved by SDS-PAGE and stained with coomassie. The lanes from left to right are: BRL protein standards, lanes 1 and 11; lysate from IPTG-induced bacteria, lane 2; the pelleted or inclusion body (IB) fraction, lane 3; sonicate supernatant, lane 4; high salt wash, lane 5; Urea-solubilized and dialysed protein, lane 6; glutathione-affinity column unbound fraction (flow through), lane 7; affinity column washes: W1, lane 8 and W2, lane 9; affinity column eluate, lane 10. Lanes 1-9 and 10-11 represent companion gels.

1 2 3 4 5 6 7 8 9 10 11

200-

-200

97-

-97

68-

~~_____~~

-68

43-

-43

29-

-29

~~_____~~

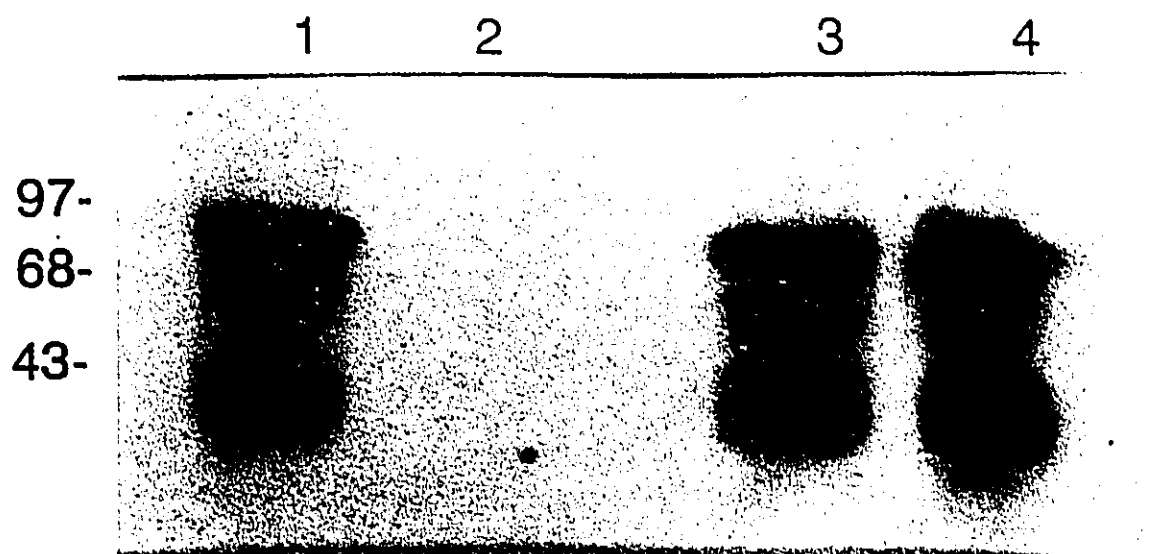
Figure 4.3B) and highly auto-phosphorylated sty kinases (Figure 4.5A). TrpE/STY-bound filters incubated with buffer (lane 1; Figure 4.5A) or bacterially expressed and renatured GST protein alone (lane 3; Figure 4.5A) retained the immunoreactivity, whereas incubating with the solubilized GST/CAP-1 fusion protein resulted in loss of the signal (lane 2; Figure 4.5A). In addition, pretreatment of the GST/CAP-1 fusion protein with the PTPase inhibitor vanadate, reestablished phosphotyrosine (PY) epitope recognition (lane 5, Figure 4.3B; lane 4, Figure 4.5A).

4.3-2 CAP-1 is a Phosphotyrosine Specific Phosphatase

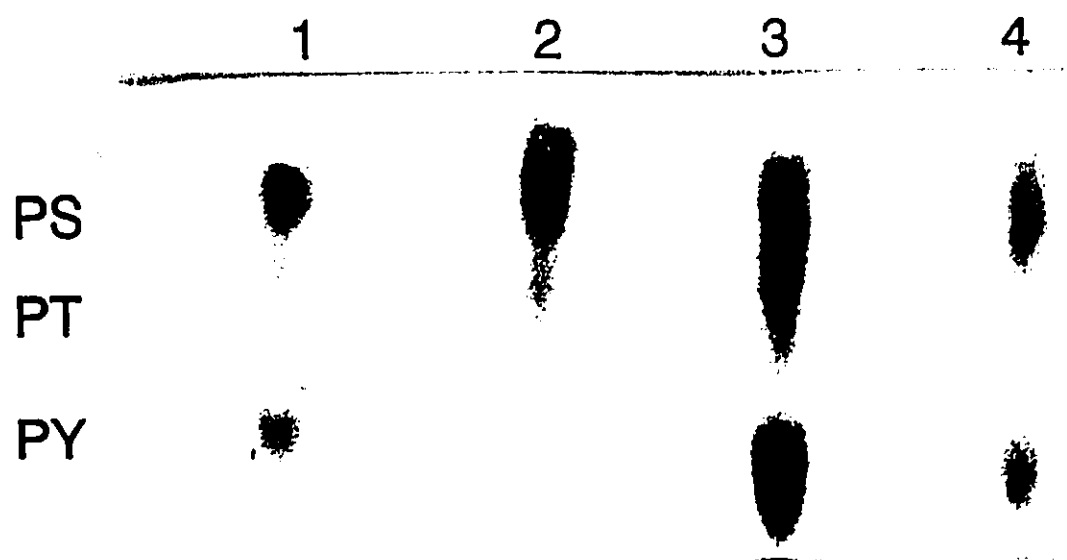
Catalytic removal of the phosphotyrosine epitope is sufficient to attribute phosphatase activity to CAP-1. However, the phosphotyrosine-specificity of this PTPase was assayed by examining the explicit removal of a ^{32}P -labeled phosphate moiety from tyrosine residue(s). This required the autophosphorylation of the trpE/STY fusion in the presence of $[\gamma\text{-}^{32}\text{P}]$ ATP to label all three hydroxyamino acids (Howell *et al*, 1991). The sty kinase was immobilized on protein A beads (with αTrpE), ^{32}P -labeled, and employed as a substrate for PTPase activity. The dephosphorylation of this STY kinase substrate was followed by acid hydrolysis and separation of radiolabeled phosphoserine (PS), phosphothreonine (PT), and phosphotyrosine (PY). The presence of phosphotyrosine-specific phosphatase activity can be identified in lanes which lack PY but retain PS and PT. One-dimensional phosphoamino acid analysis of the CAP-1 PTPase treated-STY kinase revealed a decrease in

Figure 4.5 PTPase activity of the renatured GST/CAP-1 fusion protein. A) CAP-1 activity is delineated by the absence of antiphosphotyrosine antibody (α PY Ab) immunoreactivity. The trpE/STY kinase, transferred to nitrocellulose strips, was treated with PTPase buffer (lane 1), with the renatured GST/CAP-1 fusion protein (lane 2), with renatured GST protein alone (lane 3), or with vanadate-pretreated CAP-1 fusion (Lane 4). The results were visualized by probing with the α PY Ab and ^{125} I conjugated secondary antibodies. The trpE/STY fusion and a breakdown product run at 40 and 100 kDa, respectively. B) Phosphoamino acid analysis of CAP-1 dephosphorylated STY kinase identified PY-specific phosphatase activity. α TrpE-immunoprecipitated STY kinase was in vitro labeled by autophosphorylation, treated with buffer (lane 1), the GST/CAP-1 fusion (lane 2), the renatured GST protein alone (lane 3), or with the vanadate-treated GST/CAP-1 (lane 4). The SDS PAGE-separated reaction products were electroblotted to immobilon from which phosphoamino acids were extracted and analysed by one-dimensional thin layer electrophoresis. Positions of the phosphoamino acids, detected by ninhydrin staining, are delineated as: PS, phosphoserine; PT, phosphothreonine; PY, phosphotyrosine.

A



B

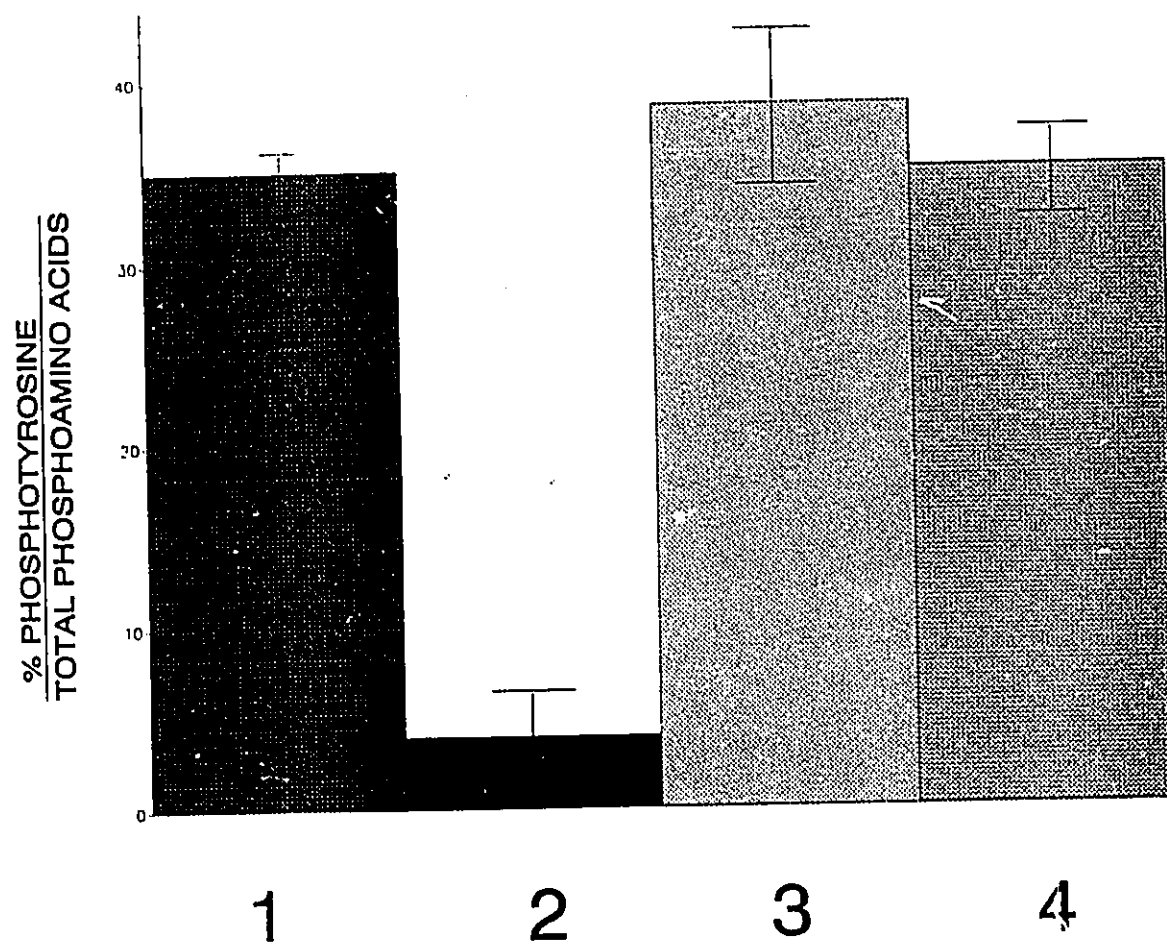


phosphotyrosine levels (lane 2; Figure 4.5B). PTPase activity was not observed when the PTPase buffer (lane 1; Figure 4.5B) or bacterially-expressed GST (lane 3; Figure 4.5B) controls were used, whereas the enzymatic activity of CAP-1 was abolished by vanadate pretreatment of the enzyme (lane 4; Figure 4.5B). This reproducible data, summarized in Figure 4.6, clearly demonstrates a preference of phosphotyrosine for this PTPase.

4.4 Discussion

The CAP-1 catalytic domain contains all of the consensus sequences thought to confer phosphotyrosine specificity to the PTPases (Charbonneau *et al*, 1989; Cool *et al*, 1989; Streuli *et al*, 1988; 1989; Table 2.7). Dual-specificity phosphatases, that is enzymes which dephosphorylate PY and PS/PT are known to exist and share sequence homology with PTPases (Guan *et al*, 1991; Moreno and Nurse, 1991; Hakes *et al*, 1993; Sheng and Charbonneau, 1993; Section 1.5-3). The bacterial expression of the catalytic domain served to determine the specificity of the CAP-1 phosphatase. Fusion of the CAP-1 catalytic domain with the soluble GST region was predicted to produce an active and soluble fusion protein (Guan and Dixon, 1991; Pharmacia); however, the bacterially expressed GST/CAP-1 was extremely insoluble. The quantity of soluble GST/CAP-1 (sonicate supernatant) was sufficient to dephosphorylate the *esk* kinase, however, activity against the *sty* kinase required solubilization of the recombinant CAP-1 fusion. This may be due to the lower level of phosphotyrosine [PY] (relative to phosphoserine

Figure 4.6 The tyrosine specific phosphatase activity of CAP-1.
This is a summary of three individual PTPase assays, a representative of which is shown in Figure 4.5B. The substrate (sty kinase) was immunoprecipitated, radiolabeled, and treated: with the PTPase buffer (lane 1); the GST/CAP-1 fusion (lane 2); the GST protein (lane 3); or with the vanadate-treated GST/CAP-1 (lane 4). The resulting phosphoamino acid analyses, carried out as indicated in Figure 4.5, were detected by autoradiography and the x-ray films were scanned. The results are presented as the percentage (average) of PY/[PY+PS+PT] with the standard error indicated by the bar. The GST/CAP-1 PTPase preparation specifically removes phosphate moieties from the tyrosine residues of radiolabeled trpE/STY.



[PS]) in *esk* (Douville *et al*, 1992) compared with that seen in the autophosphorylated sty (Howell *et al*, 1991). Affinity purification of the solubilized GST/CAP-1 was inconsistent and appeared to involve incorrect renaturation of the GST portion. Alternatively, limited proteolysis of GST/CAP-1 may be required to obtain catalytic activity; however, this does not explain the low level of GST-mediated binding to and elution from the glutathione affinity column (Figure 4.4; Data not shown).

The elimination of α PY Ab immunoreactivity upon treatment with the recombinant GST/CAP-1 fusion simply identified CAP-1 as having phosphatase activity. The CAP-1 catalytic domain was shown to remove phosphates preferentially from tyrosine residues when a substrate autophosphorylated on all three hydroxyamino acids was used (Howell *et al*, 1991). This specificity in addition to the lack of ser/thr phosphatase activity in the solubilized GST fraction would argue against the action of bacterial alkaline phosphatase (Lau *et al*, 1989). The reproducible and selective decrease in phosphotyrosine from ^{32} P-labeled sty indicates a phosphotyrosine specificity for the CAP-1 enzyme *in vivo*.

The tyrosine specific phosphatase activity observed here for the bacterially expressed CAP-1 Δ fusion may be extrapolated to include its *in vivo* function. This PTPase, predicted by its N-terminal sequence to localize to the inner face of the plasma membrane, may function in regulating the interaction between co-localizing cytoskeletal components and thereby modulate signalling events at this site (refer to Section 1.6).

CHAPTER 5
GENERAL DISCUSSION

This thesis describes the cloning and characterization of PTPases from murine embryonic (P19 stem cells) and adult sources. A PCR strategy was used to amplify novel sequences (Class I and II) residing between two degenerate PTPase-specific primers. Sequence from these PCR fragments and from several P19 stem cell-derived cDNA clones revealed sufficient homology to the PTPases consensus to indicate the cloning of two individual PTPases.

5.1 The PTP2 Homologue [MEG2] has a Putative Ligand Binding Domain

A partial Class II or PTP2 clone was shown by sequence analysis to be the murine homologue of the human MEG2 PTPase (Gu *et al.*, 1992). Although PTP2 and the Megakaryocyte derived, MEG2, were cloned from two very specialized cell lines, Northern analysis revealed this PTPase [PTP2] is ubiquitously expressed as a 5 Kb mRNA transcript in all mouse tissues and cell lines examined (Figure 2.10; Gu *et al.*, 1992).

The upstream portion of MEG2, not present in the partial PTP2 cDNA, predicts a domain having 24% and 28% identity with the phosphatidylinositol (PI) transfer protein, SEC14 and the cellular 11-cis retinol and 11-cis retinaldehyde binding protein CRALBP, respectively (Gu *et al.*, 1992). If the MEG2 N-terminal domain and "constitutive" expression of PTP2 are taken into consideration then one can predict a function for this PTPase in regulating the transfer of a hydrophobic ligand. The phospholipid transfer activity of SEC14 is thought responsible for the induction of protein secretion from the Golgi complex and in fact, PI transfer

proteins have been shown to be essential for cell growth in yeast (Aitken *et al*, 1990; Bankaitis *et al*, 1990). Subcellular localization and overexpression of sense and antisense PTP2 constructs may help define a role for this phosphatase in this or other processes.

The catalytic activity and/or subcellular localization of the PTP2/MEG2 enzyme may be governed by the binding of a hydrophobic [lipid] moiety. In support of the latter, the requirement of a lipid linkage in localization and function has been clearly documented for pp60^{v-src} (pp60^{c-src}) and has been implied for the CD45 and PTP1B PTPases (Frangioni *et al*, 1992; Guan *et al*, 1990; Resh and Ling, 1990; Sefton *et al*, 1982; Takeda and Maizel, 1990). Immunoprecipitation of the endogenous PTP2 or overexpressed PTP2myc with an α PTP2/MAP (Dr. L. Desjardine and R.Marius, personal communications) or by an α myc antibody, respectively would allow the examination of the lipid nature and content. More importantly, this material or *in vitro* transcribed and translated full length PTP2 could be analyzed for binding to and/or regulation by phospholipid-containing vesicles or inositol trisphosphate (IP₃), as has been recently reported for several PTPases (Stader and Hofer, 1992; Zhao *et al*, 1993). Only with the possession of the full length PTP2 cDNA or of a specific anti-PTP2 antibody will it be possible to elucidate the regulation and function of this enzyme.

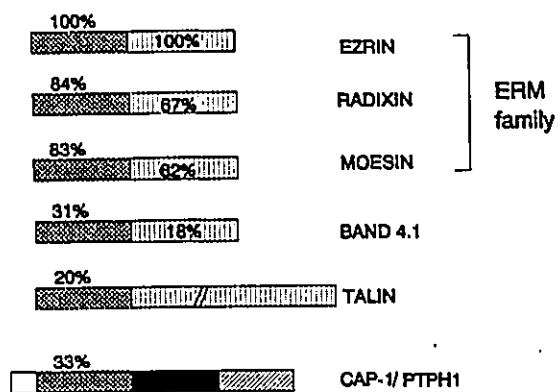
5.2 The CAP-1 PTPase is a Member of the Band 4.1 Superfamily

The composite CAP-1 cDNA contains an open reading frame of 942 amino acids and predicts a nontransmembrane enzyme of about 107 KDa. The N-terminal portion of this PTPase shares significant identity to a homologous domain within the band 4.1 superfamily, which includes the cytoskeleton associated proteins: band 4.1, ezrin, radixin, moesin and talin (Figure 5.1A; Section 1.6). The Class I PTPase is therefore referred to as CAP-1 for Cytoskeletal Associated Protein related PTPase. This PTPase is virtually identical to the human PTPH1 (91.6%) and shares 55% identity with the MEG1 PTPase (Gu et al, 1991; Yang and Tonks, 1991). The predicted structural design of CAP-1/PTPH1 and MEG1 reveals a C-terminal localized PTPase domain, an N-terminal band 4.1 domain, and a central putative regulatory domain (Gu et al, 1991; Yang and Tonks, 1991; Figure 5.1B).

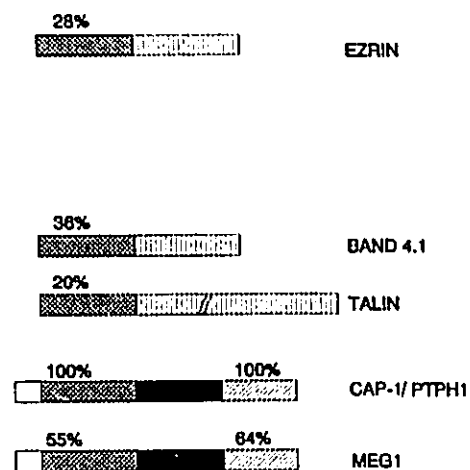
CAP-1 appears to be a member of a family of phosphatases, including MEG-1 and PTPH1, (Gu et al, 1991; Yang and Tonks, 1991) that contain amino terminal sequences thought to localize proteins to cytoskeletal:plasma membrane linkage sites (Figure 5.1). This domain is essential for the interaction of certain cytoskeletal associated proteins with plasma membrane anchor proteins (Algrain et al, 1993; Anderson and Lovrien, 1984; Bretscher 1983; 1989; Furthmayr et al, 1992; Gould et al, 1986; Horwitz et al, 1986; Lankes and Furthmayr, 1991; Sato et al, 1991; Tsukita et al, 1989). The exact binding interface involved in linking band 4.1 with the cytoplasmic domain of the anion exchanger (band 3) has been

Figure 5.1 Band 4.1 superfamily and the ezrin, radixin, and moesin (ERM) family. The structures of the CAP PTPases: CAP-1, PTPH1, and MEG1 are compared with ezrin, radixin, moesin, talin, and human erythrocyte band 4.1. The regions of homology, within the first column were obtained by comparing sequence identity with the murine ezrin. Whereas the second group depicts a comparison of band 4.1 superfamily members with CAP-1. The protein homology regions are delineated as patterned boxes. This diagram was modified from Tsukita et al, 1992.

A)



B)



protein 4.1 homology region
 putative regulatory domain
 PTPase catalytic domain
 cytoskeleton association domain

identified (Jöns and Drenckhahn, 1992). This short 5 residue motif, as seen below, is conserved in the homologous region of CAP-1/PTPH1 and MEG1. The band 4.1 sequence has three charged amino acids (acidic/-) positioned immediately after a nonpolar residue (Leu or Val) and just before a tyrosine residue. The positioning of a Lys (basic/+) or Gln residue at +3 and of a nonhydrophobic amino acid at +1 [in the CAP-1/PTPH1 or MEG1 motif] may provide protein specificity to the interaction of these PTPases with their cognate transmembrane molecule(s).

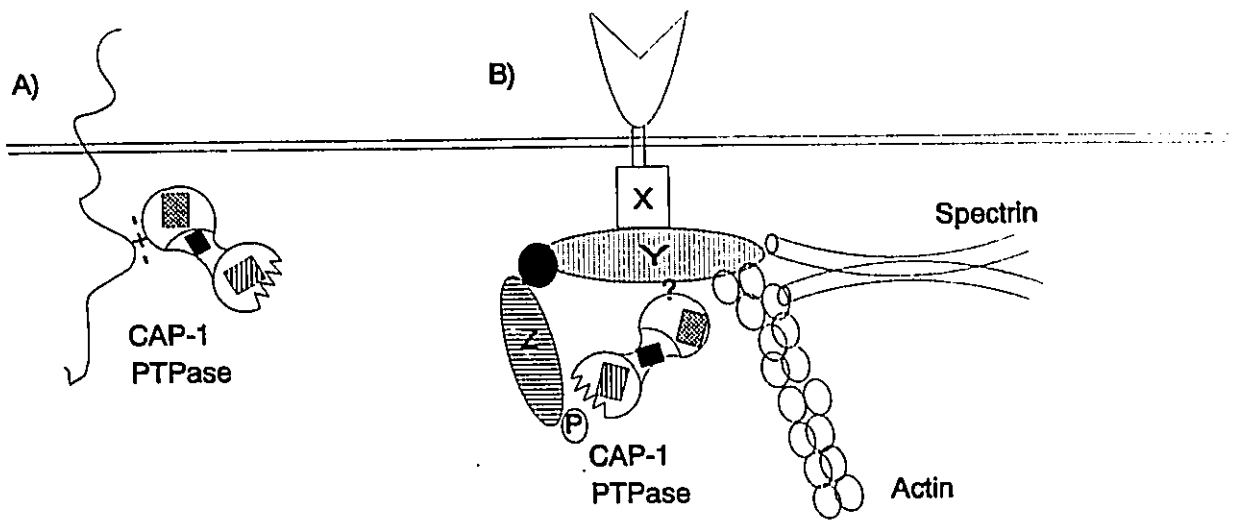
	N-terminal Extension	
	MOTIF #1	MOTIF #2
Human Band 4.1	L E E D Y	L D E E I
Xenopus Band 4.1	V E E D Y	L E D D V
Murine CAP-1/Human PTPH1	T E K E Y	
Human MEG1	T E{Q}E Y	
Consensus	XE[+/.](-)Y	

The L/VEEDY motif of band 4.1 and two oppositely charged sequences (IRRRY/LRRRY) present in the cytoplasmic domain of band 3, were shown to specifically inhibit the interaction of these two molecules (Jöns and Drenckhahn, 1992). It would be possible to test the importance of this sequence in CAP-1 subcellular localization/protein interaction by including this or nonsense peptides in coimmunoprecipitation or cellular fractionation (Western) experiments. This should determine whether the TEKEY motif is required for the association of CAP-1 with a protein or

structure. Alternatively, overexpression of CAP-1myc mutants which have site-directed changes in this region may determine whether this N-terminal sequence is essential for the observed pattern of CAP-specific localization. It is not known whether the related stretch of IRRL found in the glycophrin A and B cytoplasmic region are essential for the interaction of band 4.1 with this molecule (Blanchard et al, 1987; Tomita and Marchesi, 1975). The fact that the LEEDY motif is not conserved in the slightly less related ERM family members (ezrin, radixin, and moesin), indicates a different type of interaction for the ERM protein(s) with its proposed receptor(s), i.e. with leukosialin (Baecher et al, 1990; Brown et al, 1981; Cyster et al, 1991; Killeen et al, 1987). CAP-1 also shares significant identity with the ERM member, ezrin (Figure 5.1). Therefore, it is possible that the CAP-1 enzyme is able to interact with several integral membrane or cytoskeletal proteins via this (TEKEY) and/or other regions of the amino terminal sequence (Figure 5.2).

The construction and overexpression of N-terminal deletions and site directed mutants in CAP-1 may be instrumental in determining sequences which are both essential and sufficient for the targeting and function of this PTPase. Ultimately, the search for interacting or docking component(s), may lead to the development of anti-idiotypic antibodies against an essential primary sequence or the use of a specific motif or domain in affinity purification or cloning procedures; these strategies have been used previously to characterize and identify interacting

Figure 5.2 Models for possible CAP-1 PTPase function at the cytoskeleton/membrane interface. The subcellular localization and substrate specificity of the CAP-1 PTPase is proposed to involve the interaction via the band 4.1 domain of CAP-1 with cytoskeleton/membrane colocalizing components. A) The first model predicts a direct association between a motif found within the N-terminal region and the cytoplasmic domain of an integral membrane protein. The limited conservation of the LEEDY motif of band 4.1 (TEKEY in CAP-1) would imply an ionic association as was observed with the band 4.1/band 3 interaction. B) Alternatively, this PTPase may localize to the innerface of the membrane by an indirect interaction with other associating cytoskeletal/membrane proteins (i.e. protein Y). In both models, the CAP-1 PTPase could function to dephosphorylate and modulate the relative binding affinities and/or activity (i.e. Kinase activity) of colocalizing substrates (i.e. protein Z).



 protein 4.1
homology
region

 putative
regulatory
domain

 PTPase
catalytic
domain

proteins (Argraves *et al.*, 1989; Jöns and Drenckhahn, 1992). Although there is a lot of work to be done before this point, the information gleaned along the way should prove interesting and may provide a clue as to the exact function(s) for this PTPase.

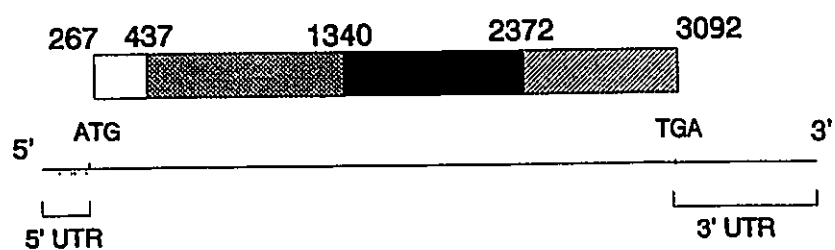
5.3 A Specific Function for the Skeletal Muscle CAP-1 Isoform?

The expression pattern of PTPH1 in human tissues is unknown (Yang and Tonks, 1991); however, this report identifies multiple CAP-1 mRNA species in a wide variety of mouse tissues (Figure 2.6). Two major transcripts, with sizes of 5 and 7 Kb were seen in brain, heart, lungs, liver, and kidney, whereas in adult leg muscle, these widely expressed transcripts appear as two reproducibly shorter mRNAs (Figures 2.6 and 2.11). CAP-1 leg muscle (LM) cDNAs were cloned and shown to have specific sequence at their 5' end (Figure 3.3); this cDNA predicts a truncated protein which lacks part of the N-terminal domain (Figure 3.2; Figure 5.3). If the LM-CAP-1 isoform initiates at methionine 1049 (Figure 2.8), the resulting protein would retain only 97 residues or roughly 1/3 of the band 4.1 domain. A computer search revealed short stretches of identity within a region which overlaps [25 amino acids] the latter portion of the band 4.1 domain. This region (335-385 aa in CAP-1) shares 27% identity with the intermediate filament, keratin type II (Eckert and Rorke, 1988; Hanukoglu and Fuchs, 1982; Marchuk *et al.*, 1984). The significance of these homologies are unknown; however, a truncated protein lacking most (2/3) of the band 4.1 domain may localize to structures which depend upon interactions within the

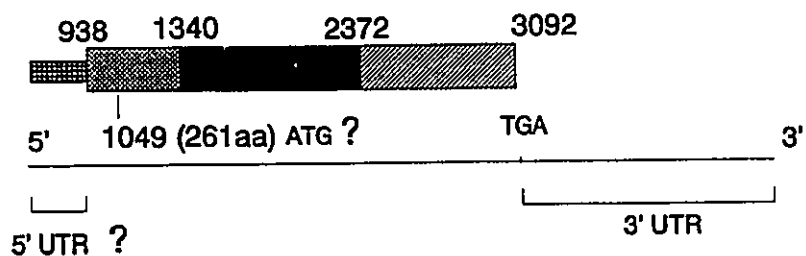
Figure 5.3 The predicted structural design of the CAP-1 PTPases. The key to the putative amino acid homology regions (enclosed in patterned boxes) is outlined in the bottom portion. Specific nucleotide positions are given to delineate the deduced protein domains. The thin line represents composite P19/kidney- or leg muscle- derived cDNAs with proposed 5' and 3' untranslated regions (UTRs).

CAP-1 cDNAs

Kidney/P19



Leg Muscle



1 2 3 4 Kb

Protein 4.1
homology
region
(CAP-1)



Putative
regulatory
domain



PTPase
catalytic
domain



Skeletal
Muscle-
specific



"unmasked" C-terminal (1/3) region. This finding suggests a specific subcellular localization and function for the LM-CAP-1 isoform in the highly organized cytoskeleton/ membrane structure of the skeletal muscle.

The mechanism responsible for generating this pattern of CAP-1 expression in leg muscle, as yet unknown, possibly involves alternative splicing and/or differential promoter usage. Alternative inclusion of a C-terminal noncatalytic domain is reported to generate a nuclear versus a cytoplasmic/ "reticular" localization pattern for a *Drosophila* PTPase (McLaughlin and Dixon, 1993). In addition, a lineage specific splicing event is responsible for producing band 4.1 isoforms which retain (erythroid) or lose (lymphoid) a 35 amino acid stretch known to harbor the LEEDY motif discussed above (Tang et al, 1988). Alternatively, embryonic-specific promoter usage is also known to give rise to a truncated PDGF receptor which lacks the PDGF binding domain (Vu et al, 1989). These examples suggest several mechanisms whereby tissue-specific changes in protein structure may lead to a different localization, protein-protein interaction, and subsequent function for the CAP-1 or LM-CAP-1 enzyme.

5.4 Deregulation of the CAP-1 PTPase: A Piece of the Puzzle?

Although in vitro analysis reveals the CAP-1 enzyme to act exclusively upon phosphotyrosine, it would be interesting to determine whether the CAP-1 phosphatase exerts its function by specifically dephosphorylating associated proteins at

cytoskeleton/membrane linkage sites. Preliminary studies have analysed the expression and localization of a myc tagged CAP-1 in mammalian cells. Several morphological changes, namely the formation of membranous projections or the presence of "giant cells" observed upon CAP-1 overexpression in COS-1 and NIH 3T3 cells appear specific to CAP-1 function (Figures A⁵.5; A⁵.6). Further documentation of this phenotype may provide a clue as to the normal function of the CAP-1 enzyme. Subsequent analysis should focus on localizing the "normal" or endogenous CAP-1 by overexpressing a PTPase minus CAP-1myc mutant of this enzyme (Appendix II) or by employing an affinity purified α CAP-1/MAP antibody (refer to preliminary experiments in Appendix III), respectively.

Underlying and associated with the plasma membrane is a highly organized and surprisingly flexible structure, often referred to as the cortical cytoskeleton. These proteins are known to determine cell shape and through their interactions with various integral membrane components appear to govern cellular events which occur at the plasma membrane (reviewed by Bretscher, 1991). Phosphotyrosine-containing proteins are often concentrated at intracellular junctions and in focal adhesion contacts in both normal and transformed cells where they are thought to regulate aspects of cell-cell interaction, cell morphology, growth and differentiation (Section 1.6 and references therein). It seems likely that phosphatases such as CAP-1 with the potential to associate at the plasma membrane:cytoskeletal interface are essential components of

the signal transduction network regulating these events.

The disruption or deregulation of PTPase [CAP-1] expression may be instrumental in the cause or promotion of disease. The mapping of several PTPases to regions frequently deleted in tumors in addition to the identification of an ERM family member, Merlin, as the neurofibromatosis 2 tumor suppressor gene product suggests that CAP-1 may itself be an anti-oncogene (reviewed by Charbonneau and Tonks, 1992; Jirik *et al*, 1992; LaForgia *et al*, 1991; Streuli *et al*, 1992; Trofatter *et al*, 1993). It would be interesting to assess the integrity and expression of the CAP-1 gene in human tumours.

5.5 Conclusions

This thesis has outlined the cloning and characterization of two separate PTPases from murine embryonic and adult sources. The majority of this report has been devoted towards analyzing the CAP-1 PTPase. The entire coding sequence for the CAP-1 PTPase was obtained from both kidney/P19 and leg muscle. The existence of tissue-specific CAP-1 transcripts has been documented here and the relevance of the proposed truncated leg muscle specific isoform (LM-CAP-1) to its skeletal function has been discussed.

CAP-1 is a member of the band 4.1 superfamily of proteins which contain sequences thought to localize proteins to cytoskeletal: plasma membrane linkage sites. The results of *in vitro* PTPase assays presented here are consistent with the CAP-1 enzyme belonging to a family of PTPases which exclusively act upon

phosphotyrosine. The deduced structure of CAP-1 with its putative N-terminal localization domain along with the C-terminal catalytic region predicts a very interesting function for this enzyme.

The introduction of the CAP-1myc construct into mammalian cells resulted in the expression and subsequent immunoprecipitation of a CAP-1myc specific protein and in the identification of a discontinuous pattern of immunofluorescence staining. The morphogenetic nature of CAP-1, indicated by the appearance of membranous structures and "giant" cells upon transfection with CAP-1myc, may provide a clue as to the function of the endogenous CAP-1 PTPase. Further investigation into the nature of this PTPase should provide a most interesting vantage point from which to observe specific signalling events which occur at the cytoskeleton/membrane border.

APPENDIX I

Oligonucleotides used as primers in sequencing, myc epitope insertion, and polymerase chain reaction (PCR).

#	Sequence (5' to 3')	Position in CAP-1	Orientation
1	CACTGACTGACTTACTCTG	* 51-4	S
2	TATCAGTCCCCAATACT	* 51-4	S
3 ₇	ATACTGAGTCTCTTGGG	* 51-4	S
4 ₁₂	ACCGATACAAAGATGTG	2374-2390	S
5	GACAACTGTGAAGTGGT	3096-3112	S
6	CCTGAACATACATCATG	3775-3759	AS
7 ₃	CCCAAGAGACTCAGTAT	* 51-4	AS
8	TGACGCTACACTTGCCA	* 51-4	AS
9	AATGGGTGCCTGGTCGATGT	* 51-4	S
11	TGGCCTTTGCAGGGCTGGCA	**GST	S
12 ₄	CACATCTTTGTATCGGT	2390-2374	AS
13 ₁₆	GCCAGTTCTCTCGAGTG	2125-2109	AS
14 ₁₅	CCATCGTCACTCTTATC	1864-1848	AS
15 ₁₄	GATAAGAGTGACGATGG	1848-1864	S
16 ₁₃	CACTCGAGAGAACTGGC	2109-2125	S
17	GTA CTGGTGACATTTGG	2624-2608	AS
18	ACCTCACATACTTCACA	2841-2825	AS
19	CACTTCACAGTTGTCCT	3110-3096	AS
20	ACCTGTCCGACCTATTC	2903-2887	AS
22	GTGACTAATCTAGGAGT	*** PTP2	S

* 51-4 is a partial CAP-1 cDNA (Table 2-6).

** This primer binds to a sequence found 55 nucleotides upstream of the multicloning site of GEX-3X (Pharmacia).

*** Primer #22 recognizes a 17 bp stretch of DNA found in PTP2 (378-384 bp; Figure 2-9).

APPENDIX I

#	Sequence (5' to 3')	Position in CAP-1	Orientation
23	GACAGCGACTCTGAAGT	1622-1639	S
24	ACTGGATGACTTCTGGG	1705-1721	AS
25 ₂₆	GAGCCTAGAGTCCAGTA	1323-1339	AS
26 ₂₅	TACTGGACTCTAGGCTC	1339-1323	S
27	TTGCTAGGGACTCA	125-140	S
28 ₃₀	CCGTTATGACCTCCCG	349-364	S
29	CTAGGTTGTGCAGATC	1029-1044	AS
30 ₂₈	CGGGAGGTCATAACGG	364-349	AS
31	CAAGCAAACCCCTCAG	613-628	S
32	CTGAGGGGTTTGCTTG	628-613	AS
33	AGCCAGTTCATACCAG	873-888	S
34	TTCTTCATACACCAGC	1155-1170	S
35	GATCCCAGTGAGCAAAAGCTGATTTCTGAGGAGGATCT	MYC/CAP-1*	
36	GATCACAGATCCTCCTCAGAAATCAGCTTTTGCTCACT	MYC/CAP-1*	
37	GGGCT <u>CG</u> AGTGCTGGAATAGGTCG	2776-2886	S**
38	GGGCT <u>CG</u> AGTGAACGAGGGCAGGC	2886-2776	AS**
39	ATTTTCGGTAGACTGC	1092-1077	AS
40	ACTGCTTTAGCCCACT	LM	41
	AGTGCCTTTAGTGAGG	LM	42
	ATTAGCTACTGGTAAC	LM	

trpE: AAAGCTCGCTCAAGTAT

* These myc epitope-encoding primers are delineated in Figure A⁵-2.

** Primers #37 and #38 were used in a PCR strategy to create a PTPase minus mutant; a 2 bp substitution (underlined) changed Cys to a Ser (Figure A³-2).

APPENDIX II

Design and Generation of PTPase Minus CAP-1 Constructs

A².1 Introduction

Introduction of CAP-1 in mammalian cells produced a relative increase in bimultinucleation; this phenotype is often generated upon the overexpression or deregulation of an active PTPase (Cool *et al*, 1992; Woodford-Thomas *et al*, 1992). The production of stable CAP-1 expressing cell lines and elucidation of the "normal" pattern of CAP-1 localization may require transfection of a construct exhibiting a deletion or mutation in the PTPase domain of this enzyme. Detection of CAP-specific staining, with these plasmids as transfectants, would require affinity purification and/or production of a high titer CAP-specific polyclonal antibody (refer to the discussion, Appendix IV). Alternatively, modification of both CAP-1 constructs to incorporate the myc epitope would allow the affirmation and extension of experiments outlined in Appendix V.

A².2 Materials and Methods: Construction of Plasmids

A².2-1 Production of a CAP-1 PTPase Minus Deletion Mutant

A PTPase minus mutant was generated by deleting a BspEII fragment encompassing nucleotides 1885 to 2974 in CAP-1 (Figure 2.8). This deletion mutant is lacking the CAP-1 PTPase catalytic domain. The pMT21/CAP-1 construct (pMT29) was grown in DAM minus bacteria, isolated, digested with BspEII, and recircularized after removal of the 1089 bp fragment (Figure A².1). Individual pMT29 Δ clones were checked by restriction enzyme digestion, while the disrupted coding region was seen to be in frame by sequencing

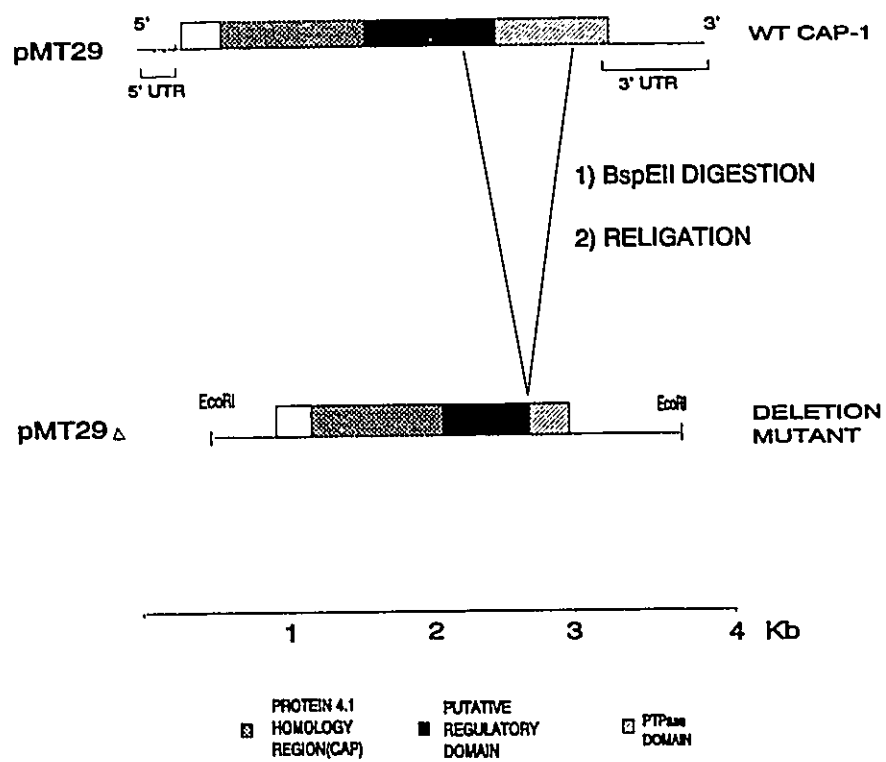
analysis.

A^{3.2-2} Creation of the PTPase Minus Mutant of CAP-1

A PTPase minus CAP-1 was generated by mutating the essential cysteine to a serine using a PCR-mediated procedure. Two overlapping primers (50 pmol #37 and #38; Appendix I) were used to prime synthesis off opposing strands using 10 ng of the 4-1 plasmid (CAP-1Δ/pTZ19R, Table 2.4) as template. The PCR program featured 25 cycles of: a 1 minute melting step at 95°C; annealing at 60°C for 1 minute; and a polymerase reaction for 5 minutes at 72°C. The final block added a extra 10 minutes at 72°C with the Vent DNA polymerase (Biolabs) to ensure a completed product. The 4.1 Kb linear PCR fragment was purified (GENECLEAN), digested at the engineered XhoI site, and recircularized by ligation. The presence of the two bp substitution and the absence of subsequent changes were verified by XhoI or PstI digestion and by sequencing analysis of the region to be subcloned. A composite PTPase minus CAP-1/pTZ19R (X2S2-15) was constructed by ligating the CAP-1 2850 bp SalI insert of CAP-1/pTZ19R^{6,10} (Section A^{5.2-2}) with the 3766 bp Sal/CIP treated vector of 4-1/X2S2 (Figure A^{2.2}).

The 4-1/X2S2 vector was treated with BamHI/CIP and ligated together with the phosphorylated myc insert (described previously, Section A^{5.2-2}) to produce Xmyc-7. The 2850 bp SalI fragment of CAP-1/pTZ19R^{6,10} was inserted into the 3766 bp SalI/CIP treated Xmyc-7 vector in the correct orientation to generate a PTPase minus CAP-1**myc/pTZ19R construct (Xmyc7-93). This CAP-1**myc insert can now

Figure A'.1 Production of a CAP-1 PTPase deletion mutant. The generation of the CAP-1/pMT21 deletion mutant, pMT29Δ, is shown here as the removal of a BspEII fragment from CAP-1/pMT21. The 5' and 3' UTRs and deduced protein homology regions of CAP-1 are illustrated as before.

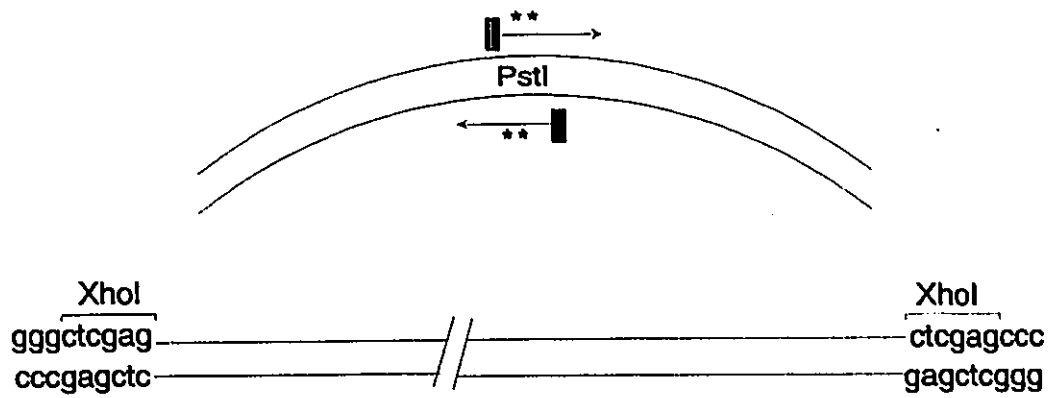


be inserted into the EcoRI site of the pECE and/or pMT21 mammalian expression vectors (Sections 5.2-3).

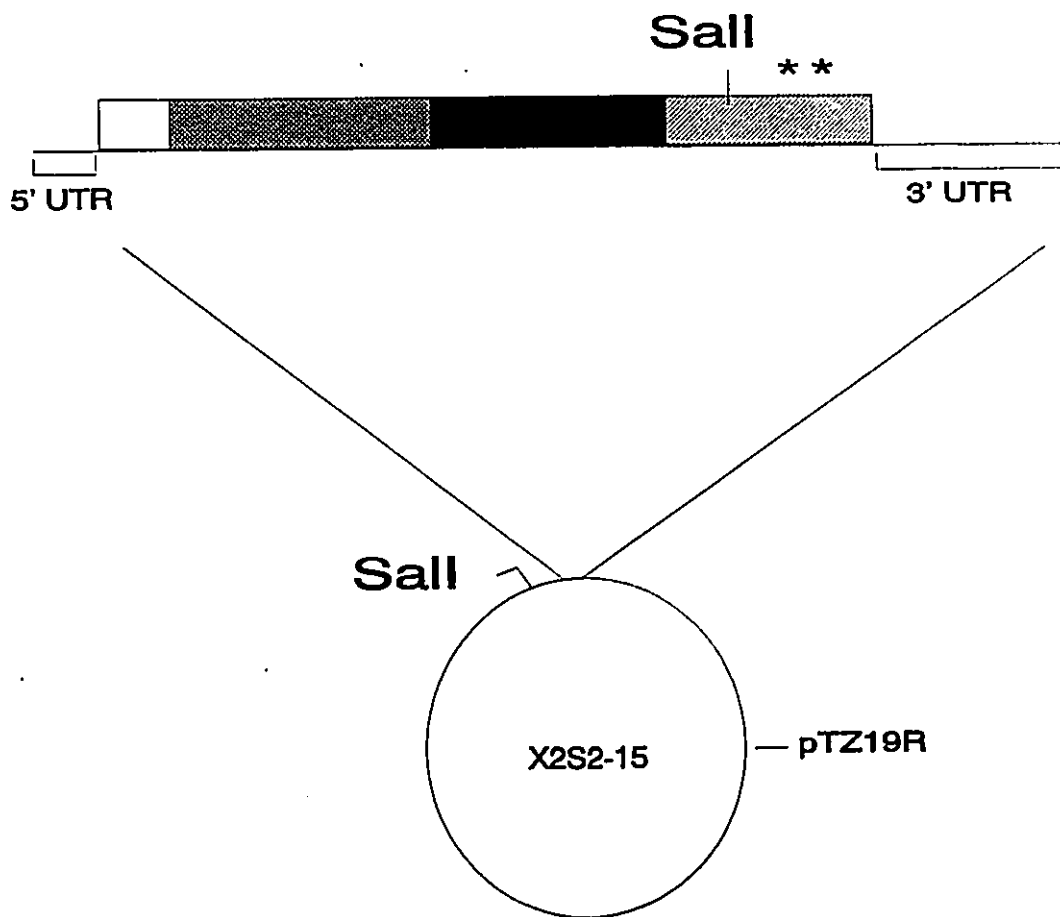
A².3 Summary

The CAP-1 PTPase encoding cDNA was altered to generate PTPase minus mutants; once transfected into mammalian cells these constructs can direct the expression of mutant CAP-1myc fusion protein to be used in immunoprecipitation and immunofluorescence studies. The deletion mutant, pMT29Δ, is missing the C-terminal portion of the putative regulatory region as well as the initial two-thirds of the catalytic domain (Figure A².1). This sequence predicts the production of a catalytically inactive 67 KDa protein. Mutation of the essential residue [Cys], required for catalysis, to a Ser [HCSAG to HSSAG] was carried out by PCR-generated substitution of two nucleotides (Figure A².2). The myc epitope was inserted into the HSSAG background to permit the investigation of exogenous CAP-1 subcellular localization and phosphorylation state with the known αmyc monoclonal antibody. These constructs should prove useful in further characterization of CAP-1 function.

Figure A².2 Use of a PCR Strategy to Generate a PTPase Minus CAP-1.
A PCR strategy was used to generate site directed mutagenesis of the catalytic region of CAP-1; an essential Cys in the HCSAG sequence was mutated to a Ser [HSSAG]. In this procedure, a PstI site was altered to XhoI by the priming of a PCR reaction from two mutant oligonucleotides. These primers have substitutions, denoted by **, in two adjacent nucleotides (gc to cg). This PCR reaction used a circular P19 CAP-1 cDNA/pTZ19R construct as template. Digestion with XhoI, religation, and insertion of the upstream CAP-1 sequence generated the full length CAP-1**/pTZ19R construct, X2S2-15.



- 1) XhoI digestion
- 2) religation
- 3) insertion of upstream
Sall CAP-1 fragment



APPENDIX III

Generation of a CAP-1/MAP Specific Polyclonal Antibody

A³.1 Introduction

Northern analysis has identified multiple CAP-1 mRNA species in a variety of mouse tissues (Chapter 2 and 3). These CAP-1 transcripts may be translated into alternative enzyme isoforms. The generation of a CAP-1 specific polyclonal antibody is required to determine whether this pattern and distribution of CAP-1 mRNA expression is reflected at the protein level. The novel multiple antigen peptide (MAP) system is known to provide a consistent and highly effective approach for peptide-based antibody production (Tam et al, 1989). An animal, immunized with a CAP-1 directed MAP, produced antibody (antibodies) which reacts specifically against the GST/CAP-1 fusion. This antiserum was also found to detect a mostly insoluble protein of the expected molecular weight (110-120 kDa) from CAP-1 transfected and untransfected cells.

A³.2 Materials and Methods

A³.2-1 Synthesis of a CAP-Specific Synthetic Peptide

A CAP-1 specific synthetic peptide was generated in context of the multiple antigen peptide (MAP) system (Tam, 1988; 1989). This CAP-1/MAP has an octa-branched peptide arrangement (last 14 residues in CAP-1; Figure 2.8) uniformly synthesized as a single unit on a 7 lysyl core ("tree"). The automated MAP synthesis (Applied Biosystems; Model 431-A) and manual cleavage was performed by Mr. R. Marius using "tBoc" (t-butyloxycarbonyl) resin and "Fmoc" (9-fluorenylmethoxycarbonyl) chemistry as outlined in the

Applied Biosystems' manual. The CAP-1/MAP was cleaved from the "tBoc" resin by TFMSA/TFA and upon solubilization in 5% NH_4HCO_3 at 20°C, was desalted on a gravity column. The pre-equilibrated G50 Sepharose column was loaded with the resolved CAP-1/MAP and 500 μl fractions were collected. The peptide concentration was monitored using the Biorad protein determination miniassay. The CAP-1/MAP-containing fractions were combined and lyophilized in a speed vacuum apparatus (without heat). A fraction of this solid was sent to Christina Lyons at Queen's University for amino acid composition analysis.

A³.2-2 Immunization of the CAP-1/MAP Antigen into Animals

The CAP-1/MAP antigen was injected into a New Zealand white male rabbit. This animal received approximately 150 μg intradermally, while 750 μg was introduced via an intramuscular route. PBS solubilized synthetic peptide (CAP-1/MAP) was emulsified in Freund's complete adjuvant (Pierce) for all initial intramuscular injections while the booster needles contained a 1:1 mixture of Freund's incomplete adjuvant (Pierce) plus antigen. In all cases, CAP-1/MAP + RIBI was injected at multiple intradermal locations with about 100 μl per site. The initial injection (at day 0) was followed by booster injections at 6 week intervals. Blood was collected from the marginal ear vein and the serum was prepared, diluted, and used in Western blotting analysis as described in Section A⁴.2-2. The injections and bleedings were performed by Dr. J.C. Bell et al (refer to the Acknowledgements)

using standard animal care procedures (Harlow and Lane, 1989).

A³.2-3 Specific Immunoreactivity of the α CAP-1/MAP Antibodies

Bacterial extracts expressing the GST/CAP-1 fusion protein (10 μ g) were run on a 10% SDS-PAGE and transferred to nitrocellulose (Section 4.2-3). Blood was collected from a rabbit a week following the second CAP-1/MAP booster injection. Triplicate filters were exposed to the subsequent antisera (1:50 dilution) with or without preadsorption to 30 μ g of the CAP-1/MAP (Section A³.2-1) or esk/MAP (Douville and Bell, unpublished) peptides for 1 hour at 4°C. Specific immunoresponsive bands were detected by exposing the ¹²⁵I-conjugated protein A (NEN)-treated filters to x-ray film.

A³.2-4 CAP-1 Expression in COS-1 Cell Extracts

Whole cell lysate and 1% Triton X-100 soluble ("Frack's") fractions (100 μ g) from pMT2 (CAP-1myc) and pMT21 vector transfected cells were separated on 10% SDS-PAGE. This protein was then electrophoretically transferred in duplicate and exposed to a 1:10 dilution of preimmune or immune (from a boosted rabbit) sera in 3% milk-containing TBST for 1 hour. The results were detected with an α rabbit alkaline phosphatase-conjugated secondary antibody (1/3000) as outlined in Section 4.2-3.

A³.3 Results

A³.3-1 Specific Immunoreactivity of the α CAP-1/MAP Antisera

A CAP-1 specific peptide was designed and used to generate a

polyclonal antibody. The final 14 carboxyl terminal amino acids of CAP-1 were synthesized on a lysine tree and the MAP-multiple antigen peptide-was injected into a rabbit. Sera, prepared from the CAP-1/MAP-injected animal was found to respond to this 24 kDa synthetic MAP peptide (data not shown). This antisera was also screened for a specific response against the GST/CAP-1 fusion protein (lane 1, Figure A³.1A). This immunoreactivity was competed out by pretreatment with the CAP-1/MAP (lane 3, Figure A³.1A) but not with an unrelated *esk*/MAP (lane 2, Figure A³.1A).

In anticipation of low endogenous CAP-1 expression, pMT2 (pMT2/CAP-1myc) overexpressed COS-1 cells were analysed for α CAP immunoreactivity. As expected, there was relatively more reactivity observed in the fractions obtained from the transfected cells (Figure A³.1B). The majority of both the exogenous and endogenous 110-120 kDa CAP-1 protein was found in the total (lanes 2 and 4, respectively, Figure A³.1B), versus the soluble fraction (lanes 1 and 3, respectively; Figure A³.1B). The nonimmune antibody did not detect these or any other bands (data not shown).

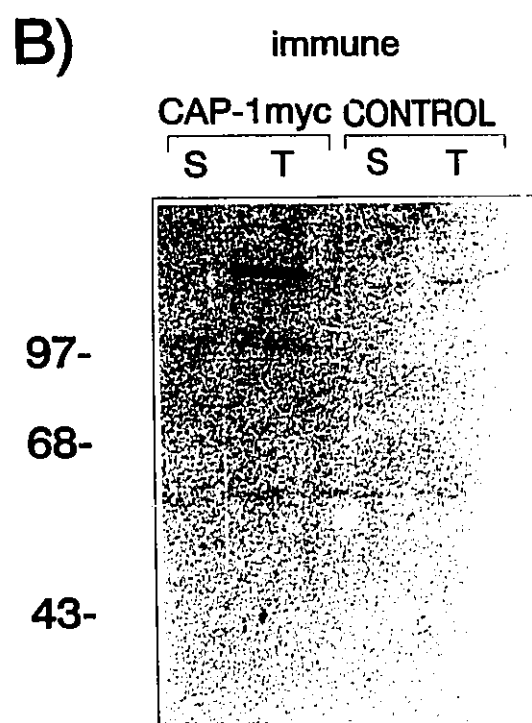
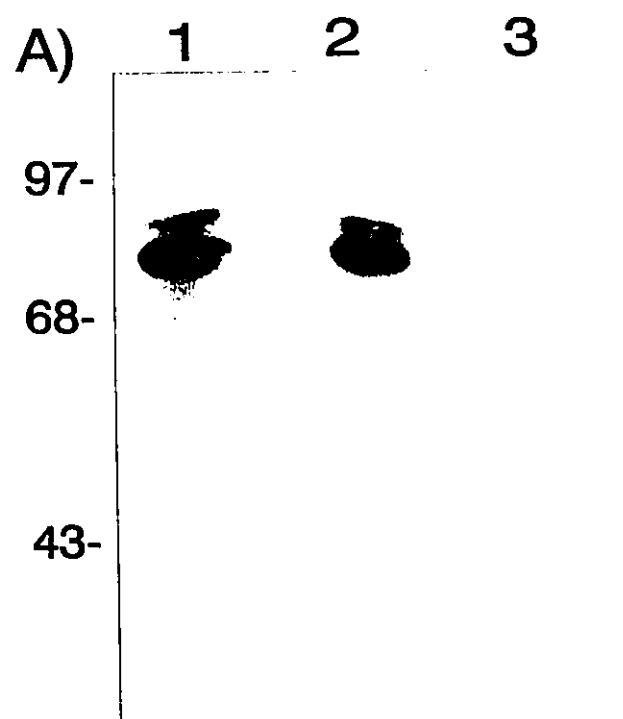
A³.4 Discussion

A CAP-1/MAP was used to raise polyclonal antibodies against the C-terminal region of CAP-1. This antisera was found to react specifically in a preliminary competition assay. Utilization of cleaved CAP-1 (or nonspecific *esk*) synthetic peptide in these experiments will ensure that the lack of immunoreactivity towards the GST/CAP-1 fusion is due to specific interaction of the peptide

with the α CAP-1 antibody [antibodies].

This antisera also recognized a 110-120 kDa band which is predicted to be the endogenous CAP-1 and exogenous CAP-1myc proteins. In this immunoblot analysis the myc epitope did not appear to interfere with the interaction between the CAP-1 epitope and the α CAP-1/MAP antisera. This result could be substantiated by competition experiments similar to these discussed above. In addition, transfection of a construct which harbors a deletion in the CAP-1 PTPase domain, would be useful in both Western and immunoprecipitation analysis. This deduced 67 KDa protein would produce a shift in α CAP-1/MAP immunoreactivity about 30 KDa smaller than that of the endogenous and full length exogenous CAP-1. Detection of a CAP-1 specific band or staining pattern in immunoprecipitation or immunofluorescence experiments, respectively would require high titer α CAP-1 antibodies or the affinity purification of this α CAP-1/MAP antisera. Specific recognition of endogenous CAP-1, should be useful in corroborating and extending the α myc tag experiments presented in Appendix V. Deletion mutants, including the one described above, may be employed along with these antibodies to define the N-terminal sequence(s) of CAP-1 required for the subcellular localization of this PTPase.

Figure A'.1 Specific immunoreactivity of the α CAP-1/MAP antisera.
A) GST/CAP-1 Δ fusion protein was prepared, resolved on SDS-PAGE, and transferred to nitrocellulose (Sections 4.2-2 and 4.2-3). These strips were treated with three separate preparations of the primary antibody. The diluted antisera used in this experiment were either incubated alone (lane 1), or pretreated with *esk*/MAP (lane 2), or CAP-1/MAP (lane 3) for one hour. The results were detected with the ^{125} I-conjugated secondary antibody. B) The immunoreactivity of the α CAP-1/MAP (immune) antibody was tested in a Western analysis. Triton X-100 soluble [S] or total lysate [T] fractions were obtained from CAP-1myc (pMT2) and vector control (pMT21) transfected COS-1 cells. The bands were visualized with an alkaline phosphatase-conjugated anti-rabbit [2 $^{\circ}$] antibody.



APPENDIX IV

THE BACTERIAL EXPRESSION OF CAP-1 AND PTP2 AS Type FUSIONS:

POLYCLONAL ANTIBODY PRODUCTION

A⁴.1 Introduction

The pattern of tissue-specific CAP-1 transcripts outlined in Chapter 2 and 3 may be reflected at the protein level. The investigation of these putative PTPase isoforms require the generation of CAP-specific antibodies. Many protocols utilize bacterially expressed fusion proteins as antigen. The trpE and FLAG systems were used due to the availability of commercial antibodies (i.e. α trpE or α FLAG) to the prokaryotic portion. These tools will allow monitoring of the rate of induction or of the expressed fusion during purification. The use of truncated CAP-1 fusions as immunogen may initiate the investigation of cellular CAP-1 protein expression and function.

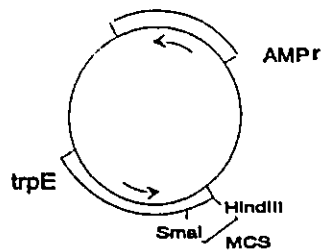
A⁴.2 Materials and Methods

A⁴.2-1 Construction of TrpE/PTPase-Encoding Plasmids

Partial PTPase clones were inserted in frame into the pATH expression vector (a generous gift from Dr. T. Pawson; Diekmann and Tzagoloff, 1985). This system is designed to generate bacterially expressed trpE fusion proteins (Sadowski et al, 1986). The EcoRI-digested 1.3 Kb cDNA (2453 to 3776 in CAP-1; Figure 2.8) was inserted into the EcoRI site of pATH1 to produce a trpE fusion with the C-terminal 217 amino acids of CAP-1 (trpE/CAP-1 Δ ; Figure A⁴.1). Restriction enzyme mapping and junction sequence analysis with the trpE primer (Appendix I) identified both sense [1-2 (+)] and antisense [1-11 (-)] constructs, the latter of which has a stop codon placed just after the trpE sequence. A partial 2.5 Kb cDNA

Figure A'.1 Construction of the trpE/CAP-1Δ fusion protein.

A partial CAP-1 cDNA (2453-3776 in CAP-1; Figure 2.8) was inserted into the EcoRI site of pATH1. The nucleotide and deduced protein sequence is given for the multicloning cloning site (MCS) of pATH1 and for the 5' border of the partial CAP-1 cDNA. The junction amino acid sequence (single letter code) of trpE and CAP-1Δ is provided with the extra residue underlined. The pictorial representation of the constructed plasmid includes estimated protein sizes, 3' UTR or untranslated regions, and the location of major CAP-1 and trpE stop codons. This illustration has been modified from Diekmann and Tzagoloff, 1985 and Sambrook et al, 1989.



pATH1 MCS

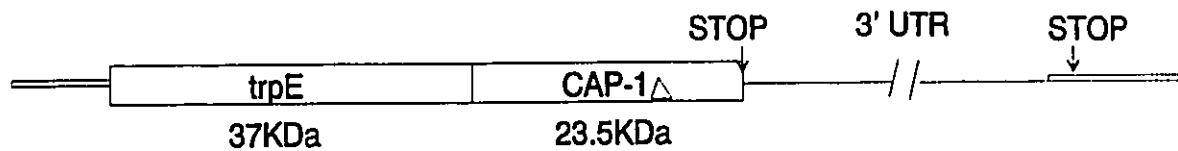
$\overbrace{\text{...ATC CCC GGG}}^{\text{SmaI}}$
 $\overbrace{\text{CGA GCT CGA}}^{\text{SacI}}$
 $\overbrace{\text{ATT CGA...}}^{\text{EcoRI}}$

Partial CAP-1 cDNA

5' GAATTC G GTG AAC ATG...
 EcoRI P V N M

trpE/CAP-1 Δ (+)

...I P G R A R I P V N M...



(Figure 2.9) of PTP2 was also inserted into the EcoRI site of pATH1 expression vector to generate the 2-8 (+) and 2-10 (-) constructs. The trpE/PTP2 Δ fusion predicts a combined protein size of 64,800 daltons. These partial CAP-1 and PTP2 cDNAs were cloned from the P19-derived library; there is an extra cytosine (C) in the EcoRI site of these λ gt11 recombinant phage (Figure A⁴.1).

A⁴.2-2 TrpE/CAP-1 Δ Fusion Protein Expression and Induction

These trpE/PTPase fusion proteins were expressed and induced according to a procedures outlined in Sadowski *et al* (1986) with a few minor modifications. Bacterial lysates were often fractionated into supernatant and pellet by centrifugation at 8K for 10 minutes. Aliquots of the supernatant and resuspended pellet fractions were resolved on 10% SDS-PAGE and transferred to nitrocellulose as indicated in Sections 4.2-2 and 4.2-3.

The trpE/PTPase clones were characterized by Western blotting analysis. The nitrocellulose strips were treated for 30 minutes in blocking buffer: 20% fetal calf serum (FCS) in TBST (10 mM Tris-HCl, pH 8.0; 150 mM NaCl; 0.05% Tween 20). The filters were then incubated with a 1:500 dilution (0.2 μ g/ml) of the anti-trpE mouse monoclonal primary antibody (α trpE Ab; Oncogene Science) in blocking solution. The α trpE Ab-reactive protein bands were identified using a goat anti-mouse secondary antibody (2^o Ab) alkaline phosphatase conjugate (IMMUNOSELECT; Gibco-BRL) and visualized by a color reaction using 5-bromo-4-chloro-3-indoyl phosphate (BCIP) and Nitro Blue tetrazolium (NBT) in alkaline

phosphatase buffer (AP buffer: 100 mM Tris, 9.5 pH ; 100 mM NaCl; 5 mM MgCl₂; substrates are from Gibco-BRL).

A⁴.2-3 Gel Purification of TrpE/CAP-1A and Antibody Production

The expression/induction protocol was scaled-up to generate large quantities of gel-purified fusion protein for use as antigen in polyclonal antibody production. A 250 ml-indoyleacrylic acid (IAA; Aldrich) induced culture was processed to yield 10 mls of bacterial lysate; an aliquot, or approximately 10%, of this was separated by 10% SDS-PAGE on a preparative 30-40 ml gel. A vertical SDS-PAGE strip was stained and then aligned, via several notches, with the unstained gel to ascertain the position of the fusion protein. Protein was liberated from the thin horizontal gel slices by electroeluting in a flatbed apparatus at 100 volts for 2 hours with 1X SDS Tank buffer (25 mM Tris base; 192 mM glycine, pH 8.3; 0.1% SDS). The eluted fusion protein plus co-electrophoresing protein contaminants were transferred to a fresh dialysis bag (Gibco BRL) and dialysed overnight against 20 mM NH₄HCO₃. The protein was dessicated by spinning in a speed vacuum apparatus and was resuspended in PBS (Phosphate Buffered Saline; Sambrook *et al*, 1989). The pooled eluates were evaluated by coomassie and silver reagent staining of SDS-PAGE and by Western blotting analysis (Section 4.2-3).

About 200 µg of the gel-purified fusion protein was mixed with Freund's complete adjuvant (Pierce) and inoculated into a New Zealand white male rabbit. Intramuscular and subcutaneous

injections were made in all four limbs and underneath the skin at the nape of the neck, respectively. At 26 day intervals, the animal received booster injections via the subcutaneous protocol with 50-100 μ g of a more stringently gel purified preparation. This antigen mixture was derived from the insoluble material outlined in Figure A'.2 to diminish the degree of contamination and from a 2 hour IAA-induced fusion protein to maximize expression levels. Blood, obtained from the rabbit's ear vein was allowed to clot for 1 hour at room temperature or overnight at 4°C. Sera (6-7 mls) was obtained from 20 mls of clotted blood by spinning for 10 minutes in a clinical centrifuge.

Dilutions of the antibody-containing sera were used to probe trpE/PTPase fusion and control extracts using the Western blotting protocol described above. Attempts were made to remove non-specific bacterial or α trpE-reactive antibodies by adsorbing immune and preimmune sera against filters coated with antisense pATH/CAP-1 (1-11) expressing extracts or by incubating with these bacterial lysates directly.

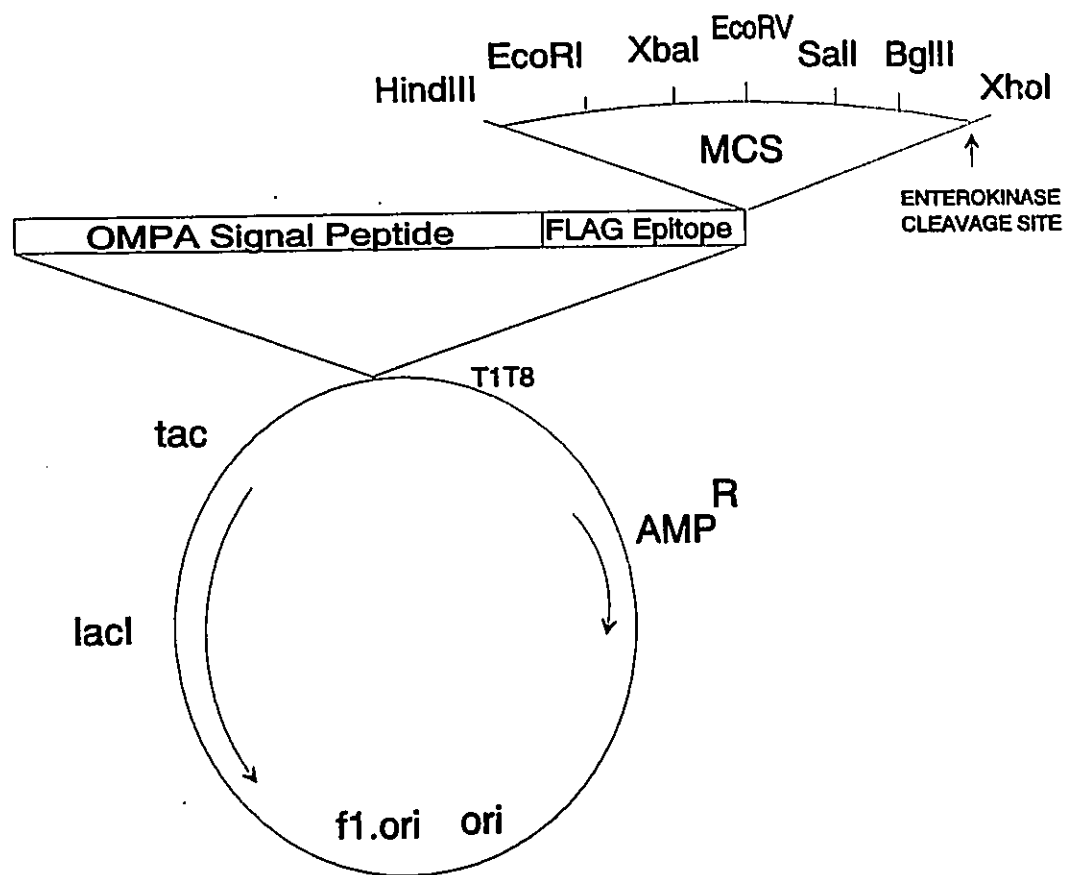
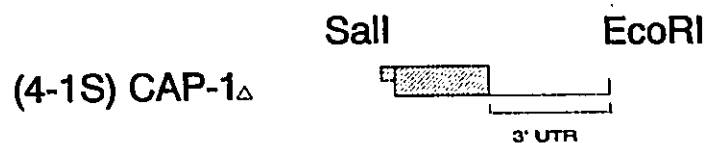
A'.2-4 Construction and Analysis of FLAG/CAP-1

Truncated CAP-1 and CAP-1 myc cDNAs were inserted downstream of the α FLAG immuno-responsive peptide. FLAG-1 was linearized with XhoI, treated with klenow (Section 2.2-2), and subsequently digested with SalI. First, the EcoRI site of the truncated CAP-1 myc (B8myc) and CAP-1 constructs (4-1S) was blunt-ended by incubating with klenow at 37°C for 30 minutes. These SalI liberated

980 or 950 bp fragments were then inserted into the CIP treated FLAG-1 vector (Figure A⁴.2). Positive clones were identified by miniprep restriction enzyme and sequencing analysis.

The FLAG/CAP-1Δ myc and FLAG/CAP-1Δ fusion proteins were verified by Western analysis using the commercially available αFLAG (International Biotechnologies, Inc.) and αmyc (Cambridge Research Biochemicals Ltd.) antibodies. FLAG construct-containing bacteria were grown to an OD₆₀₀ of 0.4 to 0.5 and induced for 4 hours with 0.8 mM IPTG. The cellular localization of the FLAG/CAP-1Δ fusions were determined by examination of the bacterial periplasmic, cytoplasmic, and membrane fractions (Sambrook *et al*, 1989). Bacterial pellets were treated with 1mg/ml lysozyme in 30 mM Tris-HCl, pH 8.0; 20% sucrose; 1.0 mM EDTA; and 200 μg/ml PMSF for 10 minutes on ice. A brief 1 minute spin at 12,000 xg released the periplasmic fraction into the supernatant. The pelleted material was lysed by freezing and thawing in 100 mM Tris-HCl, pH 8. Centrifugation at 13,000 xg for 5 minutes fractionated this suspension into soluble cytoplasmic proteins, while the sample buffer-solubilized pellet contained membrane proteins and insoluble inclusion body material. Aliquots of these fractions were separated on 15% SDS-PAGE and transferred to nitrocellulose as described in Section 4.2-3. One set of these filters were blocked with 3% BSA (Bovine Serum Albumin) in TBS + CaCl₂ and incubated with 10 μg/ml of αFLAG for 1 hour. Alternatively, 20% FCS + TBST blocked filters were treated with 10 μg/ml (1:1000 dilution) of the αmyc 9E10 antibody. Immunoreactive proteins were detected following specific

Figure A⁴.2 Construction of FLAG/CAP-1Δ and FLAG/CAP-1Δmyc fusions.
The SalI/blunt-end (EcoRI) fragments of 4-1S or B8myc were inserted downstream of FLAG into the SalI and blunted (XhoI) sites of FLAG-1. This diagrammatical representation of FLAG-1, which includes the: OMPA signal peptide, αFLAG M1 epitope, enterokinase cleavage site, and FLAG-1 multicloning site (MCS), was modified from that outlined in the FLAG system handbook; ICI.



PROTEIN 4.1
HOMOLOGY
REGION(CAP)

PUTATIVE
REGULATORY
DOMAIN

PTPase
DOMAIN

MYC
EPIOTOPE

¹²⁵I-conjugated secondary antibody binding after exposure to x-ray film.

A⁴.3 Results

A⁴.3-1 Induction of TrpE/CAP-1Δ and TrpE/PTP2Δ Fusion Proteins

CAP-1 and PTP2 are widely expressed at the mRNA level; however, subsequent experiments are required to examine the protein expression of these enzymes in mouse development and in adult tissues. This dictates the production of polyclonal antibodies with specificity for these enzymes. Initial attempts to obtain a CAP-specific antibody, involved the generation of a trpE/CAP-1Δ fusion protein (Figure A⁴.1). Induction of fusion protein from the sense [1-2 (+)] and antisense constructs [1-11 (-)] was verified by Western blot analysis using an αtrpE primary antibody. The trpE/CAP-1Δ fusion migrates to a position of 60,500 daltons on a 10% SDS PAGE (lane 4, Figure A⁴.3A). Expression from bacteria harboring the antisense (1-11) construct generated a band slightly below 43 kDa (lane 3, Figure A⁴.3A); this is a little larger than the relative size of trpE which runs at 37,000 daltons (data not shown). Practically all of the trpE/CAP-1Δ fusion is found in the insoluble pellet with low levels of αtrpE immunoreactivity present in the sonicate supernatant (lane 3 and 4, Figure A⁴.3B). Similarly, the insoluble trpE/PTP2Δ fusion protein (lane 2, Figure A⁴.3B) is visualized as a major αtrpE-reacting band near the 68 KDa marker (lane 2, Figure A⁴.3A), while the antisense construct directs a trpE protein migrating at about 37 KDa (lane 1, Figure

Figure A⁴.3 Expression of trpE/PTPase fusion proteins. Expression from the sense (+) and antisense (-) trpE/PTPase constructs was visualized by probing with the α trpE antibody A);B). A) Western analysis revealed the bacterially expressed trpE/PTP2 Δ (-), lane 1 and (+), lane 2; bacterial lysate-derived trpE/CAP-1 Δ (-), lane 3 and (+), lane 4; and gel-purified trpE/CAP-1 Δ , lane 5. B) The pATH constructs were induced and separated into soluble (sonicate supernatant) and insoluble (pellet) fractions. The soluble trpE/PTP2 Δ was run in lane 1, while the insoluble was shown in lane 2. The trpE/CAP-1 Δ was divided into soluble, lane 3 versus insoluble, lane 4 components. The migration position of the BRL molecular weight standards are shown.

A)

1 2 3 4 5

97-

68-

43-

29-

B)

1 2 3 4

A⁴.3A). The maximal level of trpE/PTPase expression was found to be after a 2 hour induction with IAA (lane 2, Figure A⁴.4). The percentage of soluble trpE fusion did not change appreciably with the use of different induction times (data not shown).

A⁴.3-2 Polyclonal Antibody Production

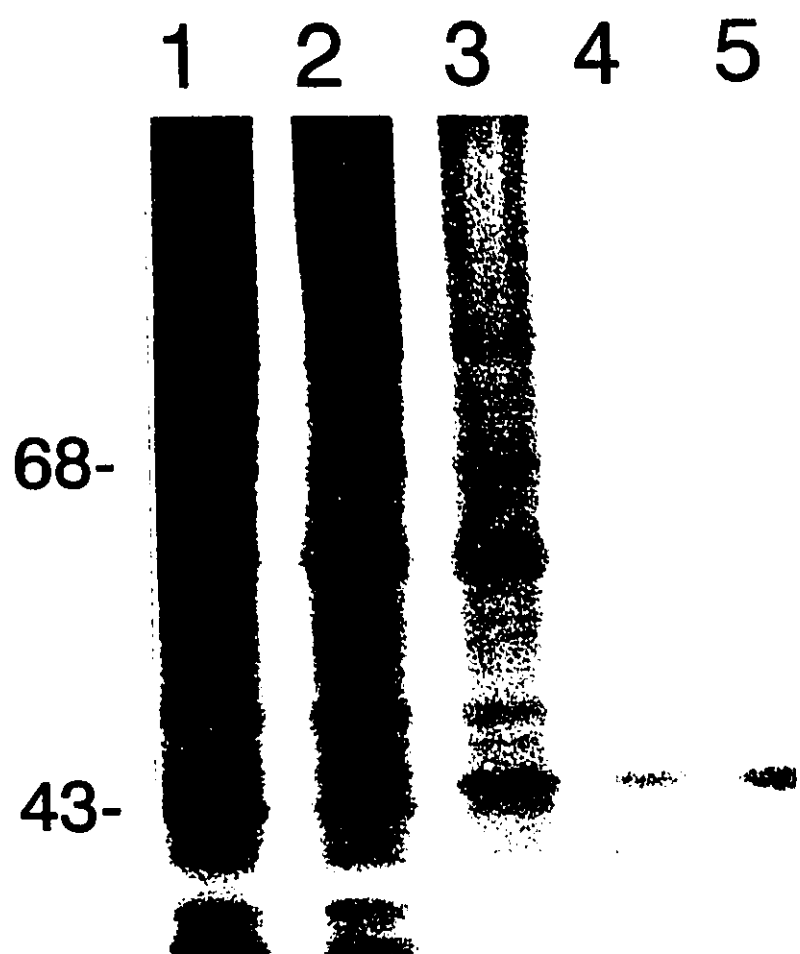
Induction of a CAP-specific response involved the injection [initial and booster] of a New Zealand white rabbit with gel-purified TrpE/CAP-1 Δ . This antigen preparation may have been contaminated with comigrating bacterial proteins. However, the major proteins detected by Western blot and silver stain analysis are the trpE/CAP-1 Δ fusion and a breakdown product (lane 5, Figure A⁴.3A, lane 4, Figure A⁴.4). The resulting serum (diluted to 1:10,000) was found to contain a very low titer of α CAP-1 reactive antibodies mixed with extremely high titers of α trpE immunoreactivity. Adsorption of antisera against bacterial and trpE protein(s) did reveal a strong immunoreactive protein migrating at 60,000 daltons. This band, however, was identified in trpE/CAP-1 as well as trpE/PTP2 (antisense) lanes indicating the presence of antibodies against contaminating bacterial proteins (data not shown).

A⁴.3-3 Analysis of the FLAG/CAP-1 Δ myc Fusion

Introduction of a myc epitope at the predicted C-terminal end of CAP-1, generated a mechanism by which to tag exogenous CAP-1 PTPase upon expression in mammalian cells. Truncated CAP-1myc cDNA

Figure A⁴.4 Induction of the trpE/CAP-1Δ fusion protein.

Induction of the trpE/CAP-1Δ fusion was detected by silver staining of an SDS-PAGE. Bacteria, expressing the trpE/CAP-1Δ fusion, received a one, lane 1; two, lane 2; four, lane 3; and six, lane 4; hour(s) induction period. The gel-purified trpE/CAP-1Δ is shown in lane 5. The migration position of the BRL molecular weight standards are shown.



was inserted downstream of FLAG to test the myc epitope in context of a FLAG/CAP-1 Δ myc fusion. This bacterially induced CAP-1, "sandwiched" between the FLAG and myc epitopes, was shown to be recognized by both the α FLAG (Figure A^{4.5A}) and α myc (Figure A^{4.5B}) antibodies in Western blot analysis. Although the FLAG/CAP-1 and FLAG/BAP (control) fusions were responsive to α FLAG, these fusion proteins were not recognized by α myc (data not shown; lanes 4 and 5, Figure A^{4.5B}). This specific recognition of the myc epitope predicts successful analysis of the full length CAP-1myc upon introduction into mammalian cells.

The bacterial expression of yet another CAP-1 fusion could also be useful in providing material for antibody production. The majority of the FLAG/CAP-1 Δ myc fusion was found in the insoluble or membrane fraction (lane A^{4.5A/B}). Cytosolic and periplasmic localized CAP-1 Δ fusion was detected with α myc immunoblotting analysis (lanes 2 and 3, Figure A^{4.5B}), however, the relative amount of soluble protein, revealed by coomassie staining (data not shown), was found to be minor. Since the FLAG/CAP-1 Δ was also identified to be mostly insoluble, this system, like that of trpE and possibly GST (Chapter 4), is perhaps not conducive for the preparation of a CAP-1 specific antigen.

A^{4.4} Discussion

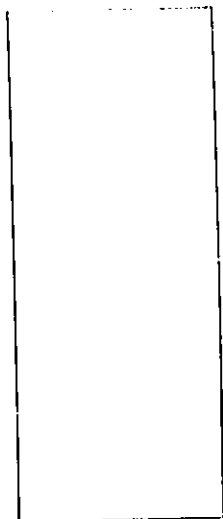
The trpE expression system employed here has been used successfully to raise antibodies against the PTKs. We originally thought that fusion of trpE to the C-terminal residues of CAP-1

Figure A'.5 Recognition of the myc epitope in context of a FLAG/CAP-1Δmyc fusion The FLAG/CAP-1Δmyc and control FLAG/BAP (bacterial alkaline phosphatase) fusion proteins were induced and fractionated. The SDS-PAGE separated P-periplasmic, C-cytoplasmic, and M-membrane fractions were electroblotted to nitrocellulose and detected with the A) αFLAG and B) αmyc antibodies.

A)

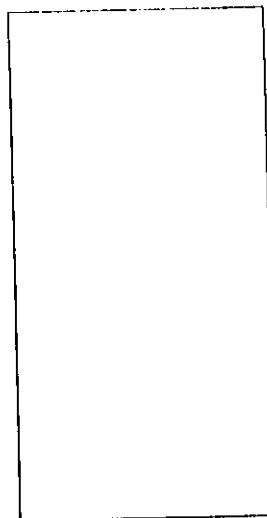
FLAG/
BAP

P C



FLAG/
CAP-1

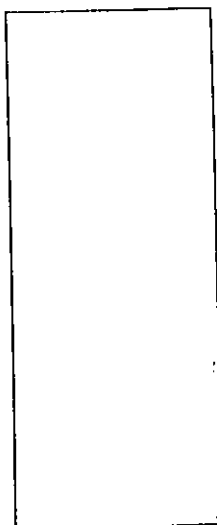
P C M



B)

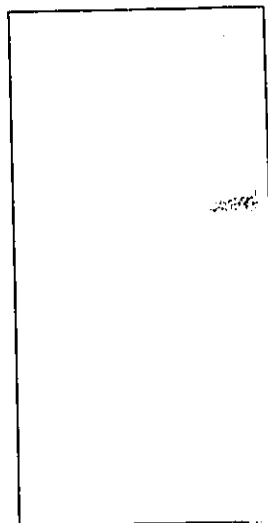
FLAG/
BAP

P C



FLAG/
CAP-1

P C M



PTPase domain would contribute to the solubility of trpE (Section 1.5-1; Guan and Dixon, 1991). Instead, the majority of the trpE/CAP-1 (and trpE/PTP2) fusion was found in the insoluble pellet. Subsequent gel purification generated a partially proteolyzed and a putative denatured product. Antigens, prepared in this manner, generally yield antisera that react preferentially with the denatured versus the native form of proteins (Sambrook et al, 1989); however, the antisera should still function as a powerful tool in the analysis of CAP-1 protein expression. Western analysis did detect the presence of a very high titer of α trpE antibodies in the antisera after boosting. This α trpE immunoreactivity was adsorbed out by the bacterial trpE lysate, however, the band recognized at 60 kDa was actually identified as comigrating bacterial protein.

Although, a great percentage of the injected (electroeluted) material was identified as trpE/CAP-1, the immunogenicity of CAP-1 was probably much less than that of trpE or of the contaminating bacterial protein. Adsorption against both the trpE and bacterial proteins and the subsequent use of solubilized trpE/CAP-1 in an affinity purification step would appear futile once the individual antibody titers are taken into consideration. The lack of detectable α CAP-1 antibodies in this antisera as well as the insolubility of trpE/CAP-1 and trpE/PTP2 impeded the immunization of another animal with these antigens. The high level of FLAG/CAP-1 Δ insolubility also prevented the use of this material in antibody production. CAP-1 antibodies were elicited by injecting animals with

the CAP-1/MAP synthetic peptide (Appendix III).

APPENDIX V

Immunofluorescent and Morphological Analysis of CAP-1myc-Expressing Cells

A⁵.1 Introduction

The N-terminal protein 4.1-like domain of CAP-1 predicts a function for this enzyme at the cytoskeleton/plasma membrane interface. Insertion of a small epitope C-terminal to CAP-1 would provide a means to probe the subcellular localization of exogenous CAP-1. The "myc tag" has been used successfully to target the location of int1- and of several luminal endoplasmic reticulum (ER)-myc fusion proteins by whole mount immunocytochemistry and immunofluorescence, respectively (Evan et al, 1985; McMahon and Moon, 1989; Munro and Pelham, 1987). The versatility of this tagging system should also allow the immunoprecipitation of the overexpressed CAP-1/myc PTPase (Kaptain et al, 1991).

This CAP-1myc fusion was transiently expressed in COS-1 cells to elucidate the localization and possible function of CAP-1. A CAP-1myc specific protein close to the predicted molecular weight (110-120 kDa) was immunoprecipitated from these ³⁵S-Met labeled cells. Concurrently, indirect immunofluorescence with an α myc antibody revealed a punctate, non-nuclear pattern of exogenous CAP-1myc expression in transfected COS-1 cells. Morphological changes, such as an increase in binucleation as well as the appearance of membranous structures and "giant" cells, were observed in COS-1 cells and in stable NIH 3T3 clones transfected with the CAP-1myc construct(s). The latter two morphological changes appear to be specific to CAP-1, as opposed to the general response of cells upon overexpression of PTPases (i.e. multinucleation; Cool et al, 1992; Woodford-Thomas et al, 1992). These changes in plasma membrane/

cytoskeletal structure may provide a clue as to the function of the endogenous CAP-1 enzyme.

A⁵.2 Materials and Methods

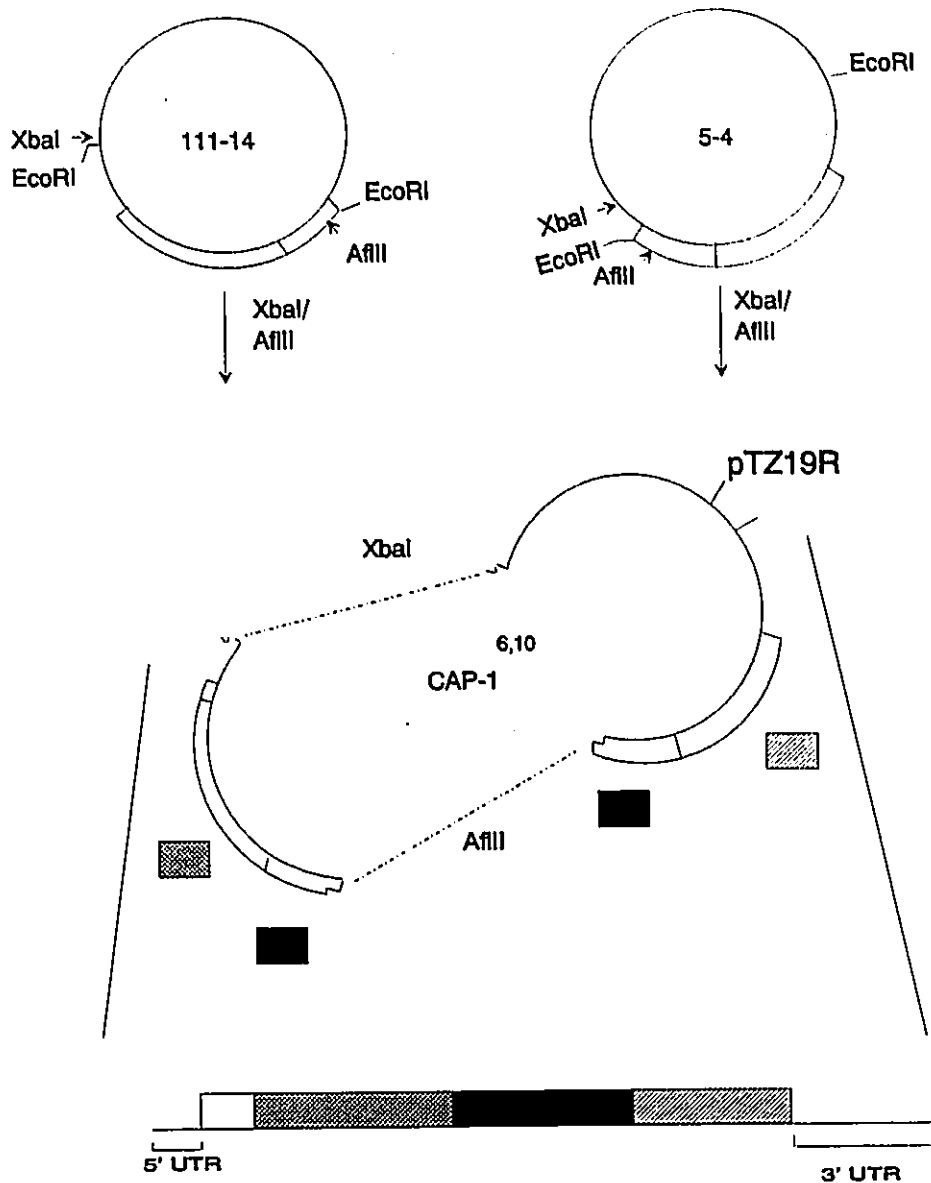
A⁵.2-1 Generation of the Composite CAP-1 cDNA Clone

The 3' portion of CAP-1 present in the 2.0 Kb P19 cDNA/pTZ19R plasmid (5-4, Table 2.4) and the subcloned 5' kidney cDNA (111-14, Table 2.6) were digested with AflIII (1926 in CAP-1; Figure 2.8) and XbaI (in the pTZ19R MCS, multicloning site). The 1956 bp fragment of 111-14 and a 4810 bp region of the CIP-treated 5-4 vector were ligated together to create a composite CAP-1 cDNA in pTZ19R (Figure A⁵.1).

A⁵.2-2 Myc Epitope-Tagging of CAP-1

Myc epitope-encoding oligonucleotides were annealed and inserted into the C-terminal BamHI site of CAP-1 (3083 in CAP-1, Figure 2.8). Approximately 2 μ g of the #35 and #36 primers (Appendix I) were 5'-end phosphorylated by T4 PNK in 50 mM Tris-HCl, pH 7.6; 10 mM MgCl₂; 5 mM DTT; 0.1 mM EDTA; 0.1 Spermidine; and 0.5 mM ATP for 30 minutes at 37°C. These complementary oligonucleotides were annealed by slow cooling at room temperature for 30 minutes following heat treatment at 65°C in TE (10 mM Tris-HCl, pH 8.0; 1 mM EDTA). BamHI cohesive ends, generated upon interaction of primers 35 and 36, promoted the ligation of 0.0175 pmol of this insert with 0.035 pmol of a BamHI/CIP treated partial cDNA/pTZ19R construct (4-1S; 2850 to 3776 in CAP-1, Figure 2.8).

Figure A⁵.1 Generation of the composite CAP-1 cDNA/pTZ19R plasmid.
A 1956 bp XbaI/AflIII fragment consisting of the 5' kidney cDNA (from 111-14) was ligated into the CIP-treated plasmid ,5-4, also having XbaI and AflIII ends. The composite cDNA is illustrated below with patterned boxes denoting deduced protein homology regions, while the single line represents 5' and 3' untranslated regions (UTRs).



PROTEIN 4.1	PUTATIVE	
■ HOMOLGY	REGULATORY	■ PTPase
REGION(CAP)	DOMAIN	DOMAIN

This plasmid (4-1S) was made by removal of a 430 bp SalI fragment from 4-1 (Table 2.4); SalI digests at the pTZ19R MCS and at 2850 in CAP-1 (Figure 2.8). Individual clones were identified as having a single myc insert in the correct orientation by sequencing analysis (Section 2.2-5). This truncated CAP-1 myc/pTZ19R construct (B8myc) was linearized with SalI and combined with the upstream 2880 bp fragment of the composite CAP-1 cDNA (1 to 2850 in CAP-1, Figure 2.8) to generate a myc tagged composite CAP-1 cDNA in pTZ19R (Myc-10, Figure A⁵.2).

A⁵.2-3 Insertion of CAP-1 into Mammalian Expression Vectors

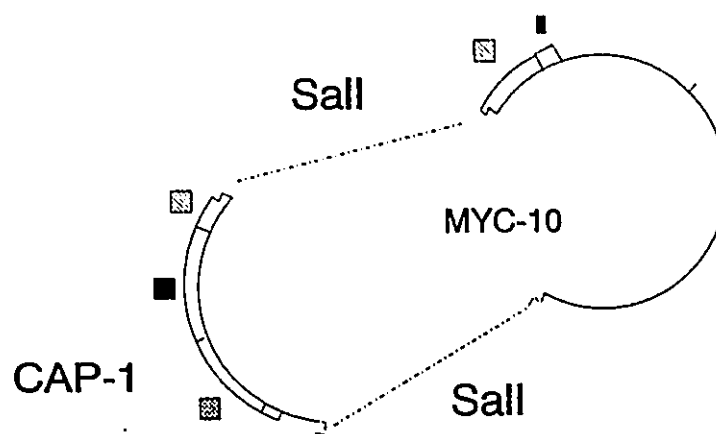
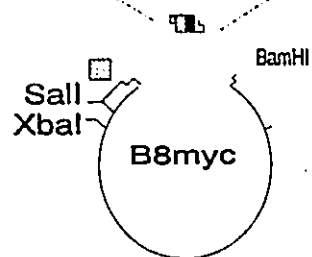
The EcoRI 3776 bp insert from the composite CAP-1myc/pTZ19R constructs were ligated together with EcoRI/CIP treated pMT21 (modified from Sambrook et al, 1989) and pECE vectors (Ellis et al, 1986). Insert positives, cloned in the correct orientation, were verified by restriction enzyme and sequencing analysis (Figure A⁵.3).

A⁵.2-4 Large Scale Plasmid Preparation

Plasmids utilized in this Section were prepared according to a modified large scale plasmid procedure outlined by Maniatis et al (1982). Briefly, the plasmid-containing bacteria were grown overnight in 500 mls of LB + 100 µg/ml of ampicillin. Bacterial pellets were lysed by a 10 minute incubation with 1 mg/ml of lysozyme in Solution I, followed by alkali lysis using Solutions II and III as outlined in Maniatis et al, 1982. The plasmid DNA was

Figure A⁵.2 Insertion of the myc epitope tag into CAP-1. The myc-epitope encoding oligonucleotides, once annealed, generate an insert with BamHI cohesive ends. This myc epitope was ligated into the BamHI site of the truncated CAP-1/pTZ19R construct, 4-1S to produce the construct, B8myc. A composite CAP-1myc cDNA was generated upon the insertion of the upstream portion or SalI fragment from the CAP-1/pTZ19R^{6,10} construct (Figure 5.1). The SalI enzyme digests at the multicloning site (MCS) in pTZ19R and at 2850 bp in CAP-1.

. . P S E Q K L I S E E D L S T O P
 gatccacgtgagcaaaagctgat t t ctgaggaggatctgt
 ggtaact og t t t t gactaagact cc tctagacactag
 BamHI BamHI
 ENDS ENDS



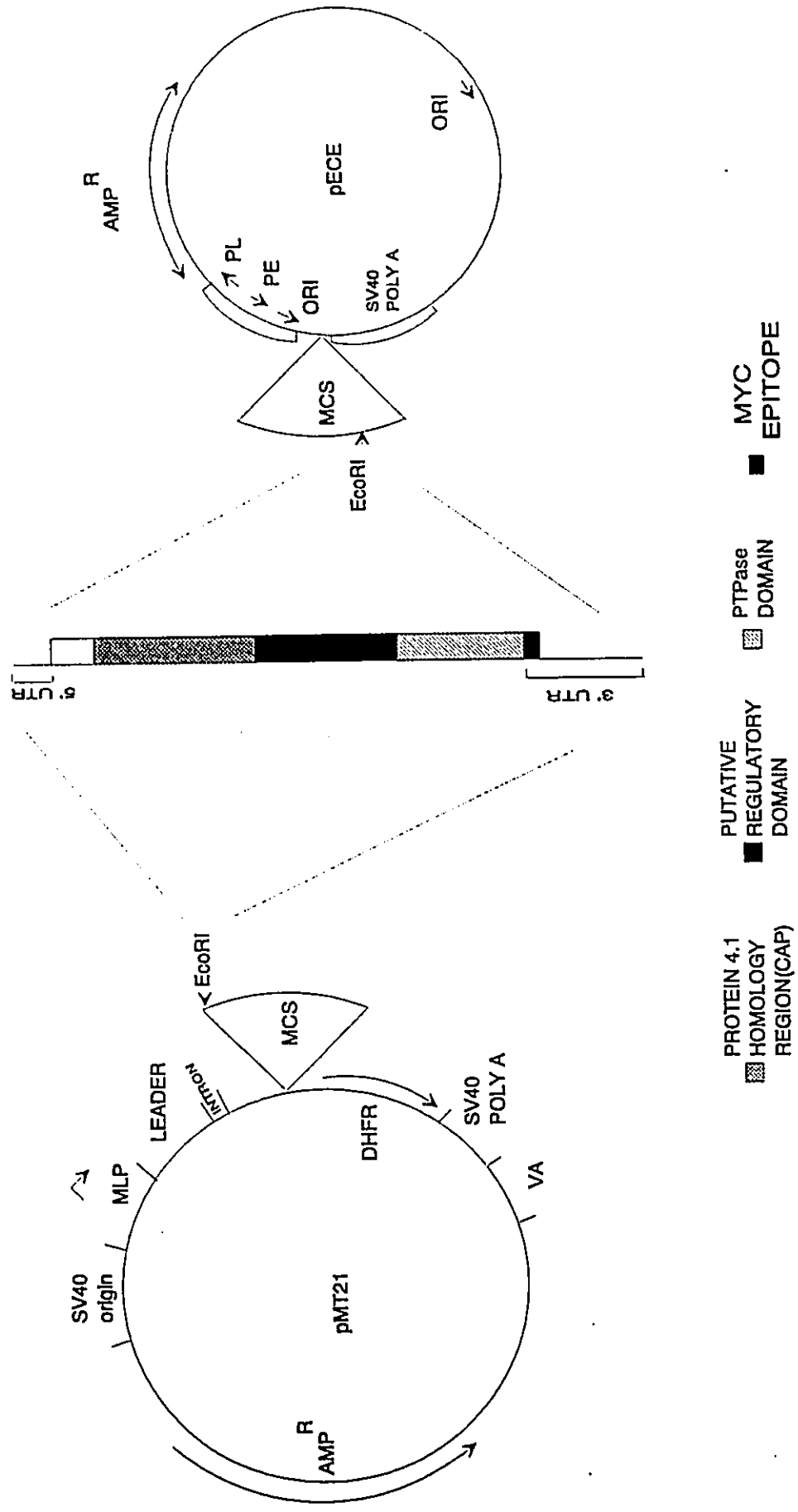
PROTEIN 4.1
 HOMOLOGY REGION(CAP)

PUTATIVE
 REGULATORY DOMAIN

PTPase
 DOMAIN

MYC
 EPI TOPE

Figure A⁵.3 Insertion of CAP-1 into mammalian expression vectors.
The insertion of CAP-1myc into the mammalian expression system is represented here in a simple diagram. The pECE and pMT21 vectors are as illustrated as given in Ellis et al, 1986 and modified from Sambrook et al, 1989, respectively. The 5' and 3' UTRs and deduced protein homology regions of CAP-1 are illustrated as before.



precipitated by isopropanol (0.6 volume), followed by several rounds of precipitation, first with 2.5 mM NH_4OAc to remove the salts and crude material, and subsequently with 2 volumes of cold ethanol to obtain the DNA. This TE-resuspended DNA (7 mls) was treated with 10 $\mu\text{g/ml}$ of RNAase and then precipitated by a 30 minute incubation on ice upon the addition of 2.5 mls of 5M NaCl and 3 mls of 1.5 M NaCl; 30% PEG (polyethylene glycol). The plasmid DNA was, in this and all precipitation steps, collected by a 20 minute centrifugation at 8000 xg. The resuspended DNA was then incubated with 50 $\mu\text{g/ml}$ of proteinase K in 10 mM Tris-HCl, pH 8.0; 5 mM EDTA; 0.5% SDS for 30 minutes at 37°C. The proteolyzed material was then removed by several Phenol/Chloroform steps and the DNA was subjected to a final ethanol precipitation. The cDNA constructs obtained from this procedure was now ready to use upon estimation of the DNA concentration from $\text{OD}_{260/280}$ values.

A⁵.2-5 Transient Transfection of CAP-1 Constructs into COS-1 Cells

COS-1 cells were transiently transfected according to a DEAE Dextran/Chloroquin protocol with minor modifications (Kaufman et al, 1989). COS-1 cells were trypsinized with 1.5 mls of 0.025% trypsin in PBS + 1 mM EDTA and seeded at 60,000/ 60 mm dish in αMEM (Gibco-BRL) + 10% calf serum. These cells were allowed to recover for 16-24 hours at 37°C in 5% CO_2 . The following day, these cells were washed 2X with serum-free αMEM and covered with 0.25 mg/ml of DEAE Dextran (3.6 ml) in αMEM . A suspension of plasmid DNA (4 μg) in 400 μl of sterile 1M Tris-HCl, pH 7.3 was dripped over the cells

and gently mixed. Following a 12 hour incubation in the DNA/DEAE Dextran mixture, the cells were washed 2X with serum-free α MEM. The cells were then shocked with 1ml per dish of 10% DMSO in HBS (42 mM HEPES, pH 7.05; 0.27 M NaCl; 10 mM KCl; 1.9 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$; 11 mM dextrose); after 3 minutes residual DMSO was removed by washing with serum-free α MEM. Subsequently, these shocked cells were treated with 0.1 mM chloroquin in α MEM + 10% serum. Following incubation for 2 1/2 hours at 37°C, the cells were rinsed as before and grown for 24 to 48 hours in media + serum. These CAP-1 expressing COS-1 cells were then analyzed via Western blotting, immunofluorescence, and metabolic labeling.

A⁵.2-6 Immunofluorescence of CAP-1 myc

Transfected COS-1 cells were grown to subconfluence on coverslips precoated with 0.1% gelatin. The cells on these coverslips, after washing 2X with PBS, were fixed in -20 °C methanol for 5 minutes and allowed to air dry. Following a 15 minute rehydration in Stockholm PBS (137 mM NaCl; 8.1 mM Na_2HPO_4 ; 1.8 mM NaH_2PO_4 ; 2.7 mM KCl; pH 7.2), the fixed cells were permeabilized with 50 μ l of PBS + 0.2% Triton X-100 and incubated with or without the 9E10 α myc antibody (5 μ g/ml) in a humidity chamber at room temperature for 1 hour. These permeabilized cells were treated with a 1/50 dilution of the α mouse fluorescein-conjugated secondary antibody (Dako) for 30 minutes at 22°C. Unbound primary and secondary antibodies were removed by washing 3X with PBS for 5 minute intervals following each antibody incubation.

These coverslips were subsequently covered in mounting medium and stored at -20°C. This antifade medium was made by adding 50 mg p-phenylenediamine dissolved in 5 mls of 100 mM PBS, pH 7.2-7.4 to 45 mls of glycerin, pH 8.0 [pH was adjusted by a 0.5 M sodium carbonate/sodium bicarbonate solution, pH 9.0]. Fluorescent cells were detected by Zeiss (ZF) microscopy and photography was carried out with the Zeiss camera using 400 HC color film.

A⁵.2-7 Immunoprecipitation of CAP-1myc from ³⁵S-Met Labeled Cells

COS-1 cells were transiently transfected with CAP-1myc or expression vector controls as described in Section A⁵.2-5. Metabolic labeling, after 48 hours, featured a 1 1/2 hour incubation in Met-free medium (ICN Biochemicals Inc.), followed by growth in 1ml of Met-free medium +10% serum with 100 µCi ³⁵S-Met (NEN) for 6 1/2 hours at 37°C. After removal of excess radioactivity with PBS, cells were harvested in 500 µl of a lysis buffer ("Frack's" solution; 10 mM Tris-HCl, pH 7.5; 1 mM EDTA; 150 mM NaCl; 1% Triton X-100; 200 µg/ml PMSF; 5 µg/ml leupeptin; 2 µg/ml aprotinin) and lysed by passing through a needle. The triton X-100 soluble material was collected by brief centrifugation at 13,000 xg. A fraction of this labeled material (75 µg) was examined by immunoblotting.

Immunoprecipitation of exogenous CAP-1myc involved rotation of the αmyc antibody (2.5 µg) with 300 µl (990 µg) of the "Frack's" solubilized lysate for 2 hours at 4°C. Meanwhile, 3 µl (7.2 µg) of an affinity-purified Rabbit αmouse antibody was incubated with 50

μ l of prewashed protein A Sepharose beads (Pharmacia) for 30 minutes. Following removal of the unbound secondary antibody with "Frack's", the surface of the beads were blocked with 0.7% BSA (Fraction V; Boehringer Mannheim) and once again pre-equilibrated with "Frack's". These pretreated beads were incubated with the α myc-bound COS-1 proteins for 2 hours at 4°C. Washed beads were boiled in 50 μ l of sample buffer for 3-5 minutes. The eluted immunoprecipitated proteins were separated on 10% SDS-PAGE and exposed to x-ray film.

A⁵.2-8 Stable Transfection of CAP-1myc Constructs

The CAP-1myc constructs were stably expressed in NIH 3T3 cells using a standard Calcium Phosphate transfection procedure (Graham and van der Eb, 1973). Briefly, 500,000 cells were seeded into a 60 mm dish and allowed to recover in α MEM + 10% serum. The following day, approximately 12 μ g of the pE3 or pMT2 CAP-1myc constructs were introduced along with 1.4 μ g of PGK/Puromycin (P. Laird, personal communications) into these cells. A DNA/125 mM CaCl_2 , final suspension was added dropwise to an equal volume of 2X BBS solution (50 mM BES; 280 mM NaCl; 1.5 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$; pH 6.96) while gently mixing and the precipitate was allowed to form at room temperature for 20 minutes. This CaPO_4 /DNA precipitate was added dropwise to the cells and carefully mixed thoroughly. Following 8 hours incubation, these cells were washed with PBS and fresh medium was added. Two days later, the cells were trypsinized and seeded at 10^5 /150 mm dish. The medium + 4 μ g/ml puromycin was added at this

time and was changed every 3-4 days until the puromycin resistant colonies were ready to be picked. Several colonies were selected and grown in 24 well dishes and ultimately seeded into a 60 or 100 mm dish for subsequent analysis.

A^{5.3} Results

A^{5.3-1} Immunoprecipitation of the myc-tagged CAP-1 PTPase

Transiently expressed CAP-1myc protein was identified in metabolically labeled COS-1 cell extracts. Immunoprecipitation with the 9E10 α myc antibody revealed a 110-120 kDa band present in pMT2 and pE3 or CAP-1myc-transfected cells (lanes 2 and 3, respectively, Figure A^{5.4}); this band was not observed in the vector control (lane 1, Figure A^{5.4}). A faint signal was also detected at 110-120 kDa by the α myc antibody in a Western analysis; in a similar study, a vanadate-sensitive phosphatase was immunoprecipitated with the α myc antibody (data not shown).

A^{5.3-2} Indirect Immunofluorescence of CAP-1myc in COS-1 cells

The CAP-1myc constructs were transiently expressed in COS-1 cells in an effort to determine their localization and possible function. Immunofluorescence using antibodies directed against the myc tag revealed a discontinuous pattern of staining in the cytoplasm (Figure A^{5.5}). In addition, a subpopulation of the cells appears to have developed microvillar or membrane extensions. The highest level of expression correlated with a filamentous-like pattern extending into a membrane projection (Figure A^{5.5B'}). The

Figure A³.4 Immunoprecipitation of CAP-1myc from COS-1 cells.
COS-1 cells were transiently transfected with the vector pMT21 alone, lane 1; with pMT2, CAP-1myc in pMT21, lane 2; or with pE3, CAP-1myc in pECE, lane 3. These cells were labeled with ³⁵S-Met and the lysed protein was immunoprecipitated with the α myc antibody. The CAP-1myc 110-120 kDa band is indicated as an arrow. The migration position of the BRL protein standards are shown.

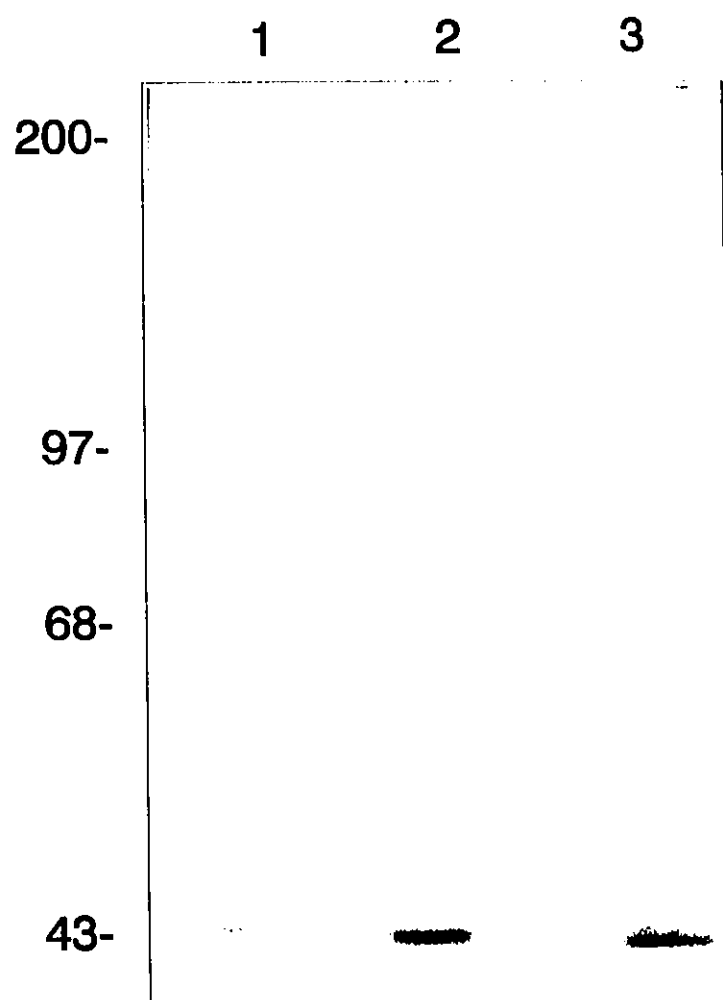
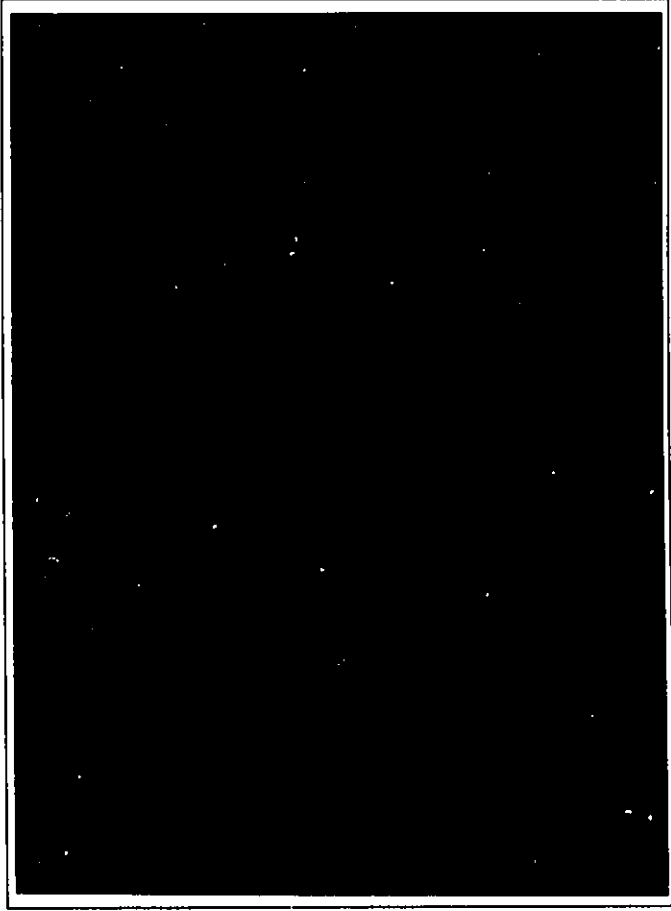
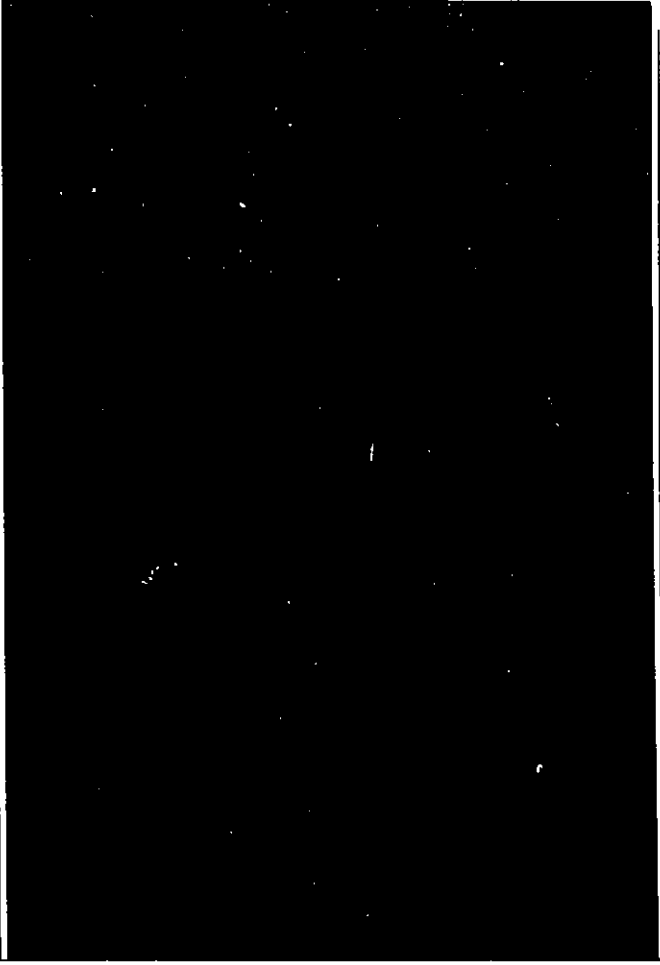


Figure A⁵.5 Indirect immunofluorescence of COS-1/CAP-1myc cells. COS-1 cells were transiently transfected with the CAP-1myc [pMT2] construct. These cells were stained with a primary α myc monoclonal followed by a FITC-labeled anti-mouse secondary antibody. The results were visualized on a Zeiss Fluorescence microscope. The first field of view (A/A') was examined at 40X (A) and 100X (A') magnification. The 40X objective was utilized to examine the cells observed in B, while the same field was seen a magnification of 100X in B.

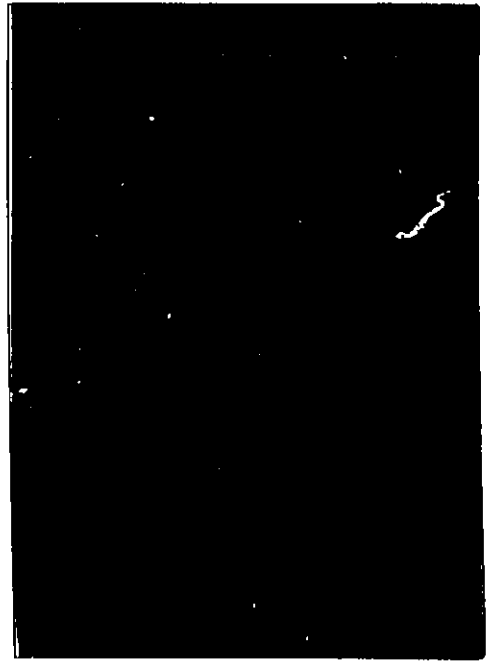
A



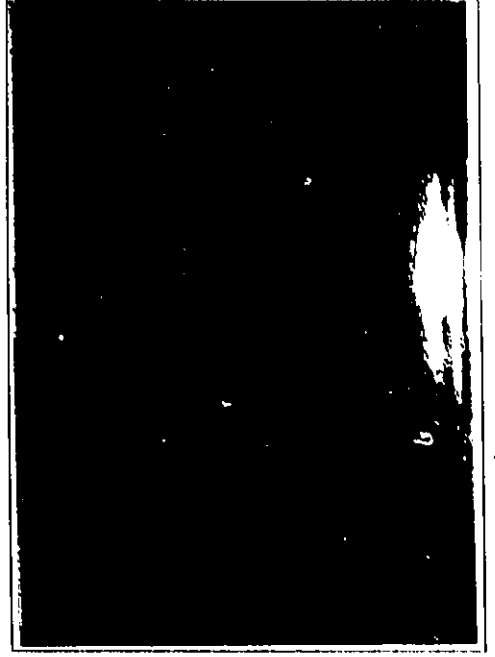
B



A'



B'



CAP-1 expression is not found directly within the submembranous region of the filopodia-like extensions (Figure A⁵.5A').

Overexpression of CAP-1myc in COS-1 cells induced several gross morphological changes. COS-1 cells overexpressing moderate to high levels of CAP-1 tend to exhibit a bi- and multinucleated phenotype (Figure A⁵.5B). This increase in the relative # of nuclei/cell upon the overexpression of an active PTPase has been documented previously and appears to be due to a disruption in cytokinesis; this phenotype also exhibits asynchronous nuclear division (Cool *et al*, 1992; Woodford-Thomas *et al*, 1991). There is evidence for these events occurring here (Figure A⁵.5B).

A⁵.3-3 Stable CAP-1myc clones Exhibit Morphological Changes

NIH 3T3 cells, were stably transfected with CAP-1myc (pECE or pMT21) constructs. A percentage of the selected clones were found to exhibit abnormal properties. Several aspects of the morphological changes observed in the transient COS-1/CAP-1myc cells were also observed in these clones. These clones appeared to contain relatively high levels of bi- and multinucleated cells (Figure A⁵.6); a count of several randomly chosen fields revealed that about 24% and 36% of the cells have 2 or more nuclei, this is considerably higher than that seen in untransfected NIH 3T3 cells (4% and 5%). In addition, a subpopulation of cells maintain a "giant-cell" phenotype which exhibits a large cytoplasmic area and membrane outgrowths (Figure A⁵.6A and B). These morphological changes are not simply an exaggerated version of the "normal" NIH

Figure A⁵.6 Morphological changes induced in NIH 3T3/CAP-1myc clones. NIH 3T3 cells were transfected with both CAP-1myc constructs [PE3 and pMT2]. Phase contrast microscopy was used to document the morphological changes observed in two individual clones (A/B and C, respectively). The cells shown in D are a representative field of untransfected NIH 3T3 cells.

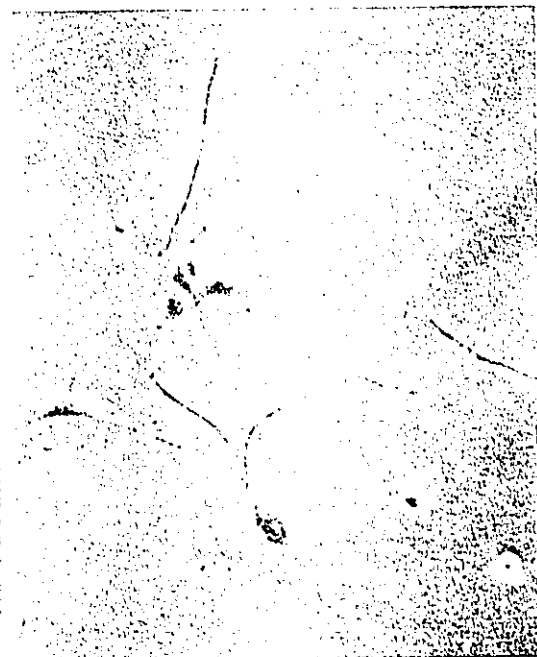
A



B



C



D



3T3 morphology; closer examination of this phenotype may provide a clue as to the function of the endogenous CAP-1 PTPase.

A⁵.4 Discussion

A CAP-1myc fusion was utilized to tag the expression of this enzyme in mammalian cells. The α myc monoclonal antibody (MAb) successfully immunoprecipitated a protein close to the predicted molecular weight (110-120 kDa) specifically from the CAP-1myc transfected COS-1 cells. It would be of interest to investigate the phosphorylation state of the immunoprecipitated CAP-1 from in vivo (orthophosphate) labeled cells. The presence of many putative phosphorylation sites in the central domain of CAP-1/PTPH1 is indicative of a putative regulatory function for this domain (Yang and Tonks, 1991; Chapter 2). The PTPases, CD45 and PTP1B, are both phosphoproteins (Autero and Gahmberg, 1987; Patarroyo and Gahmberg, 1984; Flint et al, 1993). Increased phosphorylation of PTP1B on serine residues is found to accompany entry into mitosis; however it is not known whether this modifies the substrate specificity or activity of the phosphatase (Flint et al, 1993). The level of endogenous PTPase activity has been shown to increase as cells in culture reach confluence indicating a role for certain dephosphorylation events in contact inhibition and growth control (Pallen and Tong, 1991; Ramponi et al, 1992). A preliminary study has attributed phosphatase activity to α myc immunoprecipitated protein from the CAP-1myc transfected cells. Substantiation of this result may also involve examination of PTPase activity in CAP-1myc

expressing cells at various stages of confluence and during the cells cycle. This will initiate elucidation of the specificity and normal regulation of the endogenous CAP-1.

CAP-1myc transfected cells appear to exhibit two classes of morphological changes. The observed increase in bimultinucleated cells seem to reflect a general event precipitated by the overexpression and/or deregulation of a PTPase (Cool *et al*, 1992; Woodford-Thomas *et al*, 1992). The formation of membranous projections as seen in CAP-1 expressing COS-1 cells and the presence of "giant cells" in several pMT2- and PE3-generated NIH 3T3 clones is quite possibly unique to CAP-1. It would be interesting to see if this phenomenon is a result of the dephosphorylation of a cytoskeleton/membrane protein found normally associated with this enzyme.

The immunofluorescence pattern of the exogenous CAP-1 reveals a conspicuous absence of staining at the tips or underlying membrane region of these processes (Figure A⁵.5A/A'). The CAP-1 N-terminal domain similarity to the membrane association region of the protein 4.1 family, would predict a submembranous location for this enzyme. It is possible that the high level of CAP-1 expression or the gross alteration of morphology is preventing a clear estimation of the true subcellular location for this enzyme. At a technical level, electroporation of the CAP-1myc construct or alternatively the introduction of CAP-1myc into an inducible system may improve the outcome somewhat.

Introduction of CAP-1 PTPase minus mutant constructs (Appendix

II) into these cells should eliminate the general PTPase-induced changes (i.e. multinucleation). This would also indicate whether the formation of the membrane processes require the CAP-1 specific dephosphorylation of a cytoskeletal/plasma membrane component. There is precedence for the involvement of dephosphorylation events in regulating the motive forces that govern the specialized structure and function of the cell. For instance, ezrin phosphorylation (at Ser and Tyr) is correlated with its redistribution into newly formed membrane ruffles and as well, the nature of the mutated serine residue in GAP-43 is related to the extent of cell spreading, generation of irregular membrane extensions or appearance of filopodia (Bretscher, 1989; Widmer and Caroni, 1993).

Investigation of the extent of cytoskeletal disruption, in both the COS-1/ and NIH 3T3/CAP-1myc cells, may provide a clue as to its normal function. Analysis of CAP-1myc staining with the Confocal microscope will allow a more definite localization of CAP-1 expression and will document more clearly the cytoskeletal and membrane changes which occur upon CAP-1 expression. These experiments would require the double staining of the CAP-1myc expressing cells with the α myc antibody and with stains or antibodies specific to components of the cytoskeleton (i.e. actin, tubulin). This would also reaffirm that the morphology of the NIH 3T3 clones is related to the expression of the CAP-1 PTPase. Elucidation of the "normal" function of CAP-1 will require determination of the subcellular localization of the endogenous

CAP-1 with a CAP-specific antibody (Appendix III).

REFERENCES

- Abraham, N., M.C. Miceli, J.R. Parnes and A. Veillette. 1991. Enhancement of T-cell responsiveness by the lymphocytic-specific tyrosine protein kinase p56^{lck}. *Nature* 350:62-66.
- Adachi, M., M. Sekiya, T. Miyachi, K. Matsuno, Y. Hinoda, K. Imai and A. Yachi. 1992. Molecular cloning of a novel protein-tyrosine phosphatase SH- PTP3 with sequence similarity to the src-homology region 2. *FEBS Lett.* 314:335-339.
- Adamson, E.D. 1987. Oncogenes in Development. *Development* 99:447-471.
- Ahmad, S., D. Banville, Z. Zhao, E.H. Fischer and S.-H. Shen. 1993. A widely expressed human-tyrosine phosphatase containing src homology 2 domains. *Proc. Natl. Acad. Sci. USA* 90:2197-2201.
- Aitken, J.F., G.P.H. van Heusden, M. Temkin and W. Dowhan. 1990. The gene encoding the phosphatidylinositol transfer protein is essential for cell growth. *J. Biol. Chem.* 265:4711-4717.
- Algrain, M., O. Turunen, A. Vaheri, D. Louvard and M. Arpin. 1993. Ezrin contains cytoskeleton and membrane binding domains accounting for its proposed role as a membrane-cytoskeletal linker. *J. Cell Biol.* 120:129-139.
- Anderson, D., Koch, C.A., Grey, L., Ellis, C., Moran, M.F., and T. Pawson. 1990. Binding of SH2 domains of phospholipase C γ 1, GAP, and src to activated growth factor receptor. *Science* 250:979-982.
- Anderson, R.A. and R.E. Lovrien. 1984. Glycophorin is linked by band 4.1 protein to the human erythrocyte membrane skeleton. *Nature* 307:655-658.
- Anderson, R.A. and V.T. Marchesi. 1985. Regulation of the association of membrane skeletal protein 4.1 with glycophorin by a polyphosphoinositide. *Nature* 318:295-298.
- Argaves, W.S., K. Dickerson, W.H. Burgess and E. Kuoslahti. 1989. Fibulin, a novel protein that interacts with the fibronectin receptor β subunit cytoplasmic domain. *Cell* 58:623-629.
- Auffray, C. and F. Rougeon. 1980. Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumor RNA. *Eur. J. Biochem.* 107:303-314.
- Autero, M. and C.G. Gahmberg. 1987. Phorbol diesters increase the

phosphorylation of the leukocyte common antigen CD45 in human T cells. *Eur.J.Immunol.* 17:1503-1506.

Baecher, C.M., K.S. Dorfman, M.-G. Mattei and J.G. Frelinger. 1990.cDNA cloning and localization of the mouse leukosialin gene (Ly48) to chromosome 7. *Immunogenetics* 31:307-314.

Banerjee, U., P.J. Renfranz, J.A. Pollock and S. Benzer. 1987.Molecular characterization and expression of *sevenless*, a gene involved in neuronal pattern formation in the *Drosophila* eye. *Cell* 49:281-291.

Baniyash, M., P. Garcia-Morales, E. Luong, L.E. Samelson and R.D. Klausner. 1988.The T cell antigen receptor ζ chain is tyrosine phosphorylated upon activation. *J.Biol.Chem.* 34:18225-18230.

Bankaitis, V.A., J.R. Aitken, A.E. Cleves and W. Dowhan. 1990.An essential role for a phospholipid transfer protein in yeast golgi function. *Nature* 347:561-562.

Bankaitis, V.A., D.E. Malehorn, S.D. Emr and R. Greene. 1989.The *Saccharomyces cerevisiae* SEC14 gene encodes a cytosolic factor that is required for transport of secretory proteins from the yeast golgi complex. *J.Cell Biol.* 108:1271-1281.

Barbacid, M., F. Lamballe, D. Pulido and R. Klein. 1991.The trk family of tyrosine kinase receptors. *Biochimica Et Biophysica Acta* 1072:115-127.

Barclay, A.N., D.I. Jackson, A.C. Willis and A.F. Williams. 1987.Lymphocyte specific heterogeneity in the rat leukocyte common antigen (T200) is due to differences in polypeptides sequences near the NH₂ terminus. *EMBO J.* 6:1259-1264.

Basler, K. and E. Hafen. 1988.Control of photoreceptor cell fate by the *sevenless* protein requires a functional tyrosine kinase domain. *Cell* 54:299-311.

Beach, D.,Basilico,C.,and J. Newport. 1988.Current communications in molecular biology: cell cycle control in eukaryotes. Cold Spring Harbor Symp. Quant. Biol. 128.

Beach, D.,Durkacz,B.,and P. Nurse. 1982.Functionally homologous cell cycle control genes in budding and fission yeast. *Nature* 300:706-709.

Ben-David, Y., K. Letwin, L. Tannock, A. Bernstein and T. Pawson. 1991.A mammalian protein kinase with potential for serine/threonine and tyrosine phosphorylation is related to cell cycle regulators. *EMBO J.* 10:317-325.

- Birnboim, H.C. and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nuc. Acids Res.* 7:1513-1523.
- Bishop, J.M.** 1991. Molecular themes in oncogenesis. *Cell* 64:235-248.
- Bliska, J.B., K.L. Guan, J.E. Dixon and S. Falkow.** 1991. Tyrosine phosphate hydrolysis of host proteins by an essential *Yersinia* virulence determinant. *Proc. Natl. Acad. Sci. USA* 88:1187-1191.
- Bolen, J.B. and A. Veillette.** 1989. A function for the lck proto-oncogene. *Trends in Biochem. Sci.* 14:404-407.
- Bourguignon, L.Y.W., S.J. Suchard, M.L. Nagpal and Jr. J.R. Glenney.** 1985. A T-lymphoma transmembrane glycoprotein (gp180) is linked to the cytoskeletal protein, Fodrin. *J. Cell Biol.* 101:477-487.
- Bowtell, D.D.L., M.A. Simon and G.M. Rubin.** 1989. Ommatidia in the developing *Drosophila* eye require and can respond to sevenless for only a restricted period. *Cell* 56:931-936.
- Brautigan, D.L., P. Bornstein and B. Gallis.** 1981. Phosphotyrosyl-protein phosphatase. Specific inhibition by Zn^{2+} . *J. Biol. Chem.* 256:6519-6522.
- Bretscher, A.** 1983. Purification of an 80,000-dalton protein that is a component of the isolated microvillus cytoskeleton, and its localization in nonmuscle cells. *J. Cell Biol.* 97:425-432.
- Bretscher, A.** 1989. Rapid phosphorylation and reorganization of ezrin and spectrin accompany morphological changes induced in A-431 cells by epidermal growth factor. *J. Cell Biol.* 108:921-930.
- Bretscher, A.** 1991. Microfilament structure and function in the cortical cytoskeleton. *Annu. Rev. Cell Biol.* 7:337-374.
- Brown, W.R.A., A.N. Barclay, C.A. Sunderland and A.F. Williams.** 1981. Identification of a glycophorin-like molecule at the cell surface of rat thymocytes. *Nature* 289:456-460.
- Brown-Shimer, S., K.A. Johnson, D.E. Hill and A.M. Bruskin.** 1992. Effect of protein tyrosine phosphatase 1B expression on transformation by the human *neu* oncogene. *Cancer Res.* 52:478-482.
- Brugge, J., P. Cotton, A. Lustig, W. Yonemoto, L. Lipsich, P. Coussens, J.N. Barrett, D. Nonner and R.W. Keane.** 1987. Characterization of the altered form of the c-src gene product in neuronal cells. *Genes and Dev* 1:287-296.

Brugge, J.S., P.C. Cotton, A.E. Queral, J.N. Barrett, D. Nonner and R.W. Keane. 1985. Neurons express high levels of a structurally modified activated form of pp60^{c-src}. *Nature* 316:554-557.

Burridge, K. and K. Fath. 1989. Focal contacts: transmembrane links between the extracellular matrix and the cytoskeleton. *BioEssays* 10:104-108.

Burridge, K., C.E. Turner and L.H. Romer. 1992. Tyrosine phosphorylation of paxillin and pp125^{PAK} accompanies cell adhesion to extracellular matrix: A role in cytoskeletal assembly. *J. Cell Biol.* 119:893-903.

Camici, G., G. Manao, G. Cappugi, A. Modesti, M. Stefani and G. Ramponi. 1989. The complete amino acid sequence of the low molecular weight cytosolic acid phosphatase. *J. Biol. Chem.* 264:2560-2567.

Cantley, L.C., K.R. Auger, C. Carpenter, B. Duckworth, A. Graziani, R. Kapeller and S. Soltoff. 1991. Oncogenes and signal transduction. *Cell* 64:281-302.

Carlin, C.R., J. McGrath, D.B. Searls, D. Solter and B. Knowles. 1983. Phosphoproteins of early mouse embryos and teratocarcinoma-derived cell lines. *CSH Confer. Cell Prolif.* 10:245-255.

Carpenter, G., Jr. King, L. and S. Cohen. 1979. Rapid enhancement of protein phosphorylation in A-431 cell membrane preparations by epidermal growth factor. *J. Biol. Chem.* 254:4884-4891.

Chan, C.P., B. Gallis, D.K. Blumenthal, C.J. Pallen, J.H. Wang and E.G. Krebs. 1986. Characterization of the phosphotyrosyl protein phosphatase activity of calmodulin-dependent protein phosphatase. *J. Biol. Chem.* 261:9890-9895.

Charbonneau, E.H., N.K. Tonks, K.A. Walsh and E.H. Fischer. 1988. The leukocyte common antigen (CD45): A putative receptor-linked protein tyrosine phosphatase. *Proc. Natl. Acad. Sci. USA* 85:7182-7186.

Charbonneau, H. and N.K. Tonks. 1992. 1002 Protein Phosphatases? *Annu. Rev. Cell Biol.* 8:463-493.

Charbonneau, H., N.K. Tonks, S. Kumar, C.D. Diltz, M. Harrylock, D.E. Cool, E.G. Krebs, E.H. Fischer and K.A. Walsh. 1989. Human placenta protein-tyrosine-phosphatase: amino acid sequence and relationship to a family of receptor-like proteins. *Proc. Natl. Acad. Sci. USA* 86:5252-5256.

Charles, C.H., A.S. Abler and L.F. Lau. 1992. cDNA sequence of a growth factor-inducible immediate early gene and characterization

of its encoded protein. *Oncogene* 7:187-190.

Chernoff, J., A.R. Schievella, C.A. Jost, R.L. Erikson and B.G. Neel. 1990. Cloning of a cDNA for a major human protein-tyrosine-phosphatase. *Proc. Natl. Acad. Sci. USA* 87:2735-2739.

Chernoff, J., M.A. Sells and H.-C. Li. 1984. Characterization of phosphotyrosyl-protein phosphatase activity associated with calcineurin. *Biochem. Biophys. Res. Commun.* 121:141-148.

Cho, H., S.E. Ramer, M. Itoh, E. Kitas, W. Bannwarth, P. Burn, H. Saito and C.T. Walsh. 1992. Catalytic domains of the LAR and CD45 protein tyrosine phosphatases from *Escherichia coli* expression systems: Purification and characterization for specificity and mechanism. *Biochemistry* 31:133-138.

Cho, H., S.E. Ramer, M. Itoh, D.G. Winkler, E. Kitas, W. Bannwarth, P. Burn, H. Saito and C.T. Walsh. 1991. Purification and characterization of a soluble catalytic fragment of the human transmembrane leukocyte antigen related (LAR) protein tyrosine phosphatase from an *Escherichia coli* expression system. *Biochemistry* 30:6210-6216.

Cicchetti, P., B.J. Mayer, G. Thiel and D. Baltimore. 1992. Identification of a protein that binds to the SH3 region of Abl and is similar to Bcr and GAP-rho. *Science* 257:803-806.

Cicirelli, M.F., N.K. Tonks, C.D. Diltz, J.E. Weiel, E.H. Fischer and E.G. Krebs. 1990. Microinjection of a protein-tyrosine-phosphatase inhibits insulin action in *Xenopus* oocytes. *Proc. Natl. Acad. Sci. USA* 87:5514-5518.

Clark, E.A. and J.A. Ledbetter. 1989. Leukocyte cell surface enzymology: CD45 (LCA, T200) is a protein tyrosine phosphatase. *Immunology Today* 10:225-228.

Cohen, C.M. and S.F. Foley. 1980. Spectrin-dependent and -independent association of F-actin with the erythrocyte membrane. *J. Cell Biol.* 86:694-698.

Cohen, C.M., J.M. Tyler and D. Branton. 1980. Spectrin-actin associations studied by electron microscopy of shadowed preparations. *Cell* 21:875-883.

Cohen, P. 1982. The role of protein phosphorylation in neural and hormonal control of cellular activity. *Nature* 296:613-620.

Cohen, P. 1989. The structure and regulation of protein phosphatases. *Annu. Rev. Biochem.* 58:453-508.

Cohen, P. and P.T.W. Cohen. 1989. Protein phosphatases come of age. *J.Biol.Chem.* 264:21435-21438.

Conboy, J., Y.W. Kan, S.B. Shohet and N. Mohandas. 1986. Molecular cloning of protein 4.1, a major structural element of the human erythrocyte membrane skeleton. *Proc.Natl.Acad.Sci.USA* 83:9512-9516.

Cool, D.E., P.R. Andreassen, N.K. Tonks, E.G. Krebs, E.H. Fischer and R.L. Margolis. 1992. Cytokinetic failure and asynchronous nuclear division in BHK cells overexpressing a truncated protein-tyrosine-phosphatase. *Proc.Natl.Acad.Sci.USA* 89:5422-5426.

Cool, D.E., N.K. Tonks, H. Charbonneau, E.H. Fischer and E.G. Krebs. 1990. Expression of a human T-cell protein-tyrosine-phosphatase in baby hamster kidney cells. *Proc.Natl.Acad.Sci.USA* 87:7280-7284.

Cool, D.E., N.K. Tonks, H. Charbonneau, K.A. Walsh, E.H. Fischer and E.G. Krebs. 1989. cDNA isolated from a human T-cell library encodes a member of the protein-tyrosine-phosphatase family. *Proc.Natl.Acad.Sci.USA* 86:5257-5261.

Cooper, J.A., B.M. Sefton and T. Hunter. 1983. Detection and quantification of phosphotyrosine in proteins. *Methods In Enzymology* 99:387-405.

Crabb, J.W., V.P. Gaur, G.G. Garwin, S.V. Marx, C. Chapline, C.M. Johnson and J.C. Saari. 1991. Topological and epitope mapping of the cellular retinaldehyde-binding protein from retina. *J.Biol.Chem.* 266:16674-16683.

Crabb, J.W., S. Goldflam, S.E. Harris and J.C. Saari. 1988. Cloning of the cellular retinaldehyde-binding protein from bovine and human retina and comparison of the protein structures. *J.Biol.Chem.* 263:18688-18692.

Cunningham, B.A., J.J. Hemperly, B.A. Murray, E.A. Prediger, R. Brackenbury and G.M. Edelman. 1987. Neural cell adhesion molecule: structure, immunoglobulin-like domains, cell surface modulation, and alternative RNA splicing. *Science* 236:799-806.

Cyster, J.G., D.M. Shotton and A.F. Williams. 1991. The dimensions of the T lymphocyte glycoprotein leukosialin and identification of linear epitopes that can be modified by glycosylation. *EMBO J.* 10:893-902.

Dang, C.V. and W.M.F. Lee. 1988. Identification of the human c-myc protein nuclear translocation signal. *Mol.Cell.Biol.* 8:4048-4054.

- Daum, G., N.F. Zander, B. Morse, D. Hurwitz, J. Schlessinger and E.H. Fischer. 1991.Characterization of a human recombinant receptor-linked protein tyrosine phosphatase. J.Biol.Chem. 266:12211-12215.
- David-Pfeuty, T. and S.J. Singer. 1980.Alterd distributions of the cytoskeletal proteins vinculin and alpha-actinin in cultered fibroblasts transformed by Rous sarcoma virus. Proc.Natl.Acad.Sci.USA 77:6687-6691.
- Davis, S., M.L. Lu, S.H. Lo, S. Lin, J.A. Butler, B.J. Druker, T.M. Roberts, Q. An and L.B. Chen. 1991.Presence of an SH2 domain in the actin-binding protein tensin. Science 252:712-715.
- Den Hertog, J., C.E.G.M. Pals, L.J.C. Jonk and W. Kruijer. 1992.Differential expression of a novel murine non-receptor protein tyrosine phosphatase during differentiation of P19 embryonal carcinoma cells. Biochem.Biophys.Res.Comm. 184:1241-1249.
- DeRobertis, E.D.P. and E.M.F DeRobertis, Jr. Cell and Molecular Biology, Philadelphia:Sanders College, 1980. Ed. 7th
- Devault, A., J.C. Cavadore, D. Fesquet, J.C. Labbé, T. Lorca, A. Picard, U. Strausfeld and M. Dorée. 1991.Concerted roles of cyclin A ,cdc25⁺ mitotic inducer, and type 2A phosphatase inactivating the cyclin B1 cdc2 protein kinase at the G2/M phase transition. Cold Spring Harbor Symp.Quant.Biol. 56:503-513.
- Diekmann, C.L. and A. Tzagoloff. 1985.Assembly of the mitochondrial membrane system. CBP6, a yeast nuclear gene necessary for synthesis of cytochrome b. J.Biol.Chem. 260:1513-1520.
- Douville, E.M.J., D.E.H. Afar, B.W. Howell, K. Letwin, L. Tannock, Y. Ben-David, T. Pawson and J.C. Bell. 1992.Multiple cDNAs encoding the esk kinase predict transmembrane and intracellular enzyme isoforms. Mol.Cell.Biol. 12:2681-2689.
- Dunphy, W.G. and A. Kumagai. 1991.The cdc25 protein contains an intrinsic phosphatase activity. Cell 67:189-196.
- Dunphy, W.G. and J.W. Newport. 1989.Fission yeast p13 blocks mitotic activation and tyrosine dephosphorylation of the Xenopus cdc2 protein kinase. Cell 58:181-191.
- Eckert, R.L. and E.A. Rorke. 1988.The sequence of the human epidermal 58-kD (#5) type II keratin reveals an absense of 5' upstream sequence conservation between coexpressed epidermal keratins. DNA 7:337-345.
- Edgar, B.A. and P.H. O'Farrell. 1990.The three postblastoderm cell cycles of Drosophila embryogenesis are regulated in G2 by *string*.

Cell 62:469-480.

Edwards, A.M., M. Arquint, P.E. Braun, J.C. Roder, R.J. Dunn, T. Pawson and J.C. Bell. 1988. Myelin-associated glycoprotein, a cell adhesion molecule of oligodendrocytes, is phosphorylated in brain. *Mol. Cell. Biol.* 8:2655-2658.

Eiseman, E. and J.B. Bolen. 1990. Src-related tyrosine protein kinases as signaling components in hematopoietic cells. *Cancer cells* 2:303-310.

Elkins, T., K. Zinn, L. McAllister, F.M. Hoffman and C.S. Goodman. 1990. Genetic analysis of a *Drosophila* neural cell adhesion molecule: Interaction of fasciclin I and the Abelson tyrosine kinase mutations. *Cell* 60:565-575.

Ellis, L., E. Clauser, D.O. Morgan, M. Edery, R.A. Roth and W.J. Rutter. 1986. Replacement of insulin receptor tyrosine residues 1162 and 1163 compromises insulin-stimulated kinase activity and uptake of 2-Deoxyglucose. *Cell* 45:721-732.

Emmrich, F. 1988. Cross-linking of CD4 and CD8 with the T-cell receptor complex: quaternary complex formation and T-cell repertoire selection. *Immunology Today* 9:296-300.

Escobedo, J.A., S. Navankasattusas, W.M. Kavanaugh, D. Milfay, V.A. Fried and L.T. Williams. 1991. cDNA cloning of a novel 85 kd protein that has SH2 domains and regulates binding of PI3-kinase to the PDGF β receptor. *Cell* 65:75-82.

Evan, G.I., G.K. Lewis, G. Ramsay and J.M. Bishop. 1985. Isolation of monoclonal antibodies specific for human *c-myc* proto-oncogene product. *Mol. Cell. Biol.* 5:3610-3616.

Evans, M.J. and M.H. Kaufman. 1981. Establishment in culture of pluripotential cells from mouse embryos. *Nature* 292:154-156.

Fazioli, F., W.T. Wong, S.J. Ullrich, K. Sakaguchi, E. Appella and P.P. Di Fiore. 1993. The ezrin-like family of tyrosine kinase substrates: Receptor-specific pattern of tyrosine phosphorylation and relationship to malignant transformation. *Oncogene* 8:1335-1345.

Featherstone, C. and P. Russell. 1991. Fission yeast p107^{wee1} mitotic inhibitor is a tyrosine/serine kinase. *Nature* 349:808-811.

Feng, G.-S., C.-C. Hui and T. Pawson. 1993. SH2-containing phosphotyrosine phosphatase as a target of protein-tyrosine kinases. *Science* 259:1607-1611.

Firtel, R.A. 1991. Signal transduction pathways controlling

multicellular development in *Dictyostelium*. Trends Genet. 7:381-388.

Fischer, E.H., H. Charbonneau and N.K. Tonks. 1991. Protein tyrosine phosphatases: A diverse family of intracellular and transmembrane enzymes. Science 253:401-406.

Flint, A.J., M.F.G.B. Gebbink, Jr. Franza, B.R., D.E. Hill and N.K. Tonks. 1993. Multi-site phosphorylation of the protein tyrosine phosphatase, PTP1B: identification of cell cycle regulated and phorbol ester stimulated sites of phosphorylation. EMBO J. 12:1937-1946.

Fowler, V. and D.L. Taylor. 1980. Spectrin plus band 4.1 cross-link actin. Regulation by micromolar calcium. J. Cell Biol. 85:361-376.

Frangioni, J.V., P.H. Beahm, V. Shifrin, C.A. Jost and B.G. Neel. 1992. The nontransmembrane tyrosine phosphatase PTP-1B localizes to the endoplasmic reticulum via its 35 amino acid C-terminal sequence. Cell 68:545-560.

Freeman, Jr, R.M., J. Plutzky and B.G. Neel. 1992. Identification of a human src homology 2-containing protein-tyrosine-phosphatase: a putative homolog of *Drosophila* corkscrew. Proc. Natl. Acad. Sci. USA 89:11239-11243.

Fu, X.-Y. 1992. A transcription factor with SH2 and SH3 domains is directly activated by an interferon α -induced cytoplasmic protein tyrosine kinase(s). Cell 70:323-335.

Funayama, N., A. Nagafuchi, N. Sato, Sa. Tsukita and Sh. Tsukita. 1991. Radixin is a novel member of the band 4.1 family. J. Cell. Biol. 115:1039-1048.

Furthmayr, H., W. Lankes and M. Amieva. 1992. Moesin, a new cytoskeletal constituent of filopodia: Its role in cellular functions. Kidney Int. 41:665-670.

Galaktionov, K. and D. Beach. 1991. Specific activation of cdc25 tyrosine phosphatases by B-type cyclins: evidence for multiple roles of mitotic cyclins. Cell 67:1181-1194.

Garcia-Bustos, J., J. Heitman and M.N. Hall. 1991. Nuclear protein localization. Biochim. Biophys. Acta 1071:83-101.

Gautier, J., T. Matsukawa, P. Nurse and J. Maller. 1989. Dephosphorylation and activation of *Xenopus* p34^{cdc2} protein kinase during the cell cycle. Nature 339:626-629.

Gautier, J., M.J. Solomon, R.N. Booher, J.F. Bazan and M.W.

- Kirschner. 1991.cdc25 is a specific tyrosine phosphatase that directly activates p34^{cdc2}. Cell 67:197-211.
- Gebbink, M.F.B.G., I. van Etten, G. Hateboer, R. Suijkerbuijk, R.L. Beijersbergen, A. Geurts van Kessel and W.H. Moolenaar. 1991.Cloning, expression and chromosomal localization of a new putative receptor-like protein tyrosine phosphatase. FEBS Lett. 290:123-130.
- Girard, F., U. Strausfeld, J.-C. Cavadore, P. Russell, A. Fernández and N.J.C. Lamb. 1992.cdc25 is a nuclear protein expressed constitutively throughout the cell cycle in nontransformed mammalian cells. J.Cell.Biol. 118:785-794.
- Girard, F., U. Strausfeld, A. Fernández, and N.Y.C. Lamb. 1991.Cyclin A is required for the onset of DNA replication in mammalian fibroblasts. Cell 67:1169-1179.
- Gould, K., and P. Nurse. 1989.Tyrosine phosphorylation of the fission yeast cdc2⁺ protein kinase regulates entry into mitosis. Nature 342:39-45.
- Gould, K.L., A. Bretscher, F.S. Esch and T. Hunter. 1989.cDNA cloning and sequencing of the protein-tyrosine kinase substrate, ezrin, reveals homology to band 4.1. EMBO J. 8:4133-4142.
- Gould, K.L., J.A. Cooper, A. Bretscher and T. Hunter. 1986.The protein-tyrosine kinase substrate, p81, is homologous to a chicken microvillar core protein. J.Cell.Biol. 99:83s-91s.
- Gould, K.L. and T. Hunter. 1988.Platelet-derived growth factor induces multisite phosphorylation of pp60^{c-src} and increases its protein-tyrosine kinase activity. Mol.Cell.Biol. 8:3345-3356.
- Gould, K.L., S. Moreno, N.K. Tonks and P. Nurse. 1990.Complementation of the mitotic activator, p80^{cdc25}, by a human protein-tyrosine phosphatase. Science 250:1573-1576.
- Graham, F.L. and A.J. van der Eb. 1973.A new technique for the assay of infectivity of human adenovirus 5 DNA. Virol. 52:456-467.
- Grantham, R., C. Gautier, M. Guoy, M. Jacobzone and R. Mercier. 1981.Codon catalog usage is a genome strategy modulated for gene expressivity. Nuc.Acids Res. 9:r43-r75.
- Gu, M., I. Warshawsky and P.W. Majerus. 1992.Cloning and expression of a cytosolic megakaryocyte protein- tyrosine -phosphatase with sequence homology to retinaldehyde- binding protein and yeast SEC14p. Proc.Natl.Acad.Sci.USA 89:2980-2984.
- Gu, M.X., J.D. York, I. Warshawsky and P.W. Majerus.

1991. Identification, cloning, and expression of a cytosolic megakaryocyte protein-tyrosine-phosphatase with sequence homology to cytoskeletal protein 4.1. *Proc.Natl.Acad.Sci.USA* 88:5867-5871.

Guan, K.L., S.S. Broyles and J.E. Dixon. 1991a. A Tyr/Ser protein phosphatase encoded by vaccinia virus. *Nature* 350:359-362.

Guan, K.L., R.J. Deschenes, H. Qiu and J.E. Dixon. 1991b. Cloning and expression of a yeast protein tyrosine phosphatase. *J.Biol.Chem.* 266:12964-12970.

Guan, K.L. and J.E. Dixon. 1990. Protein tyrosine phosphatase activity of an essential virulence determinant in *Yersinia*. *Science* 249:553-556.

Guan, K.L. and J.E. Dixon. 1991a. Evidence for protein-tyrosine-phosphatase catalysis proceeding via a cysteine-phosphate intermediate. *J.Biol.Chem.* 266:17026-17030.

Guan, K.L. and J.E. Dixon. 1991b. Eukaryotic proteins expressed in *Escherichia coli*: An improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. *Analytical Biochemistry* 192:262-267.

Guan, K.L., R.S. Haun, S.J. Watson, R.L. Geahlen and J.E. Dixon. 1990. Cloning and expression of a protein-tyrosine-phosphatase. *Proc.Natl.Acad.Sci.USA* 87:1501-1505.

Haars, R., M. Kronenberg, W.M. Gallatin, I.L. Weissman, F.L. Owen and L. Hood. 1986. Rearrangement and expression of T cell antigen receptor and γ genes during thymic development. *J.Exp.Med.* 164:1-24.

Hakes, D.J., K.J. Martell, W.-G. Zhao, R.F. Massung, J.J. Esposito and J.E. Dixon. 1993. A protein phosphatase related to the vaccinia virus VH1 is encoded in the genomes of several orthopoxviruses and a baculovirus. *Proc.Natl.Acad.Sci.USA* 90:4017-4021.

Hanukoglu, I. and E. Fuchs. 1982. The cDNA sequence of a human epidermal keratin: divergence of sequence but conservation of structure among intermediate filament proteins. *Cell* 31:243-252.

Hao, Q.-L., N. Heisterkamp and J. Groffen. 1989. Isolation and sequence analysis of a novel human tyrosine kinase gene. *Mol.Cell.Biol.* 9:1587-1593.

Hariharan, I.K., P.-T. Chuang and G.M. Rubin. 1991. Cloning and characterization of a receptor-class phosphotyrosine phosphatase gene expressed on central nervous system axons in *Drosophila melanogaster*. *Proc.Natl.Acad.Sci.USA* 88:11266-11270.

Harlow, E. and D. Lane. *Antibodies. A Laboratory manual*, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1988.

Hatakeyama, M., T. Kono, N. Kobayashi, A. Kawahara, S.D. Levin, R.M. Perlmutter and T. Taniguchi. 1991. Interaction of the IL-2 receptor with the src-family kinase p56^{lck}: identification of novel intermolecular association. *Science* 252:1523-1528.

Henkemeyer, M., S.R. West, F.B. Gertler and F.M. Hoffmann. 1990. A novel tyrosine kinase-independent function of *Drosophila* *abl* correlates with proper subcellular localization. *Cell* 63:949-960.

Himeno, M., H. Fujita, Y. Noguchi, A. Kono and K. Kato. 1989. Isolation and sequencing of a cDNA clone encoding acid phosphatase in rat liver lysosomes. *Biochem. Biophys. Res. Commun.* 162:1044-1053.

Hirano, S., N. Kimoto, Y. Shimoyama, S. Hirohashi and M. Takeichi. 1992. Identification of a neural α -catenin as a key regulator of cadherin function and multicellular organization. *Cell* 70:293-301.

Hirst, R., A. Horwitz, C. Buck and L. Rohrschneider. 1986. Phosphorylation of the fibronectin receptor complex in cells transformed by oncogenes that encode tyrosine kinases. *Proc. Natl. Acad. Sci. USA* 83:6470-6474.

Horvath, A.R., M.A. Elmore and S. Kellie. 1990. Differential tyrosine-specific phosphorylation of integrin in Rous sarcoma virus transformed cells with differing transformed phenotypes. *Oncogene* 5:1349-1357.

Horvath, A.R., L. Muszbek and S. Kellie. 1992. Translocation of pp60^{c-src} to the cytoskeleton during platelet aggregation. *EMBO J.* 11:855-861.

Horwitz, A., K. Duggan, C. Buck, M.C. Beckerle and K. Burridge. 1986. Interaction of plasma membrane fibronectin receptor with talin-a transmembrane linkage. *Nature* 320:531-533.

Howard, P.K., B.M. Sefton and R.A. Firtel. 1992. Analysis of a spatially regulated phosphotyrosine phosphatase identifies tyrosine phosphorylation as a key regulatory pathway in *Dictyostelium*. *Cell* 71:637-647.

Howell, B.W., D.E.H. Afar, J. Lew, E.M.J. Douville, P.L.E. Icely, D.A. Gray and J.C. Bell. 1991. STY, a tyrosine-phosphorylating enzyme with sequence homology to serine/threonine kinases. *Mol. Cell. Biol.* 11:568-572.

Hunt, T. 1991. Cell cycle gets more cyclins. *Nature* 350:462-463.

Hunter, T. 1982. Phosphotyrosine-a new protein modification. Trends Biochem. Sci. 7:246-249.

Hunter, T. 1987. A thousand and one protein kinases. Cell 50:823-829.

Hunter, T. and B.M. Sefton. 1980. Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc. Natl. Acad. Sci. USA 77:1311-1315.

Hunter, T., B.M. Sefton and K. Beemon. 1979. Studies on the structure and function of the avian sarcoma transforming gene product. Cold Spring Harbor Symp. Quant. Biol. 44:931-941.

Icely, P.L., P. Gros, J.J.M. Bergeron, A. Devault, D.E.H. Afar and J.C. Bell. 1991. TIK, a novel serine/threonine kinase, is recognized by antibodies directed against phosphotyrosine. J. Biol. Chem. 266:16073-16077.

Igarashi, M., S. Saito and Y. Komiya. 1990. Vinculin is one of the major endogenous substrates for intrinsic tyrosine kinase in neuronal growth cones isolated from fetal rat brain. Eur. J. Biochem. 193:551-558.

Ingebritsen, T.S. 1989. Phosphotyrosyl-protein phosphatases II. Identification and characterization of two heat-stable protein inhibitors. J. Biol. Chem. 264:7754-7759.

Ingebritsen, T.S., V.M. Ingebritsen and S.K. Lewis. 1988. Coordinate expression of phosphotyrosyl-protein phosphatase and phosphatase inhibitor activities in animal cells. FASEB J. 2:A549.

Itoh, M., M. Streuli, N.X. Krueger and H. Saito. 1992. Purification and characterization of the catalytic domains of the human receptor-linked protein tyrosine phosphatases HPTP β , leukocyte common antigen (LCA), and leukocyte common antigen-related molecule (LAR). J. Biol. Chem. 267:12356-12363.

Itoh, M., S. Yonemura, A. Nagafuchi, Sa. Tsukita and Sh. Tsukita. 1991. A 220-kD undercoat-constitutive protein: Its specific localization at cadherin-based cell-cell adhesion sites. J. Cell Biol. 115:1449-1462.

Jiang, Y.-P., H. Wang, P. D'Eustachio, J.M. Musacchio, J. Schlessinger and J. Sap. 1993. Cloning and characterization of R-PTP- κ , a new member of the receptor protein tyrosine phosphatase family with a proteolytically cleaved cellular adhesion molecule-like extracellular region. Mol. Cell. Biol. 13:2942-2951.

Jirik, F.R., L.L. Anderson and A.M. Duncan. 1992. The human protein-tyrosine phosphatase PTP alpha/LRP gene (PTPA) is assigned

to chromosome 20p13. Cytogenetics & Cell Genetics 60:117-118.

Jirik, F.R., K.W. Harder, I.G. Melhado, L.L. Anderson and A.M. Duncan. 1992. The gene for leukocyte antigen-related tyrosine phosphatase (LAR) is localized to human chromosome 1p32, a region frequently deleted in tumors of neuroectodermal origin. Cytogenetics & Cell Genetics 61:266-268.

Jirik, F.R., N.M. Janzen, I.G. Melhado and K.W. Harder. 1990. Cloning and chromosomal assignment of a widely expressed human receptor-like protein-tyrosine phosphatase. FEBS Lett. 273:239-242.

Johnson, P., L. Greenbaum, K. Bottomly and I.S. Trowbridge. 1989. Identification of the alternatively spliced exons of murine CD45 (T200) required for reactivity with B220 and other T200-restricted antibodies. J. Exp. Med. 169:1179-1184.

Jöns, T. and D. Drenckhahn. 1992. Identification of the binding interface involved in linkage of cytoskeletal protein 4.1 to the erythrocyte anion exchanger. EMBO J. 11:2863-2867.

June, C.H., M.C. Fletcher, J.A. Ledbetter and L.E. Samelson. 1990. Increases in tyrosine phosphorylation are detectable before phospholipase C activation after T cell receptor stimulation. J. Immunol. 144:1591-1599.

Justement, L.B., K.S. Campbell, N.C. Chien and J.C. Cambier. 1991. Regulation of B cell antigen receptor signal transduction and phosphorylation by CD45. Science 252:1839-1842.

Kamps, M.P. and B.M. Sefton. 1989. Acid and base hydrolysis of phosphoproteins bound to immobilized facilitates analysis of phosphoamino acids in gel-fractionated proteins. Anal. Biochem. 176:22-27.

Kanner, S.B., A.B. Reynolds, R.R. Vines and J.T. Parsons. 1990. Monoclonal antibodies to individual tyrosine-phosphorylated protein substrates of oncogene-encoded tyrosine kinases. Proc. Natl. Acad. Sci. USA 87:3328-3332.

Kaplan, R., B. Morse, K. Huebner, C. Croce, R. Howk, M. Ravera, G. Ricca, M. Jaye and J. Schlessinger. 1990. Cloning of three human tyrosine phosphatases reveals a multigene family of receptor-linked protein-tyrosine-phosphatases expressed in brain. Proc. Natl. Acad. Sci. USA 87:7000-7004.

Kaptain, S., W.E. Downey, C. Tang, C. Philpott, D. Haile, D.G. Orloff, J.B. Harford, T.A. Rouault and R.D. Klausner. 1991. A regulated RNA binding protein also possesses aconitase activity. Proc. Natl. Acad. Sci. USA 88:10109-10113.

- Kaufman, R.J., M.V. Davies, V.K. Pathak and J.W.B. Hershey.** 1989. The phosphorylation state of eukaryotic initiation factor 2 alters the translation efficiency of specific mRNAs. *Mol. Cell. Biol.* 9:946-958.
- Kazlauskas, A., C. Ellis, T. Pawson and J.A. Cooper.** 1990. Binding of GAP to activated PDGF receptors. *Science* 247:1578-1581.
- Keyse, S.M. and E.A. Emslie.** 1992. Oxidative stress and heat shock induce a human gene encoding a protein-tyrosine phosphatase. *Nature* 359:644-647.
- Killeen, N., A.N. Barclay, A.C. Willis and A.F. Williams.** 1987. The sequence of rat leukosialin antigen (W3/13) reveals a molecule with O-linked glycosylation of one third of its extracellular amino acids. *EMBO J.* 6:4029-4034.
- Klarlund, J.K.** 1985. Transformation of cells by an inhibitor of phosphatases acting on phosphotyrosine in proteins. *Cell* 41:707-717.
- Klein, R., D. Conway, L.F. Parada and M. Barbacid.** 1990. The trkB tyrosine protein kinase gene codes for a second neurogenic receptor that lacks the catalytic kinase domain. *Cell* 61:647-656.
- Kobayashi, N., T. Kono, M. Hatakeyama, Y. Minami, T. Miyazaki, R.M. Perlmutter and T. Taniguchi.** 1993. Functional coupling of the src-family protein tyrosine kinases p59^{lck} and p53/56^{lck} with the interleukin 2 receptor: implications for redundancy and pleiotrophism in cytokine signal transduction. *Proc. Natl. Acad. Sci. USA* 90:4201-4205.
- Koch, C.A., D. Anderson, M.F. Moran, C. Ellis and T. Pawson.** 1991. SH2 and SH3 domains: Elements that control interactions of cytoplasmic signaling proteins. *Science* 252:668-674.
- Kondo, K., T. Watanabe, H. Sasaki, Y. Uehara and M. Oishi.** 1989. Induction of in vitro differentiation of mouse embryonal carcinoma (F9) and erythroleukemia (MEL) cells by herbomycin A, an inhibitor of protein phosphorylation. *J. Cell Biol.* 109:285-293.
- Koretzky, G.A., J. Picus, T. Schultz and A. Weiss.** 1991. Tyrosine phosphatase CD45 is required for T-cell antigen receptor and CD2-mediated activation of a protein tyrosine kinase and interleukin 2 production. *Proc. Natl. Acad. Sci. USA* 88:2037-2041.
- Koretzky, G.A., J. Picus, M.J. Thomas and A. Weiss.** 1990. Tyrosine phosphatase is essential for coupling T-cell antigen receptor to the phosphatidyl inositol pathway. *Nature* 346:66-68.
- Kozak, M.** 1986. Point mutations define a sequence flanking the AUG

initiator codon that modulates translation by eukaryotic ribosomes. *Cell* 44:283-292.

Kraft, R., J. Tardiff, K.S. Krauter and L.A. Leinwand. 1988. Using mini-prep plasmid DNA for sequencing double stranded templates with sequenase. *BioTech.* 6:544-546.

Krek, W. and E.A. Nigg. 1991a. Differential phosphorylation of vertebrate p34^{cdc2} kinase at the G1/S and G2/M transitions of the cell cycle: identification of major phosphorylation sites. *EMBO J.* 10:305-316.

Krek, W. and E.A. Nigg. 1991b. Mutations of p34^{cdc2} phosphorylation sites induce premature mitotic events in Hela cells: evidence for a double block to p34cdc2 kinase activation in vertebrates. *EMBO J.* 10:3331-3341.

Krueger, N.X. and H. Saito. 1992. A human transmembrane protein-tyrosine-phosphatase, PTP γ , is expressed in brain and has an N-terminal receptor domain homologous to carbonic anhydrases. *Proc. Natl. Acad. Sci. USA* 89:7417-7421.

Krueger, N.X., M. Streuli and H. Saito. 1990. Structural diversity and evolution of human receptor-like protein tyrosine phosphatases. *EMBO J.* 9:3241-3252.

Kumagai, A. and W.G. Dunphy. 1991a. The cdc25 protein controls tyrosine dephosphorylation of the cdc2 protein in a cell-free system. *Cell* 64:903-914.

Kumagai, A. and W.G. Dunphy. 1991b. Molecular mechanism of the final steps in the activation of MPF. *Cold Spring Harbor Symp. Quant. Biol.* 56:585-589.

Kupfer, A., S.J. Singer, jr. Janeway, C.A. and S.L. Swain. 1987. Co-clustering of CD4 (L3T4) molecule with the T-cell receptor is induced by specific direct interaction of helper T cells and antigen presenting cells. *Proc. Natl. Acad. Sci. USA* 84:5888-5892.

LaForgia, S., B. Morse, J. Levy, G. Barnea, L.A. Cannizzaro, F. Li, P.C. Nowell, L. Boghosian-Sell, J. Glick, A. Weston, C.C. Harris, H. Drabkin, D. Patterson, C.M. Croce, J. Schlessinger and K. Huebner. 1991. Receptor protein-tyrosine phosphatase gamma is a candidate tumor suppressor gene at human chromosome region 3p21. *Proc. Natl. Acad. Sci. USA* 88:5036-5040.

Lankes, W.T. and H. Furthmayr. 1991. Moesin: A member of the protein 4.1-talin-ezrin family of proteins. *Proc. Natl. Acad. Sci. USA* 88:8297-8301.

Lau, K.-H.W., J.R. Farley and D.J. Baylink. 1985. Phosphotyrosyl-specific protein phosphatase activity of a

bovine skeletal acid phosphatase isoenzyme. Comparison with the phosphotyrosyl protein phosphatase activity of skeletal alkaline phosphatase. *J.Biol.Chem.* 260:4653-4660.

Lau, K.-H.W., J.R. Farley and D.J. Baylink. 1989. Phosphotyrosyl protein phosphatases. *Biochem.J.* 257:23-36.

Ledbetter, J.A., C.H. June, L.S. Grosmaire and P.S. Rabinovitch. 1987. Crosslinking of surface antigens causes mobilization of intracellular ionized calcium in T lymphocytes. *Proc.Natl.Acad.Sci.USA* 84:1384-1388.

Ledbetter, J.A., G.L. Schieven, F.M. Uckun and J.B. Imboden. 1991. CD45 cross-linking regulates phospholipase C activation and tyrosine phosphorylation of specific substrates in CD3/Ti-stimulated T cells. *J.Immunol.* 146:1577-1583.

Ledbetter, J.A., N.K. Tonks, E.H. Fischer and E.A. Clark. 1988. CD45 regulates signal transduction and lymphocyte activation by specific association with receptor molecules on T or B cells. *Proc.Natl.Acad.Sci.USA* 85:8628-8632.

Lee, M. and P. Nurse. 1987. Complementation used to clone a human homolog of the fission yeast cell cycle control gene *cdc2*. *Nature* 327:31-35.

Lehto, V.-P., V.-M. Wasenius, P. Salven and M. Sarcaste. 1988. Transforming and membrane proteins. *Nature* 334:388.

Leis, J.F. and N.O. Kaplan. 1982. An acid phosphatase in the plasma membranes of human astrocytoma showing marked specificity towards phosphotyrosine protein. *Proc.Natl.Acad.Sci.USA* 79:6507-6511.

Letwin, K., S.-P. Yee and T. Pawson. 1988. Novel protein-tyrosine kinase cDNAs related to *fps/fes* and *eph* cloned using anti-phosphotyrosine antibody. *Oncogene* 3:621-627.

Lewin, B. 1990. Driving the cell cycle: M phase Kinase, its partners, and substrates. *Cell* 61:743-752.

Li, H.-C., J. Chernoff, L.B. Chen and A. Kirschonbaum. 1984. A phosphotyrosyl-protein phosphatase activity associated with acid phosphatase from human prostate gland. *Eur.J.Biochem.* 138:45-51.

Lin, M.-F. and G.M. Clinton. 1988. The epidermal growth factor receptor from prostate cells is dephosphorylated by a prostate-specific phosphotyrosyl phosphatase. *Mol.Cell.Biol.* 8:5477-5485.

Lindberg, R.A., A.M. Quinn and T. Hunter. 1992. Dual-specificity protein kinases: will any hydroxyl do? *Trends Biochem. Sci.*

17:114-119.

Lipfert, L., B. Haimovich, M.D. Schaller, B.S. Cobb, J.T. Parsons and J.S. Brugge. 1992. Integrin-dependent phosphorylation and activation of the protein tyrosine kinase pp125^{PAK} in platelets. *J. Cell Biol.* 119:905-912.

Lokeshwar, V.B. and L.Y.W. Bourguignon. 1992. Tyrosine phosphatase activity of lymphoma CD45 (GP180) is regulated by a direct interaction with the cytoskeleton. *J. Biol. Chem.* 267:21551-21557.

Lombroso, P.J., G. Murdoch and M. Lerner. 1991. Molecular characterization of a protein-tyrosine-phosphatase enriched in striatum. *Proc. Natl. Acad. Sci. USA* 88:7242-7246.

Luna, E.J. 1991. Molecular links between the cytoskeleton and membranes. *Curr. Opin. Cell Biol.* 3:120-126.

Lundgren, K., Walworth, N., Bocher, R., Dembski, M., Kirschner, M., and D. Beach. 1991. m1k1 and weel cooperate in the inhibitory tyrosine phosphorylation of cdc2. *Cell* 64:1111-1122.

Maher, P.A., E.B. Pasquale, J.Y.J. Wang and S.J. Singer. 1985. Phosphotyrosine-containing proteins are concentrated in focal adhesions and intercellular junctions in normal cells. *Proc. Natl. Acad. Sci. USA* 82:6576-6580.

Maness, P.F., M. Aubry, C.G. Shores, L. Frame and K.H. Pfenninger. 1988. c-src gene product in developing brain is enriched in nerve growth cone membranes. *Proc. Natl. Acad. Sci. USA* 85:5001-5005.

Maness, P.F. and W.T. Matten. Tyrosine phosphorylation of membrane-associated tubulin in nerve growth cones enriched in p60c-src. In: *Proto-oncogenes in Cell Development*, Chichester, England: John Wiley and Sons, 1990, p. 57-78.

Maniatis, T, E.F. Fritsch, and J. Sambrook. *Molecular Cloning A Laboratory Manual*, Cold Spring Harbor Laboratory, 1982.

Marchuk, D., S. McCrohon and E. Fuchs. 1984. Remarkable conservation of structure among intermediate filament genes. *Cell* 39:491-498.

Marmur, J. 1962. A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J. Molec. Biol.* 3:208-218.

Martin, G.R. 1980. Teratocarcinomas and mammalian embryogenesis. *Science* 209:768-776.

Martin, G.R. 1981. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. USA* 78:7634-7638.

Martin-Zanca, D., M. Barbacid and L.F. Parada. 1990. Expression of the trk proto-oncogene is restricted to the sensory cranial and spinal ganglia of neural crest origin in mouse development. *Genes Develop.* 4:683-694.

Matasuyoshi, N., M. Hamaguchi, S. Taniguchi, A. Nagafuchi, S. Tsukita and M. Takeichi. 1992. Cadherin-mediated cell-cell adhesion is perturbed by v-src tyrosine phosphorylation in metastatic fibroblasts. *J. Biol. Chem.* 118:703-714.

Matsushime, H., M.F. Roussel, and C.J. Sherr. 1991. Novel mammalian cyclins (CY1 genes) expressed during G1. Cold Spring Harbor Symp. Quant. Biol. 56:69-74.

Matten, W.T., M. Aubry, J. West and P.F. Maness. 1990. Tubulin is phosphorylated at tyrosine by pp60^{c-src} in nerve growth cone membranes. *J. Cell Biol.* 111:1959-1970.

Matthews, J.R., D.B. Bowne, E. Flores and M.L. Thomas. 1992. Characterization of hematopoietic intracellular protein tyrosine phosphatases: Description of a phosphatase containing an SH2 domain and another enriched in Proline-, Glutamic acid-, Serine-, and Threonine-rich sequences. *Mol. Cell. Biol.* 12:2396-2405.

Matthews, R.J., E.D. Cahir and M.L. Thomas. 1990. Identification of an additional member of the protein-tyrosine-phosphatase family: evidence for alternative splicing in the tyrosine phosphatase domain. *Proc. Natl. Acad. Sci. USA* 87:4444-4448.

Matthews, R.J., E. Flores and M.L. Thomas. 1991. Protein tyrosine phosphatase domains from the protochordate *Styela plicata*. *Immunogenetics* 33:33-41.

Matthews, R.J., J.T. Pingel, C.M. Meyer and M.L. Thomas. 1989. Studies on the leukocyte-common antigen: structure, function, and evolutionary conservation. Cold Spring Harbor Symp. Quant. Biol. 54:675-682.

Mayer, B.J., and H. Hanafusa. 1990. Association of the v-crck oncogene product with phosphotyrosine containing proteins and protein kinase activity. *Proc. Natl. Acad. Sci. USA* 87:2638-2642.

Mayer, B.J., Jackson, P.K. and D.C. Baltimore. 1991. The noncatalytic src homology region 2 segment of abl tyrosine kinase binds to tyrosine-phosphorylated cellular proteins with high affinity. *Proc. Natl. Acad. Sci. USA* 88:627-631.

McBurney, M.W., E.M.V. Jones-Villeneuve, M.K.S. Edwards and P.J. Anderson. 1982. Control of muscle and neuronal differentiation in a cultured embryonal carcinoma cell line. *Nature* 299:165-167.

McCarty, D.R., T. Hattori, C.B. Carson, V. Vasil, M. Lazar and I.K. Vasil. 1991. The viviparous-1 developmental gene of maize encodes a novel transcriptional activator. *Cell* 66:895-905.

McCormick, F. 1993. How receptors turn ras on. *Nature* 363:15-16.

McLaughlin, S. and J.E. Dixon. 1993. Alternative splicing gives rise to a nuclear protein tyrosine phosphatase in *Drosophila*. *J. Biol. Chem.* 268:6839-6842.

McMahon, A.P. and R.T. Moon. 1989. Ectopic expression of the proto-oncogene int-1 in *Xenopus* embryos leads to duplication of the embryonic axis. *Cell* 58:1075-1084.

Meyerson, M., Faha, B., Su, L.-K., Harlow, E., and L.-H. Tsai. 1991. The cyclin-dependent kinase family. *Cold Spring Harbor Symp. Quant. Biol.* 56:177-186.

Millar, J.B.A., C.H. McGowan, R. Jones, K. Sadhu, A. Bueno, H. Richardson, and P. Russell. 1991a. cdc25 M-phase. *Cold Spring Harbor Symp. Quant. Biol.* 56:577-581.

Millar, J.B.A., C.H. McGowan, G. Lenaers, R. Jones and P. Russell. 1991b. p80cdc25 mitotic inducer is the tyrosine phosphatase that activates p34cdc2 kinase in fission yeast. *EMBO J.* 10:4301-4309.

Millar, J.B.A., J. Blevitt, L. Gerace, K. Sadhu, C. Featherstone, and P. Russell. 1991c. p55^{cdc25} is a nuclear protein required for the initiation of mitosis in human cells. *Proc. Natl. Acad. Sci. USA* 88:10500-10504.

Millar, J.B.A. and P. Russell. 1992. The cdc25 M-phase inducer: An unconventional protein phosphatase. *Cell* 68:407-410.

Miyasaka, H. and S.S.-L. Li. 1992. The cDNA cloning, nucleotide sequence and expression of an intracellular protein tyrosine phosphatase from mouse testis. *Biochem. Biophys. Res. Commun.* 185:818-825.

Moos, M., R. Tacke, H. Scherer, D. Teplow, K. Fruh and M. Schachner. 1988. Neural adhesion molecule L1 as a member of the immunoglobulin superfamily with binding domains similar to fibronectin. *Nature* 334:701-703.

Moreno, S. and P. Nurse. 1991. Clues to action of cdc25 protein. *Nature* 351:194.

Morla, A.O., G. Draetta, D. Beach and J.Y.J. Wang. 1989. Reversible tyrosine phosphorylation of cdc2: dephosphorylation accompanies activation during entry into mitosis. *Cell* 58:193-203.

Mosinger Jr., B., U. Tillmann, H. Westphal and M.L. Tremblay. 1992. Cloning and characterization of a mouse cDNA encoding a cytoplasmic protein-tyrosine-phosphatase. *Proc. Natl. Acad. Sci. USA* 89:499-503.

Mueller, T.J. and M. Morrison. Glycoconnectin (PAS2), a membrane attachment site for the human erythrocyte cytoskeleton. In: *Erythrocyte Membrane 2: Recent Clinical and Experimental Advances*, edited by Krukenberg, W.C., Eaton, J.W. and Brewer, G.J. New York: Alan R. Liss, 1981, p. 95-116.

Munro, S. and H.R.B. Pelham. 1987. A C-terminal signal prevents secretion of luminal ER proteins. *Cell* 48:899-907.

Murakami, M., M. Hibi, N. Nakagawa, T. Nakagawa, K. Yasukawa, K. Yamanishi, T. Taga and T. Kishimoto. 1993. IL-6 induced homodimerization of gp130 and associated activation of a tyrosine kinase. *Science* 260:1808-1810.

Murray, A.W. 1992. Creative blocks: cell-cycle checkpoints and feedback controls. *Nature* 359:599-604.

Mustelin, T. and A. Altman. 1990. Dephosphorylation and activation of the T cell tyrosine kinase pp56^{lck} by the leukocyte common antigen (CD45). *Oncogene* 5:809-813.

Mustelin, T., K.M. Coggeshall and A. Altman. 1989. Rapid activation of the T-cell tyrosine protein kinase pp56^{lck} by the CD45 phosphotyrosine phosphatase. *Proc. Natl. Acad. Sci. USA* 86:6302-6306.

Mustelin, T., K.M. Coggeshall, N. Isakov and A. Altman. 1990. T cell antigen receptor-mediated activation of phospholipase C requires tyrosine phosphorylation. *Science* 247:1584-1587.

Mustelin, T., T. Pessa-Morikawa, M. Autero, M. Gassmann, L.C. Andersson, C.G. Gahmberg and P. Burn. 1992. Regulation of the p59^{lyn} protein tyrosine kinase by the CD45 phosphotyrosine phosphatase. *Eur. J. Immunol.* 22:1173-1178.

Nagata, A., M. Igarashi, S. Jinno, K. Suot and H. Okayama. 1991. An additional homolog of the fission yeast cdc25⁺ gene occurs in humans and is highly expressed in some cancer cells. *New Biology* 3:959-968.

Nelson, R.L. and P.E. Branton. 1984. Identification, purification, and characterization of phosphotyrosine-specific phosphatases from cultured chicken embryo fibroblasts. *Mol. Cell. Biol.* 4:1003-1012.

Nigg, E.A., Krek, W., and M. Peter. 1991. Vertebrate cdc2 kinase : its regulation by phosphorylation and its mitotic targets. *Cold Spring Harbor Symp. Quant. Biol.* 56:539-547.

Nishibe, S., M.I. Wahl, S.M.T. Hernández-Sotomayor, N.K. Tonks, S.G. Rhee and G. Carpenter. 1990. Increase of the catalytic activity of phospholipase-C γ 1 by tyrosine phosphorylation. *Science* 250:1253-1256.

Norton, P.A., R.O. Hynes and D.J.G. Rees. 1990. Sevenless: seven found? *Cell* 61:15.

Nurse, P. 1975. Genetic control of cell size at cell division in fission yeast. *Nature* 256:547-551.

Nurse, P. 1990. Universal control mechanism regulating onset of M phase. *Nature* 344:503-508.

Ostergaard, H.L., D.A. Shackelford, T.R. Hurley, P. Johnson, R. Hyman, B.M. Sefton and I.S. Trowbridge. 1989. Expression of CD45 alters phosphorylation of the lck-encoded tyrosine protein kinase in murine lymphoma T-cell lines. *Proc. Natl. Acad. Sci. USA* 86:8959-8963.

Ostergaard, H.L. and I.S. Trowbridge. 1990. Coclustering CD45 with CD4 or CD8 alters the phosphorylation and kinase activity of p56^{lck}. *J. Exp. Med.* 172:347-350.

Ostergaard, H.L. and I.S. Trowbridge. 1991. Negative regulation of CD45 protein tyrosine phosphatase activity by ionomycin in T cells. *Science* 253:1423-1425.

Ostu, M., I. Hiles, I. Gout, M.J. Fry, F. Ruiz-Larrea, G. Panayotou, A. Thompson, R. Dhand, J. Hsuan, N. Totty, A.D. Smith, S.J. Morgan, S.A. Courtneidge, P.J. Parker and M.D. Waterfield. 1991. Characterization of two 85 kd proteins that associate with receptor tyrosine kinases, middle-T/pp60^{c-src} and PI3-kinase. *Cell* 65:91-104.

Ottillie, S., J. Chernoff, G. Hannig, C.S. Hoffman and R.L. Erikson. 1991. A fission-yeast gene encoding a protein with features of protein-tyrosine-phosphatases. *Proc. Natl. Acad. Sci. USA* 88:3455-3459.

Pallen, C.J., D.S. Lai, H.P. Chai, I. Boulet and P.H. Tong. 1991. Purification and characterization of a higher-molecular-mass form of protein phosphotyrosine phosphatase (PTP1B) from placental membranes. *Biochem. J.* 276:315-323.

Pallen, C.J. and P.H. Tong. 1991. Elevation of membrane tyrosine phosphatase activity in density-dependent growth-arrested fibroblasts. *Proc. Natl. Acad. Sci. USA* 88:6996-7000.

Pallen, C.J., K.A. Valentine, J.A. Wang and M.D. Hollenberg. 1985. Calcineurin-mediated dephosphorylation of the human placental

membrane receptor for epidermal growth factor urogastrone. *Biochemistry* 24:4727-4730.

Parker, L.L., S. Atherson-Fessler, M.S. Lee, S. Ogg, J.L. Falk, K.I. Swenson and H. Piwnica-Worms. 1991. Cyclin promotes the tyrosine phosphorylation of p34^{cdc2} in a weel⁺ dependent manner. *EMBO J.* 10:1255-1263.

Pasquale, E.B., P.A. Maher and S.J. Singer. 1986. Talin is phosphorylated on tyrosine in chicken embryo fibroblasts transformed by Rous sarcoma virus. *Proc. Natl. Acad. Sci. USA* 83:5507-5511.

Pasternack, G.R., R.A. Anderson, T.L. Leto and V.T. Marchesi. 1985. Interactions between protein 4.1 and band 3: an alternative binding site for an element of the membrane skeleton. *J. Biol. Chem.* 260:3676-3683.

Patarroyo, M. and C.G. Gahmberg. 1984. Phorbol 12,13-dibutyrate enhances lateral redistribution of membrane glycoproteins in human blood lymphocytes. *Eur. J. Immunol.* 14:781-787.

Patthy, L. 1990. Homology of a domain of the growth factor hormone/prolactin receptor family with the type III modules of fibronectin. *Cell* 61:13-14.

Pawson, T. and A. Bernstein. 1990. Receptor tyrosine kinases: Genetic evidence for their role in *Drosophila* and mouse development. *Trends Genet.* 6:350-356.

Pawson, T. and G.D. Gish. 1992. SH2 and SH3 domains: from structure to function. *Cell* 71:359-362.

Pei, D., B.G. Neel and C.T. Walsh. 1993. Overexpression, purification, and characterization of SHPTP1, a Src-homology 2-containing protein-tyrosine-phosphatase. *Proc. Natl. Acad. Sci. USA* 90:1092-1096.

Perkins, L.A., I. Larsen and N. Perrimon. 1992. corkscrew Encodes a putative protein tyrosine phosphatase that functions to transduce the terminal signal from the receptor tyrosine kinase torso. *Cell* 70:225-236.

Peter, M., J. Nakagawa, M. Dorée, J.C. Labbe and E.A. Nigg. 1990. Identification of major nucleolar proteins as candidate mitotic substrates of cdc2 kinase. *Cell* 60:791-801.

Pines, J. and T. Hunter. 1991a. Human cell division : the involvement of cyclins A and B1 , and multiple cdc2s. *Cold Spring Harbor Symp. Quant. Biol.* 56:449-463.

Pines, J. and T. Hunter. 1991b. Human cyclins A and B are differentially located in the cell and undergo cell cycle dependent nuclear transport. *J.Cell.Biol.* 115:1-17.

Pingel, J.T. and M.L. Thomas. 1989.Evidence that the leukocyte-common-antigen is required for antigen-induced T lymphocyte proliferation. *Cell* 58:1055-1065.

Piwnicka-Worms, H., S. Atherton-Fessler, M.S. Lee, S. Ogg, K.I. Swenson, and L. Parker. 1991.p107 weel is a serine/threonine and tyrosine kinase that promotes the phosphorylation of the cyclin p34^{cdc2} complex. *Cold Spring Harbor Symp. Quant. Biol.* 56:567-576.

Plutzky, J., B.G. Neel and R.D. Rosenberg. 1992.Isolation of a src homology 2-containing tyrosine phosphatase. *Proc.Natl.Acad.Sci.USA* 89:1123-1127.

Polmann, R., C. Krentler, B. Schmidt, W. Schroder, G. Lorkowski, J. Culley, G. Mersmann, C. Geier, A. Waheed, S. Gottschalk, K.-H. Grzeschik, A. Hasilik and K. von Figura. 1988.Human lysosomal acid phosphatase: cloning, expression and chromosomal assignment. *EMBO J.* 7:2343-2350.

Pot, D.A., T.A. Woodford, E. Remboutsika, R.S. Haun and J.E. Dixon. 1991.Cloning, bacterial expression, purification, and characterization of the cytoplasmic domain of rat LAR, a receptor-like protein tyrosine phosphatase. *J.Biol.Chem.* 266:19688-19696.

Powrie, F. and D. Mason. 1988.Phenotype and functional heterogeneity of CD4⁺ T cells. *Immunology Today* 9:274-277.

Qian, L., L. Theodor, M. Carter, M.N. Vu, A.W. Sasaki and M.F. Wilkinson. 1993.T Cell receptor- β mRNA splicing: regulation of unusual splicing intermediates. *Mol.Cell.Biol.* 13:1686-1696.

Radke, K. and G.S. Martin. 1979.Transformation by rous sarcoma virus: effects of src-gene expression on the synthesis and phosphorylation of cellular polypeptides. *Cold Spring Harbor Symp.Quant.Biol.* 44:957-982.

Ralph, S.J., M.L. Thomas, C.C. Morton and I.S. Trowbridge. 1987.Structural variants of human T200 glycoprotein (leukocyte-common antigen). *EMBO J.* 6:1251-1257.

Ramponi, G., M. Ruggiero, G. Raugeli, A. Berti, A. Modesti, D. Degl'Innocenti, L. Magnelli, C. Pazzagli, V.P. Chiarugi and G. Camici. 1992.Overexpression of a synthetic phosphotyrosine protein phosphatase gene inhibits normal and transformed cell growth. *Int. J. Cancer* 51:652-656.

- Reed, S.I., C. Wittenberg, D.J. Lew, V. Dulic, and M. Henze. 1991. G1 control in yeast and animal cells. Cold Spring Harbor Symp. Quant. Biol. 56:61-67.
- Rees, D.J.G., S.E. Ades, S.J. Singer and R.O. Hynes. 1990. Sequence and domain structure of talin. Nature 347:685-689.
- Rijksen, G., M.C.W. Völler and E.J.J. Van Zoelen. 1993. The role of protein tyrosine phosphatases in density-dependent growth control of normal rat kidney cells. FEBS Lett. 322:83-87.
- Rogers, S., K. Wells and M. Rechsteiner. 1986. Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. Science 234:364-368.
- Rotenberg, S.A. and D.L. Brautigen. 1987. Membrane protein phosphotyrosine phosphatase in rabbit kidney. Biochem.J. 243:747-754.
- Rudd, C.E., J.M. Trevillyan, J.D. Dasgupta, J.L. Wong and S.F. Schlossman. 1988. The CD4 receptor is complexed in detergent lysates to a protein-tyrosine kinase (pp58) from human T lymphocytes. Proc.Natl.Acad.Sci.USA 85:5190-5194.
- Rudnicki, M.A., K.R. Reuhl and M.W. McBurney. 1989. Cell lines with developmental potential restricted to mesodermal lineages isolated from differentiating cultures of pluripotential P19 embryonal carcinoma cells. Development 107:361-372.
- Russell, P., and P. Nurse. 1986. cdc25⁺ functions as an inducer in the mitotic control of fission yeast. Cell 45:145-153.
- Russell, P. and P. Nurse. 1987. Negative regulation of mitosis by weel⁺, a gene encoding a protein kinase homolog. Cell 49:559-567.
- Rutishauser, U., A. Acheson, A.K. Hall, D.M. Mann, and J. Sunshine. 1988. The neural adhesion molecule (N-CAM) as a regulator of cell-cell interactions. Science 240:53-57.
- Sadhu, K., I. Reed, H. Richardson, and P. Russell. 1990. Human homolog of fission yeast cdc25 mitotic inducer is predominantly expressed in G2. Proc.Natl.Acad.Sci.USA 87:5139-5143.
- Sadowski, I., J.C. Stone and T. Pawson. 1986. A noncatalytic domain conserved among cytoplasmic protein-tyrosine kinases modifies the kinase function and transforming activity of Fujinami sarcoma virus P130^{gag-fps}. Mol.Cell.Biol. 6:4396-4408.
- Saga, Y., J.-S. Tung, F.-W. Shen and E.A. Boyse. 1986. Sequences of Ly-5 cDNA: isoform-related diversity of Ly-5 mRNA. Proc.Natl.Acad.Sci.USA 83:6940-6944.

Saga, Y., J.-S. Tung, F.-W. Shen and E.A. Boyse. 1987. Alternative use of 5' exons on the specification of Ly-5 isoforms distinguishing hematopoietic cell lineages. *Proc.Natl.Acad.Sci.USA* 84:5364-5368.

Saito, H. and M. Streuli. 1991. Molecular characterization of protein tyrosine phosphatases. *Cell Growth Diff* 2:59-65.

Salama, S.R., A.E. Cleves, D.E. Malehorn, E.A. Whitters and V.A. Bankaitis. 1990. Cloning and characterization of *Kluyveromyces lactis* SEC14, a gene whose product stimulates golgi function in *Saccharomyces cerevisiae*. *J. Bacteriol.* 172:4510-4521.

Sambrook, J., Fritsch, E.F. and T. Maniatis. *Molecular cloning: A Laboratory Manual*, Cold Spring Harbor, NY: Cold Spring Harbor laboratory, 1989. Ed. 2nd

Samelson, L.E., J.B. Harford and R.D. Klausner. 1985. Identification of the components of the murine T cell antigen receptor complex. *Cell* 43:223-231.

Samelson, L.E., Fletcher, M.C., Ledbetter, J.A., and C.H. June. 1990a. Activation of tyrosine phosphorylation in human T cells via the CD2 pathway. Regulation by the CD45 tyrosine phosphatase. *J. Immunol.* 145:2448-2454.

Samelson, L.E., A.F. Phillips, E.T. Luong and R.D. Klausner. 1990b. Association of the fyn protein-tyrosine kinase with the T-cell antigen receptor. *Proc.Natl.Acad.Sci.USA* 87:4358-4362.

Sanders, M.E., M.W. Makgoba and S. Shaw. 1988. Human naive and memory T cells: reinterpretation of helper-induced and suppressor-induced subsets. *Immunology Today* 9:195-199.

Sanger, F., S. Nicklen and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc.Natl.Acad.Sci.USA* 74:5463-5467.

Sap, J., P. D'Eustachio, D. Givol and J. Schlessinger. 1990. Cloning and expression of a widely expressed receptor tyrosine phosphatase. *Proc.Natl.Acad.Sci.USA* 87:6112-6116.

Sato, N., S. Yomemura, T. Obinata, Sa. Tsukita and Sh. Tsukita. 1991. Radixin, a barbed end-capping actin-modulating protein, is concentrated at the cleavage furrow during cytokinesis. *J. Cell Biol.* 113:321-330.

Schaller, M.D., C.A. Borgman, B.S. Cobb, R.R. Vines, A.B. Reynolds and J.T. Parsons. 1992. pp125^{PAK}, a structurally distinctive protein-tyrosine kinase associated with focal adhesions. *Proc.Natl.Acad.Sci.USA* 89:5192-5196.

- Scott, R.E.M., D.J. Autelitano, D.L. Lugo, M. Blum, J.L. Roberts and J.E. Pintar. 1990. Developmental changes in levels of proopiomelanocortin intron A-containing heterogeneous nuclear RNA and mature messenger RNA in the anterior and neurointermediate lobes of the rat pituitary. *Mol. Endocrinol.* 4:812-82.
- Sefton, B.M., T. Hunter and W.C. Raschke. 1981. Evidence that the abelson virus protein functions in vivo as a protein kinase that phosphorylates tyrosine. *Proc. Natl. Acad. Sci. USA* 78:1552-1556.
- Sejersen, T., J. Sumegi and N.R. Ringertz. 1985. Expression of cellular oncogenes in teratoma-derived cell lines. *Exp. Cell Res.* 160:19-30.
- Shaw, A. and M.L. Thomas. 1991. Coordinate interactions of protein tyrosine kinases and protein tyrosine phosphatases in T-cell receptor-mediated signalling. *Curr. Opin. Cell Biol.* 3:862-868.
- Shaw, A.S., J. Chalupny, J.A. Whitney, C. Hammond, K.E. Anrein, F. Kavathas, B.M. Sefton and J.K. Rose. 1990. Short repeated sequences in the cytoplasmic domains of CD4 and CD8 mediate binding to the amino-terminal domain of the p56^{lck} tyrosine protein kinase. *Mol. Cell. Biol.* 10:1853-1862.
- Shen, S.-H., L. Bastien, B.I. Posner and P. Chrétien. 1991. A protein-tyrosine phosphatase with sequence similarity to the SH2 domain of the protein-tyrosine kinases. *Nature* 352:736-739.
- Sheng, Z. and H. Charbonneau. 1993. The baculovirus *Autographa californica* encodes a protein tyrosine phosphatase. *J. Biol. Chem.* 268:4728-4733.
- Siegfried, E., L. Ambrosio and N. Perrimon. 1990. Serine/threonine protein kinases in *Drosophila*. *Trends Genet.* 6:357-362.
- Skolnik, E.Y., B. Margolis, M. Mohammadi, E. Lowenstein, R. Fischer, A. Drepps, A. Ullrich and J. Schlessinger. 1991. Cloning of PI3 kinase-associated p85 utilizing a novel method for expression/cloning of target proteins for receptor tyrosine kinases. *Cell* 65:83-90.
- Smith, C.W.J., J.G. Patton and B. Ginard Nadal. 1989. Alternative splicing in the control of gene expression. *Annu. Rev. Genet.* 23:527-577.
- Smith, D.B. and K.S. Johnson. 1988. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene* 67:31-40.
- Solomon, M.J., J. Gauthier, T. Lee, M.W. and Kirschner. 1991. Control of p34cdc2 activation. Cold Spring Harbor

Symp.Quant.Biol. 56:427-435.

Stader, C. and H.W. Hofer. 1992.A major lienal phosphotyrosine phosphatase is inhibited by phospholipids and inositol trisphosphate. *Biochem.Biophys.Res.Comm.* 189:1404-1409.

Stahl, M.L., C.R. Ferenz, K.L. Kelleher, R.W. Kriz and J.L. Knopf. 1988.Sequence similarity of phospholipase C with the non-catalytic region of src. *Nature* 332:269-272.

Stern, D.F., P. Zheng, D.R. Beidler and C. Zerillo. 1991.Spkl, a new kinase from *Saccharomyces cerevisiae*, phosphorylates proteins on serine, threonine and tyrosine. *Mol.Cell.Biol.* 11:987-1001.

Stover, D.R., H. Charbonneau, N.K. Tonks and K.A. Walsh. 1991.Protein-tyrosine-phosphatase CD45 is phosphorylated transiently on tyrosine upon activation of Jurkat T cells. *Proc.Natl.Acad.Sci.USA* 88:7704-7707.

Strausfeld, U., J.C. Labbé, D. Fesquet, J.C. Cavadore, A. Picard, K. Sadhu, P. Russell and M. Dorée. 1991.Dephosphorylation and activation of a p34^{cdc2}/cyclin B complex in vitro by human cdc25 protein. *Nature* 351:242-245.

Streuli, M., L.R. Hall, Y. Saga, S.F. Schlossman and H. Saito. 1987.Differential usage of three exons generates at least five different mRNAs encoding human leukocyte common antigens. *J.Exp.Med.* 166:1548-1566.

Streuli, M., N.X. Kreuger, T. Thai, M. Tang and H. Saito. 1990.Distinct functional roles of the two intracellular phosphatase like domains of the receptor-linked protein tyrosine phosphatases LCA and LAR. *EMBO J.* 9:2399-2407.

Streuli, M., N.X. Krueger, P.D. Ariniello, M. Tang, J.M. Munro, W.A. Blattler, D.A. Adler, C.M. Distèche and H. Saito. 1992.Expression of the receptor-linked protein tyrosine phosphatase LAR: Proteolytic cleavage and shedding of the CAM-like extracellular region. *EMBO J.* 11:897-907.

Streuli, M., N.X. Krueger, L.R. Hall, S.F. Schlossman and H. Saito. 1988.A new family of the immunoglobulin superfamily that has a cytoplasmic region homologous to the leukocyte common antigen. *J.Exp.Med.* 168:1553-1562.

Streuli, M., N.X. Krueger, A.Y.M. Tsai and H. Saito. 1989.A family of receptor-linked protein tyrosine phosphatases in human and *Drosophila*. *Proc.Natl.Acad.Sci.USA* 86:8698-8702.

Streuli, M. and H. Saito. 1989.Regulation of tissue-specific alternative splicing: exon-specific cis-elements govern the

splicing of the leukocyte common antigen pre-mRNA. EMBO J. 8:787-796.

Strickland, S. 1981. Mouse teratocarcinoma cells: prospects for the study of embryogenesis and neoplasia. Cell 24:277-278.

Subrahmanyam, G., P.J. Bertics and R.A. Anderson. 1991. Phosphorylation of protein 4.1 on tyrosine-418 modulates its function in vitro. Proc.Natl.Acad.Sci.USA 88:5222-5226.

Suh, P.-g., S.H. Ryu, J.H. Moon, H.W. Suh and S.G. Rhee. 1988. Inositol phospholipid-specific phospholipase C: complete cDNA and protein sequences and sequence homology to tyrosine kinase-related oncogene products. Proc.Natl.Acad.Sci.USA 85:5419-5423.

Swarup, G., S. Cohen and D.L. Garbers. 1981. Selective dephosphorylation of proteins containing phosphotyrosine by alkaline phosphatases. J.Biol.Chem. 256:8197-8201.

Swarup, G., S. Kamatkar, V. Radha and V. Rema. 1991. Molecular cloning and expression of a protein-tyrosine phosphatase showing homology with transcription factors Fos and Jun. FEBS Lett. 280:65-69.

Swarup, G. and G. Subrahmanyam. 1989. Purification and characterization of a protein-phosphotyrosine phosphatase from rat spleen which dephosphorylates and inactivates a tissue specific protein kinase. J.Biol.Chem. 264:7801-7808.

Taborsky, G. 1974. Phosphoproteins Adv.Protein Chem. 28:1-210.

Takeda, A. and A.L. Maizel. 1990. An unusual form of lipid linkage to the CD45 peptide. Science 250:676-679.

Takeda, A., J.J. Wu and A.L. Maizel. 1992. Evidence for monomeric and dimeric forms of CD45 associated with a 30-kDa phosphorylated protein. J.Biol.Chem. 267:16651-16659.

Takeichi, M. 1988. The cadherins: cell-cell adhesion molecules controlling morphogenesis. Development 102:639-655.

Takeichi, M. 1991. Cadherin cell adhesion receptors as a morphogenetic regulator. Science 251:1451-1455.

Tam, J.P. 1988. Synthetic peptide vaccine design: Synthesis and properties of a high-density multiple antigenic peptide system. Proc.Natl.Acad.Sci.USA 85:5409-5413.

Tam, J.P. Multiple antigen peptide system: a novel design for synthetic peptide vaccine and immunoassay. In: Synthetic

peptides: Approaches to Biological problems, edited by Tam, J.P. and Kaiser, E.T., New York: Alan R. Liss, Inc., 1989, p. 3-18.

Tamura, S., Y. Suzuki, K. Kikuchi and S. Tsuiki. 1986. Identification and characterization of Mg^{2+} -dependent phosphotyrosyl protein phosphatase from rat liver cytosol. *Biochem. Biophys. Res. Commun.* 140:212-218.

Tan, J.C., K. Nocka, P. Ray, P. Traktman and P. Besmer. 1990. The dominant W^{42} spotting phenotype results from a missense mutation in the c-kit receptor kinase. *Science* 247:209-212.

Tan, X., D.R. Stover and K.A. Walsh. 1993. Demonstration of protein tyrosine phosphatase activity in the second of two homologous domains of CD45. *J. Biol. Chem.* 268:6835-6838.

Tang, T.K., T.L. Leto, I. Correias, M.A. Alonso, V.T. Marchesi and E.J. Benz, Jr. 1988. Selective expression of a erythroid-specific isoform of protein 4.1. *Proc. Natl. Acad. Sci. USA* 85:3713-3717.

Tapley, P., A. Horwitz, C. Buck, K. Burridge, K. Duggan and L. Rohrschneider. 1989. Integrins isolated from Rous sarcoma virus-transformed chicken embryo fibroblasts. *Oncogene* 4:325-333.

Thomas, M.L., A.N. Barclay, J. Gagnon and A.F. Williams. 1985. Evidence from cDNA clones that the rat leukocyte-common antigen (T200) spans the lipid bilayer and contains a cytoplasmic domain of 80,000 Mr. *Cell* 41:83-93.

Thomas, M.L., P.J. Reynolds, A. Chain, Y. Ben-Neriah and I.S. Trowbridge. 1987. B-cell variant of mouse T200 (Ly-5): Evidence for alternative mRNA splicing. *Proc. Natl. Acad. Sci. USA* 84:5360-5363.

Thuriaux, P., P. Nurse, and B. Carter. 1978. Mutants altered in the control co-ordinating cell division with cell growth in the fission yeast *Schizosaccharomyces pombe*. *Mol. Gen. Genet* 161:215-220.

Tian, S.-S., P. Tsoulfas and K. Zinn. 1991. Three receptor-linked protein-tyrosine-phosphatases are selectively expressed on central nervous system axons in the *Drosophila* embryo. *Cell* 67:675-685.

Tomita, M. and V.T. Marchesi. 1975. Amino-acid sequence and oligosaccharide attachment sites of human erythrocyte glycophorin. *Proc. Natl. Acad. Sci. USA* 72:2964-2968.

Tonks, N.K., C.D. Diltz and E.H. Fischer. 1988a. Purification of the major protein-tyrosine-phosphatases of human placenta. *J. Biol. Chem.* 263:6722-6730.

Tonks, N.K., C.D. Diltz and E.H. Fischer. 1988b. Characterization of the major protein-tyrosine-phosphatases of human placenta.

J.Biol.Chem. 263:6731-6737.

Tonks, N.K., H. Charbonneau, C.D. Diltz, E.H. Fischer and K.A. Walsh. 1988c. Demonstration that the leukocyte common antigen CD45 is a protein tyrosine phosphatase. *Biochemistry* 27:8695-8701.

Tonks, N.K., M.F. Cicirelli, C.D. Diltz, E.G. Krebs and E.H. Fischer. 1990a. Effect of microinjection of a low-M_r human placenta protein tyrosine phosphatase on induction of meiotic cell division in *Xenopus* oocytes. *Mol.Cell.Biol.* 10:458-463.

Tonks, N.K., C.D. Diltz and E.H. Fischer. 1990b. CD45, an integral membrane protein tyrosine phosphatase. Characterization of enzyme activity. *J.Biol.Chem.* 265:10674-10680.

Tonks, N.K., Q. Yang and P. Guida, Jr.. 1991. Structure, regulation, and function of protein tyrosine phosphatases. Cold Spring Harbor Symp.Quant.Biol. 56:265-273.

Trofatter, J.A., M.M. MacCollin, J.L. Rutter, J.R. Murrell, M.P. Duyao, D.M. Parry, R. Eldridge, N. Kley, A.G. Menon, K. Pulaski, V.H. Haase, C.M. Ambrose, D. Munroe, C. Bove, J.L. Haines, R.L. Martuza, M.E. MacDonald, B.R. Seizinger, M.P. Short, A.J. Buckler and J.F. Gusella. 1993. A novel moesin-, ezrin-, radixin-like gene is a candidate for the neurofibromatosis 2 tumor suppressor. *Cell* 72:791-800.

Trowbridge, I.S. 1991. CD45. A prototype for transmembrane protein tyrosine phosphatases. *J.Biol.Chem.* 266:23517-23520.

Tsukita, Sa., Y. Hieda and Sh. Tsukita. 1989. A new 82 KD barbed end capping protein localized in the cell-to-cell adherens junction: purification and characterization. *J.Cell.Biol.* 108:2369-2382.

Tsukita, Sh., Sa. Tsukita, A. Nagafuchi and S. Yonemura. 1992. Molecular linkage between cadherins and actin filaments in cell-cell adherens junctions. *Curr.Opin.Cell Biol.* 4:834-839.

Turner, C.E. 1991. Paxillin is a major phosphotyrosine-containing protein during embryonic development. *J.Cell.Biol.* 115:201-207.

Turner, C.E., J.R. Glenney, Jr. and K. Burridge. 1990. Paxillin, a new vinculin-binding protein present in focal adhesions. *J.Cell.Biol.* 111:1059-1068.

Turner, J.M., M.H. Brodsky, B.A. Irvine, S.D. Levin, R.M. Perlmutter and D.R. Littman. 1990. Interaction of the unique N-terminal region of tyrosine kinase p56^{lck} with cytoplasmic domains of CD4 and CD8 is mediated by cysteine motifs. *Cell* 60:755-765.

Turunen, O., R. Winqist, R. Pakkanen, K.-H. Grzeschik, T. Wahlstrom

- and A. Vaheri. 1989. Cytovillin, a microvillar Mr 75,000 protein. cDNA sequence, prokaryotic expression, and chromosomal localization. *J. Biol. Chem.* 264:16727-16732.
- Tyers, M., I. Fitch, G. Tokiwa, C. Dahmann, R. Nash, M. Linskens and B. Futcher. 1991. Characterization of G1 and mitotic cyclins of budding yeast. *Cold Spring Harbor Symp. Quant. Biol.* 56:21-32.
- Ullrich, A. and J. Schlessinger. 1990. Signal transduction by receptors with tyrosine kinase activity. *Cell* 61:203-212.
- Ullman, K.S., J.P. Northrop, C.L. Verweij and G.R. Crabtree. 1990. Transmission of signals from the T lymphocyte antigen receptor to the genes responsible for cell proliferation and immune function: The missing link. *Annu. Rev. Immunol.* 8:421-452.
- Ungewickell, E., P.M. Bennet, R. Calvert, V. Ohanian and W.B. Gratzer. 1979. In vitro formation of a complex between cytoskeletal proteins of the human erythrocyte. *Nature* 280:811-814.
- van Lohuizen, M., S. Verbeek, B. Scheijen, E. Weintjens, H. van der Gulden and A. Berns. 1991. Identification of cooperating oncogenes in E μ -myc transgenic mice by provirus tagging. *Cell* 65:737-752.
- Veillette, A., M.A. Bookman, E.M. Horak and J.B. Bolen. 1988. The CD4 and CD8 T cell surface antigens are associated with the internal tyrosine-protein kinase p56^{lck}. *Cell* 55:301-308.
- Veillette, A., M.A. Bookman, E.M. Horak, L.E. Samelson and J.B. Bolen. 1989. Signal transduction through the CD4 receptor involves the activation of the internal membrane tyrosine-protein kinase p56^{lck}. *Nature* 338:257-259.
- Veillette, A. and D. Davidson. 1992. Src-related protein tyrosine kinases and T-cell receptor signalling. *Trends Genet.* 8:61-66.
- Velazquez, L., M. Fellous, G.R. Stark and S. Pellegrini. 1992. A protein tyrosine kinase in the interferon α/β signaling pathway. *Cell* 70:313-322.
- Vogel, W., R. Lammers, J. Huang and A. Ullrich. 1993. Activation of a phosphotyrosine phosphatase by tyrosine phosphorylation. *Science* 259:1611-1614.
- Vu, T.H., G.R. Martin, P. Lee, D. Mark, A. Wang and L.T. Williams. 1989. Developmentally regulated use of alternative promoters creates a novel platelet-derived growth factor receptor transcript in mouse teratocarcinoma and embryonic stem cells. *Mol. Cell. Biol.* 9:4563-4567.
- Waheed, A., P.M. Laidler, Y.-Y.P. Wo and R.L. Van Etten.

1988. Purification and physiobiochemical characterization of a human placental acid phosphatase possessing phosphotyrosyl protein phosphatase activity. *Biochemistry* 27:4265-4273.
- Wang, Y. and C.J. Pallen. 1991. The receptor-like protein tyrosine phosphatase HPTP α has two active catalytic domains with distinct substrate specificities. *EMBO J.* 10:3231-3237.
- Wang, Y. and C.J. Pallen. 1992. Expression and characterization of wild type, truncated, and mutant forms of the intracellular region of the receptor-like protein tyrosine phosphatase HPTP β . *J. Biol. Chem.* 267:16696-16702.
- Wary, K.K., Z. Lou, A.M. Buchberg, L.D. Siracusa, T. Druck, S. LaForgia and K. Huebner. 1993. A homozygous deletion within the carbonic anhydrase-like domain of the Ptp ^{prg} gene in murine L-cells. *Cancer Res.* 53:1498-1502.
- Weaver, C.T., J.T. Pingel, J.O. Nelson and M.L. Thomas. 1991. CD8⁺ T-cell clones deficient in the expression of the CD45 protein tyrosine phosphatase have impaired responses to T-cell receptor stimuli. *Mol. Cell. Biol.* 11:4415-4422.
- Widmer, F. and P. Caroni. 1993. Phosphorylation-site mutagenesis of the growth-associated protein GAP-43 modulates its effects on cell spreading and morphology. *J. Cell Biol.* 120:503-512.
- Williams, L.T., Escobedo, J.A., Fantl, W.J., Turck, C.W., and A. Klippel. 1991. Interactions of growth factor receptors with cytoplasmic signaling molecules. Cold Spring Harbor Symp. Quant. Biol. 56:243-250.
- Wo, Y.-Y.P., M.-M. Zhou, P. Stevis, J.P. Davis, Z.-Y. Zhang and R.L. Van Etten. 1992. Cloning, expression, and catalytic mechanism of the low molecular weight phosphotyrosyl protein phosphatase from bovine heart. *Biochemistry* 31:1712-1721.
- Wolfe, L.C., S.E. Lux and V. Ohanian. 1980. Regulation of spectrin-actin binding by protein 4.1 and polyphosphates. *J. Cell Biol.* 87:203a.
- Woodford, T.A., K. Guan and J.E. Dixon. 1991. Expression of rat PTP1 in normal and transformed cells. *Adv Prot. Phosphatases* 6:503-524.
- Woodford-Thomas, T.A., J.D. Rhodes and J.E. Dixon. 1992. Expression of a protein tyrosine phosphatase in normal and v-src -transformed mouse 3T3 fibroblasts. *J. Cell Biol.* 117:401-414.
- Yamada, A., M. Streuli, H. Saito, D.M. Rothstein, S.F. Schlossman and C. Morimoto. 1990. Effect of activation of protein kinase C on

CD45 isoform expression and CD45 protein tyrosine phosphatase activity in T cells. *Eur.J.Immunol.* 20:1655-1660.

Yamanashi, Y., T. Kakiuchi, J. Mizuguchi, T. Yamamoto and K. Toyoshima. 1991. Association of B cell antigen receptor with protein tyrosine kinase Lyn. *Science* 251:192-194.

Yang, Q., D. Co, J. Sommercorn and N.K. Tonks. 1993. Cloning and expression of PTP-PEST. A novel, human, nontransmembrane protein tyrosine phosphatase. *J.Biol.Chem.* 268:6622-6628.

Yang, Q. and N.K. Tonks. 1991. Isolation of a cDNA clone encoding a human protein-tyrosine phosphatase with homology to the cytoskeletal-associated proteins band 4.1, ezrin, and talin. *Proc.Natl.Acad.Sci.USA* 88:5949-5953.

Yang, X., K.T. Seow, S.M. Bahri, S.H. Oon and W. Chia. 1991. Two *Drosophila* receptor-like tyrosine phosphatase genes are expressed in a subset of developing axons and pioneer neurons in the embryonic CNS. *Cell* 67:661-673.

Yi, T., J.L. Cleveland and J.N. Ihle. 1992. Protein tyrosine phosphatase containing SH2 domains: Characterization, preferential expression in hematopoietic cells, and localization to human chromosome 12p12-p13. *Mol.Cell.Biol.* 12:836-846.

Yu, Q., T. Lenardo and R.A. Weinberg. 1992. The N-terminal and C-terminal domains of a receptor tyrosine phosphatase are associated by non-covalent linkage. *Oncogene* 7:1051-1057.

Zachary, I. and E. Rozengurt. 1992. Focal adhesion kinase (p125^{FAK}): A point of convergence in the action of neuropeptides, integrins, and oncogenes. *Cell* 71:891-894.

Zander, N.F., D.E. Cool, C.D. Diltz, L.R. Rohrschneider, E.G. Krebs and E.H. Fischer. 1993. Suppression of v-fms-induced transformation by overexpression of a truncated T-cell protein tyrosine phosphatase. *Oncogene* 8:1175-1182.

Zander, N.F., J.A. Lorenzen, D.E. Cool, N.K. Tonks, G. Daum, E.G. Krebs and E.H. Fischer. 1991. Purification and characterization of a human recombinant T-cell protein-tyrosine-phosphatase from a baculovirus expression system. *Biochemistry* 30:6964-6970.

Zanke, B., H. Suzuki, K. Kishihara, L. Mizzen, M. Minden, A. Pawson and T.W. Mak. 1992. Cloning and expression of an inducible lymphoid-specific, protein tyrosine phosphatase (HePTPase). *Eur.J.Immunol.* 22:235-239.

Zhao, Z., S.-H. Shen and E.H. Fischer. 1993. Stimulation by phospholipids of a protein-tyrosine-phosphatase containing two src

homology 2 domains. Proc.Natl.Acad.Sci.USA **90**:4251-4255.

Zheng, X.M., Y. Wang and C.J. Pallen. 1992.Cell transformation and activation of pp60^{c-src} by overexpression of a protein tyrosine phosphatase. Nature **359**:336-339.