

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600



Université d'Ottawa • University of Ottawa

**Molecular Cloning, Characterization and Expression of
A Novel Family of Tail-Anchored Membrane Proteins
from the Myocardium**

Jeffrey T. Wigle

Thesis Submitted to the Department of Pharmacology in partial fulfillment of the
requirements for the degree of Doctor of Philosophy

University of Ottawa

Ottawa, Ontario, Canada

October 1997

©Jeffrey T. Wigle



National Library
of Canada

Acquisitions and
Bibliographic Services

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale
du Canada

Acquisitions et
services bibliographiques

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-28383-6

ABSTRACT

The process of Excitation-Contraction (E-C) coupling in cardiac muscle is dependent on calcium. Many proteins that regulate calcium homeostasis in the cardiocyte are also calcium binding proteins. Two cDNAs were previously isolated by screening a rabbit heart cDNA expression library with an antiserum that recognized two calcium binding polypeptides of the sarcolemma.

These cDNAs and other related cDNAs, which were isolated from cardiac cDNA libraries, were shown to encode a novel family of sarcolemmal associated proteins (SLAPs). The SLAP cDNAs had divergent 5' termini and could potentially encode polypeptides of 37, 46, and 74 kDa molecular designated SLAP1, SLAP2 and SLAP3 respectively. Three SLAP transcripts of 5.9, 4.6 and 3.5 kb were found in heart. The 5.9 kb transcript was ubiquitously expressed while the 3.5 kb and 4.6 kb transcripts were predominantly expressed in cardiac, soleus and smooth muscle. SLAP was encoded by a single gene that mapped to chromosome 3p14.3-21.2 and thus the various transcripts were likely generated by alternative splicing or alternative promoter usage. The primary structure of SLAP predicted that it would have large regions of coiled-coil structure, which included an eleven heptad acidic amphipathic α -helical segment containing two leucine zippers, and a carboxyl terminal transmembrane domain. Three SLAPs of 81, 45 and 35 kDa were shown to be associated with the cardiac sarcolemmal membranes. SLAPs could only be solubilized from cardiac membrane with detergents suggesting that they were integral membrane proteins. Immunohistochemical localization of SLAP in cardiac muscle revealed that SLAP associated with the sarcolemma and displayed a reticular pattern of staining that resembled the transverse-tubules and/or the

sarcoplasmic reticulum. Expression studies showed that MYC-epitope tagged SLAP localized to regions of juxtaposition between neighbouring cell membranes although an intracellular pool of the protein was also present in cells undergoing apparent cleavage.

SLAPs appeared to be the mammalian orthologues of TOP_{AP}, a topographically graded protein expressed in the developing chick retina and tectum (Savitt et al., 1995). Immunofluorescence studies with specific antibodies raised against SLAP fusion proteins showed that SLAP expression was developmentally regulated in the rat retina where it is enriched in Mueller glial cells. However, no evidence of a topographical gradient of SLAP distribution was found in either the mammalian retina, superior colliculus or the lateral geniculate nucleus. In the neonatal rat brain SLAP was selectively expressed in neurons of the hippocampus as well as in the support cells of the nerve fiber layer of the olfactory bulb. SLAP also shared 44% homology with USO1, a yeast protein involved in the fusion of vesicles between the endoplasmic reticulum and the Golgi (Sapperstein et al., 1996). SLAP expression was found to inhibit the fusion of myoblast fusion without effecting their differentiation. In view of the role of tail-anchored-membrane proteins in membrane fusion and the similarity of SLAPs to USO1, we propose that SLAPs may define a novel family of proteins involved in mediating membrane-membrane interactions.

ACKNOWLEDGEMENTS

I would like to thank the many people who have aided me personally or professionally in my thesis work. This list is only as complete as my incomplete memory. I would like to thank Dr. Balwant Tuana for the opportunity to pursue exciting, novel molecular research and the freedom to learn independently. As well, I would like to thank Dr. W.A. Staines for showing me the wonders of the microscope and general guidance. The members of the lab past and present who have helped immeasurably VP, MS, IR, EP, PW and especially my longtime hockey playing/beer drinking/Liberal companion JLL- Thanks for everything John. I would also like to thank the fellow students who have kept me relatively sane: NK (monkey), SC, TG (Mr. Utrophin), the CRG hockey group and the beer-clubbers but especially CT (HRP, Mrs. Lam) and M.L. for all their help in this last hectic year. The general guidance and assistance of the faculty (Drs. Christine Pratt, George Robertson and Bernard Jasmin) was much appreciated. As well, the insight of Ms. Ute Davis and Ms. Babben Tinner-Staines helped steer me clear of many pitfalls. I would like to thank the Muscular Dystrophy Association of Canada for granting me a studentship.

I dedicate this thesis to my family.

- My parents Dr. Donald and Elizabeth Wigle who encouraged and supported me through the entire thesis process.
- My grandmother for all her love and attention.
- My brother, Jason and sister, Jackie for their continued support.
- My sons Theodore and Liam who make everything right.
- My wife Nichola who I will never be able to thank enough.

PREFACE

The work presented in this thesis has been/ will be published in the following papers:

Wigle, J.T., Demchyshyn, L., Pratt, M.A.C., Staines, W.A., and Tuana, B.S. (1997) Molecular Cloning, Expression and Chromosomal Assignment of SLAPs: A Family of Acidic Amphipathic α -helical Proteins Associated with the Sarcolemmal Membrane. *J. Biol. Chem.*, (in press).

Wigle, J.T., Demchyshyn, L., Pratt, C., Staines, W.A., and Tuana, B.S. (1997) SLAP Expression is Developmentally Regulated in the Mammalian Retina and Brain (Manuscript in Preparation).

Wigle, J.T., and Tuana, B.S. (1997) Ectopic Expression of SLAP in Myoblasts Inhibits Myotube Formation but not Differentiation (Manuscript in Preparation).

Wiesolweiski, P., Wigle, J.T., and Tuana, B.S. (1997) Genomic Organization and Splicing (Manuscript in Preparation)

ABSTRACT	I
ACKNOWLEDGEMENTS	III
PREFACE	IV
LIST OF FIGURES	IX
LIST OF ABBREVIATIONS	XII
INTRODUCTION	1
The Heart: Structure and Function	1
Cardiac Membrane Systems and Excitation-Contraction Coupling	2
Molecular Components of Cardiac Membranes Involved in E-C coupling ..	7
Molecular Organization of E-C Coupling Units	12
Ontogeny of E-C Coupling Units	13
Calcium Binding Proteins and Excitation-Contraction Coupling	15
Statement of the Problem	17
MATERIALS AND METHODS	18

mRNA Isolation	18
λ gt-11 cDNA library Construction	18
Screening of oligo-dT primed cDNA library	19
Screening of Random primed cDNA library	19
Template Generation for DNA Sequencing	19
Plasmid Isolation	20
Isolation of ssDNA templates for Sequencing	20
Preparation of Purified Plasmid DNA for DNA sequencing	21
5' Rapid Amplification of cDNA Ends (5' RACE)	21
Genbank Accession Numbers of SLAP Sequences	22
Northern Blot Analysis of SLAP Expression in Rabbit and Rat Tissues ...	22
Southern Blot Hybridization	23
Fluorescence <i>In Situ</i> Hybridization (FISH)	23
Antiserum Production to SLAP fusion protein	24
Affinity Purification of Antiserum	25
Subcellular Fractionation of Myocardial Proteins	25
Extraction of Sarcolemmal Membranes	26
In Vitro Transcription/Translation	27
Primary Antibodies Used in Immunohistochemical Staining	27
Cell Culture and Expression of 6MYC Tagged Recombinant SLAP Protein	28
Immunohistochemistry of Live P19 Cells	29

Differentiation of P19 cells	30
Immunohistochemical Staining of Differentiated P19 cells	30
Expression of SLAP1 in P19 cells	31
Trypsin Digestion of SLAP1 Expressing P19 Cells	32
Preparation of Myocardium for Immunohistochemical Staining	32
Single Immunohistochemical Staining of Myocardium	33
Double Immunohistochemical Staining of Myocardium	33
Adsorption of Antiserum	34
Skeletal Muscle Immunohistochemical Staining	35
Double Immunohistochemical Staining for SLAP and Fiber Specific Markers	35
Immunohistochemical Staining of Retina and Brains	35
Double Immunohistochemical Staining of Brain Cryosections for SLAP and a Neuronal Marker	36
C2C12 Transfections	36
Immunohistochemical Staining of C2C12 cells	37
Determination of Fusion Index	37
Northern Blot Analysis of C2C12 Cultures	39
RESULTS	40
Molecular Cloning and Sequence Analysis of Sarcolemmal Associated Protein	40

SLAP Encodes an Acidic Amphipathic α-helical Tail Anchored Membrane Protein	43
Tissue Specific Expression of SLAP Transcripts	54
Mapping the Termini of the Two Muscle Specific SLAP Transcripts	57
Chromosomal Assignment of the SLAP Gene	59
Biochemical Characterization of SLAP Protein	61
Membrane Association of SLAP	66
Immunohistochemical Localization of SLAP in Muscle	69
SLAP Staining Was Fibre Type Specific in Skeletal Muscle	76
SLAP Expression in the Developing CNS	78
SLAP is related to a Topographically Graded Retinal Protein (TOP_{AP}) ..	78
SLAP Expression is Developmentally Regulated in the Rat Retina	82
SLAP is Expressed in a Population of Neonatal Migratory Neurons	83
SLAP Targeting and Membrane fusion	89
 DISCUSSION	 114
 REFERENCES	 139

LIST OF FIGURES

Figure 1. Excitation-Contraction Coupling in Cardiac Muscle.	6
Figure 2. cDNA Cloning of Sarcolemmal Associated Protein (SLAP).	41
Figure 3. Nucleotide and Deduced Amino Acid Sequence of SLAP.	44
Figure 4. Conservation of the SLAP 3' Untranslated Region (UTR).	45
Figure 5. In Vitro Transcription/Translation of SLAP From Two Potential Translation Start Sites.	47
Figure 6. Secondary Structure Analysis of the SLAP protein.	49
Figure 7. Acidic Amphipathic Nature of the Leucine Zipper Region of SLAP. ...	51
Figure 8. Homology Between SLAP and USO1.	53
Figure 9. Tissue Specific Expression of SLAP Transcripts.	55
Figure 10. Expression of SLAP During Muscle Development.	56
Figure 11. Mapping the 5' End of the Smaller Cardiac Transcripts.	58
Figure 12. SLAPs Have Divergent 5' Termini.	60
Figure 13. Genomic Organization of SLAP.	62
Figure 14. Chromosomal Assignment of the SLAP Gene.	63
Figure 15. Generation of Specific anti-SLAP Antiserum.	65
Figure 16. Immunoreactivity of the Affinity Purified anti-SLAP Antiserum.	67
Figure 17. Membrane Specific Association of SLAPs.	68
Figure 18. SLAPs are Integral Membrane Proteins.	70
Figure 19. SLAP Staining in the Developing and Adult Myocardium.	71

Figure 20. SLAP Staining Compared to the Staining Pattern of Other Membrane Markers.	73
Figure 21. Myocardium Stained for SLAP and Membrane Markers.	74
Figure 22. Specificity of Anti-SLAP Antiserum.	75
Figure 23. SLAP Localization in the Developing and Adult Skeletal Muscles.	77
Figure 24. Muscle Fiber Specific Nature of SLAP.	79
Figure 25. Co-localization of SLAP and the Slow Form of Ca ²⁺ ATPase.	80
Figure 26. Alternative SLAP Transmembrane Domains.	81
Figure 27. SLAP Expression in Developing Rat Retina.	84
Figure 28. SLAP Staining in the Developing Hippocampus.	86
Figure 29. SLAP Expression in the Olfactory Bulb.	87
Figure 30. Olfactory Bulb Cells Stained for SLAP and a Neural Marker.	88
Figure 31. Ectopic Expression of SLAP1 Produces a 35 kDa protein.	90
Figure 32. Trypsin Digestion of SLAP1 Expressing Cells.	92
Figure 33. Subcellular Localization of Recombinant Epitope Tagged SLAP.	93
Figure 34. Subcellular Localization of SLAP Examined by Confocal Laser Microscopy.	94
Figure 35. DNA Condensation in Bipolar SLAP Positive Cells.	97
Figure 36. Staining For SLAP in Permeabilized and Unpermeabilized Cells.	98
Figure 37. DMSO Induces Expression of Cardiac Actin in P19 cells.	99
Figure 38. SLAP Transcript Expression in Differentiated P19 Cells.	102
Figure 39. SLAP in Differentiated P19 Cell Cultures.	103

Figure 40. Double Immunofluorescence Analysis of Differentiated P19 cells. . . .	104
Figure 41. SLAP Localization in Myoblasts.	107
Figure 42. Localization of SLAP in C2C12 Cells.	108
Figure 43. Fusion of SLAP Transfected C2C12 Cells.	109
Figure 44. Fusion Index of SLAP Expressing Myoblasts.	110
Figure 45. Myogenin Expression in Differentiated C2C12 Cells.	112
Figure 46. Expression of SLAP Transcripts in C2C12 Cells.	113
Figure 47. Proposed Role of SLAPs in Modulating Excitation-Contraction	
Coupling.	135

LIST OF ABBREVIATIONS

ATPase	Adenosine Triphosphatase
bp	Base pair
BrdU	5-bromo-2'-deoxyuridine
cDNA	Complementary DNA
DAPI	4'6'-diamino-2-phenylindole
DMSO	Dimethyl Sulfoxide
DEPC	Diethylpyrocarbonate
EDTA	Ethylenediaminetetracetic acid
EGTA	Ethyleneglycol-bis(β -aminoethyl ether)-N,N'-tetracetic acid
FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate
IgG	Immunoglobulin
kb	kilobase
kDa	kilodalton
MEM	Minimal Essential Medium
MOPS	Morpholino propane sulfonic acid
NP-40	nonyl phenoxy polyethoxy ethanol
nt	Nucleotide
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffered Saline

SDS	Sodium Dodecyl Sulfate
SSC	Standard Citrate Saline
TBS	Tris-Buffered Saline
Tris	Tris(hydroxymethyl)aminomethane
Tween-20	Polyoxyethylene sorbitan monolaurate
UTR	Untranslated Region

INTRODUCTION

The Heart: Structure and Function

The pivotal role of the heart in supporting life was summarized more than 350 years ago by the English scientist William Harvey who said “The heart, with the veins and blood they contain, is to be regarded as the beginning and the author, the fountain and original of all things in the body, the primary cause of life”(Opie, 1991). The heart is composed of cells of various types such as: the pacemaker cells of the sinoatrial and the atrioventricular nodes, the conducting cells of the Purkinje system, endothelial and smooth muscle cells of the coronary blood vessels and the cardiocytes, the contractile cells of the heart. It is the co-ordinated contraction of the cardiocytes that generates the force required to pump the blood through the circulatory system. This co-ordinated contraction is possible since the cardiocytes are electrically connected via gap junctions, which allow for the entire myocardium to depolarize rapidly. The gap junctions are localized in the intercalated discs that are the specialized cell membrane regions formed at the junction of two adjoining cardiocytes. The contractile proteins account for approximately 50% of the volume of the cardiocyte and are ordered in such a way to give the cell a striated appearance (Page and McCallister, 1973). The dark or A bands seen under a light microscope are composed largely of myosin whereas the light or I bands seen under the microscope are composed of the thin filaments (actin, tropomyosin and the troponins (TroponinC, TroponinI and TroponinT)). The dark band in the middle of the I band is called the Z-disc and is composed largely of α -actinin. The cardiocytes are triggered to contract in a process called excitation-contraction (E-C) coupling in which the depolarization of the cardiac cell membrane, also called the

sarcolemma, leads to muscle contraction. The sarcolemmal depolarization increases intracellular calcium levels, which in cardiac muscle is required to induce further calcium release from the sarcoplasmic reticulum (SR). The rise of the intracellular calcium level is sensed by the calcium binding protein Troponin C that allows the myofibrillar proteins to contract. The cytoplasmic calcium levels must be lowered quickly so the cardiocytes can relax before the next contraction. The cardiocyte has adapted to the demands imposed by the contraction-relaxation cycle by developing an exquisite ability to regulate intracellular calcium levels. The proteins of these cardiac membranes have evolved to regulate calcium influx and efflux as well as mediating signal transduction that fine tunes intracellular calcium levels in response to physiological demands.

Cardiac Membrane Systems and Excitation-Contraction Coupling

The sarcolemma consists of the lipid bilayer and the glycocalyx, which is a 40 nm thick coat of glycopospholipids and glycoproteins (Opie, 1991). The glycoalyx can become detached from the cardiocyte in calcium free solution and this disruption of the glycocalyx has been implicated in the deleterious effects observed when cardiocytes are reperfused with medium containing calcium (Frank et al., 1982). The cardiac cell membrane (sarcolemma) has several important and diverse roles in cell function that are largely mediated by membrane associated proteins. The lipid bilayer prevents the entry of many compounds (macromolecules, ions) into the cell but membrane proteins such as ion channels, transporters and pumps allow for the controlled flow of diverse molecules across the cell membrane. This selective permeability barrier allows for the formation of an electrochemical gradient necessary for electrically excitable cells and to insulate cells from

sudden changes in their environment. Plasma membrane proteins, such as those of the dystrophin complex, are believed to mediate interaction of the sub-sarcolemmal cytoskeleton network with extracellular matrix proteins such as laminin (Gee et al., 1994). These interactions are believed to maintain the integrity of the cardiac cell membrane during contraction since mutations in dystrophin have been implicated in human cardiomyopathies (Ortiz-Lopez et al., 1997; Melacini et al., 1996). The connexins are cell membrane proteins that form the gap junctions and thus serve to couple the myocardium electrically (Gros and Jongsma, 1996). Membrane proteins, such as receptors, enzymes and anchoring proteins, are involved in transducing signals across the plasma membrane, which allows the cardiocyte to respond to changes in demand or its environment. The lipid bilayer of the cell membrane contains many proteins which are associated with it in an integral or peripheral manner. Integral membrane proteins can not be solubilized from cell membranes except by detergent extractions. These integral membrane proteins have either a transmembrane segment that traverses the lipid bilayer or they have had lipid moieties attached post-translationally. Peripheral proteins are localized to the plasma membrane by associating with plasma membrane proteins. This protein-protein association can be disrupted by various pHs and the peripherally associated proteins can therefore be extracted from membrane fractions without the use of detergents.

The cardiac sarcolemma plays a key role in transmitting the depolarization signal throughout the individual cardiocytes and the myocardium as well as regulating the trans-sarcolemmal flow of calcium. There is 10,000 fold more calcium in extracellular fluid than in the cytoplasm of a resting cardiocyte. This large calcium gradient is maintained by

sarcolemmal membrane proteins such as the sodium calcium exchanger and the calcium adenosinetriphosphatase (ATPase). The depolarization of the membrane activates the dihydropyridine sensitive L-Type calcium channel (DHPR) and permits calcium to flow into the cardiocyte down its large electrochemical gradient. The sarcolemma invaginates periodically to form structures called the Transverse-tubules (T-tubules) which allows the sarcolemma to be in close contact over a large area with the internal storage site for calcium - the sarcoplasmic reticulum.

In cardiocytes, the sarcoplasmic reticulum (SR) structure of is an elaborate internal membrane compartment that comprises up to 2% of the total cell volume (Opie, 1991). The main functions of the SR are the storage, release and uptake of calcium. The SR can be divided into longitudinal, junctional and corbular compartments (Jorgensen et al., 1988b). The longitudinal SR surrounds the myofibrils forming a lattice network throughout the cardiocyte and is enriched in the SR calcium ATPase and its regulatory protein phospholamban (Jorgensen et al., 1982; Jorgensen and Jones, 1987). The junctional SR is a specialized compartment that is in very close contact with either the T-tubules or the sarcolemma and is enriched in proteins such as calcium binding protein calsequestrin, junctin, triadin and the ryanodine receptor/ SR calcium release channel (RyR) (Flucher and Franzini-Armstrong, 1996). The corbular SR is enriched in the RyR and calsequestrin but unlike the junctional SR it is not apposed to either the sarcolemma or the T-tubules (Jorgensen et al., 1988b).

The T-tubules greatly increase the surface area of the plasma membrane of the myocyte and allow for the electrical depolarization to be transmitted deep into the myocyte.

The T-tubular system is more developed in the ventricular cardiocyte than in the atrial cardiocyte. In cardiocytes, each T-tubule is apposed to only one junctional SR in a structure called the diad. T-tubules are enriched in DHPR and the junctional SR is enriched in RyR when compared with the sarcolemma and the longitudinal SR respectively (Flucher and Franzini-Armstrong, 1996). Excitation of the sarcolemma is tightly linked to the contraction of the muscle by a process called excitation-contraction coupling (E-C coupling). Depolarization of the sarcolemma travels into the T-tubules and leads to the activation of DHPR calcium channel, which allows for an influx of external calcium (Fig. 1A) (Fleischer and Inui, 1989). The influx of external calcium through the DHPR triggers the RyR, located in the adjoining junctional SR, to release calcium from the SR stores and it is this release of calcium that induces muscle contraction. The mechanism of E-C coupling in skeletal and cardiac muscle are similar but have some important differences. In cardiac muscle the influx of external calcium is required for the activation of the RyR but in skeletal muscle the voltage induced conformational change of the DHPR alone seems sufficient to mediate E-C coupling (Fabiato, 1985; Armstrong et al., 1972). In contrast to cardiac muscle, most of the DHPR molecules in skeletal muscle function as voltage sensors, rather than as calcium channels (Schwartz et al., 1985). In fast twitch skeletal muscle, each T-tubule is apposed to two junctional SR membranes in a complex called a triad instead of being apposed by a single junctional SR as happens in cardiac muscle. After contraction, the intracellular calcium levels of the cardiocyte must be reset quickly

Figure 1. Excitation-Contraction Coupling in Cardiac Muscle.

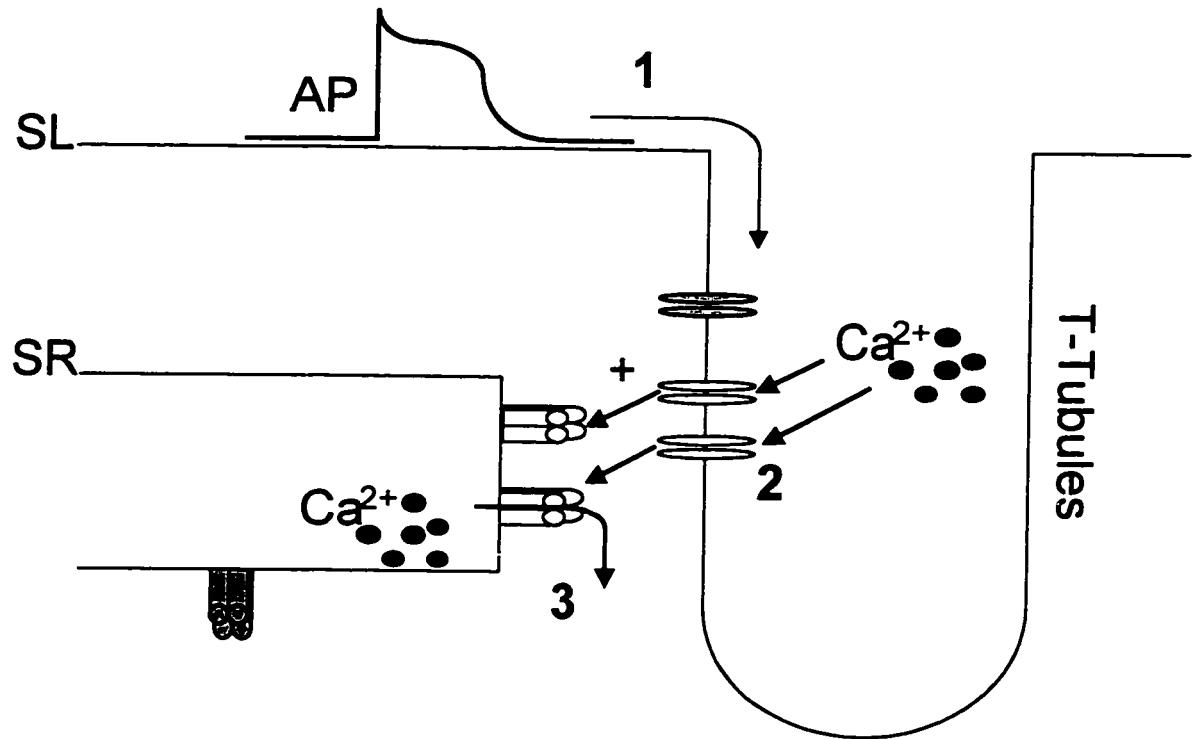
A) Calcium influx and muscle contraction.

The wave of depolarization (AP) travels down the sarcolemma (SL) and into the invaginations of the Transverse tubules (1) where the voltage gated calcium channels are opened and calcium can flow into the cell (2). The calcium entry induces further calcium release from the sarcoplasmic reticulum (SR) by activating the ryanodine receptor calcium channel (3) and it is the release of the internal calcium that induces the cardiocyte to contract.

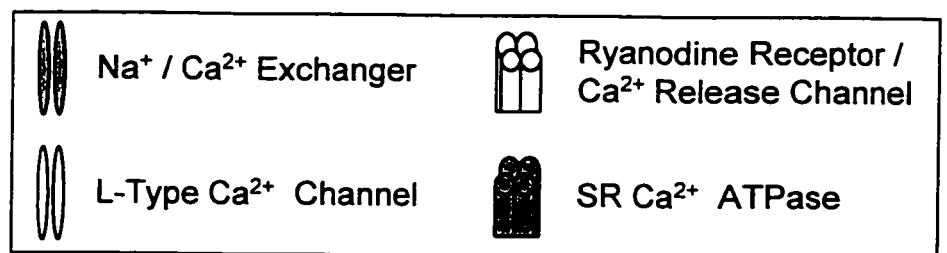
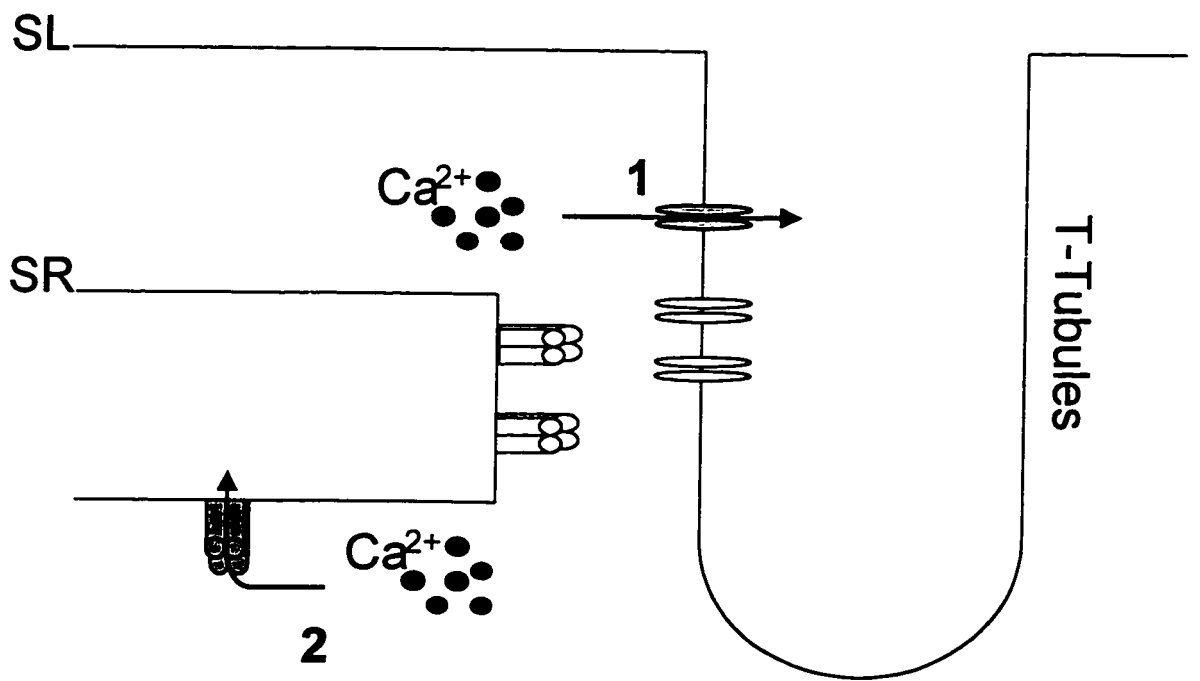
B) Calcium efflux and muscle relaxation.

After the muscle contracts the elevated calcium is pumped out of the cytoplasm by the plasma membrane sodium/calcium exchanger (1) or by the calcium ATPase of the SR (2).

A



B



so that the muscle can relax before the next contraction cycle starts (Fig. 1B). The elevated intracellular calcium levels result from calcium influx from across either the SR or the sarcolemma. The external influx of calcium can be extruded from the myocytes by either the sodium calcium exchanger or the sarcolemmal Ca^{2+} ATPase (Barry and Bridge, 1993). The large amount of calcium depleted from the SR during muscle contraction is replenished by the calcium ATPase of the SR, which is distributed in the longitudinal SR but not the junctional SR (Jorgensen et al., 1982).

Molecular Components of Cardiac Membranes Involved in E-C coupling

External calcium entry is mediated by the L-type calcium channels of the sarcolemma. Cardiac DHPR exists as a multimeric complex of four different subunits designated α_1 , α_2 , β , and δ in a ratio of 1:1:1:1 (Catterall, 1991). The α_2 and the δ subunits are disulphide linked and are derived by proteolytic processing of a common precursor protein (De Jongh et al., 1990). Unlike the α_2 and the δ subunits, the other two calcium channel subunits are encoded by distinct genes. The functional calcium pore of the DHPR is encoded by the α_1 subunit and the other subunits act to modify channel properties (Catterall, 1991). Secondary structure analysis of the α_1 subunit predicted it to have four hydrophobic domains each containing six transmembrane segments. In each hydrophobic domain, the fourth transmembrane segment contains positively charged amino acids and is the putative voltage sensor of the calcium channel. Upon depolarization of the sarcolemma the S4 segment undergoes a conformation change and there is an outward movement of a positive gating charge (Catterall, 1993). The α_1 subunit of cardiac muscle (designated α_{1C}) has an overall 66% homology with the α_1 subunit of skeletal muscle (designated α_{1S})

(Mikami et al., 1989). The transmembrane domains are extremely well conserved between the two isoforms, but the intracellular loops are much less conserved. Differences in the properties of the two calcium channel isoforms can be largely attributed to these divergent intracellular loops. For instance, in skeletal muscle the depolarization induced conformational change of the α_{1S} subunit is sufficient to activate E-C coupling but in cardiac muscle E-C coupling requires extracellular calcium to enter the cell as well (Armstrong et al., 1972; Tanabe et al., 1990a). In dysgenic myotubes, skeletal E-C coupling can be restored by expressing the α_{1S} (Tanabe et al., 1988). Expression of the α_{1C} subunit in dysgenic myotubes can convert the mode of E-C coupling to a cardiac, calcium dependent mechanism (Tanabe et al., 1990b). Expression of skeletal/cardiac α_1 subunit chimeras in these cells demonstrated that it was the intracellular loop between domains II and III of the calcium channels which was responsible for dictating the mode of E-C coupling (Tanabe et al., 1990a). The segment between transmembrane segments S5 and S6 lines the pore of the calcium channel (Striessnig et al., 1990). Mutation of glutamate residues in these segments of domain III and IV can change the selectivity filter of the channel and make it more permeable to sodium compared with calcium (Sather et al., 1993).

The α_1 subunit binds the β subunit through a motif located in the intracellular loop between domain I and II (Pragnell et al., 1994). *In vitro* experiments showed expression of the cardiac β_2 subunit directed the cardiac α_{1C} subunit to the plasma membrane and increased the number of dihydropyridine binding sites (Chien et al., 1995). Studies of myotubes derived from mice null for the skeletal specific β_1 subunit showed that skeletal α_1 subunit was not localized to the sarcolemma and that L-type calcium current was dramatically

decreased in the absence of the β_1 subunit (Gregg et al., 1996). In myotubes derived from dysgenic mice (lacking the skeletal α_1 subunit) the β_1 subunit still localizes to the plasma membrane. The α_2/δ subunits are common to both cardiac and skeletal muscle. They are transmembrane proteins and their co-expression with α_1 subunit has been shown to increase calcium channel current and to accelerate the activation of the channel (Mikami et al., 1989).

The calcium that enters the cardiocyte through the DHPR is thought to trigger calcium release from the RyR via a calcium induced calcium release mechanism (Fabiato, 1985). The external calcium that enters the cardiocyte is transported out by the sodium calcium exchanger and the sarcolemmal ATPase (Barry and Bridge, 1993). The sodium calcium exchanger transports three sodium ions into the cell for each calcium ion it extrudes (Reeves and Hale, 1984). The exchanger uses the large gradient of sodium ions (lower intracellularly) to drive calcium ions out of the cell against its concentration gradient and does not directly utilize ATP. However, the sodium gradient is maintained by the sodium potassium ATPase, which does use ATP. The activity of the exchanger is believed to be activated by the calcium binding protein calmodulin, which removes a source of autoinhibition (Li et al., 1991). The sodium calcium exchanger has been shown to be concentrated in the T-tubules through its association with the cytoskeletal protein ankyrin (Frank et al., 1992; Li et al., 1993). In cardiocytes the majority of calcium transport is performed by the sodium calcium exchanger and the sarcolemmal ATPase probably has a modulatory role only (Cannell, 1991). The RyR is a large 500 kDa protein that assembles into a homotetramer to form the high conductance calcium release channel of the SR (Franzini-Armstrong and Protasi, 1997). This homotetramer appears as a cloverleaf

structure seen in electron-microscopy images of the junctional SR. Three isoforms of the RyR have been cloned: RyR1 (found in skeletal muscle), RyR2 (found in cardiac muscle and brain) and RyR3 (found in smooth and skeletal muscle) (McPherson and Campbell, 1993). The RyR protein can be divided into a large cytoplasmic foot domain and a carboxyl-terminal calcium channel domain. The carboxyl terminus of the RyR contains four transmembrane domains that form the calcium pore and the binding site for the plant alkaloid ryanodine (Franzini-Armstrong and Protasi, 1997). The RyR interacts with the sarcoplasmic reticulum proteins junctin, calsequestrin, and triadin (Zhang et al., 1997). The skeletal and the cardiac RyRs have been shown to bind the FK506 binding protein tightly (FKBP12) (Timmerman et al., 1996). FKBP12 apparently inhibits the calcium current of the skeletal RyR but does not appear to affect the current of the cardiac RyR (Barg et al., 1997). The amino terminal portion of the RyR protein is cytoplasmic and contains sites for calcium binding, calmodulin binding, phosphorylation and other modulatory sites (McPherson and Campbell, 1993). The cytoplasmic portion of the RyR is very large and forms the foot structure seen in electron microscopy analysis of junctional SR membranes.

The calcium released from the SR serves to trigger the contraction of the cardiocytes. In steady state conditions the amount of calcium released from the SR during contraction equals the amount of calcium pumped into the SR during relaxation. The SR Ca^{2+} ATPase replenishes the SR Ca^{2+} stores thereby helping to lower the intracellular calcium levels and allowing the cardiocyte to relax. The activity of the SR Ca^{2+} ATPase is inhibited by the unphosphorylated form of the protein phospholamban (Sasaki et al., 1992). Phosphorylation of phospholamban by cAMP dependent protein kinase or Ca^{2+} /Calmodulin protein kinase

removes the inhibition by phospholamban (Fujii et al., 1989) and thereby activates the Ca^{2+} ATPase.

Modulating the activity of the proteins involved in E-C coupling can greatly affect myocardial contractility. For example, adrenergic agonist stimulate myocardial contractility by increasing calcium influx through the DHPR and by enhancing calcium uptake by the SR Ca^{2+} ATPase. Binding of agonists to the β_1 adrenoreceptor leads to an increase in intracellular cAMP and the increased activity of the cAMP dependent protein kinase (PKA). The activated kinase phosphorylates the DHPR which increases calcium entry and it also phosphorylates phospholamban which leads to increased activity of the SR Ca^{2+} ATPase (McDonald et al., 1994; Sasaki et al., 1992). The L-type calcium current was not affected by addition of PKA activators in cells transfected with a mutant cAMP kinase anchoring protein (AKAP-79) which did not bind PKA (Gao et al., 1997). Therefore, the regulation of the DHPR by PKA phosphorylation apparently requires that PKA be targeted to the membrane by the AKAP-79 scaffold protein. The adrenergic agonists increase myocardial contractility since more calcium enters the cell to trigger release from the SR and there is more calcium in the SR to be released. As well, the relaxation of the cardiocyte is enhanced because the rate of uptake of the SR Ca^{2+} ATPase is increased. The cardiac glycoside digoxin alters myocardial contractility by altering the activity of the sodium calcium exchanger. Digoxin inhibits the sodium potassium ATPase of the sarcolemma (Smith, 1988) and inhibition of this pump reduces the sodium gradient that drives the sodium calcium exchanger thus more calcium accumulates inside the cell (Sheu and Fozzard, 1982). This elevated intracellular calcium level results in increased contractility of the myocardium.

Molecular Organization of E-C Coupling Units

The concentration of DHPR in the T-tubules and the RyR in the junctional SR are necessary for effective E-C coupling so that the depolarization sensed by the DHPR can be efficiently transmitted to the RyR. Electron microscopy analysis revealed the presence of large particles in the junctional SR that were called feet structures. These have been shown to be composed of RyR tetramers since purified cardiac and skeletal RyR receptors form the foot structure and the foot structures are absent from the SR in dyspedic mice which are null for the skeletal muscle RyR (Inui et al., 1987b; Inui et al., 1987a; Takekura et al., 1995a). The DHPR is not required for the localization of RyR since feet structures were found to be localized correctly to the junctional SR in dysgenic myoblasts, which are null for α_{1S} (Powell et al., 1996). The DHPR is concentrated in the T-tubular compartment of the sarcolemma in both heart and skeletal muscle (Sun et al., 1995; Protasi et al., 1997). The cardiac DHPR has been shown by confocal microscopy to be situated in patches directly across from concentrations of RyR in the SR (Carl et al., 1995). Cardiac E-C coupling is initiated via a calcium induced release model so the two calcium channels do not have to be as closely linked physically as in skeletal muscle (Fabiato, 1985). However, the close proximity of the DHPR to the RyR allows for the opening of a single DHPR to be sufficient to trigger calcium release from a cluster of adjacent RyRs (Cheng et al., 1996). In skeletal muscle the DHPR form tetrameric structures called tetrads. Analysis of freeze fracture images of triads from the swimbladder (fast muscle) of the toadfish revealed a stoichiometric ratio of 2 RyR foot structures to each DHPR tetrad (Block et al., 1988). Furthermore, the RyR are arrayed in such a manner that half of the foot structures are apparently directly coupled to tetrads and

the other half would be uncoupled. This finding led to the proposal that the tetrads coupled to foot structures are probably physically linked which would allow for E-C coupling to occur without external calcium influx. The uncoupled RyR could be stimulated by a calcium induced calcium release mechanism similar to that in cardiac muscle. Indeed a component of the E-C coupling in skeletal muscle has been shown experimentally by the microinjection of calcium chelators to be calcium dependent (Jacquemond et al., 1991). The relevance of this hypothesis of E-C coupling outside of the toadfish is still unclear since binding studies have determined the ratio of tetrads to foot structure in rabbit skeletal muscle to be 4:1 foot structures:tetrads possibly indicating a significant physiological role for external calcium in skeletal muscle E-C coupling (Anderson et al., 1994). As well, the ratio of DHPR charge movement to RyR varies between muscle types with it being twice as large in fast twitch skeletal muscle as compared to slow twitch skeletal muscle (Cullen et al., 1984; Appelt et al., 1989). Perhaps the fast twitch skeletal muscle E-C coupling is more dependent on physically linked channels than slow-twitch E-C coupling. Studies on dyspedic mice have shown that the formation of the DHPR into tetrads is dependent on the expression of the RyR while their localization to the T-tubules is independent of the RyR (Nakai et al., 1996). Both calcium channels are concentrated at the triad/diad structures in skeletal and cardiac muscle but the proteins that direct and anchor them to their appropriate sites are unknown.

Ontogeny of E-C Coupling Units

The triad membrane structure and the localization of the DHPR and RyR in the junctional membranes is essential for efficient E-C coupling. The T-tubular membrane and the SR membrane associate tightly with each other during subcellular fractionation and

purified T-tubular membranes and SR membranes will even re-associate *in vitro* (Flucher and Franzini-Armstrong, 1996). The precise arrangement of these two distinct membrane systems has been proposed to be mediated through direct interactions between the DHPR and the RyR. The physical association between DHPR and RyR is supported by the finding that bacterially expressed fusion proteins containing the II-III intracellular loop of the DHPR from either skeletal or cardiac muscle can activate the skeletal RyR *in vitro* (Lu et al., 1994). As well, the assembly of DHPR into tetrads in skeletal muscle and the calcium current through the DHPR is dependent on the expression of the RyR (Nakai et al., 1996). There are now several lines of evidence that suggest that the association between T-Tubules and the junctional SR is not mediated by a direct interaction between the two calcium channels. For instance, recent work with transgenic mice has shown that DHPR and RyR are not required for the formation of the triad structure. In both dysgenic mice and dyspedic mice triads still form appropriately (Flucher et al., 1992; Powell et al., 1996). Expression of the RyR appears to dictate the precise width of the cleft between the T-Tubular membrane and the SR rather than the formation of the triad structure itself since the triads form in dyspedic mice but the gap between the T-Tubules and the SR is narrower and irregular (Takekura et al., 1995a). As well, studies of *in vitro* muscle development have shown that the coupling between T-tubules and SR occur before the appearance of either the DHPR or RyR (Protasi et al., 1997). Neither triads nor peripheral couplings form in Chinese hamster ovary cells expressing the skeletal RyR and the skeletal DHPR suggesting another a role for other proteins in the coupling of the cell membrane to the sarcoplasmic reticulum (Takekura et al., 1995b). Finally, in the myocardium there is no evidence for direct coupling of the DHPR and the RyR

yet the diad structure still forms between SR and T-tubules. The formation of cardiac diads suggests that the association of the junctional SR and the T-tubules is not mediated by a direct interaction between the two calcium channel proteins but via other diad proteins.

A protein called triadin that localizes to the junctional SR was proposed as a putative linker molecule between the T-tubules and the SR (Caswell et al., 1991). However, topological and proteolytic analysis predicted that the majority of the triadin protein would reside in the SR lumen not the cytoplasm which led to the proposal that triadin functions to anchor the SR calcium binding protein calsequestrin to the junctional SR (Knudson et al., 1993). Fusion protein fragments of triadin bind both calsequestrin and RyR but not the DHPR indicating that triadin may act to link the RyR to calsequestrin rather than to the DHPR (Guo and Campbell, 1995). Probably there are proteins in the sarcolemma and the SR which mediate the association between these two muscle membrane systems that have yet to be characterized.

Calcium Binding Proteins and Excitation-Contraction Coupling

Many of the important regulators of E-C coupling are calcium binding proteins. The contraction of the myofibrils is a calcium dependent process and the key calcium sensor protein in this process is Troponin-C (Tn-C). In situations of low intracellular calcium, myosin can not bind to actin because its site is blocked by the tropomyosin/Troponin complex. When calcium levels rise following excitation of the cardiocyte, Troponin-C binds calcium and the tropomyosin/troponin complex undergoes a conformational change such that myosin can bind actin and the myofibrils can contract. The two major calcium channels involved in E-C coupling are themselves calcium binding proteins. The DHPR binds

calcium through an EF hand motif which is believed to modulate the calcium dependent inactivation of the channel (de Leon et al., 1995). The Ryanodine receptor contains a calcium binding site and binding of calcium to this site increases the open probability of the channel (Chen et al., 1992). The calcium efflux proteins (Ca^{2+} ATPase and sodium calcium exchanger) are also calcium binding proteins as although they do not contain a discernible EF hand. A Ca^{2+} / calmodulin dependent protein kinase has been shown to associate with heart membrane fractions (Tuana et al., 1987a). Phosphorylation of SR vesicles by the endogenous Ca^{2+} / calmodulin dependent protein kinase has been implicated in the closing of the RyR calcium channel (Wang and Best, 1992; Hain et al., 1995). This inhibition likely occurs indirectly since the endogenous Ca^{2+} / calmodulin dependent protein kinase does not phosphorylate the RyR itself but it does phosphorylate the junctional SR protein triadin which is a potential modulator of the RyR (Damiani et al., 1995). Studies have shown that the amount of calcium bound to the cardiac sarcolemma is directly proportional to myocardial contractility (Langer, 1987). The sarcolemmal calcium is bound mostly to the phospholipids but some is bound to membrane proteins. The role of sarcolemmal bound calcium in modulating E-C coupling has not yet been fully elucidated.

Calcium binding proteins clearly play critical roles in modulating E-C coupling. However, the molecular and functional properties of the calcium binding proteins of the sarcolemma have not yet been fully characterized. Previous studies utilizing ^{45}Ca binding assays identified two calcium binding polypeptides of 125 and 97 kDa in cardiac sarcolemma (Tuana et al., 1991). These polypeptides were shown to be glycoproteins that co-purified with the DHPR. Antiserum raised to purified canine cardiac sarcolemma recognized two

major antigenic polypeptides of a 125 and 97 kDa in sarcolemmal membrane fractions from a variety of species. These antigenic polypeptides were glycosylated and identified as the putative calcium binding polypeptides of the sarcolemma. Although the calcium binding proteins co-purified with the DHPR of the myocardium, the relationship between the calcium binding proteins and the subunits of the receptor remain unclear.

Statement of the Problem

In view of the importance of calcium and calcium binding proteins in myocardial function, a molecular approach was used to further characterize the calcium binding polypeptides of the sarcolemma. The anti-sarcolemmal antiserum that apparently recognized the calcium binding polypeptides was used to screen a rabbit cardiac cDNA expression library to isolate cDNAs that would encode the potential calcium binding polypeptides. Two novel cDNAs, designated LD1 and LD2, were isolated from screening the rabbit heart library (Demchyshyn, 1990).

The purpose of this study was to further characterize these cDNAs and the polypeptides they encoded. The molecular characterization of these clones has now led to the definition of a novel family of sarcolemmal associated proteins referred to as the SLAPs. SLAPs are expressed in a tissue specific manner and may represent a new family of proteins involved in membrane function.

MATERIALS AND METHODS

mRNA Isolation

Rabbits were sacrificed by cervical dislocation and their hearts were excised and snap frozen in liquid nitrogen. Total heart RNA was isolated by the guanidine thiocyanate method (Chirgwin et al., 1979). Messenger RNA (mRNA) was selected from total RNA by two purifications on oligo-dT cellulose by a protocol modified from Aviv and Leder (Aviv and Leder, 1972). Total RNA was diluted to a concentration of 2 mg/mL in diethylpyrocarbontate (DEPC) treated water and boiled for two minutes and then chilled on ice for ten minutes with an equal volume of 2X loading buffer (40 mM Tris-HCL pH 7.6, 1M NaCl, 0.2mM EDTA). SDS was added at 0.5% final concentration and the RNA mixture was added to oligo dT cellulose (Boheringer-Manheim) that had been pre-equilibrated with 1X loading buffer. This slurry was mixed for 4 hours at 37°C and then poured into a column. The column was washed with 10 bed volumes of 1X loading buffer and the mRNA was eluted with one bed volume of sterile water. Eluted mRNA was concentrated by ethanol precipitation and redissolved in sterile water.

λ gt-11 cDNA library Construction

For both the oligo dt primed library and the random primed libraries the cDNA was constructed using rabbit mRNA as the template for synthesis. Using the Superscript cDNA system (Gibco-BRL), cDNA synthesis for the first library was primed with oligo dT primers. Using the Superscript Choice Synthesis system (Gibco-BRL), random primed cDNA for the second library was synthesized. The cDNA for both libraries was size selected for inserts greater than 0.5 kb and the size selected cDNA was cloned into λ gt-11 (Amersham).

Screening of oligo-dT primed cDNA library

800 000 plaques of the oligo-dT primed rabbit heart expression library were screened with a 1.1 kb radiolabelled EcoRI/XbaI fragment from the LD1 clone. The cDNA probe was radiolabelled with α -³²P-dCTP by using the Rediprime kit (Amersham). A single positive clone was identified on a tertiary screen and was purified and subcloned into pBluescript II KS+ by standard methods (Sambrook et al., 1989).

Screening of Random primed cDNA library

250 000 plaques of the random primed rabbit heart cDNA expression library were screened with a radiolabelled Aval/EcoRI cDNA fragment from the LD1 clone which was radiolabelled as described above. Twenty positive clones were identified in a tertiary screen and three clones with inserts >2 kb were identified. These phage were purified and their inserts were subcloned into pBluescript II KS+ by standard methods (Sambrook et al., 1989).

Template Generation for DNA Sequencing

Nested deletions were made of the various plasmids using the Erase-A-Base kit (Promega). Briefly, plasmids were digested unidirectionally with ExonucleaseIII for varying times and then blunted with S1 Nuclease. The blunted, linearized plasmids were re-ligated and then transformed into bacteria. Plasmid DNA was isolated from the clones and the deleted plasmid DNA was linearized by restriction enzyme digestion and size fractionated on an agarose gel. Plasmids with inserts of varying sizes were identified and prepared for either single stranded or double stranded DNA sequencing. Sequence from the various templates were overlapped and areas of sequence that were ambiguous or not adequately covered by overlapping deletions were resolved by either sequencing with oligonucleotide

primers (University of Ottawa Biotechnology Institute) or by excising inserts from the plasmids with restriction enzymes and then sequencing. DNA sequence was analyzed by using the following programs: Seqaid II (University of Kansas), Genetics Computer Group, WWW BLAST Server(Altschul et al., 1990), COILS(Lupas et al., 1991), PSORT (Nakai and Kanehisa, 1992) SignalP WWW server (Nielsen et al., 1997).

Plasmid Isolation

Plasmids were routinely purified from overnight cultures by the alkaline lysis miniprep (Birnboim and Doly, 1979). For manual sequencing, plasmid DNA was purified by using the Spin Miniprep Kit (Qiagen) following manufactures protocol. Plasmid DNA for transfection assays was purified from 1 L of overnight culture by using the Maxi column (Qiagen) following manufacturers directions.

Isolation of ssDNA templates for Sequencing

Colonies were picked with sterile toothpicks and grown in LB/AMP (Luria Broth with 100 µg/mL of ampicillin (Sigma)) until an OD of 0.5 at 660 nm. Then 50 µL of M13K07 phage (Promega) was added and the cultures were incubated for a further 1 hour. 100 µl of this culture was added to LB/KAN (LB supplemented with 70 µg/mL of kanamycin sulphate (Boheringer Mannheim)) and cultures were grown overnight at 37°C. Cultures were centrifuged for 5 minutes at 5000 g. The supernatant was transferred to a clean eppendorf containing 1/5 volume of 20% PEG-8000/2.5M NaCl . The tubes were vortexed and incubated at room temperature for 30 minutes. The eppendorfs were centrifuged at 12000g for 15 minutes and the supernatant was discarded. The phage particles were resuspended in 180 µl TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and 20 µl 3M Sodium Acetate (pH 5.2).

The resuspended phage particles were extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, v:v:). DNA was ethanol precipitated, resuspended in TE and used for dideoxy chain termination DNA sequencing (Sanger et al., 1977) using Sequenase DNA polymerase (Amersham).

Preparation of Purified Plasmid DNA for DNA sequencing

Qiagen Purified plasmid DNA (5 µg) was denatured with 0.1 volume of 0.2 M NaOH for 10 minutes at room temperature. The denatured plasmid DNA was neutralized with 0.1 volumes of 3M Sodium Acetate (pH 5.2) and ethanol precipitated. The DNA pellet was washed twice with 70% cold ethanol and resuspend in TE. This DNA was then used for dideoxy chain terminator sequencing (Sanger et al., 1977) using Sequenase DNA polymerase (Amersham).

5' Rapid Amplification of cDNA Ends (5' RACE)

5' RACE was performed using the 5' RACE system (Gibco-BRL) following the manufacturers protocol. cDNA was synthesized from 1 µg of total heart RNA using a primer specific to SLAP RTPRIMER (5'-gccttggtgcttctctt-3'). The cDNA was tailed with polydCTP residues at the 3' end by terminal deoxynucleotide transferase. PCR was performed on the tailed cDNA using the SLAP specific primer PCRPRIM (5'-ctgacaccgaagctggaaact-3') and the Gibco-BRL anchor primer (5'-ctactactactaggccacgcgtcgactagtagcggiiggggiiggggiig-3'). Cycling conditions were 94°C for 4 minutes, then 35 cycles of 94°C for 1 minute, 57°C for 30 seconds, 72°C for 2 minutes and a final incubation at 72°C for 10 minutes. The PCR products were resolved by agarose gel electrophoresis, excised and purified using the GeneClean II resin (Biocan). The purified PCR products were subcloned using the TA cloning kit (Invitrogen)

into the pCR vector. Eight independent clones were sequenced and 2 clones were sequenced completely on both strands.

Genbank Accession Numbers of SLAP Sequences

The sequences for the various SLAP cDNAs were submitted to the Genbank database. The Genbank accession numbers for SLAP, SLAP2 and SLAP3 are U21155 , U21156 and U21157 respectively.

Northern Blot Analysis of SLAP Expression in Rabbit and Rat Tissues

Rabbits were sacrificed by cervical dislocation. Rat pups and adult rats were sacrificed by decapitation. Various tissues were excised and snap frozen in liquid nitrogen until use. Total RNA from the tissues was isolated by using Trizol Reagent (Gibco/BRL) following the manufacturer's protocol. Aliquots of total RNA (20 µg/lane) were electrophoresed through 1% agarose gel containing 2.2% formaldehyde (Formaldehyde electrophoresis buffer: 0.75% formaldehyde, 40 mM morpholinepropanesulfonic acid (MOPS), 10 mM Sodium Acetate, 1 mM EDTA) and then were transferred onto Magna nylon membranes (MSI) using 10X SSC (1.5 M NaCl, 0.15 sodium citrate, pH 7.0) (Sambrook et al., 1989). The blots were rinsed with DEPC treated water and then the RNA was cross-linked to the blot by UV radiation. The filters were pre-hybridized for 1 hour at 42°C in hybridization solution (50% deionized formamide, 0.1% SDS, 200 µg/mL sheared salmon/herring sperm DNA, 6X SSC (0.9 M NaCl, 0.09 M Sodium citrate pH 7.0), 5X Denhardt's Solution (1% Ficoll, 1% polyvinylpyrrolidone, 1% bovine serum albumin). After pre-hybridization, the various SLAP restriction fragments were radiolabelled with α -³²P-dCTP by the random primer method and incubated overnight at 42°C with the blots in fresh

hybridization solution. The blots were then washed twice at low stringency (0.1 % SDS, 1X SSC (0.15 M NaCl, 0.015 M Sodium Citrate, pH 7.0), 25°C) followed by two washes at high stringency (0.1% SDS, 0.1X SSC (0.015 M NaCl, 1.5 mM Sodium Citrate pH 7.0), 65°C). The blots were then exposed to Kodak Biomax MR films for 1-3 days at -70°C with intensifying screen. Afterwards, the blots were stripped with 0.1 % SDS in boiling DEPC treated water to remove the hybridized probes. The stripped blots were then re-hybridized with either a radiolabelled cDNA probe for glyceraldehyde-3-phosphate dehydrogenase or α -tubulin to control for RNA loading.

Southern Blot Hybridization

Rabbit genomic DNA was purified from blood as described previously (Sambrook et al., 1989). 20 μ g of purified genomic DNA was digested with the indicated restriction enzymes and size fractionated on a 0.75% agarose gel. After size fractionation the DNA was denatured with alkali, neutralized, transferred to Magna nylon membranes (MSI) and UV irradiated. The blots were pre-hybridized as described above for northern blots. A 366 bp EcoRI /AvaI fragment from LD1 was radiolabelled with α -³²P dCTP by the random primer method and incubated overnight at 42°C with the blots in hybridization solution. After overnight hybridization, the blots were washed twice at low stringency (0.1 % SDS, 1X SSC, 25°C) and twice at high stringency (0.1% SDS, 0.1X SSC, 65°C). The blots were then exposed to Kodak Biomax MR films for 1-3 days at -70°C with intensifying screen.

Fluorescence *In Situ* Hybridization (FISH)

Human lymphocytes were cultured in α -minimal essential medium (MEM) supplemented with 10% fetal calf serum and phytohemagglutinin (PHA) at 37°C for 68-72

hours. The cultures were treated with 5-Bromo-2'-Deoxyuridine (BrdU) (0.18 mg/mL Sigma) to synchronize the cell population. The synchronized cells were washed three times with serum free medium to release the block and re-cultured at 37°C for 6 hours in α -MEM supplemented with thymidine (2.5 μ g/ml). Cells were harvested and fixed on slides for FISH analysis. FISH detection was as described previously (Heng et al., 1992). Essentially, slides were baked at 55°C for 1 hour. After RNase treatment, the slides were denatured in 70% formamide and 2X SSC for 2 minutes at 70°C followed by ethanol dehydration. Human SLAP probe was biotinylated with dATP using the BRL BioNick labeling kit and denatured at 75°C for 5 minutes in a hybridization mix consisting of 50% formamide and 10% dextran sulphate. The slides were incubated with the probes overnight, washed and analyzed. FISH signals and 4'6'-diamino-2-phenylindole (DAPI) banding pattern was recorded separately by photography and assignment of the FISH mapping data with chromosomal bands was achieved by superimposing the FISH signals with the DAPI banded chromosomes as described previously (Heng and Tsui, 1993).

Antiserum Production to SLAP fusion protein

A SLAP fusion protein was created by ligating a 1.1 kb blunted EcoRI/XbaI insert from LD1 to a blunted BamHI/XbaI pMAL-cRI vector (New England Biolabs). This ligation ensured that the SLAP sequence was fused in frame to the maltose binding protein domain of the vector. The SLAP fusion protein contained the carboxyl 370 amino acids of SLAP3. DH5 α bacteria containing the recombinant protein were lysed by sonication and then the protein was affinity purified on an amylose affinity column as specified by the manufacturer (New England Biolabs). Animals were bled prior to injections to obtain pre-immune serum.

The purified fusion protein, emulsified with complete Freund's adjuvant, was injected intramuscularly into a guinea pig and a rabbit. Subsequently, after two and 4 weeks the purified fusion protein was emulsified with incomplete Freund's adjuvant and injected intramuscularly. After the two additional injections, the two animals were bled and the blood was incubated at 37°C for 1 hour and then incubated at 4°C overnight. The clots were removed and the serum was centrifuged at 2000 g for 5 minutes. The cleared sera was assayed for specific immunoreactivity in western blots of rabbit cardiac subcellular fractions.

Affinity Purification of Antiserum

Total soluble protein (50 mg) from a cell lysate of DH5 α transformed with the pMAL-cRI vector alone was attached to an Affigel-15 matrix (Biorad) following manufacturers protocol. Similarly, purified SLAP fusion protein (5 mg) was attached to another Affigel-15 column. Crude guinea pig anti-SLAP antiserum (1 mL) was diluted 1:10 with 10 mM MOPS buffer (pH 7.4) and passed three times over the control cell lysate column to remove any maltose binding protein specific antibodies. The cleared antiserum was then passed three times over the SLAP fusion column. The column was then washed extensively with 10 mM MOPS (pH 7.4) and with Tris-Buffered-Saline (10 mM Tris-HCl pH 7.4, 150 mM NaCl) containing 0.05% Tween (TBS-T). The specifically bound antibodies were eluted with 0.1 M glycine (pH 2.5) and immediately neutralized with 1/10 volume of 1 M MOPS (pH 8.0). Antiserum fractions were collected after each stage of the affinity purification procedure and assayed for specific immunoreactivity in western blots of rabbit sarcolemmal proteins.

Subcellular Fractionation of Myocardial Proteins

Myofibrillar fractions were prepared from rabbit heart as described previously (Meng et al., 1996). Rabbit sarcolemma was purified as described previously (Tuana et al., 1987b). Membrane proteins were subjected to electrophoresis in a highly porous SDS-PAGE system which permits rapid and efficient transfer of proteins to nitrocellulose while maintaining a broad degree of resolution (Doucet et al., 1990). The proteins, resolved on gels, were electrophoretically transferred to nitrocellulose membranes (Towbin et al., 1979). The blots were blocked with 5% Carnation skim milk powder dissolved in TBS-T. The affinity purified anti-SLAP antibodies were used diluted to a final concentration of 1:5000 in a mixture of 1% skim milk powder dissolved in TBS-T. The blots were rinsed and then incubated with alkaline-phosphatase-conjugated anti-guinea pig IgG (1:5000, Jackson ImmunoResearch Laboratories) diluted in TBS-T. The immunoreactive bands were visualized by the addition of nitro blue tetrazolium (0.33 mg/mL) and 5-bromo-4-chloro-3-indolyl phosphate (0.16 mg/mL) dissolved in alkaline phosphatase buffer (100 mM Tris-HCl pH 9.5, 10 mM NaCl, 5 mM MgCl₂).

Extraction of Sarcolemmal Membranes

Cardiac sarcolemmal membranes (5 mg) resuspended in one of the following buffers: Phosphate Buffered Saline (PBS), PBS with 1 mM EGTA, PBS with 1 M KCl, PBS with 4 M urea, 150 mM NaCl with 50 mM glycine (pH 3.0), 150 mM NaCl with 50 mM glycine (pH 10) or PBS with 1% Triton X-100. The resuspended pellets were incubated at 4°C for 1 hour with constant mixing and then centrifuged at 180 000 g for 1 hour. Equal volumes of samples of the pellets (resuspended in 4 mL of PBS) and the supernatants were resolved on 10% SDS-PAGE gels, transferred to nitrocellulose and analyzed by immunoblotting with

the purified anti-SLAP antibodies as described above.

***In Vitro* Transcription/Translation**

The SLAP coding sequence including the first ATG was PCR amplified with INVITRO (5'ggctcgagGTCTAACGCTTGTGAAGGCAG3') and TM3' (3'ggaattcCTCTTTACAGACAGATAC3') using SLAP3 construct as template. The PCR product was digested with XhoI/EcoRI and cloned into XhoI/EcoRI digested pBluescript II KS+ to make the ATG1 plasmid. The ATG2 plasmid (which only contained the second ATG) was constructed by subcloning the KpnI/EcoRI restriction fragment of SLAP3 into KpnI/EcoRI digested pBluescript II KS+. The two SLAP plasmids were linearized by digestion with EcoRI restriction endonuclease and then were transcribed using T3 RNA polymerase. One µg of transcribed RNA was used to program a rabbit reticulocyte lysate (Promega) in the presence of ³⁵S-methionine. DEPC-treated water and a luciferase transcript were used as negative and positive controls respectively. Equal aliquots of each reaction were analyzed by SDS-PAGE followed by autoradiography.

Primary Antibodies Used in Immunohistochemical Staining

Guinea pig anti-SLAP and Rabbit anti-SLAP antiserum were produced as described above. The monoclonal antibody IID8 which is specific to the Type 2A SR calcium ATPase was purchased from Affinity Bioreagents (Jorgensen et al., 1988a). The affinity purified antibodies to the carboxyl terminus of dystrophin were prepared as described previously (Meng et al., 1996) and were a generous gift from Dr. Paul Holland. The anti-MYC 9E10 monoclonal antibody was a generous gift from Dr. John Bell (Evan et al., 1985). The TuJ1 monoclonal antibody to the neuronal specific β -tubulinIII was a generous gift of Dr. Dave

Brown (Caccamo et al., 1989). The A60 monoclonal antibody to the neuronal specific nuclear antigen NeuN was a generous gift of Dr. William Staines (Mullen et al., 1992). The BA-78 monoclonal antibody to Type I myosin and the SC-71 monoclonal antibody to type IIA myosin were both generous gifts from Dr. David Parry.

Cell Culture and Expression of 6MYC Tagged Recombinant SLAP Protein

P19 embryonal carcinoma cells were cultured in α -minimal essential medium (MEM) supplemented with 7.5% donor bovine serum and 2.5% fetal bovine serum (Cansera). A MYC tagged SLAP protein was constructed by subcloning BglII/XbaI digested SLAP3 restriction fragment (encoded all of SLAP1, SLAP2 and from amino acid 184 of SLAP3) into the six repeat human MYC epitope of Bluescript KS+ MTG vector (Roth et al., 1991). The cDNA encoding the fusion protein was excised with the appropriate restriction enzymes (KpnI/ XbaI) and subcloned into pcDNA3 (Invitrogen). P19 cells were transfected with the plasmid DNA and stable transfectants selected in G418 as described previously (Pratt et al., 1990; Chen and Okayama, 1987). P19 cells were cultured on gelatin coated glass coverslips until cells were approximately 80% confluent. The medium was removed and the cells were rinsed three times in PBS. The cells were then fixed for five minutes using a fixative consisting of 4% (w/v) paraformaldehyde and 12 % (v/v) saturated picric acid in 160 mM sodium phosphate buffer pH 6.9 (Lana's Solution). Fixed coverslips were rinsed extensively and the anti-human MYC monoclonal antibody 9E10 was diluted 1:50 in 10 mM PBS containing 0.3% Triton-X 100 (PBS-T) and added to the coverslips (Evan et al., 1985). After incubating for 3 hours at room temperature, the slides were rinsed three times in PBS and Cy3-conjugated sheep anti-mouse IgG (Sigma) diluted 1:100 in PBS-T was added to the

slides and incubated for a further 45 minutes at 37°C. Slides were washed, coverslipped using a mounting medium of PBS containing phenylaminediamine (0.1 mM) in 90% glycerol and examined using a Zeiss Axioplan microscope. Some slides were examined further by using an upright Leica confocal scanning laser microscope equipped with a 55 mW krypton/argon air cooled laser and a 63X Plan Apo oil immersion lens. The negative control for immunofluorescence studies was either mock transfected P19 cells or P19 cells transfected with empty pcDNA3 vector. For DNA staining, cells were subsequently incubated in Hoechst 33258 (1 µg/mL) for 5 minutes at room temperature after rinsing off the secondary antibodies.

Immunohistochemistry of Live P19 Cells

P19 cells transfected with the 6MCY tagged version of SLAP were cultured on glass coverslips as described above for immunohistochemistry. Live P19 cells were rinsed once in pre-warmed PBS and then incubated for 45 minutes at 37°C in one of the following solutions: MEM, anti-MCY monoclonal antibody in MEM (1:50) or rabbit anti-SLAP diluted in MEM (1:400). The cells were then rinsed three times in PBS and fixed for 5 minutes in Lana's solution. The cells that were incubated with primary antibodies were rinsed and further incubated for a 45 minutes at 37°C in either Fluorescein Isothiocyanate (FITC -labeled donkey anti-rabbit IgG (1:25, Amersham) or Cy3-labeled sheep anti-mouse IgG (1:100, Sigma) diluted in PBS-T. The cells that were incubated with MEM were rinsed and then incubated for 45 minutes at 37°C in either rabbit anti-SLAP (1:400) or anti-MYC (1:50) diluted in PBS-T. Afterwards the cells were incubated with the appropriate secondary antibodies as described previously. After incubation with secondary antibodies the cells were

washed, coverslipped as described above, and examined using a Zeiss Axioplan microscope.

Differentiation of P19 cells

P19 cells were induced to differentiate with dimethyl sulfoxide (DMSO) or retinoic acid (RA) as described previously (Rudnicki and McBurney, 1987). Briefly, P19 cells were dispersed with trypsin and 10^6 transferred to a bacterial grade Petri dish containing 10 mL of α -MEM supplemented with 5% donor bovine serum (Cansera), 5% fetal bovine serum (Cansera) and either 0.3 μ M retinoic acid (RA) or 1% DMSO. The P19 cells were cultured for 48 hours, after which time the aggregates were transferred to new bacterial grade Petri dishes containing the appropriate differentiation medium. Following another 48 hours, the aggregates were collected and transferred to tissue culture grade Petri dishes containing α -MEM supplemented with 5% donor bovine serum and 5% fetal bovine serum. Medium was changed every 48 hours thereafter until cells had been cultured for 10 days.

Immunohistochemical Staining of Differentiated P19 cells

P19 aggregates differentiated for 4 days as described above were plated onto gelatin coated glass coverslips and cultured in α -MEM supplemented with 5% donor bovine serum and 5% fetal bovine serum. P19 cells were prepared for SLAP and MYC immunohistochemical staining as described above. Control pcDNA3 transfected P19 cells differentiated with DMSO for 10 days were simultaneously stained for SLAP and the neuronal specific marked β tubulinIII (Caccamo et al., 1989). The cells were incubated overnight at 4°C with a mixture of rabbit anti-SLAP antiserum (1:400) and the TuJ1 monoclonal antibody specific to β tubulinIII (1:25) diluted in PBS-T. The cells were rinsed three times and then incubated in a mixture of FITC labeled donkey anti-rabbit IgG (1:25,

Amersham) and biotin-conjugated donkey anti-mouse IgG (1:50, Amersham) that were prepared 24 hours prior to use. After incubation for 45 minutes at 37°C, the cells were rinsed three times and incubated in streptavidin-Texas Red (1:100, Amersham) for a further 30 minutes. Slides were washed three times in PBS, coverslipped as described previously and examined using a conventional Zeiss Axioplan microscope

Expression of SLAP1 in P19 cells

The 1.4 kb EcoRI DNA insert from LD1 which contained the entire coding sequence of SLAP1 was subcloned into the pPOP expression vector. P19 cells were transfected by the calcium phosphate method with three different plasmids: pPOP-SLAP1 expression, the pgk-NeoR plasmid which encoded the bacterial neomycin resistance gene and the B17 plasmid which contained an intragenic region of the phosphoglycerate kinase-1 (pgk-1) gene which has been shown to enhance expression of transfected genes in P19 cells (Pratt et al., 1990; McBurney et al., 1994). The SLAP1 and neomycin resistance gene were inserted between the pgk-1 promoter and the pgk-1 3' UTR which contained a consensus polyadenylation signal as well. Transfected cells were treated with G418 and 24 stable clones were isolated and assayed by northern blot analysis for the expression of the exogenously expressed SLAP1 transcript. For western blot analysis, the P19 cells were cultured in a 60 mm tissue culture dish. The cells were rinsed twice with PBS and scraped with a rubber policeman in ice cold TE buffer (10 mM Tris-HCL pH 8.0, 1 mM EDTA, 1 μ M Pepstatin A, 0.2 mM phenylmethanesulfonyl fluoride, 1 mM benzamidine, 1 mM iodoacetamide) and transferred to a microcentrifuge tube. On ice, the mixture was first passed 10 times through a 23 gauge needle to rupture the cells and then centrifuged at 12 0000 g for 10 minutes at 4°C. The

pelleted proteins were resuspended in RIPA buffer (1 % NP-40, 0.5% Sodium Deoxycholate, 0.1% SDS, PBS) and were resolved by 10% SDS-PAGE and then transferred to a nitrocellulose membrane. The blots were blocked with 5% Carnation skim milk powder dissolved in TBS-T. The rabbit anti-SLAP antiserum was diluted to a final concentration of 1:5000 in a mixture of 1% skim milk dissolved in TBS-T. Horseradish peroxidase conjugated sheep anti-rabbit IgG diluted in TBS-T (1:2500, Sigma) was used as the secondary antibody and the reaction was developed using the Enhanced Chemiluminescence Kit (Amersham) and exposure to Kodak Biomax MR films.

Trypsin Digestion of SLAP1 Expressing P19 Cells

SLAP1 expressing P19 cells were cultured in 60 mm diameter tissue culture dishes. When the cells were approximately 80% confluent the medium was aspirated and the cells were rinsed twice with PBS. The cells were then incubated at 37°C for 5 minutes in one of the following solutions: 5 mg/mL Trypsin (Gibco-BRL) that was heat treated for 10 minutes, 5 mg/mL Trypsin for 5 or 10 minutes, 5 mg/mL Trypsin with 10 mg/mL soyabean trypsin inhibitor (Sigma). After incubation, the cells were detached from the tissue culture plates and transferred to a 15 mL centrifuge tube. The cells were centrifuge for 5 minutes at 2 000 g to pellet the cells. The cell pellet proteins were resuspended in RIPA buffer, and the supernatant proteins were resolved by 10 % SDS-PAGE. The proteins were then transferred to nitrocellulose and subjected to western blot analysis as described above for SLAP1 transfected P19 cells.

Preparation of Myocardium for Immunohistochemical Staining

After decapitation, adult rat hearts were removed and immediately frozen in

powdered dry ice. Sections of heart were cut at a thickness of 2-5 μm on a cryostat and then mounted onto a slide and fixed for five minutes using Lana's solution. Pregnant rats at day 18-19 of gestation were sacrificed with phenobarbital overdose and the fetuses were removed and immediately frozen in powdered dry ice. 20 μm sections of the embryos were cut on a cryostat and mounted onto slides. Embryonic sections were fixed as described for the heart cryosections.

Single Immunohistochemical Staining of Myocardium

After rinsing, the sections were incubated for 3 hours at room temperature in one of the following: a 1:400 dilution of guinea pig anti-SLAP antiserum, 1:400 dilution of rabbit anti-SLAP antiserum, 1:100 dilution of affinity purified rabbit anti-dystrophin antiserum raised to the carboxyl terminus of dystrophin (Meng et al., 1996) or a 1:500 dilution of monoclonal antibody to the slow twitch muscle/cardiac form of the SR calcium ATPase. Primary antibodies were applied to the slide mounted sections in PBS-T. Sections were rinsed three times in PBS and then incubated with one of the following: FITC-labeled donkey anti rabbit IgG (1:25 dilution, Amersham), biotin-labeled goat anti-guinea pig IgG (1:50 dilution, Vector Biosystems) or FITC-labeled sheep anti-mouse IgG (1:25 dilution, Amersham). After a 45 minute incubation at 37°C, sections were rinsed three times in PBS and then sections stained with the guinea pig serum were incubated with streptavidin-Texas Red (1:100 Amersham) in PBS for a further 45 minutes. Slides were washed, coverslipped as previously described and examined using a Zeiss Axioplan microscope.

Double Immunohistochemical Staining of Myocardium

Rinsed fixed slides were incubated in a mixture of guinea pig anti-SLAP (1:400

dilution) and either anti-dystrophin (1:100 dilution) or anti-calcium ATPase (1:500 dilution) overnight at 4°C. The primary antibody mixtures were applied in PBS-T. After incubation, the slides were rinsed three times in PBS and then incubated in a mixture of biotin-labeled anti-guinea pig IgG (Vector, 1:50) and either FITC-labeled donkey anti-rabbit IgG (Amersham, 1:25) or FITC-labeled sheep anti-mouse IgG (Amersham, 1:25) diluted in PBS-T that were prepared 24 hours prior to use. After a 45 minute incubation, the slides were rinsed three times and the section were incubated in streptavidin labeled with Cy3 (1:100, Sigma) for a further 30 minutes. The slides were washed and coverslipped as previously described.

Adsorption of Antiserum

Maltose binding protein (MBP) or MBP-SLAP fusion protein was produced in bacteria. These bacteria were then harvested and sonicated in PBS-T buffer which contained 1 μ M Pepstatin A, 0.2 mM phenylmethysulfonyl fluoride, 1 mM benzamidine and 1 mM iodacetamide. The sonicated mixture was cleared of insoluble proteins by centrifugation at 5000g for 10 minutes at 4°C after which the supernatant was retained. 2.5 μ L of rabbit anti-SLAP antiserum was diluted 1:400 in PBS-T that contained 20 mg of total soluble protein from lysates of either MBP or MBP-SLAP producing bacteria. After incubating at 4°C for 4 hours with constant agitation, these mixtures were centrifuged at 12 000g for 5 minutes. The antibodies were then applied to slide mounted fixed cryosections of myocardium and incubated overnight at 4°C. The slides were then rinsed three times in PBS and incubated in FITC-labeled donkey anti-rabbit IgG (1:25, Amersham) for 45 minutes at 37°C. Slides were rinsed three times in PBS, coverslipped as previously described and examined using a Zeiss Axioplan microscope.

Skeletal Muscle Immunohistochemical Staining

Tibialis Anterior (TA) muscles were dissected from rats sacrificed by decapitation and immediately frozen in a dry-ice/isopentane solution (Generous gift of Dr. Bernard Jasmin). Sections of TA were cut at a thickness of 18 μ m on a cryostat and fixed for five minutes using Lana's solution. Primary antibodies were applied as described for immunohistochemical staining of the myocardium.

Double Immunohistochemical Staining for SLAP and Fiber Specific Markers

Rinsed, fixed sections were incubated in a mixture of rabbit anti-SLAP antiserum (1:400 dilution) and one of the following: BA-78 (Type I Myosin) monoclonal antibody (1:10 dilution), SC-71 (Type IIA myosin) monoclonal antibody (1:2 dilution), or monoclonal antibody to slow twitch/cardiac calcium ATPase (1:500 dilution) overnight at 4°C. The slides were rinsed three times and then incubated for 45 minutes at 37°C in a mixture of FITC labeled donkey anti-rabbit IgG (1:25, Amersham) and biotin-conjugated sheep anti-mouse IgG (1:50, Amersham) that were prepared 24 hours prior to use. The slides were rinsed three times in PBS and incubated in streptavidin-Texas Red (1:100, Amersham) for a further 30 minutes. Slides were washed three times in PBS and coverslipped as described previously. The myosin/SLAP labeled sections were examined using a conventional Zeiss Axioplan microscope while the ATPase/SLAP labeled sections were examined using an upright Leica confocal scanning laser microscope equipped with a 55 mW krypton/argon air cooled laser and a 63X Plan Apo oil immersion lens.

Immunohistochemical Staining of Retina and Brains

Rats were anaesthetized with phenobarbital and perfused with 10 mM PBS to clear

the circulatory system of blood. Brains and retinae were harvested and immediately frozen in powdered dry ice. 12 μ m sections of adult brain and 18 μ m sections of neonatal brain were cut on a cryostat and mounted onto slides. 18 μ m sections of developing and adult retina were cut on a cryostat and mounted onto slides. Immunohistochemical staining for SLAP was performed as previously described for the myocardium.

Double Immunohistochemical Staining of Brain Cryosections for SLAP and a Neuronal Marker

Rinsed fixed slides were incubated overnight at 4°C in a mixture of rabbit anti-SLAP (1:400) and A60 monoclonal antibody (neuronal specific marker NeuN)(1:50) diluted in PBS-T. After incubation, the slides were rinsed three times and incubated for 45 minutes at 37°C in a mixture of FITC labeled donkey anti-rabbit IgG (1:25) and biotin-labeled donkey anti-mouse IgG(1:50) diluted in PBS-T that was prepared 24 hours prior to use. Sections were rinsed three times in PBS and incubated for a further 30 minutes in streptavidin-Texas Red (1:100, Amersham) and then rinsed three times in PBS. The slides were coverslipped and examined as described for the immunohistochemical staining of the myocardium.

C2C12 Transfections

C2C12 myoblast cells (Titus et al., 1982) were cultured in α -minimal essential medium (MEM) supplemented with 10% fetal bovine serum (Cansera). The cells were transfected with the 6MYC-SLAP expression plasmid or the empty pcDNA3 plasmid by the modified calcium phosphate protocol (Chen and Okayama, 1987) and stable clones were selected with G418. Clones were assayed for expression of the exogenous SLAP transcript by Northern blot analysis. For fusion assays, the C2C12 cells were grown on Matrigel

treated tissue culture plates until the cultures were 80% confluent then the cells were cultured in α -MEM supplemented with 10% horse serum (Gibco-BRL). The culture medium was changed every 24 hours for the first two days of differentiation and every 48 hours thereafter. The cells were cultured in differentiation medium for 6 days.

Immunohistochemical Staining of C2C12 cells

C2C12 cells were grown on matrigel coated coverslips in α -MEM supplemented with 10% FBS and then treated for 5 minutes with Lana's fixative. The coverslips were rinsed and incubated for 45 minutes at 37°C in either rabbit anti-SLAP (1:400) or anti-MYC (1:50) diluted in 10 mM PBS-T. The slides were then rinsed three times in PBS and then incubated in FITC-labeled donkey anti-rabbit IgG (1:25, Amersham) or Cy3-labeled sheep anti-mouse IgG (1:100, Sigma) for 45 minutes at 37°C. After incubation with secondary antibodies, the cells were: rinsed three times in PBS, coverslipped as described previously and examined using either an upright Leica confocal scanning laser microscope equipped with a 55 mW krypton/argon air cooled laser and a 63X Plan Apo oil immersion lens or a Zeiss Axioplan microscope.

Determination of Fusion Index

The fusion index was determined for pcDNA3 transfected C2C12 pooled clones and four independent clones transfected with the cDNA encoding the 6MYC-SLAP fusion protein. After being cultured in differentiation medium for 6 days, the cells were fixed in 4% paraformaldehyde for 10 minutes. The cells were rinsed in PBS extensively and then were stained with haemotoxylin and eosin. Briefly, cells were stained for 7 minutes with Harris Haemotoxylin/0.2% acetic acid and rinsed in warm tap water. The cells were then briefly

rinsed with 70% ethanol and then again washed with tap water. The cells were stained for 1 minute in Eosin solution and rinsed in a series of: 70% ethanol, 50% ethanol and PBS. At least 12 random fields containing at least 300 nuclei were photographed from each clone. The total nuclei were counted in each field as well as the number of nuclei in myotubes (cells with three or more nuclei). The fusion index was defined as: $(\text{number of nuclei in myotubes} / \text{total nuclei in the field}) * 100$. The fusion index for each clone was taken as average $\pm$ standard error from the mean for all fusion indices calculated for each clone. The fusion potential of the clones was examined three separate times and the results shown are from one representative experiment. The significance of the results was examined using ANOVA.

Northern Blot Analysis of C2C12 Cultures

C2C12 cells were cultured on Matrigel (Collaborative Biomedical Products) coated culture dishes either in growth medium (α -MEM supplemented with 10% fetal bovine serum) or differentiation medium (α -MEM supplemented with 10% horse serum) as described above. Total RNA from the cultures was isolated after 6 days of differentiation by using Trizol Reagent (Gibco/BRL) following the manufacturer's protocol. Aliquots of total RNA (20 μ g/lane) were electrophoresed through formaldehyde in 1% agarose gels, blotted onto Magna nylon membranes (MSI), cross-linked with UV radiation and pre-hybridized for 1 hour at 42°C in hybridization solution (Sambrook et al., 1989). The rat myogenin cDNA or the 1.4 kb EcoRI inset from LD1 were radiolabelled with α -³²P-dCTP by the random primer method and incubated with the blots overnight at 42°C in hybridization solution. After washing twice at low stringency (0.1 % SDS, 1X SSC, 25°C) the blots were washed twice at high stringency (0.1% SDS, 0.1X SSC, 65°C). The blots were then exposed to Kodak Biomax MR films either 4 hours (myogenin) or 24 hours (SLAP) at -70°C with intensifying screen. Afterwards the blots were treated with boiling 0.1% SDS/DEPC-treated water for 5 minutes to strip the hybridized radiolabelled probes. The cleaned blots were re-probed with a radiolabelled glyceraldehyde-3-phosphate dehydrogenase cDNA probe.

RESULTS

Molecular Cloning and Sequence Analysis of Sarcolemmal Associated Protein

Two cDNA clones designated LD1 and LD2 were isolated from a rabbit heart cDNA expression library by screening with antiserum raised to highly purified canine sarcolemma. Two EcoRI cDNA inserts from LD1 of 1.4 and 0.9 kb had been subcloned into the PTZ19R sequencing vector (Demchyshyn, 1990). In order to establish the identity of these cDNA clones, the nucleotide sequence of the two clones needed to be determined and analyzed. The plasmids containing the two LD1 EcoRI cDNA inserts were digested unidirectionally with exonuclease to create a series of nested deletions. These deleted plasmids were screened by restriction endonuclease digestion for insert size and templates of varying length (0.1 to 1.4 kb) were manually sequenced. The nucleotide sequences of both strands of the LD1 clone were obtained by overlapping the sequence data from the various templates. Analysis of the sequence indicated there was a large open reading frame (ORF) at the 5' end and a smaller ORF at the start of the 0.9 kb insert (Fig. 2A). The LD1 clone was polyadenylated and contained a consensus site for polyadenylation 26 bp from the site of polyadenylation. The LD2 phage clone was digested with EcoRI and a 1.4 kb insert was subcloned into pBluescript II KS+ (pBSK) and subsequently sequenced on both strands. The LD2 sequence was identical to the 900 bp EcoRI cDNA insert of LD1 but was extended by 500 bp at the 3' end (Fig. 2A). This cDNA was polyadenylated as well and had a consensus site for polyadenylation 21 bp upstream from the site of polyadenylation. The 1.45 kb insert of LD2 had only the small ORF and not

Figure 2. cDNA Cloning of Sarcolemmal Associated Protein (SLAP).

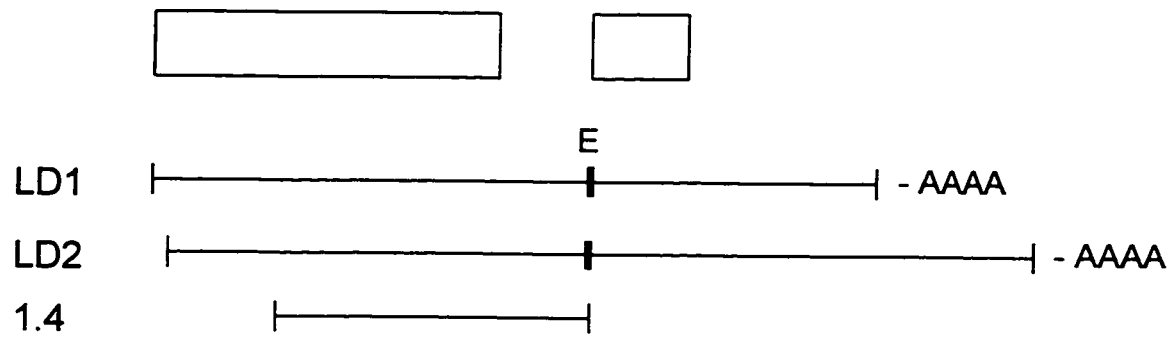
A) Map of cDNA clones from oligo-dT primed libraries.

The LD1 and LD2 cDNA clones were isolated from the primary immunoscreening of a rabbit heart oligo-dT primed λ gt-11 cDNA expression library with antibodies raised to purified sarcolemmal proteins. The two clones were identical over a 2.3 kb overlap but were polyadenylated at two different sites. The 1.4 cDNA clone was isolated by screening a rabbit heart cDNA library with a radiolabelled cDNA probe encompassing the large open reading frame (ORF) of LD1 and LD2. The potential ORFs are shown by open boxes and the location of the internal EcoRI site is denoted by E.

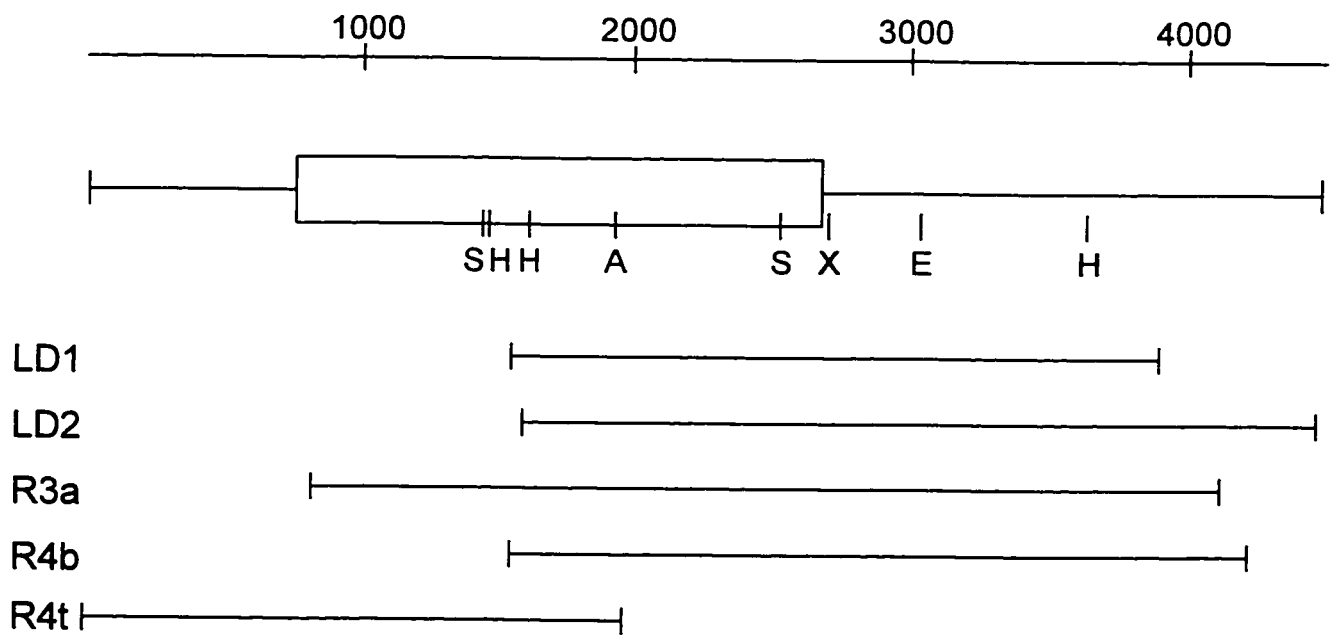
B) Map of SLAP cDNA.

The open bar denotes the open reading frame in SLAP. Restriction sites for Aval (A), EcoRI (E), HindIII (H), SacI (S) and XbaI (X) are shown. Clones LD1 (bp 1560 - 3929) and LD2 (bp 1603-4463) were isolated from the primary immunological screen of the rabbit heart oligo-dT primed λ gt-11 cDNA expression library. Clones R3a (bp 814-4116), R4b (bp 1560- 4220) and R4t (bp 1-1981) were isolated from a secondary screen of a random primed rabbit heart λ gt-11 cDNA expression library with a radiolabelled cDNA probe from the 5' end of the LD1 clone.

A



B



the large ORF found in LD1. Either the smaller ORF encoded a protein recognized by the anti-sarcolemmal antiserum or another identically sized EcoRI insert of LD2 was not identified in the original subcloning experiment. In order to resolve how the LD2 cDNA was isolated by the initial immunoscreening, the EcoRI digested DNA fragment from the LD2 phage clone was again subcloned into pBSK. In this second screening of the LD2 subclones, a unique insert of 1.42 kb was discovered as well as the previously characterized 1.45 kb insert. This second insert was sequenced completely and shown to be identical to LD1 from nt 43 to nt 1462 (Fig. 2A). The isolated cDNA clones were related and both contained a large ORF at their 5' termini.

The two isolated clones did not appear to be full length cDNAs since they were much smaller than SLAP transcripts seen in northern blots of RNA from a variety of tissues. As well, the ORF in LD1 did not contain an inframe stop codon at the 5' end so it could potentially be extended. To isolate a full length cDNA, an oligo-dT primed rabbit heart cDNA expression library was constructed in λ gt-11. Approximately 800 000 plaques of this library were screened with a cDNA probe constructed using a 1.1 kb EcoRI/ XbaI insert from LD1 as template. A tertiary screen identified a single positive clone designated 1.4 which was subcloned into pBSK and sequenced. Analysis of the sequence showed that the 1.4 clone was identical to the LD1 clone from nt 400 to nt 1460 of LD1 (Fig. 2A).

In order to obtain a full length cDNA, a different approach to constructing a cDNA library needed to be applied. Using random hexamers cDNA synthesis can be primed at random locations in the mRNA molecule whereas with oligo-dT primers, cDNA synthesis can only be primed at the 3' end of the mRNA. Therefore, the proportion of cDNAs

containing 5' sequence, especially for larger mRNAs would be higher in a random hexamer primed library compared to an oligo-dT primed library. To better represent the 5' ends of rabbit heart cDNAs, the cDNA synthesis for the second λ gt-11 expression library was primed with random hexamers instead of oligo-dT primers. Over 250 000 plaques of this library were screened with a cDNA probe constructed from the 350 bp HindIII/AvaI restriction fragment from the 5' end of LD1 (Fig. 2B). Twenty positive clones were identified in a tertiary screen and their inserts excised by restriction digests. The cDNA inserts of the positive clones were size fractionated by agarose gel electrophoresis and three clones were identified that contained inserts greater than 2 kb in length. These inserts were subcloned into pBSK and sequenced. The resulting sequence from the overlapping clones LD1, LD2, R3a, R4b and R4t was designated Sarcolemmal Associated Protein (SLAP) (Fig. 2B). The R4b clone was extended only 10 bp past the 5' end of the original LD1 clone but the other two clones were extended significantly further in the 5' direction.

SLAP Encodes an Acidic Amphipathic α -helical Tail Anchored Membrane Protein

With the full length cDNA sequence determined, the nature of the mRNA and the putative protein could be predicted. The SLAP mRNA had a 1.75 kb region of 3' untranslated (UTR) sequence (Fig. 3). The 3'UTR of homologous genes are usually not well conserved between species, however, the 3' region of SLAP appeared to be well conserved since human Expressed Sequence Tags (ESTs) have been cloned recently that are 98% identical with SLAP in this region (Fig. 4). Two distinct consensus

Figure 3. Nucleotide and Deduced Amino Acid Sequence of SLAP.

The putative initiating methionine of SLAP is shown in bold. The two predicted leucine zippers are underlined and the predicted carboxyl hydrophobic anchor is double-underlined. The polyadenylation signals of LD1 (nt 3904-3909) and LD2 (nt 4447-4452) are shown in bold and underlined. Potential PKC phosphorylation sites are double underlined, the potential PKA phosphorylation site is in italics and potential N glycosylation sites are in bold and underlined. The potential PEST site is denoted by the shaded box.

CTCCAGCCCAAACCTGCTTCGAGATTTTCTTGGCCGAAGCTGCCGAGATGTTTGGAGG
CTCTCCTTCCAGGGAGATGAGCTGAAAGCGCCAGCGGGGAGCTGTTCGATCGAGCGTGTCCGCT
TTCCAAAGAGCGCATGGGCTTGGCTGCTTCCACCCAGTGAGAGCTCGATGATGTCGCTG
GTTATGCTTTAGACGATGCGCAGTTTGTGTATATCGAAACAAAAATTACTTGAAGGTGGGTGGGGGAA
AAGAGGAGCGGTCTAAAGCTGTGAGCGAGCTGCGGATCTGGAGGAACCTCTCGTCTGCTGTAGG
CAAGGACCGGAGTCTGCTCGATGAGCTGAGCTTGGCCATCTTCACCTGCGCCCAACCTCGCATC
GCTTTTCAGGAGGCTATGCTACTGCGAGCGCCATCAAAATCGCGCCGCTCGTGGCCGCTGCGCGCC
GGCGCAGATTAATGCCCACTTGTGATCTGCAAGAGTGTCTCAGGAACCACTCTCTGCTTGGTTAGACCA
AAGACCGGAATTTTATCTTCAAGACAGATGATGATGATGATCTTATATAATGACCAAGATGGA
GTGAGGCTCTGGAAGAAGTCCACATGTGAGATTTCTTCTGGTGACATTAATCCAGTTTGGGTGAGATGT
GACGGAGAACACACGGAAGTTACCCNTGGGTGATTTGTTCTTACATAAATCTTTTCTGCCAGATGGT
ATGGAAGCCCGCTCGCTCTGATGTCTATCCATGCCCCATTAACCAAGTCTCTGTGACAAAGTTGCTGCTA
M E A R L R S D V I H A P L P S P V D K V A A N
ACACTCCAAGTATCTACTCTCAGGAACATAATCCAGCTTTCTCAGTATCTTTCAGGAGCGCTTACATCGGGA
T P S M Y S Q E L Q L L A I T Q E A L H R E
ACAGATGTTGGAAACAGAGTTAGCTACACTTCAGCGGCTACTGGCCATCACCCAGAGGCTTCAGATACC
Q M L E Q K L A T L Q R L L A I T Q E A S D T
AGTTGGCAGGCTTTAATAGATGAGATAGACTCTTATCACGGTTTAGAGTTTAGGGAACCAATTAACAGG
S W Q A L I D E D R L L S R L E V M G N Q L Q A
CATGCTCCAAAATCAACAGAGAATAGTTTACGGAGGAACTTATAGCAATTACAGGAAGATTAACACAA
C S K N Q T E D S L R K E L I A L Q E D K H N
CTATGAGACAAACAGCAAGAGACTCTGAGCGAGTCTTTCAGGAGAAAATGAAAGTTGTTAGAAAACCTT
Y E T A K E S L R R V L Q E K I E V V R K L
TCAGAAGTTGAGCGAAGCCTGAGTAATACTGAAGATGAATGTACCACCTGAAGAAATGAATGAACGAA
S E V E R S L S N T E D E C T H L K E M N E R T
CTCAGGAAGAAATTAAGCAAAATAGCCAAATAATAATAGGAGCTGTTTAATGAAATTAAGATCTATCTGA
Q E E L R E L A N K Y N G A V N E I K D L S D
TAAGCTAAAGGTAGCAGAGGGAACAAAGAGAAATCCAAACAGAGGGAACAAGCTGAGAAAAAAGAAATTA
K L K V A E G K Q E E I Q K G Q A E K E L
CAGCTAAAAATAGATGAAGAAAGGAACAGAGAGCTCCAGCGCAAAAATAGAGCTTTGCGACGCTG
Q H K I D E M E E K E Q E L Q A K I E A L Q A D
ATAATGATTTCAACCAATGAAGGCTTAACAGCTTTTACAAGAGCACTTGTCTTTCAAAGAGTGGCGGGAGCTG
N D F T T N E R L T T A L Q E H L L S K S G G D C
CACTTTATTCACTGTTTCATAGATCGCAGAGAAAGCTGATCGTGGAGGGGCACTTAACCCAAAGTGGA
T F I H A G G T I E C Q K K L I V E G H L T K V V
GAAGAAACCAAGCTTGCAAAAGAAACAGCGCAGGACCAAGAAATCTGATTTATCAGATCACTCTGAGTC
E E T K L A K E N Q A R A K E S D L S D T L S P
CAAGCAGGAAAGAGAGTGAAGCACTACGAGCGCCCAATAGATGATGAGCAAGCACTGAATGAATCTCT
S K E K S S D T T T G A Q M D E Q D L N E S L
TGCTAAAGTGTCCCTATTAAAGCTCTTTTGGAGAGAGAAAGAAAGCCTATCGAAATCAAGTTGAGGAA
A K V S L L K A L L E E A G A K A Y R N Q V E E
TCCAGTAAACAAATACAGGTTCTTCAAGCCCACTGCGAGAGGTTACACATGGAATCTGAGAAATCTCCGG
S S K Q I Q V L Q A Q L Q R L H M D I E N L R R E

[illegible]

CTGGCATCACTACTGCTGGGAAAAACGAAACCAATTTTCTCTCTTTTACCTCTTTAAACACACAGAA
TGGCAGAAACAATCTGTAGGCTTACTGCTTTAAATATATAAAATGTATCTGTCTGTAAAGGAGATATA
AAATTTGGACCTATATCTCTGTGTAGCCATATATTTTCTGTGCATATTTTCTGTAAATTTTAAATTAGTTT
GTTGAGATCTGAAATATATATGTGGGCTAAAGTAGGCTTTCTGGTACACCAATTTTATTAACATATAA
TTATGGGATTTTACTCTGAATCTGATAGCCAGATATCTATTTTCTGTATCGATTTACTACCCAAAC
CAAAACCAATACATACATGCGGAGAAAGGCCCTGTGTCTCAGTGCCTATCAGTTTGAAATATACACA
CATATATATATATTTTACATATTTAGCCATTTTACTTATATAAACCTAGCTATTTGAAATATTTGGCA
CAGATTTAGAGACACACTATTTGTGTGGGTTTTTTTTTTTAAAGGAAAAATACATTTTGAAANAATTTCT
TAGAGTGAATATTTGGGAATATGTGTGACGCTGTTCAGCTTAATGTGCACTTAAGATTTGGAGACAA
TCAGCTTGAANAACCTTCCATGAGATTTGTATTTCTTGGACCAATGTAGATTTTCTGGGAGCAAA
ACAGTAAATATACAAATTTCAAGTCAGTCAACCAACTGTGTAGTCAATAGGAATGTAGTTTATTAACCC
TCATTTTAAAGAGATATTTCTCTTAAAGCCAGTTTACTTCTCATATACAGCAATTTTAGAGAGCAT
GATTTTTTTTTCTGCACTGTCTCTCAGCACTTTTAAATGCGAAGAAATATATATCACTCTTAGAT
AAGCTTATTTGTACTAAATTTGGTATTAGTTTGTGGAGCTAAATCATGTGCGAAGTGCGCTGTGTATT
ACCAAGATGTCTGTGTGGGTATGCAAAATGGTTATTTCTTTATTTGTAAATTTGGGAGCTTTCTGT
TTCAAGAGAGCACTATCCCAAGATATATAGTGTTCATAGCAATATGAAGCACTAAACACCTGTCTGT
GTTATAAATTTAAACACAGATATGAAGTATTTAGCTATGGTTTTTGTGCGGACCATAGTTATGTCAATA
AAGTTTATTTAGGTCAATAGGATCTCAAGGAAATAGTGAATTTTCTGATCAATATGAAGATATGA
AGGAATGTGAGTGAATGCTGAATGTCTTTGGCAATACAGAGTCTTAGCCAAAATATTTGTTATATGG
ATTTTAGATAAATTTTGCTTGTGATTTGTGGCACTCATTTGTGTGTACTTTAAAGGTTATATTTTTAAGG
TATTTAGATGTCTGTTTTGGGAGGATCACTAGATTTTGTGGAGGAATTTAGTCAACAAATGATTTTAGGAA
TACTGTATATAGTTCATTTTCAATTTTGTGGAGGAAGCACTCAACACAGATGCTTAATATGCC
AGGTGTACCCAGTACTCTGTATATATAGTTATATGCAAAATATGTTCTGAAATGTCTTAACCTCAGAAATTA
CATTTTTTAAAGTAAATATGTTTTTAAATCTATTTGTGATAGATATATAGTCAACAAATGATTTCTGGAG
TACAGATTTAAACTATTTGCACTCAACACAGCTGATATGCTGATATTTAAATAAACAACTACTCTCTCCAT

Figure 4. Conservation of the SLAP 3' Untranslated Region (UTR).

A BLAST alignment of the 3' UTR of SLAP with a human Expressed Sequence Tag (EST) (Genbank accession number AA551570) cloned from a Schwannoma tumor library. The 3'UTR of SLAP is well conserved with the human sequence. Identities are indicated by dashes and nucleotide positions in SLAP and the EST are indicated at the right.

Identities = 329/334 (98%), Positives = 329/334 (98%)

```
SLAP: 4466 TAGGGAGAGTAAAGTTATTTTATTAAATCATGCACATCACGTGTGTTAGTGCAAATAGTT 4407
      |||
EST: 13 TAGGGAGAGTAAAGTTATTTTATTAAATCATGCACGTACGTGTGTTAGTGCAAATAGTT 72

SLAP: 4406 TAATCTGTACTCCAGAAATTCATTGTACTTTATATCTTTACAAAATAGATTTAAAACAA 4347
      |||
EST: 73 TAATCTGTACTCCAGAAATTCATTGTACTTTATATCTTTACAAAATAGATTTAAAACAA 132

SLAP: 4346 TTATTTACTTTAAAAAATGTAATTCTGAAGTTAAGCATTTCAGAACAATATTTGCAATAA 4287
      |||
EST: 133 TTATTTACTTTAAAAAATGTAATTCTGAAGTTAAGCATTTCAGAACAATATTTGCAATAA 192

SLAP: 4286 CCTATATACAAGAGGTACTGGGTACCACTGGCATATTAGCATTCTGTGGTGGAGTGGCTT 4227
      |||
EST: 193 CCTATATACAAGAGGTACTGGGTACCACTGGCATATTAGCATTCTGTGGTGGAGTGGCTT 252

SLAP: 4226 CCTGCAACAAAAAATGATGAAATGAACTATATGACAGTATTTCTACAAGTCATTTGTGA 4167
      |||
EST: 253 CCTGCAACAGAAAATGATGAAATGAACTATATGACAGTATTTCTACAAGTCATTTGTGA 312

SLAP: 4166 CTAATTCCTCCACAAATCTAGTGATCCTCCCAA 4133
      |||
EST: 313 CTAATTCCTCCATAAATCTAGTGATCTTCCCAA 346
```

polyadenylation sites were found in the SLAP cDNAs within 30 bp of poly-A tails (Fig. 3). The SLAP cDNA had a 209 bp of 5' UTR and a large ORF which extended from nt 209 - 2675. The first inframe methionine codon was found at nt 362 but it was in poor agreement with the Kozak consensus translation start site (Fig. 5A) (Kozak, 1991). If this methionine was used to initiate translation the encoded protein would have a predicted molecular weight of 89 kDa. A second potential methionine codon at nt 758 was in good agreement with the Kozak consensus site and if it was considered to be the SLAP translation start site the encoded protein would be 639 amino acids in length with a predicted molecular weight of 74 kDa (Fig. 5A). In order to discern whether these two sites could be used effectively to initiate translation, an *in vitro* transcription/translation reaction was performed using two different expression constructs. The ATG1 insert which contained both potential start sites and an inframe stop was PCR amplified from the SLAP plasmid and subcloned into pBSK to generate the ATG1 plasmid. The ATG2 plasmid was constructed by subcloning a KpnI/EcoRI SLAP restriction fragment which contained only the second consensus start site and the inframe stop codon into pBSK. Figure 5B shows an autoradiogram of *in vitro* translated ³⁵S -methionine labelled proteins from the various SLAP constructs and the luciferase control after size fractionation by SDS-PAGE. A protein of apparent molecular weight of 85 kDa in SDS-PAGE was produced effectively from the ATG2 construct which contained only the Kozak consensus start site (Fig. 5B, lane ATG2). Using the ATG1 construct as a template a protein of a predicted molecular weight of 94 kDa was produced but much less effectively than when the ATG2 construct was used (Fig. 5B, Lane ATG2). Although both start sites can be

Figure 5. In Vitro Transcription/Translation of SLAP From Two Potential Translation Start Sites.

A) Alignment of potential translational start sites with Kozak consensus start site.

The Kozak consensus start site for translation is shown and the residues that have been demonstrated experimentally to be critical for translation are shown in bold. The second potential SLAP ATG has both of these features while the first potential ATG has neither of them.

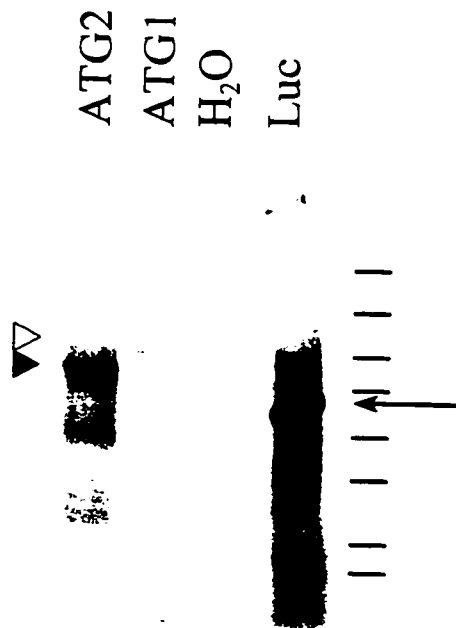
B) In vitro transcription/translation of SLAP.

Constructs containing only the Kozak consensus start site (ATG2) or a construct containing both the Kozak and the non-Kozak consensus start site (ATG1) were *in vitro* transcribed and translated and the polypeptides were electrophoretically separated by 10% SDS-PAGE. Luc, Luciferase control plasmid; H₂O, water control; ATG1, construct with both the start sites; ATG2, construct with only the kozak consensus start site. Filled arrowhead indicates polypeptide produced by ATG1, open arrowhead indicates polypeptide produced by ATG2, arrow indicates luciferase protein and the lines denote the mobility of the Protein Standards of molecular weights: 221, 133, 93, 67, 56, 42, 28 and 23 kDa.

A

ATG1	CTGTCCTCGATGC ³⁶⁵
ATG2	GCCAGAGGTATGG ⁷⁶¹
KOZAK	GCCGCCGCCATGG ⁴

B



used, the preferred initiator is likely to be at nt 758 and was considered to be the initiating methionine for the SLAP protein.

The putative SLAP protein was predicted to be acidic with a calculated pI of 4.9. The negatively charged amino acids were not evenly dispersed throughout the protein but were more concentrated in the carboxyl terminal 370 amino acids. The hydrophobic nature of a protein can be predicted by the method of Kyte-Doolittle (Kyte and Doolittle, 1982). A Kyte-Doolittle hydropathy plot of the predicted SLAP protein indicated that the protein was very hydrophilic except for a carboxyl terminal segment (aa 612-637) that was very hydrophobic (Fig. 6A). Several different algorithms that predict transmembrane domains indicated that the carboxyl hydrophobic region of SLAP was a potential transmembrane segment (Nakai and Kanehisa, 1992; Klein et al., 1985; Mohana Rao and Argos, 1986). However, a consensus signal peptide for membrane insertion was not found when the SLAP primary amino acid sequence was analyzed by the SignalP V1.1 WWW Prediction server (Nielsen et al., 1997). If the predicted SLAP protein sequence was extended to include the non-Kozak ATG, a signal peptide or an additional putative transmembrane domain would not be predicted.

The coiled-coil protein structure formed by the interweaving of two or more α -helices was first found as a dimerization motif in muscle proteins such as tropomyosin and myosin but has now been found in many other classes of proteins (Adamson et al., 1993; Lupas, 1996; Kee et al., 1995). Proteins that form coiled-coils contain a heptad repeat (**abcdefg**) where amino acids at the **a** and **d** positions are hydrophobic which gives the helices a hydrophobic face through which to dimerize (Kohn et al., 1997). Analysis of

Figure 6. Secondary Structure Analysis of the SLAP protein.

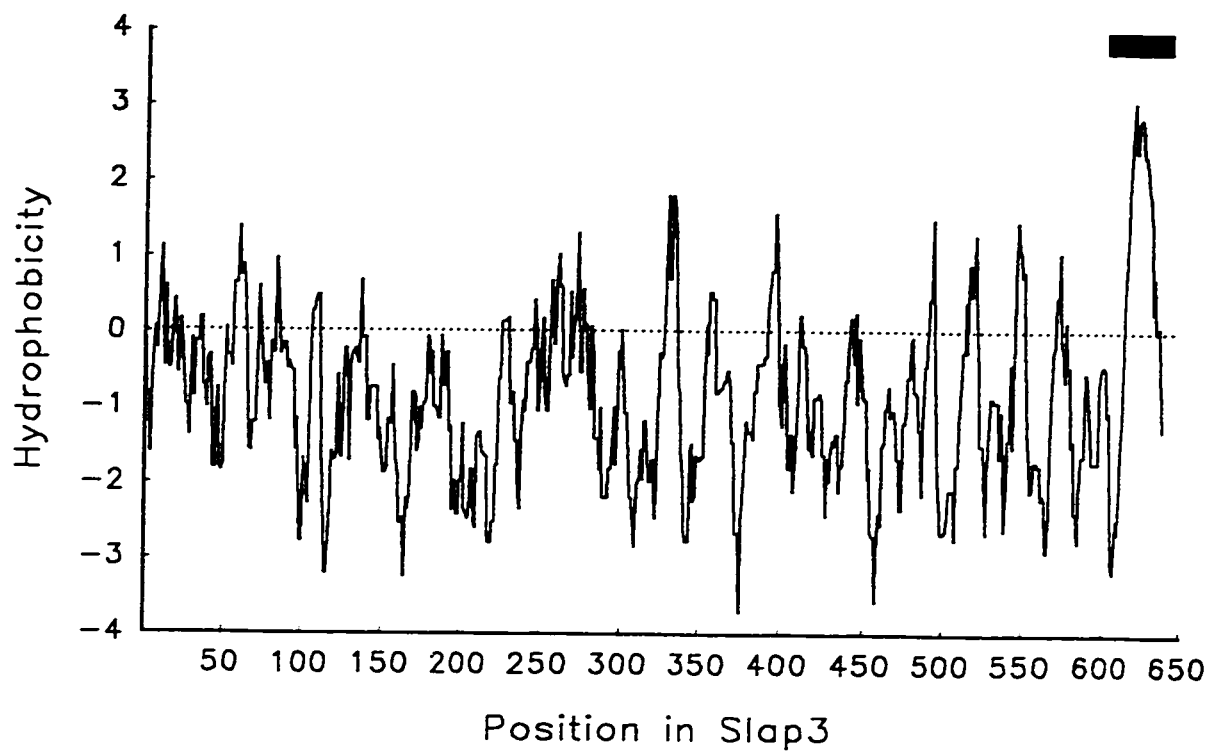
A) Hydrophobic profile of SLAP.

Kyte-Doolittle hydrophobicity plot (window of 7 residues) of SLAP shows the overall hydrophilic nature of the protein. The carboxyl terminal putative anchor domain (shown by the bold bar) is the only significantly hydrophobic stretch in the entire protein. This hydrophobic segment is predicted to be a transmembrane domain.

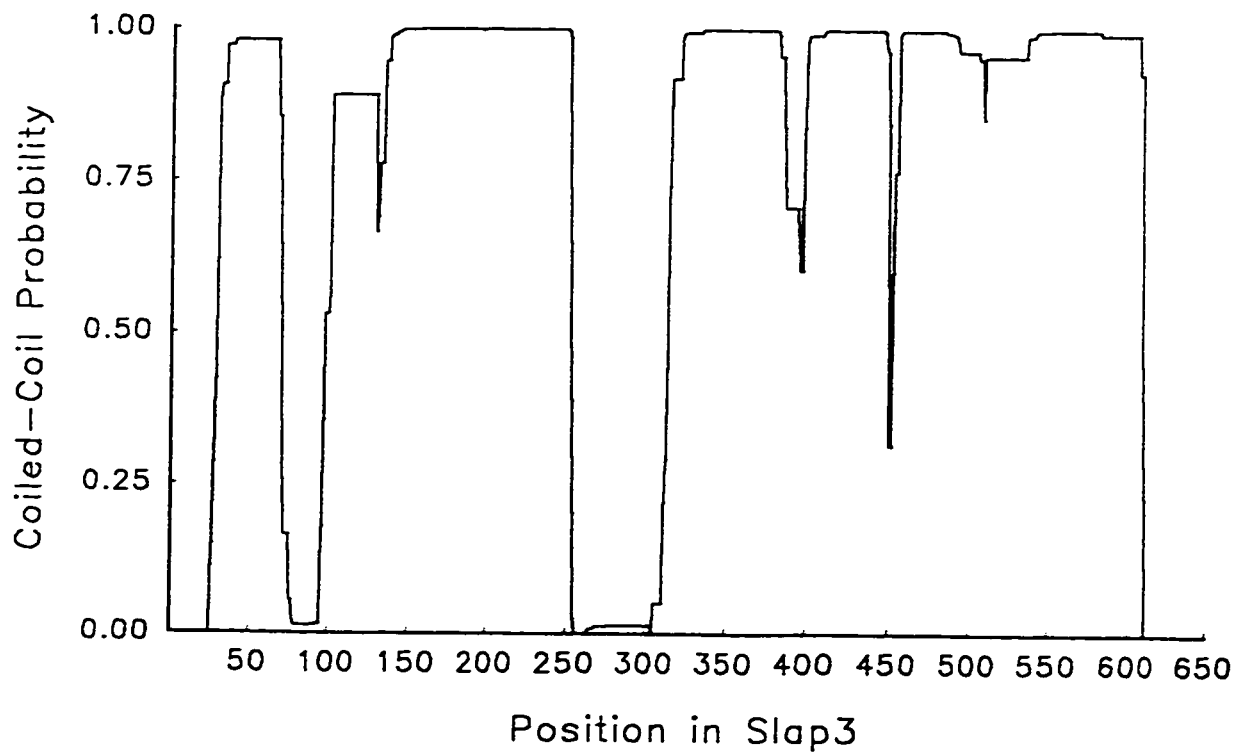
B) Tendency of SLAP to form the coiled-coil secondary structure.

The probability that SLAP could exist in a coiled-coil structure was predicted by the COILS analysis program using a window of 28 amino acids. The SLAP protein was predicted to have three large coiled-coil regions interspersed with short non-coiled-coil segments.

A



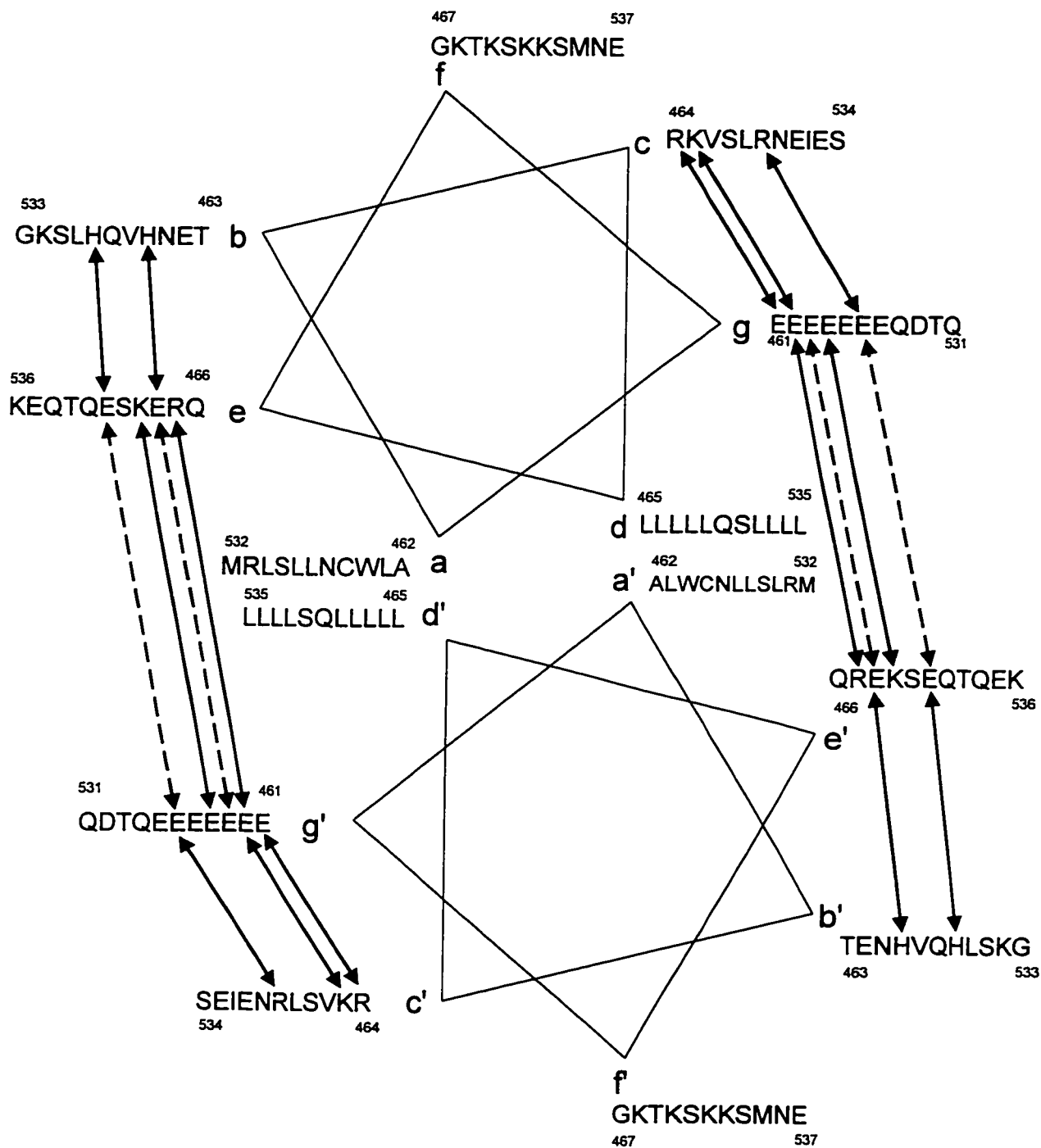
B



SLAP by a coiled-coil predictor program revealed that large segments of SLAP could exist in a coiled-coil conformation (Fig. 6B) (Lupas et al., 1991). Furthermore, two regions (aa 465-493 and aa 514 - 535) were predicted to be potential leucine zippers, a specialized form of the coiled-coil motif where a leucine is found at every seventh amino acid (Fig. 3) (O'Shea et al., 1989; O'Shea et al., 1991). These two potential leucine zippers are likely part of an eleven heptad coiled-coil structure since the region between the two leucine zippers could form a coiled-coil structure as well. In this linking region (heptads 6 and 7) there are two leucine residues at the **a** position that compensate for the glutamine and serine residues at the **d** position (Fig. 3). A helical wheel plot is a graphical representation of the α -helical structure of a coiled-coil protein. Since it takes 3.5 amino acid residues to complete a turn around the α -helix of the coiled-coil, every seventh amino acid is aligned at the same position of the helix. Figure 7 shows the alignment of the hydrophobic residues at position **a** and **d** of the α -helix which gives the tandem leucine zipper structure a hydrophobic face. The residues at position **e** and **g** of the coiled-coil structure flank the hydrophobic side and are believed to be responsible for the specificity of dimerization through the leucine zippers (O'Shea et al., 1992; Vinson et al., 1993). Seven glutamate residues align at the **g** position of the helix which gives it a negative strip. Five potential **i+3** and **i+4** intrahelical ionic attractions which could stabilize the helix were found in the structure (Fig. 7). Many coiled-coil proteins have a tendency to form homodimers and if SLAP homodimerized in a parallel manner through its leucine zipper segment four repulsive and four attractive ionic interactions would be predicted to be formed between the **i** and **i'+5** positions (Fig. 7).

Figure 7. Acidic Amphipathic Nature of the Leucine Zipper Region of SLAP.

A helical wheel plot shows the alignment of hydrophobic residues at the **a** and **d** position of the alpha helix in a segment (aa 432-538) that spans the two leucine zippers. Seven glutamates in a row line up at the **g** position giving the helix an acidic face as well as the hydrophobic one given by positions **a** and **d**. Potential **i+3** and **i+4** intrahelical attractions are shown by solid arrows. Equal number of interhelical attractions (solid arrows) and interhelical repulsions (dashed arrows) would be predicted if SLAP homodimerized in a parallel manner.



The function or localization of proteins can be altered by post-translational modification such as phosphorylation and glycosylation. Potential modification sites in a protein sequence can be identified by searching the primary amino acid sequence against the PROSITE database of consensus sites (Bairoch et al., 1997). SLAP contained potential phosphorylation sites for casein kinase II (20 sites), protein kinase C (12 sites) and a single putative cAMP dependent protein kinase site (Fig. 3). As well, two potential glycosylation sites were predicted. Since sites for lipid modification were not found in biologically relevant positions, the SLAP protein likely associates with the membrane through its carboxyl terminal hydrophobic tail. SLAP was not predicted to have any of the known calcium binding motifs, such as the EF hand. Regions of protein rich in proline, glutamate, serine and threonine (PEST domain) have been found in rapidly degraded proteins (Rechsteiner and Rogers, 1996). SLAP was predicted to contain such a PEST sequence motif between amino acids 294-306.

To better determine the potential identity of SLAP, its relationship to other proteins was analyzed by searching its sequence against previously cloned sequences deposited in the Genbank database. During most of this thesis work, SLAP showed very little homology with proteins in the Genbank database except for extended, low level homology (less than 20% identity at the amino acid level) with myosins and other coiled-coil proteins in their rod domains. As well, SLAP shared significant homology (44% similarity) with a coiled-coil yeast protein designated USO1 which is involved in the transport of vesicles from the ER to the golgi (Fig. 8) (Sapperstein et al., 1996). However, the advent of cloning and sequencing of Expressed Sequence Tags (ESTs),

Figure 8. Homology Between SLAP and USO1.

A BLAST alignment of SLAP with the yeast protein USO1 (Genbank accession number g137175), which is involved in intracellular transport. SLAP and USO1 are 44% similar over an overlap of 597 amino acids. In the middle line of the alignment, identities are indicated by the amino acid and conserved substitutions are indicated by plus signs. Gaps in sequence alignment are indicated by dashes and position of amino acid residues in SLAP and USO1 are indicated at the right.

Identities = 139/597 (23%), Positives = 264/597 (44%), Gaps = 41/597 (6%)

SLAP 40 LQEALHREQMLEQKLATLQRLLAITQEASDTSWQAL--IDEDRLLSRLEVMGNQLQACSK 97
+ E + LE +LA + L + +TS +AL + E+ + E + + +A
USO1 1077 ISELTKTREELEAELAAYKNLKNELETKLETSEKALKEVKENEHLKEEKIQLEKEATET 1136

SLAP 98 NQTEDSLRKELIALQEDKHNYETTAKESLRRVLQEKIEVVRKLSEVERSLNTEDECTHL 157
Q +SLR L +L+++ + K+ ++ ++ + ++S++ +++T+ E +
USO1 1137 KQQLNSLRANLESLEKEHEDLAAQLKKYEEQIANKERQYNEEISQLNDEITSTQQENESI 1196

SLAP 158 KEMNER-----TQEELRELANKYNGAVN-EIKDLSDKLKVAEGKQEEIQQKGQAE 206
K+ N+ T EE L A+N +IK+L K + E E E
USO1 1197 KKKNDELEGEVKAMKSTSEEQSNLKKSEIDALNLQIKELKKKNETNEASLLESIKSVESE 1256

SLAP 207 KKEQHKKIDEMEEKEQELQAKIEALQADNDFTNERTALQEHLSSKSGGCTFIHQFIEC 266
++ DE KE+E+ + L+A D ++ L +E K D I+
USO1 1257 TVKIKELQDECNFKEKEVSELEDKLSKSEDKNSKYLELQKESEKIKEELDAKTTELKIQ 1316

SLAP 267 QKKLIVEGHLTKVVEETKLAKENQARAKE-SDLSDTLSPSKEKSSDDTTDAQMDQDLNE 325
+K I K E++L++ + ++E + + L K + + + + LNE
USO1 1317 EK--ITNLSKAKEKSESELSRLKKTSSSEERKNAEEQLEKLKNEIQIKNQAFEKERKLLNE 1374

SLAP 326 SLAKVSLKALL----EERKAYRNQVEESSKQIQVLQAQLQRLHMDIENLREEKDNEIT 381
++ E+E +N+ E +K+I +++L+++ + + L EEK N I
USO1 1375 GSSTITQEYSEKINTLEDELIRLQENELKAKEIDNTRSELEKVSLSNDELLEEKQNTIK 1434

SLAP 382 STRDELLSARDEILLHQAAEKAAS-ERDT--DIASLQEELK-----KVRAELERWRKAA 433
S +DE+LS +D+I + EK S ERD D+ SL+E+L+ K + E E +K
USO1 1435 SLQDEILSYDKIT---RNDEKLLSIEDNKNRDLKESLKEQLRAAQESKAKVE-EGKKLE 1490

SLAP 434 SEYEKEVTSLQSSFQLRCQQCEDQKKEATRLQGELEKLRKEWNVLETECHSLKKENVLL 493
E KE L+ S ++ ++ E + T L+ +E +RK LE S +++ L
USO1 1491 EESSKEKAELEKSKEM-MKKLESTIESNETELKSSMETIRKSDEKLEQSKKSAEEDIKNL 1549

SLAP 494 SSELQRQEKELHNSQKQSLELTSDLSILQMTRKELNQMGSLEQHLRDSADLKILLSKA 553
E ++ S+K EL S L I + ELE ++K++ ++A KI ++
USO1 1550 QHEKSDLISRINESEKDIEELKSKLRIEAKSGSELE----TVKQE--LNNAQEKIRINAE 1603

SLAP 554 ENQAKDVQKEYEKTQTVLSELKLFEMTEQEKQSITDELKQCKDNLLQKEGNNNK 610
EN ++ + E + L + + + + ++EK+ +T LK+ + L Q+K ++
USO1 1604 ENTV--LKSKLEDIERELKDKQAEIKSNQEEKELLTSRLKELEQELDSTQQAQKSE 1658

short cDNA sequences from human tissues, has generated several clones that share more than 90% identity with SLAP. These cDNAs could potentially represent partial cDNAs of the human orthologue of SLAP. Recently, a protein called TOP_{AP} was cloned from developing chick visual system that shared an overall 72.2% sequence identity with SLAP over a 436 amino acid overlap (Savitt et al., 1995). The homology between SLAP and TOP_{AP} will be further analyzed in a later section of the thesis.

Tissue Specific Expression of SLAP Transcripts

The SLAP clones were isolated from rabbit heart cDNA libraries. In order to assay what other types of tissues expressed SLAP, a blot containing RNA from various rabbit tissues was probed with a radiolabelled cDNA SLAP probe. In rabbit heart three distinct transcripts were expressed of 5.9, 4.6 and 3.5 kb in size (Fig. 9). The largest transcript (5.9 kb) had an apparent ubiquitous pattern of expression since it was expressed at differing levels in all tissues examined. The two smaller transcripts (3.5 and 4.6 kb) were expressed in cardiac, slow twitch skeletal muscle, aorta and testes. Interestingly, fast twitch skeletal muscle only expressed the 5.9 kb SLAP transcript (Fig.9 , Fig. 10B). Many muscle proteins undergo dynamic regulation of their expression during early post-natal life (Kyselovic et al., 1994). To examine SLAP expression during development, RNA was isolated from hearts and brains of adult or 1 day old postnatal rats. A significant difference in either the SLAP transcript size or expression level was not observed between immature and adult brain (Fig. 10A). A very large transcript of 12 kb in length was seen in the developing heart but not in the adult heart (Fig. 10A). Since SLAP mRNA appeared to be highly expressed in heart and smooth muscle, this pattern of expression was more

Figure 9. Tissue Specific Expression of SLAP Transcripts.

Aliquots of total rabbit heart RNA (20 μ g) were size fractionated by formaldehyde agarose gel electrophoresis and transferred to a nylon membrane. An EcoRI/XbaI insert from LD1 was radiolabelled with α - 32 P-dCTP and used to probe the blot. Multiple transcripts of 5.9, 4.6 and 3.5 kb were seen in heart (H), slow twitch skeletal muscle (ST), aorta (A) and testes (T) but only the 5.9 kb transcript was seen in fast twitch skeletal muscle (FT). The 5.9 kb transcript was expressed to varying degrees in all rabbit tissues examined including the brain. The lines on the left side represent the mobility of the RNA molecular size standards with their sizes in kb. The arrows on the right indicate the location of the three SLAP transcripts and their calculated size in kb. The blot was stripped of the SLAP probe and probed with α -tubulin cDNA probe to control for the RNA loading (bottom panel).

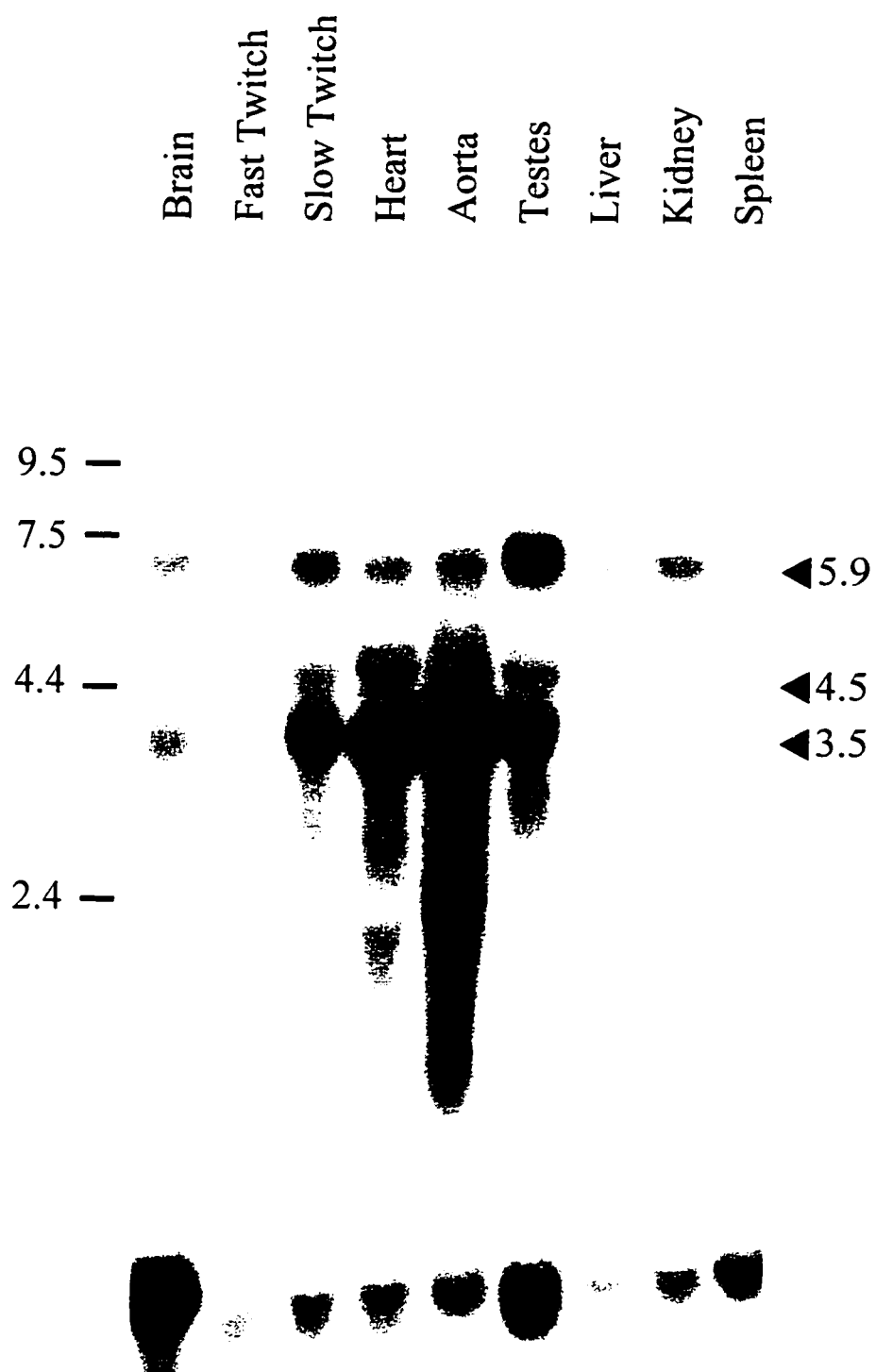


Figure 10. Expression of SLAP During Muscle Development.

Aliquots of total RNA from various tissues were size fractionated and transferred to a nylon membrane. An EcoRI/XbaI insert from LD1 was radiolabelled with α - ^{32}P -dCTP and used to probe the blot. After autoradiography the blots were stripped of the SLAP probe and reprobed with a radiolabelled GAPDH cDNA insert to control for RNA loading (bottom panels).

A) SLAP transcripts in the developing rat heart and brain.

Multiple SLAP transcripts were seen in both the developing and adult heart whereas only a single transcript was seen in both the developing and adult brain. A large transcript was detected in the developing but the adult myocardium (denoted by the open arrowhead). Filled arrowheads represent the mobility of the SLAP transcript found in adult and developing myocardium.

B) SLAP transcripts in smooth muscle.

The smaller (3.5 and 4.6 kb) SLAP transcripts were detected in all rabbit smooth muscles examined with the highest levels being in aorta and stomach. Higher levels of the 3.5 kb SLAP transcript were detected in RNA from rabbit atria versus ventricle. Mobility of SLAP transcripts are indicated by filled arrowheads.

Aorta
Uterus
Oesophagus
Stomach
Kidney
Atria
Ventricle
Heart
Slow
Fast
Brain

closely examined. The smaller “muscle specific” transcripts were expressed in all smooth muscle types examined (Fig. 10B). The level of expression of the 3.5 kb transcript seemed to be higher in the atria than in the ventricles (Fig. 10B).

Mapping the Termini of the Two Muscle Specific SLAP Transcripts

Since the deduced SLAP sequence was larger than two of the transcripts found in cardiac muscle, it was likely that the cardiac transcript did not encode the entire SLAP protein. The fact that three independently isolated clones from different cardiac cDNA libraries had nearly identical 5' ends indicated that the start of the smaller cardiac transcript was probably near the end of the LD1 clone. In order to map approximately where the 5' ends of the cardiac transcripts began cDNA inserts from different regions of SLAP were radiolabelled and used to probe Northern blots of muscle RNA. Probes were used that were from either the 5' (R3a probe) or 3' (LD1 probe) side of the 5' end of the LD1 clone and a third probe was from the SLAP 3'UTR (LD2 probe) (Fig. 11A). The probes from the 3' UTR and LD1 both recognized three transcripts in heart but only one transcript in fast twitch skeletal muscle (Fig. 11B panels LD1 and LD2). However, the R3a probe recognized only a single 5.9 kb transcript in both fast twitch muscle and heart (Fig. 11B panel R3a). The R3a probe and the LD1 probe were 132 bp apart but only the LD1 probe hybridized to the smaller cardiac transcripts.

The northern blot experiments describe above helped to define the approximate 5' ends of the SLAP transcript in cardiac muscle but to obtain the actual sequence of the 5' termini of the transcripts the method of 5' Rapid Amplification of cDNA Ends (5' RACE) was used. 5' RACE uses a gene specific internal primer for cDNA synthesis and

Figure 11. Mapping the 5' End of the Smaller Cardiac Transcripts.

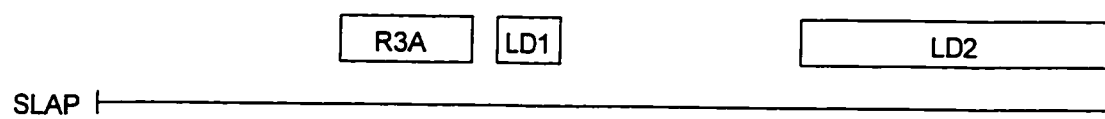
A) Map of the SLAP cDNA probes.

Location of cDNA fragments from different regions of SLAP which were used to synthesize radiolabelled probes for northern blot analysis. The size and location of the various SLAP cDNA probes are shown relative to the entire SLAP sequence.

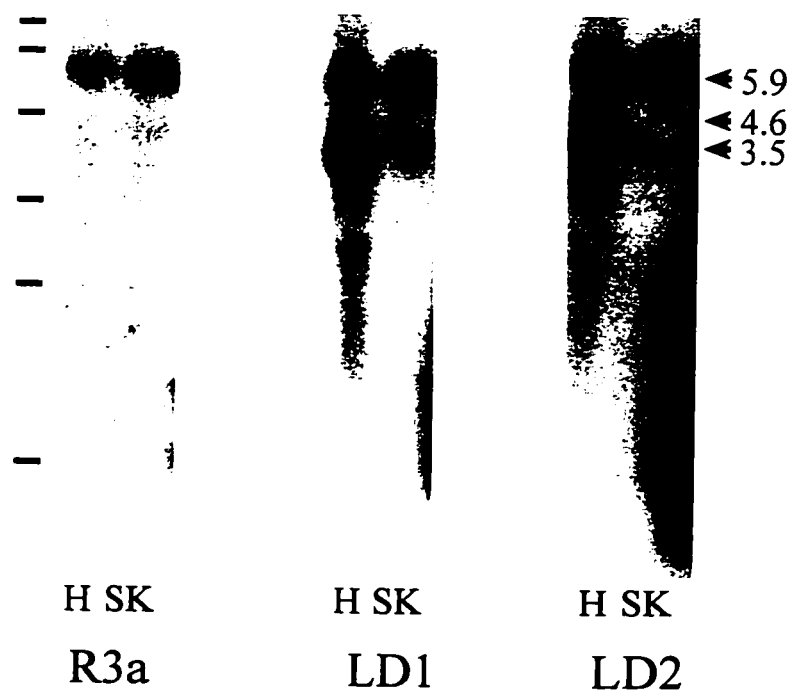
B) Tissue Specific Expression of SLAP transcripts.

Aliquots of total RNA from rabbit fast twitch skeletal muscle (SK) or heart (H) were size fractionated by formaldehyde agarose gel electrophoresis and transferred to a nylon membrane for Northern blot analysis with various SLAP cDNA probes. When a 600 bp SacI/EcoRI probe from R3a was radiolabelled and used as probe only the 5.9 kb transcript was seen in both fast twitch skeletal muscle and heart (Panel R3a). In contrast when a slightly more 3' probe was used to probe the blot, three transcripts (3.5, 4.6 and 5.9 kb) were seen in heart and only the 5.9 kb transcript in fast twitch muscle (panel LD1). A similar pattern of hybridization was observed when a probe from the SLAP 3' untranslated region was used (panel LD2). The lines on the left side represent the mobility of the RNA molecular size standards of 9.5, 7.5, 4.4, 2.4 and 1.4 kb in length. The arrows on the right indicate the location of the three SLAP transcripts and their calculated size in kb.

A



B



a nested gene specific primer with a common anchor primer for the PCR reaction. The reverse transcriptase reaction primer (RTPRIMER) was from nt 2150-2133 of SLAP and the primer for the PCR reaction was from nt 2113-2093 of SLAP. Both SLAP specific primers were situated within 600 bp of the 5' end of the LD1 clone. The substrate for cDNA synthesis was total rabbit heart RNA. The resulting PCR product was subcloned into the pCRII TA cloning vector. Restriction digests revealed that two different PCR products had been amplified and subcloned. These two cDNAs were subsequently sequenced on both strands. The first clone designated SLAP1 had an unique 5' end of 30 bp but the remainder of the clone was identical to SLAP from nt 1560 to 2113 (Fig. 12A). This clone had an inframe stop codon at nt 18 and a potential initiating methionine at position 189. The SLAP1 protein would be 322 amino acids in length and have a predicted molecular weight of 37 kDa. The larger clone, designated SLAP2, started at nt 1386 of SLAP but had a putative exon (nt 1496-1559) deleted which maintained the reading frame (Fig. 12A). A strong potential Kozak start site was found at nt 21 and the encoded protein would be 402 amino acids and have a predicted molecular weight of 46 kDa. The original SLAP sequence would now encode a protein designated SLAP3. The three different SLAP proteins would all contain the carboxyl terminal transmembrane domain as well as the leucine zipper regions (Fig. 12B). However, SLAP1 would not have the putative PEST region found in SLAP2 and SLAP3.

Chromosomal Assignment of the SLAP Gene

Since SLAP cDNA probes recognized multiple transcripts by northern blot analysis, the genomic organization of the SLAP gene was investigated by Southern blot

Figure 12. SLAPs Have Divergent 5' Termini.

A) Alignment of 5' ends of SLAP cDNAs.

The technique of Rapid Amplification of cDNA ends (RACE) was used to amplify the two alternative 5' ends of SLAP. The alignment of the 5' end of the SLAP1 transcript to SLAP2 and SLAP3 transcripts is shown. The nucleotide position in the transcripts is indicated at the right. Dashes denote gaps in sequence alignment and the region in bold denotes the unique 5' sequence of SLAP1.

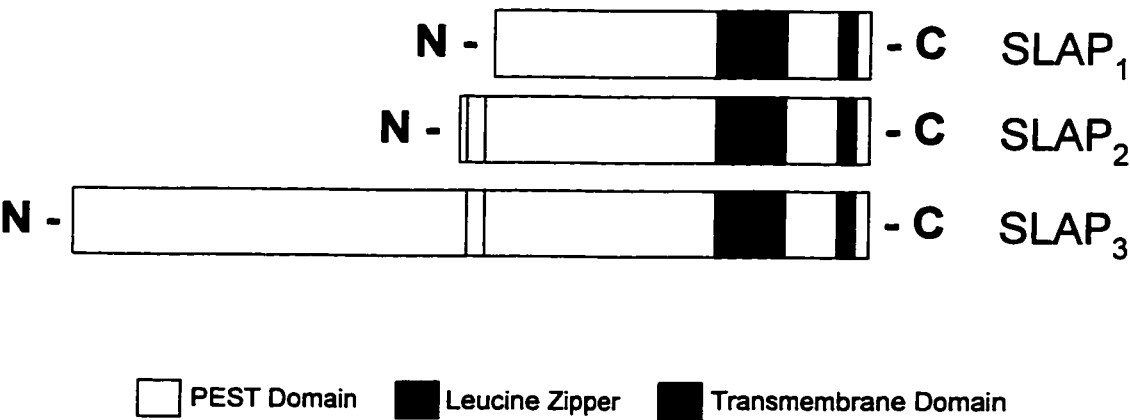
B) SLAP family.

The predicted SLAPs would contain both the carboxyl terminal transmembrane domain, as well as, the leucine zipper region. SLAP1 does not contain the putative PEST domain, which is found in both SLAP2 and SLAP3.

A

SLAP3	AAAATAGAAGCTTTGCAAGCTGATAATGATTTACCAATGAAAGGCTAAC	1485
SLAP2	AAAATAGAAGCTTTGCAAGCTGATAATGATTTACCAATGAAAGGCTAAC	100
SLAP1		
SLAP3	AGCTTTACAAGAGCACTTGCTTTCAAAGAGTGGCGGGGACTGCACTTTTA	1535
SLAP2	AGCTTTACAAG	111
SLAP1	 AGTATGGGCTTGGAT	15
SLAP3	TTCATCAGTTCATAGAATGCCAGAAGAAGCTGATCGTGGAGGGGCATCTA	1585
SLAP2	AGAAGCTGATCGTGGAGGGGCATCTA	137
SLAP1	ACTAACGTTTATTTTCTTTGGCAG AGAAGCTGATCGTGGAGGGGCATCTA	65

B



analysis and Fluorescence *In Situ* Hybridization (FISH). Rabbit genomic DNA was first digested by various restriction enzymes, size fractionated by agarose gel electrophoresis and transferred to a nylon membrane. The blot was then be hybridized with a SLAP cDNA probe to reveal the genomic organization of SLAP. When southern blot analysis was performed on rabbit genomic DNA with a SLAP cDNA probe a simple pattern was seen. Single bands were seen for the SalI (a rare cutter) and the PstI (a more frequently cutter) digested DNA (Fig. 13). Two bands were seen for the DNA digested with the other restriction enzymes. The technique of FISH allows for genes to be assigned to a chromosomal localization. To localize the human SLAP gene the cDNA of the human orthologue was isolated by screening at high stringency a human cardiac cDNA expression library with a rabbit heart cDNA probe encompassing the coding region of SLAP1. A human cDNA of 2.4 kb was isolated that was 90% identical with rabbit SLAP and was used in a FISH assay of human metaphase spreads. SLAP was localized 91 out of 100 times to one pair of chromosomes (Fig. 14). DAPI banding was used to identify the specific chromosomes and the SLAP hybridization was localized to chromosome 3. No other locus of hybridization was detected and through the compilation of ten photographs SLAP was assigned specifically to the p14.3-21.2 region of chromosome 3 (Fig. 14).

Biochemical Characterization of SLAP Protein

A specific antiserum to the SLAP proteins would greatly help in characterizing the protein's localization and the nature of its cellular association. Bacterially expressed recombinant fusion proteins can be used as antigens for antiserum production. To detect all of the putative SLAP proteins the common carboxyl terminus of the three isoforms was

Figure 13. Genomic Organization of SLAP.

Rabbit genomic DNA was digested with the indicated restriction enzymes, size fractionated by agarose gel electrophoresis and subjected to southern blot analysis. The SLAP cDNA probe used was a radiolabelled HindIII/ Aval cDNA insert from SLAP. The SLAP probe hybridized in a simple pattern to the blot of digested genomic DNA. The mobility of the DNA molecular size standards are indicated on the left with their sizes in kb.

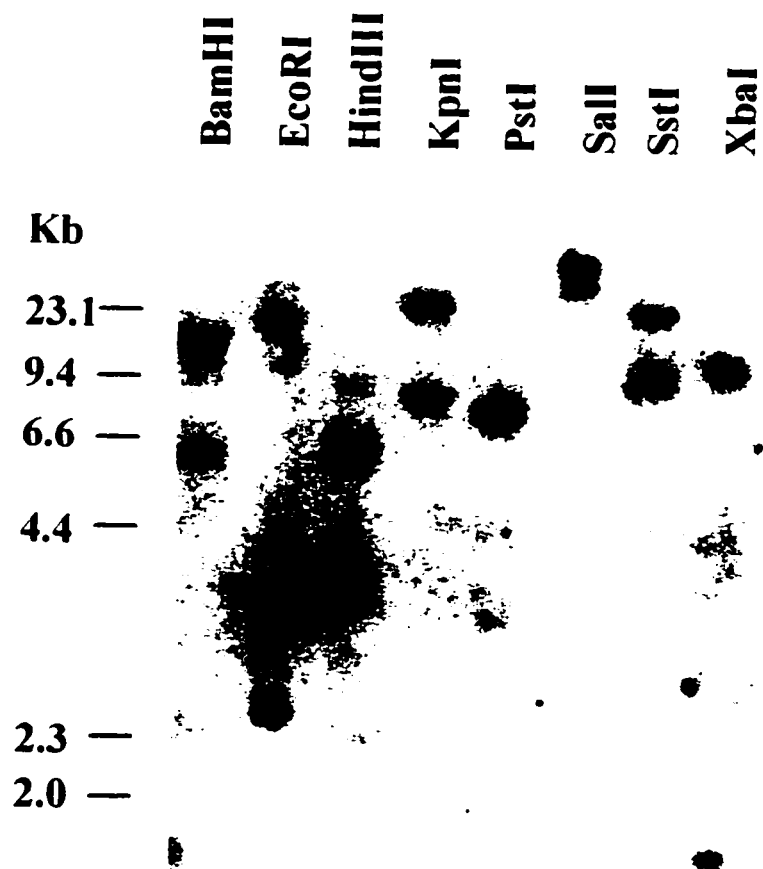
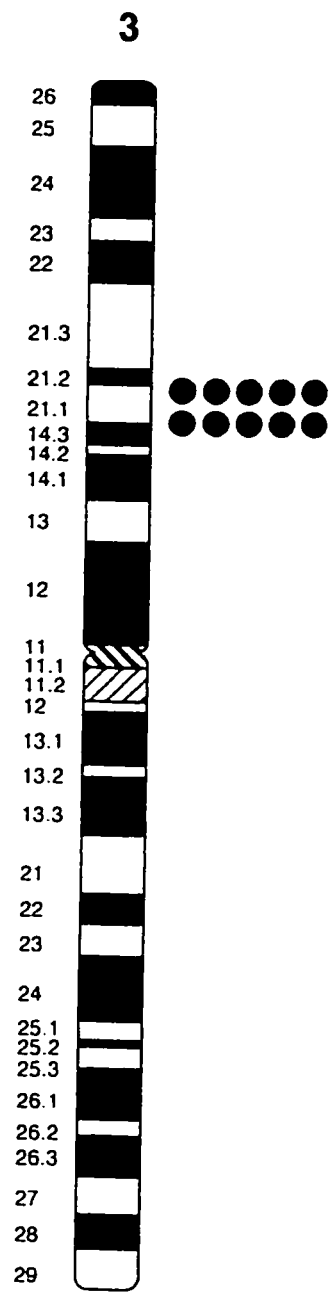
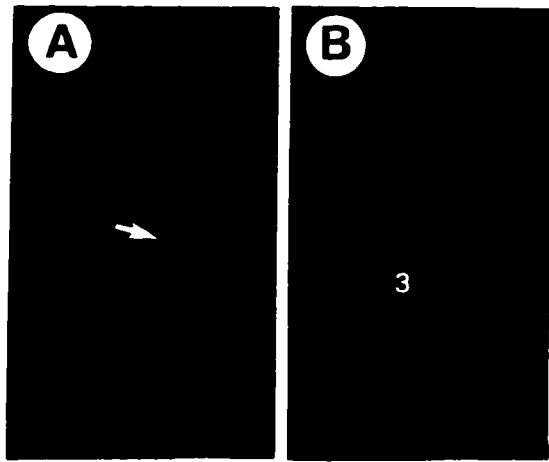


Figure 14. Chromosomal Assignment of the SLAP Gene.

The colour panels on the left are an example of the *in situ* localization of the SLAP gene to human chromosome 3. The pair of fluorescent signals on the chromosome (Panel A) show specific hybridization of the SLAP cDNA probe. The same mitotic figure was stained with DAPI to identify the chromosome as being chromosome 3 (Panel B). The figure on the right represents a map of chromosome 3 and each dot represents the results of a mapping experiment performed as in the left panels.



SLAP

used to generate antiserum. A restriction fragment of LD1 was subcloned into the carboxyl terminus of the maltose binding protein (MBP) of the pMALCRI vector (Fig. 15A). A MBP-SLAP fusion protein of 82 kDa was produced in bacteria. Supernatants from sonicated MBP-SLAP expressing bacteria were loaded onto an amylose column. The column was washed extensively and the fusion protein eluted with maltose. Purified fusion protein (1 mg) was emulsified with complete Freund's adjuvant and injected into both a rabbit and a guinea pig. After two additional boosts of the purified fusion protein emulsified with incomplete Freund's adjuvant, serum fractions were isolated from the rabbit and guinea pig. The specificity of the two antisera was assessed by western blot analysis. Rabbit heart homogenate samples were size fractionated by SDS-PAGE and transferred to nitrocellulose membranes for probing by the various antisera. Both guinea pig anti-SLAP antiserum (Fig. 15B, Gp, lane I) and rabbit anti-SLAP antiserum (Fig. 15B, Rb, lane I) recognized three polypeptides of 81, 45 and 35 kDa in homogenate samples of rabbit heart. The rabbit antiserum recognized a polypeptide of 60 kDa but this immunoreactivity was not specific towards the SLAP fusion protein since the pre-immune antiserum recognized it as well (Fig. 15B, Rb, lane PI). The guinea pig pre-immune antiserum did not recognize any polypeptides in the rabbit heart samples (Fig. 15B, Gp, lane PI).

To ensure that the reactivity of the anti-serum was specific towards the SLAP protein, guinea anti-SLAP antiserum was loaded onto a MBP column to adsorb any antibodies specific to MBP. The flowthrough from this column was then loaded onto a MBP-SLAP affinity column which adsorbed the antibodies specific to the SLAP portion of the fusion

Figure 15. Generation of Specific anti-SLAP Antiserum.

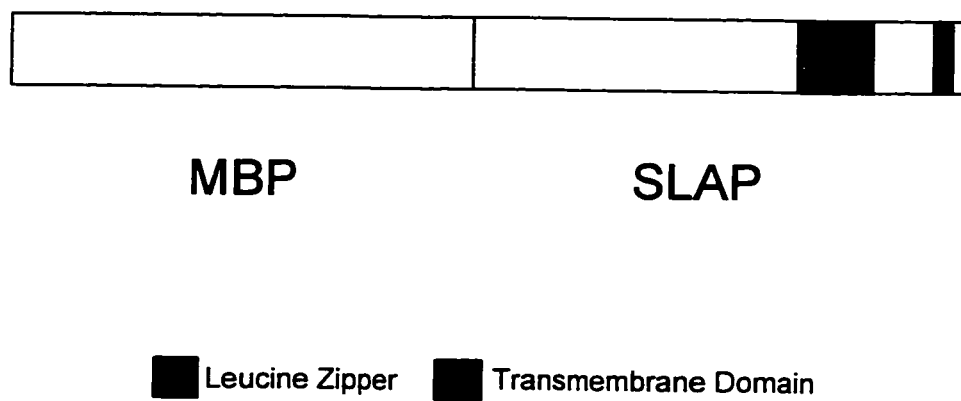
A) SLAP fusion protein.

The coding sequence for the carboxyl terminal 370 amino acids of SLAP3 were fused inframe to the Maltose Binding Protein (MBP) coding sequence. This region of SLAP3 was common to the three different SLAP isoforms and contained both the leucine zippers and the transmembrane domain.

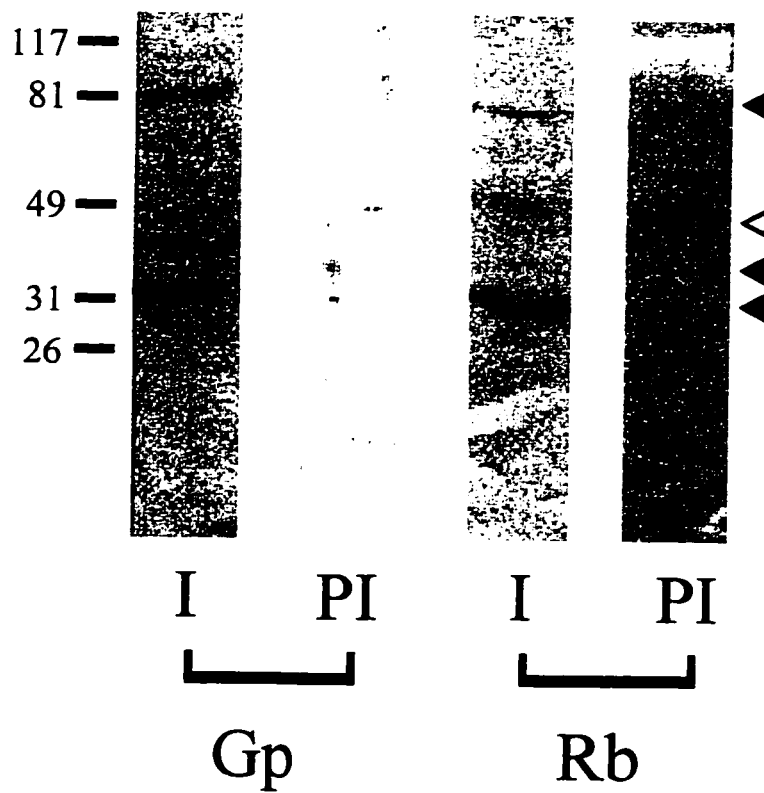
B) Characterization of rabbit anti-SLAP antiserum.

Both the guinea pig immune serum (Gp, lane I) and the rabbit immune serum (Rb, lane I) detected an abundant 35 kDa protein and two less abundant proteins of 81 and 45 kD in rabbit cardiac homogenates (indicated by filled arrowheads). Also, the rabbit immune serum recognized a 60 kDa protein (indicated by open arrowhead) that was apparently recognized by the rabbit pre-immune serum (Rb, lane PI). The guinea pig pre-immune antiserum (Gp, lane PI) did not recognize any specific proteins in the rabbit homogenate. The mobility and molecular weights in kDa of the protein molecular weight standards are indicated on the left.

A



B



protein. After washing the column extensively, the affinity purified SLAP-specific antibodies were eluted from the column by an acidic glycine buffer. The starting crude antiserum and the various adsorbed/purified antisera were then used to probe western blots of rabbit heart homogenates. Antiserum adsorbed on a MBP column (Fig. 16, lanes 2) recognized identical antigenic polypeptides when compared to the starting crude antiserum of apparent molecular weights of 35, 45, 81 and 105 kDa (Fig. 16, lane 1). Conversely, after adsorption on the MBP-SLAP column, the resulting antiserum did not recognize any polypeptides at all (Fig. 16 lane 3). The affinity purified antibodies eluted from the MBP-SLAP column recognized three rabbit heart polypeptides of 35, 45 and 81 kDa but did not recognize a polypeptide of 105 kDa that was recognized by the crude antiserum (Fig. 16, lane 4).

Membrane Association of SLAP

Originally, the cDNA encoding SLAPs were isolated by immunoscreening expression libraries with antibodies raised against purified cardiac sarcolemmal membrane proteins. To see if SLAP was associated with heart sarcolemma, rabbit heart homogenates were fractionated into their subcellular compartments and then assayed by western blot analysis for SLAP proteins using the affinity purified guinea pig antiserum as a probe. SLAP was present in the initial cardiac homogenate fractions and the crude and purified sarcolemmal fraction but not found in the cytosolic fraction (Fig. 17). The nature of SLAP's association with the membrane, either through peripheral binding or integral association, needed to be addressed particularly since the protein lacked a traditional signal sequence for membrane insertion. This question can be addressed

Figure 16. Immunoreactivity of the Affinity Purified anti-SLAP Antiserum.

Rabbit heart homogenates were size fractionated and subjected to western blot analysis using 1:5000 final dilutions of guinea pig anti-SLAP antisera that was treated as described in materials and methods. The crude antiserum (lane 1), maltose binding protein (MBP) flowthrough antiserum (lane 2) and the affinity purified antisera (lane 4) recognized three polypeptides of 81, 45 and 35 kDa. The MBP-SLAP adsorbed antiserum did not recognize any polypeptides (lane 3). The position of the three common immunoreactive polypeptides are indicated by arrows on the right. The mobility of the protein standards of molecular weights of 116, 97, 66, 45 and 31 kDa are indicated on the left.

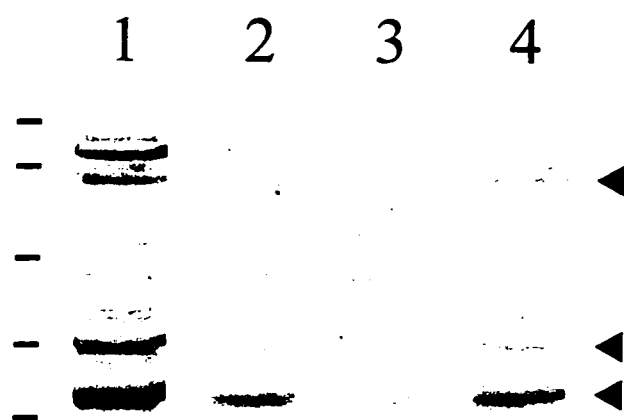
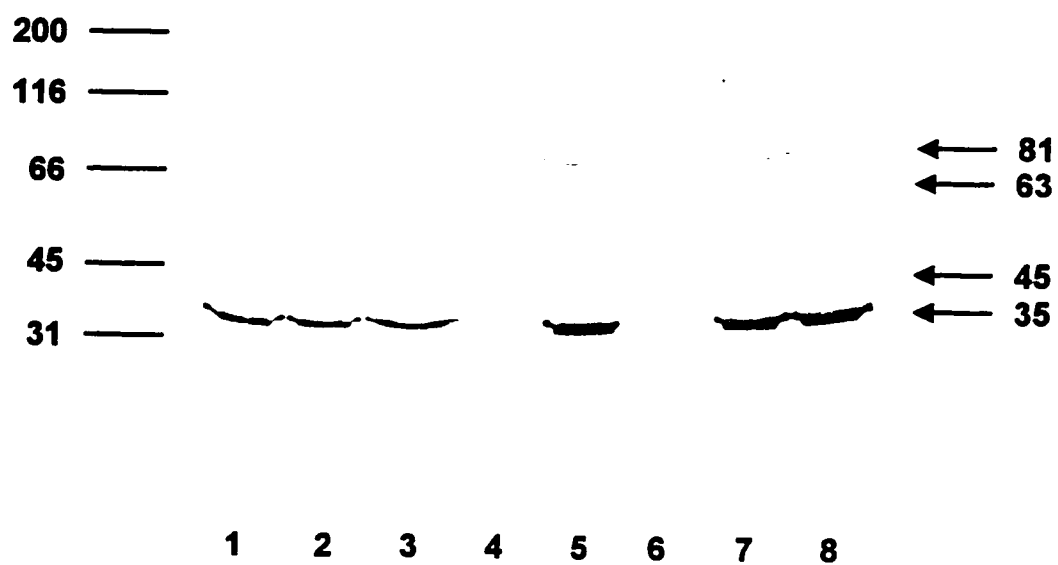


Figure 17. Membrane Specific Association of SLAPs.

The following subcellular fractions from rabbit heart: homogenate (lane 1), low speed supernatant (lane 2), low speed pellet (lane 3), cytoplasmic (lane 4), Triton-X 100 soluble extract of myofibers (lane 5), Triton-X 100 insoluble extract of myofibers (lane 6) , crude sarcolemma (lane 7) and purified sarcolemma (lane 8) were fractionated by SDS-PAGE and transferred to nitrocellulose. An immunoblot was performed using affinity purified anti-SLAP antibodies as described in material and methods. The four immunoreactive proteins found in the crude (lane 7) and purified (lane 8) sarcolemmal fractions are denoted by the arrows on right with their sizes in kilodaltons. The mobility of the molecular weight standards (in kilodaltons) are indicated on the left.



biochemically by treating membrane fractions with extraction buffers such as high salt, low pH, high pH or urea. Peripheral membrane proteins are extracted from the membranes by these treatment conditions unlike integral proteins which can only be extracted by dissolving the membrane pellets in detergents such as TritonX-100. When cardiac sarcolemmal membranes were extracted with: high salt, no calcium, low pH, high pH, and urea, SLAP remained in the pellet (Fig. 18 , top panel lanes 1-6). However TritonX-100 treatment of the membranes extracted SLAP from the pellet (Fig. 18 top panel, lane 7) into the soluble fraction (Fig. 18, bottom panel lane 7).

Immunohistochemical Localization of SLAP in Muscle

Immunofluorescence was used to study the *in situ* distribution of SLAP in cardiac muscle. Since SLAP transcripts were expressed in immature muscle, the subcellular localization of SLAPs was investigated in embryonic as well as adult muscle. In order to obtain clear data for *in situ* localization, a number of factors had to be examined including the method and time of fixation of the sample tissues. The various anti-SLAP antisera did not specifically recognize any epitopes in tissue sections that had been paraffin embedded or had been treated with gluteraldehyde. However, the SLAP anti-sera stained cryosections of rat heart that had been treated with Lana's solution (Picric acid/paraformaldehyde) for 5 minutes. Immunohistochemical staining for SLAP in cryosections of both embryonic and adult rat heart showed abundant staining (Figs. 19 A,B). Immunostaining in longitudinally oriented sections of myocardium showed that the SLAP staining was found in longitudinal and transverse bands (Fig. 19C) which was similar to the SLAP staining seen in cultured cardiac myocytes (Fig. 19D). To further

Figure 18. SLAPs are Integral Membrane Proteins.

Rabbit cardiac sarcolemmal proteins were incubated for one hour at 4°C with: PBS (lane 1), PBS/1 mM EGTA (lane 2), PBS/1 M KCl (lane 3), PBS/ 4 M Urea (lane 4), 50 mM glycine (pH 2.5)/150 mM NaCl (lane 5), 50 mM glycine (pH 10.0)/150 mM NaCl (lane 6) or PBS/1% Triton (lane 7). After incubation, the proteins were separated into pellets (Panel A) and supernatant fractions (Panel B) by centrifugation at 180 000 g. Equal volumes of pellets and supernatants from the various treatments were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with affinity purified anti-SLAP antiserum. SLAP was only extracted from the pellet by detergent extraction (lane 7). The arrows indicate the position of the major antigenic SLAP polypeptides and their molecular weights in kDa.

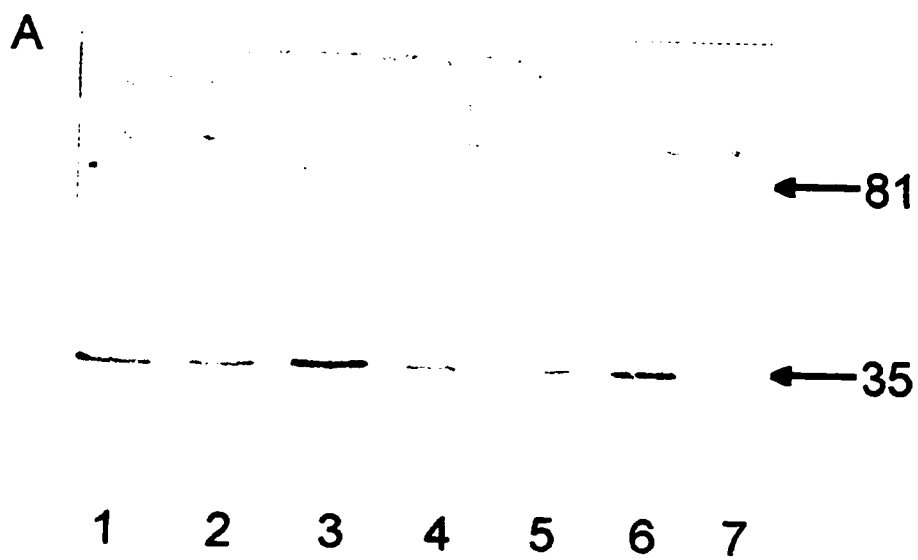
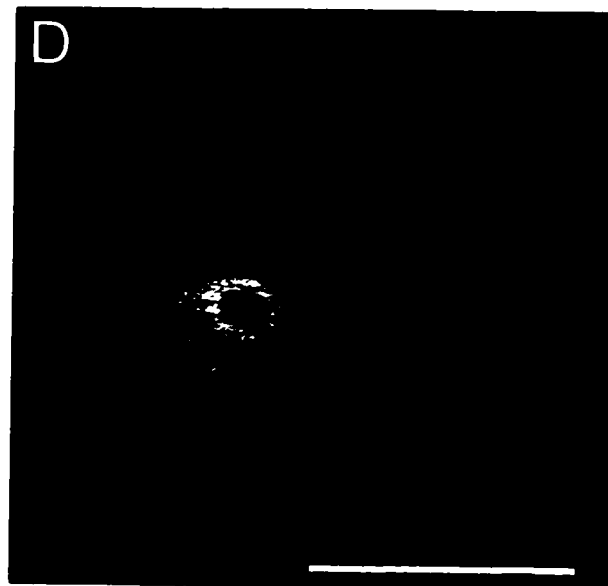
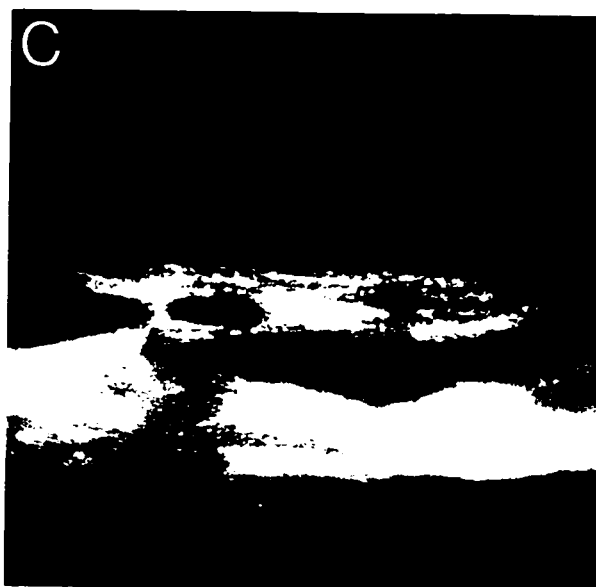
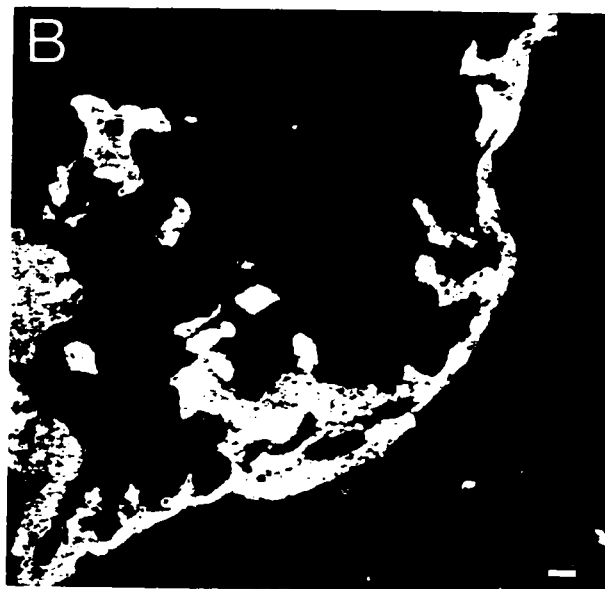
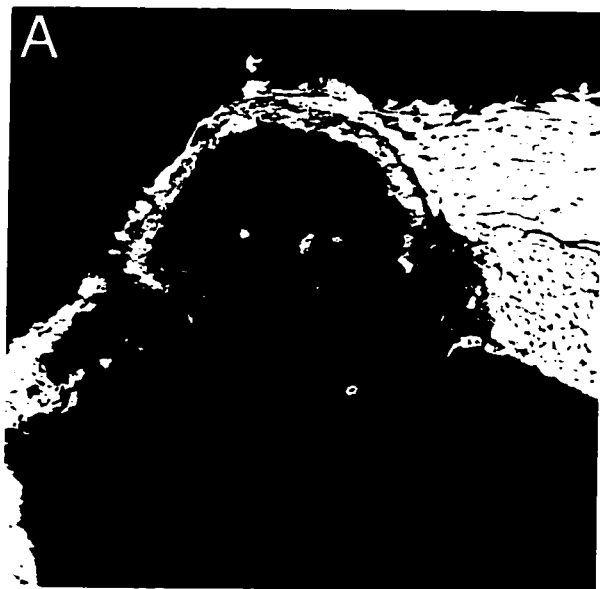


Figure 19. SLAP Staining in the Developing and Adult Myocardium.

Rabbit anti-SLAP antiserum was used to stain fixed cryosections of adult (A, C) and developing rat heart (B) as well as isolated Day 1 neonatal rat cardiomyocytes cultured for 5 days (D). SLAP staining was very strong in the adult myocardium but not the epicardium or the endocardium (A). Strong SLAP staining was seen in cryosections of fetal rat heart (B). Both longitudinal and transverse bands of SLAP staining were seen at higher magnification images of an adult myocardium section (C) or cultured neonatal cardiomyocyte (D). Scale bars equal 40 μm .



characterize the distribution of SLAP, 2 μ m cardiac cryosections were independently stained with either rabbit anti-SLAP antiserum, purified rabbit anti-dystrophin antiserum (sarcolemmal marker) or a monoclonal antibody directed towards the slow form of the SR calcium ATPase (sarcoplasmic reticulum marker). While SLAP exhibited both a reticular and sarcolemma staining pattern, dystrophin staining was localized exclusively to the sarcolemma (Fig. 20 A,B). The reticular pattern of staining seen for SLAP was similar to the pattern seen for staining of the SR calcium ATPase (Fig. 20C). The relationship between SLAP and these two markers was more closely examined by simultaneously staining the same cryosection of adult rat heart for SLAP and either dystrophin or the SR calcium ATPase. Some co-localization of the immunostaining for SLAP and dystrophin was seen but the majority of SLAP staining was localized in the reticular pattern whereas dystrophin staining was localized only to the sarcolemma (Fig. 21A and B). The calcium ATPase and SLAP were both immunostained in reticular patterns that showed substantial overlap (Fig. 21 C and D). The specificity of staining observed with the anti-SLAP antisera needed to be established. If the staining observed with the antiserum was specific to the SLAP part of the fusion protein, then it would be decreased with the pre-incubation of the antiserum with the MBP-SLAP fusion protein but not by pre-incubation with the MBP alone. Pre-adsorption of anti-SLAP antiserum with MBP-SLAP fusion protein but not with MBP dramatically reduced the amount of staining seen in heart cryosections (Fig. 22). In all subsequent immunofluorescence experiments the patterns of SLAP staining seen in tissues with either rabbit anti-SLAP antiserum, guinea pig anti-SLAP antiserum or the affinity purified guinea pig anti-SLAP antiserum was the same (data not shown).

Figure 20. SLAP Staining Compared to the Staining Pattern of Other Membrane Markers.

Very thin (2 μm) cryosections of adult rat heart were individually stained for either SLAP (A), dystrophin (B) or the calcium ATPase of the sarcoplasmic reticulum (C). SLAP staining was localized to the surface of the cardiomyocyte and to an interior reticular structure. Dystrophin staining was localized exclusively to the surface sarcolemma, whereas, the staining for the calcium ATPase was localized largely to an interior reticular pattern that resembled the pattern of staining for SLAP. Scale bar equals 10 μm .



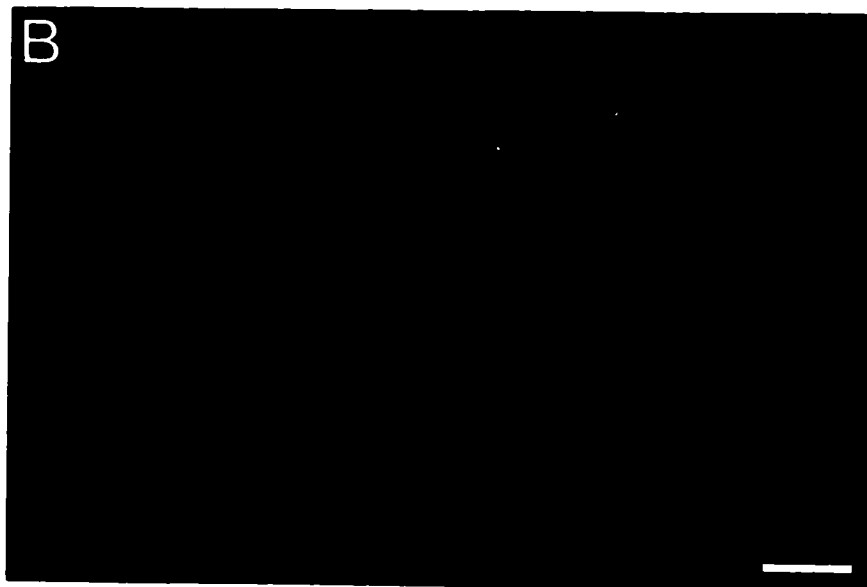
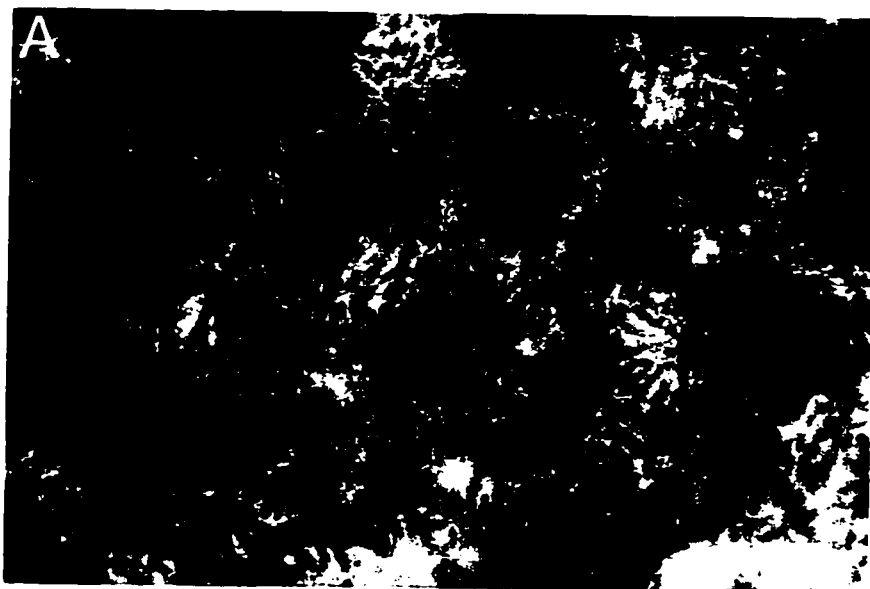
Figure 21. Myocardium Stained for SLAP and Membrane Markers.

Thin (5 μm) cryosections of adult rat heart were simultaneously stained for SLAP (A,C) and either dystrophin (B) or the SR calcium ATPase (D). Dystrophin and SLAP staining overlapped at the sarcolemma but dystrophin staining was absent from the interior of the cardiocyte (A,B). The staining for the ATPase and SLAP overlapped significantly but SLAP staining was in regions of the cardiocyte that were negative for the ATPase (C,D). Photographs were taken on a conventional Zeiss microscope and then the negatives were scanned using a Panasonic slide scanner. The digital images were adjusted for contrast and brightness in identical manners in Adobe Photoshop. The scale bar equals 10 μm .



Figure 22. Specificity of Anti-SLAP Antiserum.

Rabbit anti-SLAP antiserum was adsorbed for four hours with either MBP (A) or with MBP-SLAP fusion protein (B). The adsorbed antisera were applied to 2 μ m fixed cryosections of adult rat heart as described in Material and Methods. The SLAP staining was reduced significantly by adsorption with MBP-SLAP but not by adsorption with MBP alone. The scale bar represents 10 μ m.



SLAP Staining Was Fibre Type Specific in Skeletal Muscle

Since SLAP transcripts were expressed in other muscle types as well, the *in situ* localization of SLAP in skeletal muscle was studied by immunofluorescence staining of cryosections of skeletal muscle from rat extraocular and tibialis anterior (TA) muscles. In transverse cryosections of extraocular muscle a reticular pattern of staining was seen for SLAP which was similar to the pattern seen previously in cardiac muscle (Figs. 23A and 23B). All of the extraocular muscle fibers were not equally stained for SLAP and this apparent fiber specific staining and reticular pattern of staining was also seen in transverse cryosections of the TA muscle which were stained for SLAP (Fig. 23C). Since SLAP transcripts were found to be expressed in developing skeletal muscle, cryosections of fetal skeletal muscle (intercostal muscle) were stained for SLAP. These embryonic muscle fibers showed strong staining for SLAP but the reticular pattern of staining was not as pronounced as in the adult muscles (Fig. 23D). As well, a perinuclear localization for SLAP staining was observed in the immature muscle (Fig. 23D). In longitudinal cryosections of adult rat TA muscle, SLAP staining was largely distributed in periodic transverse bands. (Fig. 23 E and F).

Since not all of the skeletal muscle fibers were positive for SLAP staining, fiber typing of the skeletal muscle was carried out in order to account for the staining patterns seen in (Fig. 23). Skeletal muscle fibers can be classified as slow (Type I) or fast (TypeIIA or IIB) and each of these three fiber types express a unique myosin isoform. These different myosins can be discriminated by monoclonal antibodies which in turn allows for the different fiber types in skeletal muscle to be identified. Sections of ocular

Figure 23. SLAP Localization in the Developing and Adult Skeletal Muscles.

Cryosections of adult extraocular muscle (A , B) , tibialis anterior muscle (C, E, and F) and fetal intercostal muscles (D) were immunohistochemically stained for SLAP. SLAP staining presented as a reticular pattern in transverse cryosections (A, B, C) and as transverse bands in longitudinal cryosections (E2, F). Fetal skeletal muscle stained for SLAP did not demonstrate the extensive reticular staining found in the adult sections (Panel D). Images were either obtained with a conventional fluorescence microscope (A, B, C, E) or a confocal laser microscope (C, F). Scale bars equal 25 μ m.

muscle were simultaneously immunostained with SLAP and either a monoclonal antibody to the slow myosin isoform (Type I) or the fast type isoform (Type IIA) to determine which muscle fiber type expressed SLAP. All of the muscle fibers which stained for the slow myosin isoform also stained for SLAP, however, there were fibers that stained for SLAP alone (Figs. 24A and 24B). Staining with SLAP and the monoclonal antibody to the fast myosin type IIA demonstrated a similar co-localization of immunostaining (Figs. 24C and 24D). To confirm the fiber specific manner of SLAP expression, an antibody to another fiber specific skeletal muscle protein was needed. The Type IIA isoform of the SR calcium ATPase is expressed in cardiac and slow twitch (type I) skeletal muscle fibers. Sections of rat TA were double immunostained with the monoclonal antibody to the slow calcium ATPase and the rabbit anti-SLAP antiserum. All of the fibers that stained for the slow form of the calcium ATPase were also stained for SLAP but SLAP stained fibers that were not stained for the calcium ATPase (Fig. 25).

SLAP Expression in the Developing CNS

SLAP is related to a Topographically Graded Retinal Protein (TOP_{AP})

Initially the SLAPs showed very little homology with any previously cloned proteins in the databases. Recently, SLAPs were found to have homology with the TOP_{AP} antigen, a topographically graded protein cloned from the developing chick retina (Savitt et al., 1995). Like SLAP, TOP_{AP} was predicted to be a tail-anchored membrane protein which lacked a traditional signal sequence. SLAP1 shared an overall 72% amino acid identity with TOP_{AP} with only a region at the amino terminus and another at the carboxyl terminus which diverged significantly (Fig. 26A). Every leucine and glutamate residue

Figure 24. Muscle Fiber Specific Nature of SLAP.

Cryosections of fixed extraocular muscle were simultaneously immunohistochemically stained for SLAP (A, C) and slow (Type I) myosin or fast (Type IIA) myosin as described in materials and methods. Fibers that stained positively for SLAP but were negatively stained for the TypeIIA myosin isoform are indicated by arrowheads (D). Scale bar equals 25 μm .

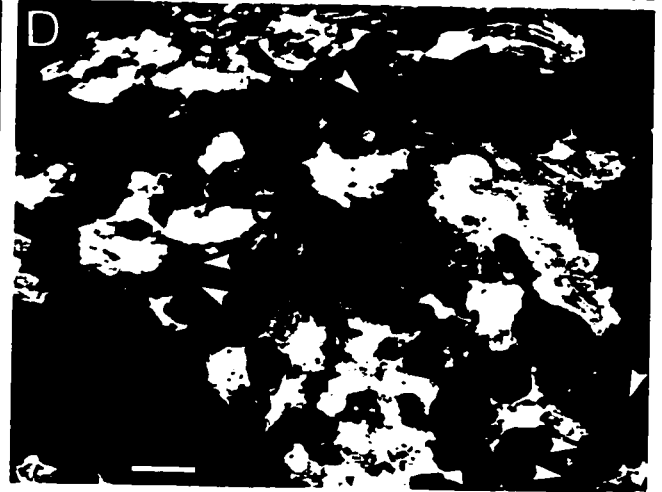
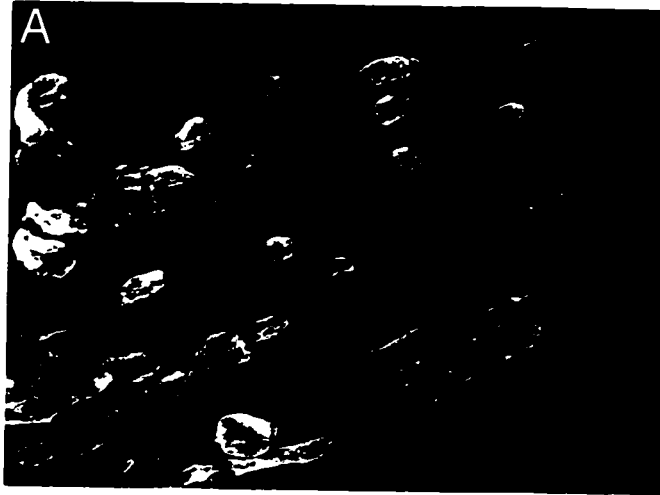
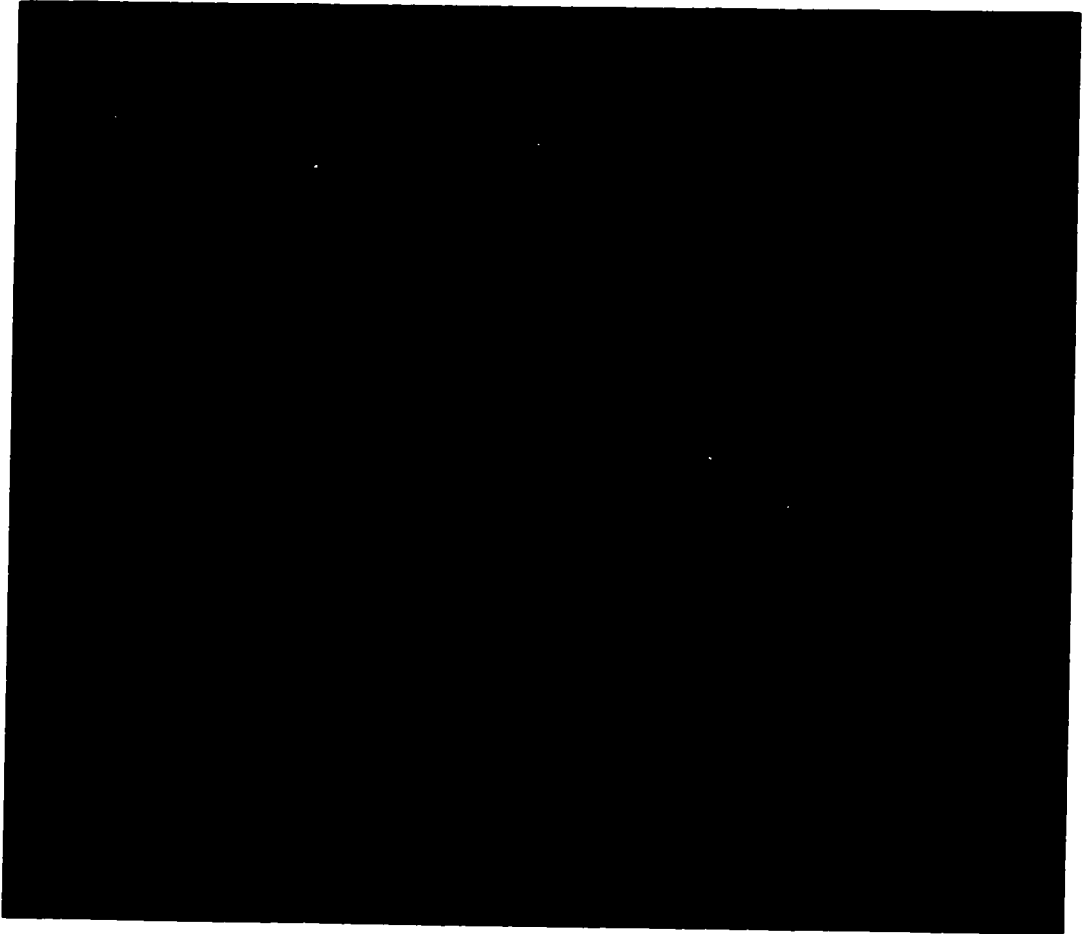


Figure 25. Co-localization of SLAP and the Slow Form of Ca^{2+} ATPase.

Cryosections of rat tibialis anterior muscle were fixed and simultaneously stained for SLAP and the Type IIA isoform of the sarcoplasmic reticulum Ca^{2+} ATPase which is specific for slow twitch muscle. Very few fibers were stained for the Ca^{2+} ATPase (red fibers, A) but all of the fibers that were positively stained for the Ca^{2+} ATPase were positively stained for SLAP as well (yellow fibers, B). However, there were SLAP positive fibers that were negatively stained for the slow Ca^{2+} ATPase (green fibers, B). Images were obtained using a confocal laser microscope. Scale bar equals 10 μm .

A



B

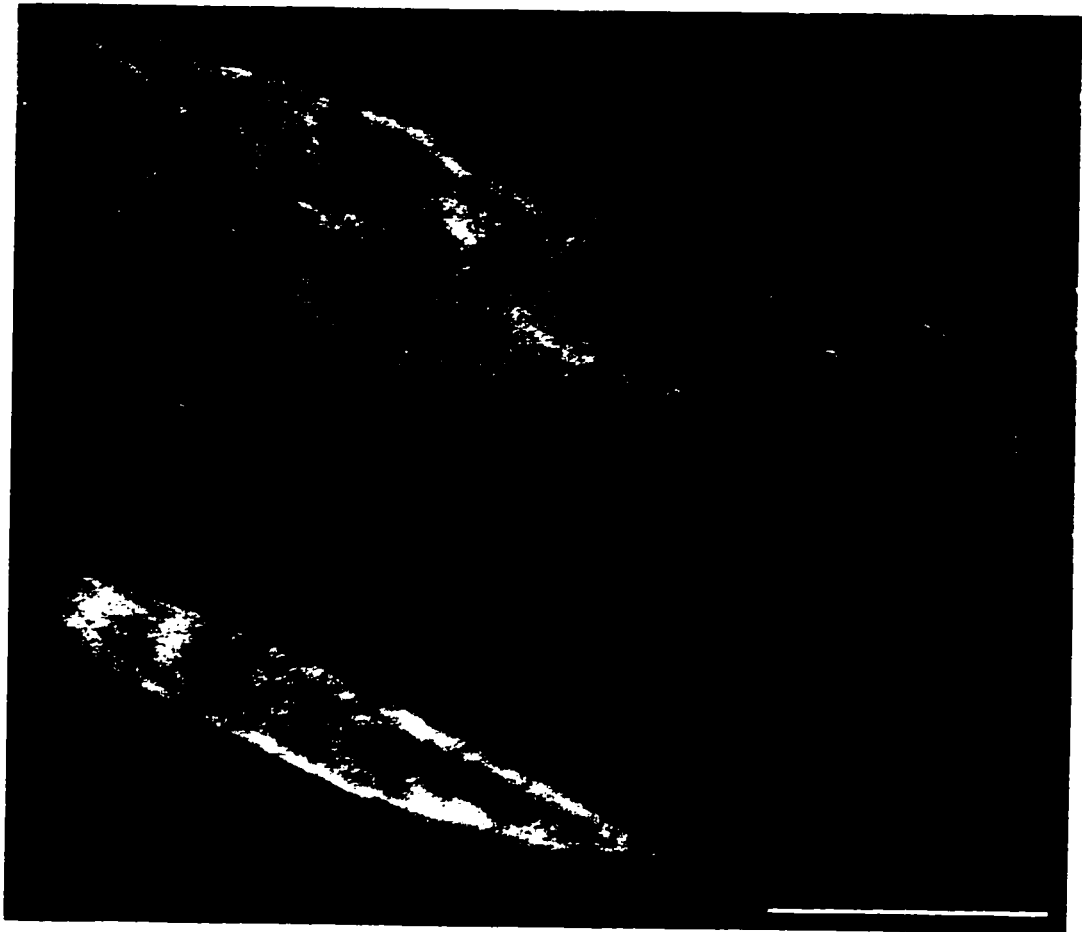


Figure 26. Alternative SLAP Transmembrane Domains.

A) Homology between SLAP and TOP_{AP}.

The GCG program BESTFIT was used to align the SLAP1 and TOP_{AP} proteins. Identical amino acids are shown by dashes and conserved residues are indicated by two dots. Gaps in alignment of the sequences are indicated by periods. The leucine residues and glutamate residues, in the area of the two leucine zippers, are conserved between the two proteins. By contrast, the degree of conservation is very low for the transmembrane domains. The area encompassing the two leucine zippers is designated by the shaded areas and the carboxyl terminal transmembrane domains are double underlined.

B) Chicken TOP_{AP} and mammalian SLAP are splice variants.

The alignment of the deduced TOP_{AP} amino acid sequence past the first stop codon (designated by an asterisk) with the SLAP1 amino acid sequence and the deduced amino acid sequence from an human Expressed Sequence Tag from melanocytes (Genbank accession number N27488). Note the homology between the three putative proteins in the SLAP transmembrane domain. Amino acids conserved in all three sequences are indicated in bold and gaps are indicated by periods.

A

1	MDEQDLNESLAKVSLK.....	17	SLAP1
1	MDDQDLNEPIAKVALLKDELQGAQSETEAKQEIQQQLHKELIEAQELARTS	50	TOP _{AP}
18	ALLEEERKAYRNQVEESSKQIQVLQAQLQRLHMDIENLREEK	59	SLAP1
51	KQKCFELQALLEEERRAYRVQVEESNKQINVLOAQLQRLQEDIENLREEK	100	TOP _{AP}
60	DNEITSTRDELLSARDEILLHQA AEKAASERDTDIASLQEELKKVRAEL	109	SLAP1
101	ESEISSTRNELVSAQNEILSLQQVAEKAASERDTDISALQEELQTVRAEL	150	TOP _{AP}
110	ERWRKAASEYEKEVTSLOSSFQLRCCQCEDQOKEEA <u>ATREQGELEKKEKKEW</u>	159	SLAP1
151	ERWRKDASDYEKEIVSLQASFQLRCCQCEEQHKEEA <u>ATREKGELEKKEKAEW</u>	200	TOP _{AP}
160	<u>NWGEHECHSEKKEWLESSE</u> ELQROQEKELHNSOKO <u>SEBETSDESLLQMK</u>	209	SLAP1
201	<u>SVGEAEQVPERKENISLASE</u> ELQROQEKELSSSOKO <u>SHALTSNTSVALEMSRK</u>	250	TOP _{AP}
210	<u>EEENOMGSEKKEQ</u> HLRDSADLKILLSKAENQAKDVQKEYEKTQTVLSELKL	259	SLAP1
251	<u>EEENOMGSEKKEQ</u> HQQDAASLKSQSEAESQAKDVQKEYERTQTVLSELKA	300	TOP _{AP}
260	KFEMTEQEKQSITDELKQCKDNLKLLQEKGN NKPWPWMPMLAALVA.VT	308	SLAP1
301	KYEMAEQEKQSITEELKQCKENLKLLQEKGN..PSILQVPVPA <u>AVFIGLIL</u>	348	TOP _{AP}
309	<u>AIVLYVPGLAR</u>	319	SLAP1
349	<u>AFLYWCYGPLW</u>	359	TOP _{AP}

B

SLAP1	FEMTEQEKQS	ITDELKQCKD	NLKLLQEKGN	N.....	291
EST	FEMTEQEKQS	ITDELKQCKN	NLKLLREKGN	NPSILQPVPA VFIGLFLAFL	50
TOP _{AP}	YEMAEQEKQS	ITEELKQCKE	NLKLLQEKGN	NPSILQPVPA VFIGLILAF	366
SLAP1		NKPWPWMPML	AALVAVTAIV	LYVPGLARASP	322
EST	FWCFGPLW*R	KKPWPWMPML	AALVAVTAIV	LYVPGLARASP	91
TOP _{AP}	YWCYGPLW*R	KRQWPWMPVM	AALVAVTAVV	LY.PGLTRASP	406

of the acidic leucine zipper region were conserved between the two proteins but the putative transmembrane domains were not conserved. This divergence of the transmembrane domains was perplexing since transmembrane segments are usually well conserved between homologous proteins even across species. However, analysis of the published sequence indicated that translation of the TOP_{AP} nucleotide sequence past the first inframe stop revealed a non-translated transmembrane domain which was very well conserved with that of SLAP (Fig. 26B). A human EST which was homologous to SLAP has been sequenced recently and this EST like TOP_{AP} contained two potential transmembrane domains, one similar to TOP_{AP} and one that was 100% identical with the SLAP transmembrane segment.

SLAP Expression is Developmentally Regulated in the Rat Retina

TOP_{AP} had been shown to be expressed in a topographic manner in the developing chick retina and tectum (the brain region which retinal ganglion cells innervate). TOP_{AP} was reported to be expressed in a manner which was temporally and spatially consistent with a putative role in axonal pathfinding of the retinal ganglion cells (Savitt et al., 1995). In view of the similarity between TOP_{AP} and SLAP, we wanted to determine if SLAP was expressed in the developing mammalian visual system in a manner that would resemble the expression pattern of TOP_{AP} in the chick. Prerequisites for a retinal ganglion cell pathfinding marker would be early expression in either the retinal ganglion cells or in their terminal field, the optic tectum. SLAP protein expression in the developing rat visual system was localized *in situ* by immunostaining cryosections of developing rat retinae and optic tecta with anti-SLAP antiserum. The retinae from fetal (E18, E19), neonatal (days P1, P2, P3, P10 and P14) and adult rats were examined immunohistochemically with anti-SLAP

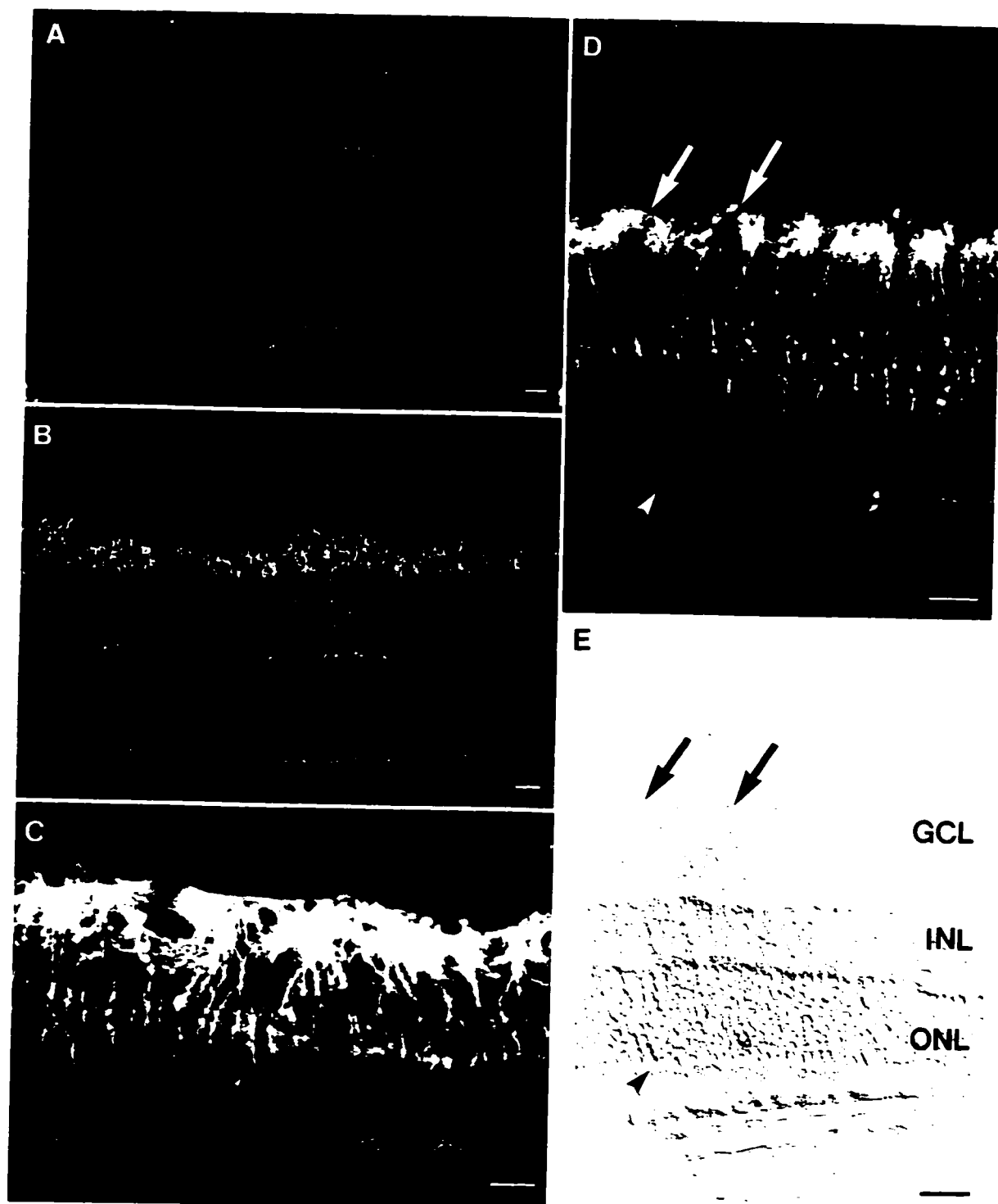
antibodies. SLAP was not expressed in the rat retina at embryonic or early postnatal timepoints of development (Fig. 27A). Retinal expression of SLAP was first seen immunohistochemically at postnatal day 10 and essentially reached adult levels of expression by postnatal day 14 (Fig. 27B, 27C and 27D). Although, in the avian retina, TOP_{AP} had been reported to be expressed in both glia and neurons (Savitt et al., 1995), SLAP expression in the rodent retina appeared to be confined solely to the Mueller glia cells (Figs. 27D and 27E). SLAP staining was uniformly distributed in Mueller cell processes which extended from the outer limiting membrane to the inner limiting membrane of the retina. The cell bodies of the retinal ganglion cells (RGCs) appeared to be negative for SLAP staining. A gradient of SLAP staining in the developing rat retina was not observed at any of the timepoints examined in this study. As well, it had been reported that TOP_{AP} was expressed in the optic tectum region of the avian brain in a topographically graded fashion (Savitt et al., 1995). To determine if there was a gradient of SLAP expression in the mammalian superior colliculus or the lateral geniculate nuclei (regions where RGCs project axons to), brain sections from adult and neonatal rats were examined using immunofluorescence techniques. Significant SLAP staining was not seen in the superior colliculus nucleus, lateral geniculate nucleus or the optic nerve of rats at any age of development examined.

SLAP is Expressed in a Population of Neonatal Migratory Neurons

Since a SLAP transcript was found in the developing and adult rat brains, we further examined SLAP expression in the developing rat CNS by immunohistochemical

Figure 27. SLAP Expression in Developing Rat Retina.

SLAP staining was not detectable in retina from postnatal day 3 rat pups (A) or from retinæ from earlier developmental timepoints. By postnatal day 10 distinct SLAP staining was clearly seen in the developing retina (B). SLAP staining was highly expressed in the adult rat retina (C). SLAP staining was localized to Mueller cells particularly within their tufted endings in the ganglion cell layer, in fibers that traversed the retina and in puncta seen at the outer limiting membrane (shown by arrowhead) (D). The cell bodies of the retinal ganglion cells (denoted by arrows) were negative for SLAP staining. Panel E shows a Nomarski image of the fluorescence image in panel D, which clearly shows the cytoarchitecture of the retina. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bar equals 25 μm .



techniques. During fetal and early neonatal development SLAP was found in a band of cells along the ventral border of the developing hippocampus (Fig. 28A) running from the subventricular zone of the hippocampus through the fornix and along the ventral boundary of the dentate gyrus. Confocal imaging of SLAP in the hippocampal neuroepithelium shows that it is present within cell soma and processes (Fig. 28B). Occasionally, individual SLAP positive cells could be seen within the dentate granule cell layer (Figs. 28C and 28D). The neuronal identity of SLAP positive cells was assayed by double immunolabelling with anti-NeuN (a neuronal specific nuclear protein) monoclonal antibodies and anti-SLAP antibodies (Mullen et al., 1992). The large majority of SLAP positive cells were negative for NeuN immunoreactivity but some were seen that stained for both NeuN and SLAP (Figs. 28E and 28F). It should be noted that SLAP expression was not detected in the adult rat hippocampus.

SLAP expressing cells were also seen in the olfactory bulbs (Fig. 29A). These cells were shown to be in the aneural olfactory nerve layer by double immunostaining for SLAP and the neuronal specific marker NeuN (Fig. 30). Cells were not found that stained both for SLAP and NeuN in the olfactory bulb. These cells displayed intense anti-SLAP immunoreactivity which was localized at the plasma membrane (Figs. 29B and 29C-F). These cells were seen in neonates as well as adult brains, but were much more numerous and more intensely immunoreactive at the earlier stages of development. The restriction of SLAP staining to the plasma membrane was more evident in the olfactory bulb cells than in the hippocampal neurons. SLAP positive cells were not seen in other layers of the olfactory bulb. We also carefully examined more caudal levels back to the anterior pole

Figure 28. SLAP Staining in the Developing Hippocampus.

SLAP immunofluorescence was carried out in brain sections on postnatal day 1 (A) and postnatal day 2 (B-F) rat pups. Panel A shows a photomontage of SLAP expression in cells in the hippocampal formation apparently migrating from the hippocampal neuroepithelium (hi) at the left of the field, across the fimbria (fi) and continuing into the ventral blade of the dentate gyrus (DG). Panel B is a confocal laser micrograph (1 μ m optical slice) of SLAP containing cells within the hippocampal neuroepithelium showing its distribution and presence in processes. SLAP is found in a small number of cells within the granule cell layer of the dentate gyrus (C), identified by NeuN immunoreactivity in the same section (D). A few cells staining for SLAP (E) could be identified as neuronal based on double labelling for NeuN immunoreactivity (F). Neurons are rare in this field because it is taken from the white matter of the fimbria, across which this neuron appears to be migrating. Scale bar equals 25 μ m.

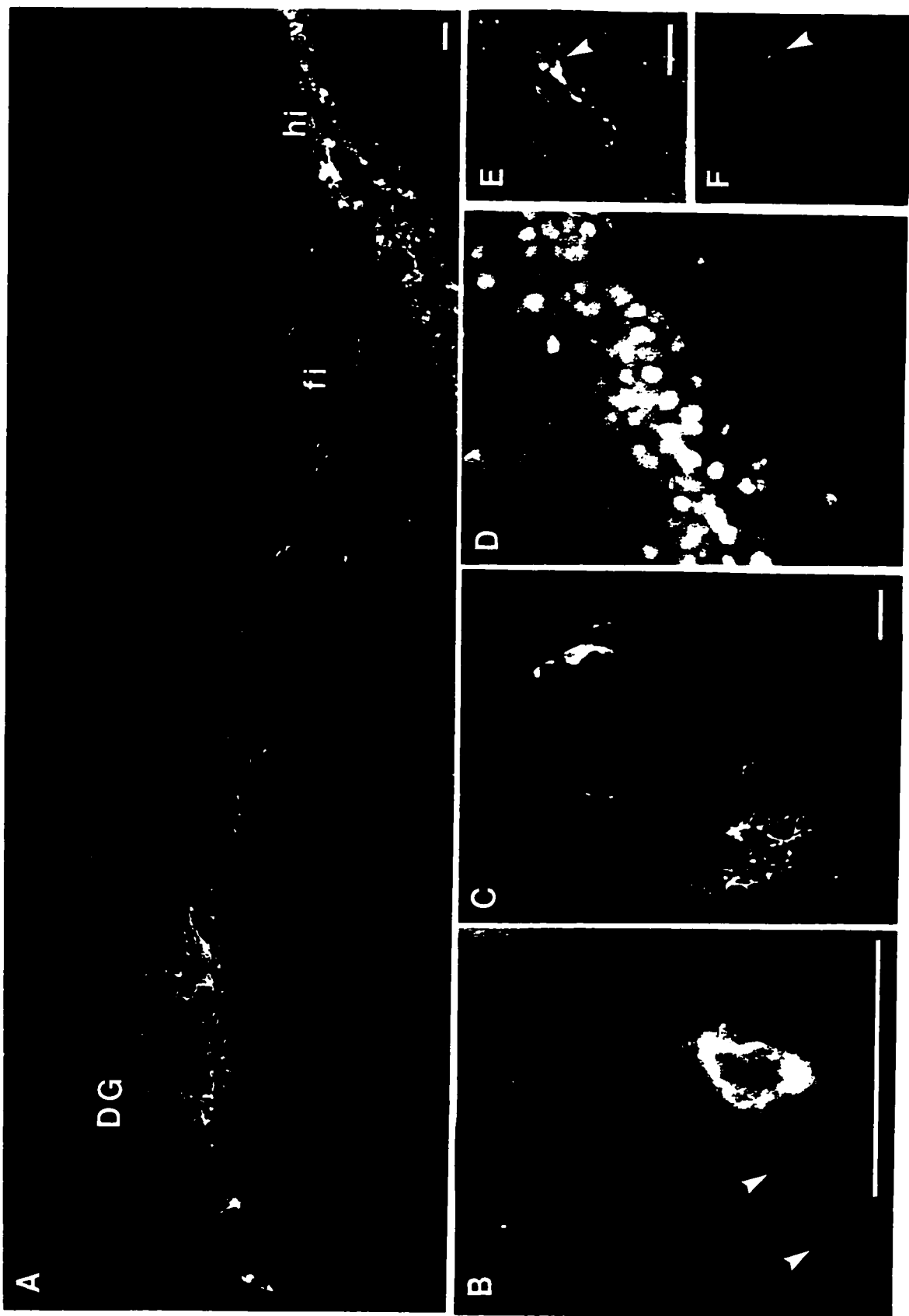


Figure 29. SLAP Expression in the Olfactory Bulb.

SLAP is expressed in cells in the olfactory nerve layer of the olfactory bulb (OB) but not in the frontal cortex (Fctx) (A). A confocal laser micrograph (1 μm optical slice) showing SLAP localization at the plasma membrane in the olfactory cells in this layer (B). Panels C-E, show a focus series illustrating the cell membrane localization of SLAP staining in the olfactory bulb cells with a single neuron highlighted by the arrowhead. Scale bar equals 25 μm .

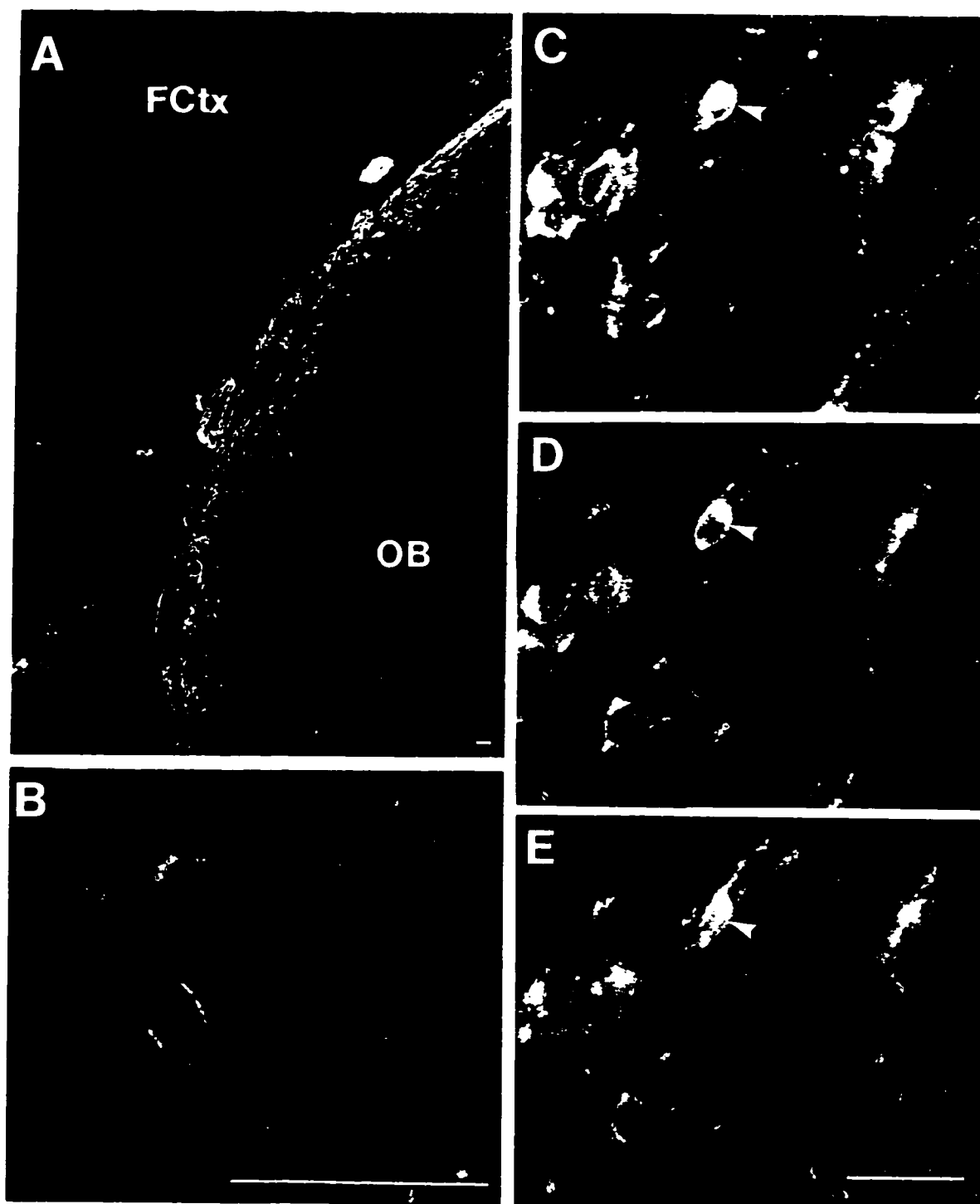
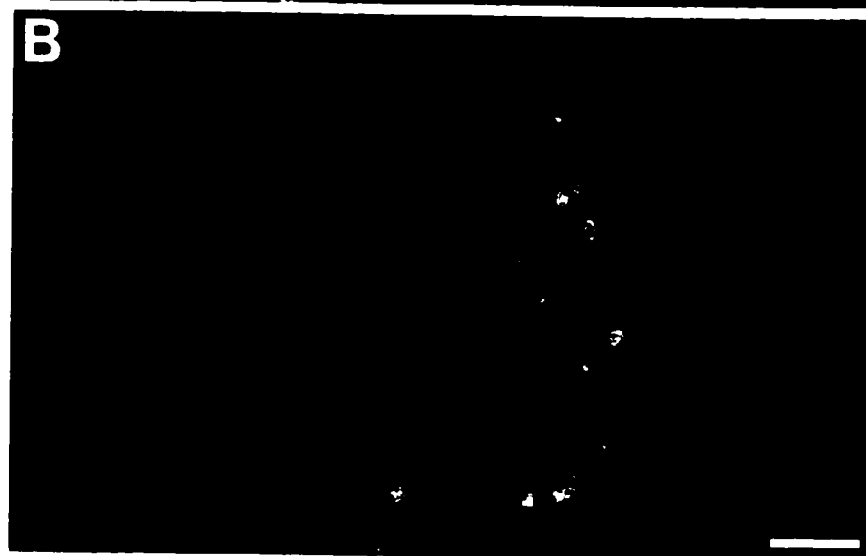
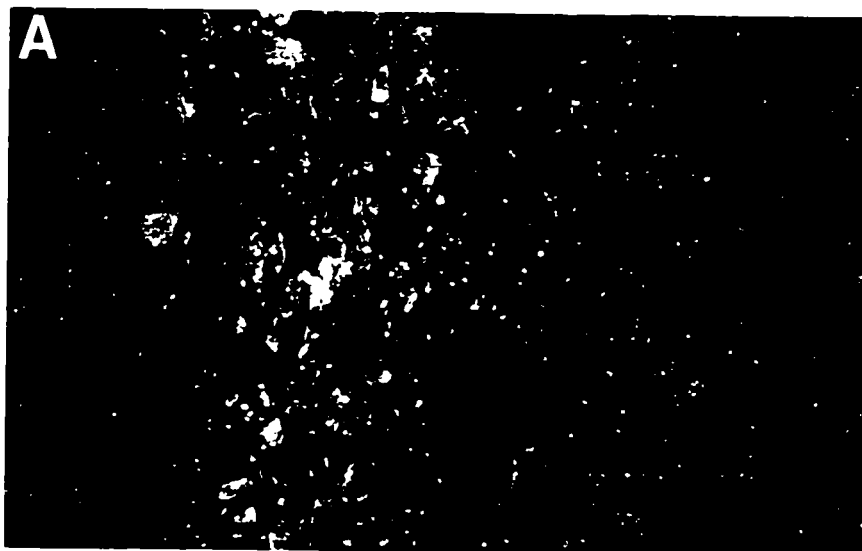


Figure 30. Olfactory Bulb Cells Stained for SLAP and a Neural Marker.

Cryosections of postnatal day 3 olfactory bulb were simultaneously stained for SLAP (A) and the neuronal specific nuclear marker NeuN (B) as described in material and methods. The SLAP positive cells were found in a layer of the olfactory bulb that was devoid of staining for NeuN. The scale bar represents 25 μm .



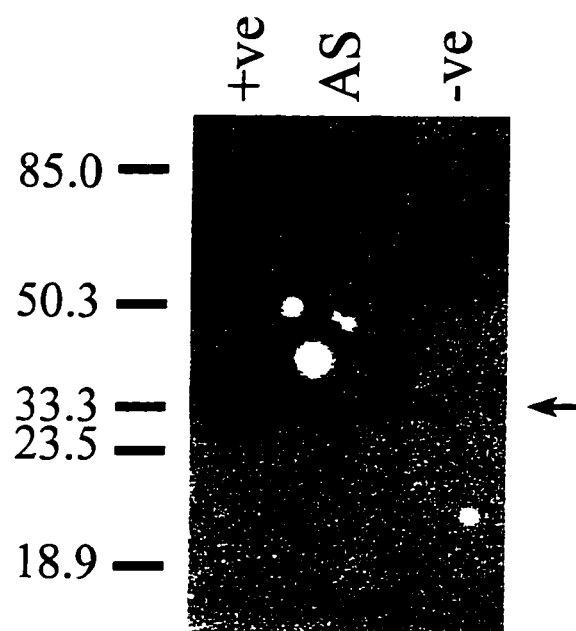
of the lateral ventricle and no SLAP positive cells were seen in the subventricular zone or any other site. SLAP positive cells were not seen in the developing cerebellum, another area of postnatal neuronal migration. In the adult rat, SLAP staining was found to be restricted to the choroid plexus, olfactory bulbs and the pineal gland (data not shown).

SLAP Targeting and Membrane fusion

In order to further study the localization and potential function of SLAP, the properties of ectopically expressed SLAP in embryonic pluripotent stem cells were investigated. The P19 embryonal carcinoma cells can be differentiated into neuroectoderm with retinoic acid (RA) treatment or along the mesoderm pathway following exposure to dimethyl sulphoxide (DMSO) (Rudnicki and McBurney, 1987). Furthermore, SLAP appeared to be expressed in early embryonic tissue and P19 cells differentiate into embryonic muscle, nerve and glia. An expression plasmid carrying the coding region of SLAP1 was transfected into P19 cells and 24 stable transfectants were isolated. Western blot analysis of the highly expressing clone clearly showed that a protein of 35 kDa reactive to the affinity purified guinea pig anti-SLAP antiserum was produced in cells transfected with the SLAP1 cDNA (Fig. 31). Although the biochemical association of SLAP with the cardiac plasma membrane had been demonstrated, the topology of SLAP, extracellular versus intracellular, had not been established. Live SLAP1 expressing P19 cells were first treated with trypsin protease to digest extracellular proteins and then the mixture was fractionated into cell pellets and supernatant. Western blot analysis of these samples for SLAP showed that there was no difference in SLAP1

Figure 31. Ectopic Expression of SLAP1 Produces a 35 kDa protein.

Crude membranes of SLAP1 sense expressing clone (+ve), SLAP1 antisense expressing clone (AS) and a non-expressing (as determined by northern blot analysis) clone (-ve) were subjected to western blot analysis using rabbit anti-SLAP antiserum as described in material and methods. A polypeptide reactive to the anti-SLAP antiserum of predicted molecular weight of 35 kDa (indicated by the arrow) was seen in the sense clone but was not seen in either the anti-sense or negative control clones. The mobility of the pre-stained protein molecular weight standards are indicated on the left with their molecular weights in kDa.



content between cells or supernatant treated with either inactivated trypsin (trypsin inhibitor. heat inactivated) or active trypsin (Fig. 32).

To further investigate the localization and membrane targeting of SLAP, P19 embryonal carcinoma cells were transfected with a SLAP expression construct in which the SLAP3 protein was tagged at amino acid 184 with the 6MYC epitope. Stable clones were isolated and their SLAP expression was assayed by western blots. The subcellular distribution of ectopically expressed SLAP in these cells was examined immunohistochemically with the monoclonal antibody to the human MYC epitope. The majority of P19 cells were flat and in these cells a clear immunofluorescent signal was seen at the membrane with the anti-MYC monoclonal antibody (Fig. 33B) indicative of SLAP being targeted to the plasma membrane of the transfected P19 cells. Interestingly, the expression of SLAP was much reduced or absent in the regions of the cell membrane that was devoid of contact with neighbouring cells. An immunohistochemical signal was not detected with anti-MYC monoclonal antibody in either the parental P19 cells or empty vector transfected P19 cells (Fig. 33D). To determine whether the SLAP staining between adjacent cells reflects the density of SLAP protein or an increased height of the cell at the point of cell-cell contact, confocal microscopy was used which takes an optical section of a fixed depth (Fig. 34A). Confocal microscopy confirmed that SLAP was concentrated at the point of cell-cell contact. Further analysis using confocal imaging revealed that two different populations of transfected P19 cells could be discriminated on the basis of their SLAP localization. In one population of cells that appeared flat, SLAP was concentrated exclusively at the plasma membranes that were juxtaposed, while in a

Figure 32. Trypsin Digestion of SLAP1 Expressing Cells.

SLAP1 expressing P19 cells were treated for 5 minutes at 37°C with heat killed trypsin (lane 1), active trypsin (lane 2), active trypsin + trypsin inhibitor (lane 4) or 10 minutes at 37°C with active trypsin (lane 3). The cell pellets (A) and the supernatant (B) fractions were isolated. The samples were resolved by 10% SDS-PAGE and subjected to western blot analysis using rabbit anti-SLAP antiserum as described in material and methods. The amount of SLAP1 protein in the cell pellets did not vary with any of the trypsin treatments. The mobility of the SLAP1 protein is indicated by an arrow and that of the protein molecular weight standards are indicated on the left with their molecular weights in kDa.

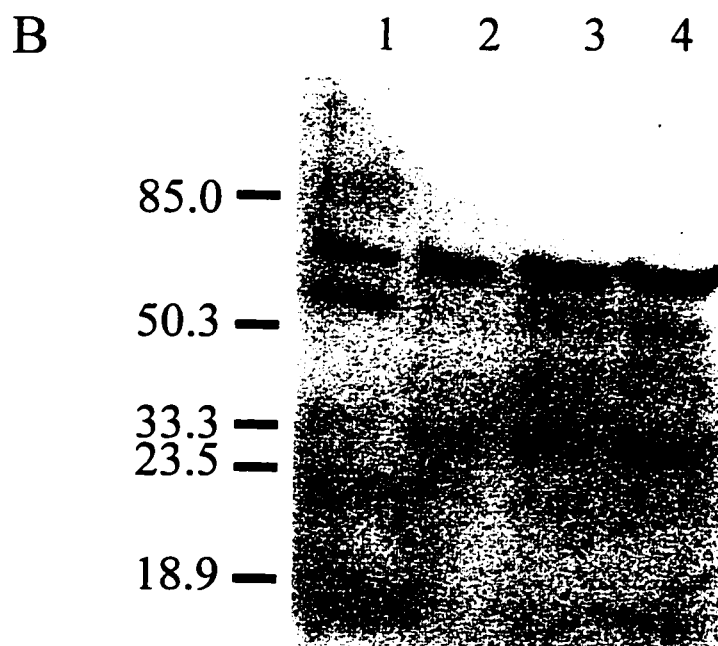
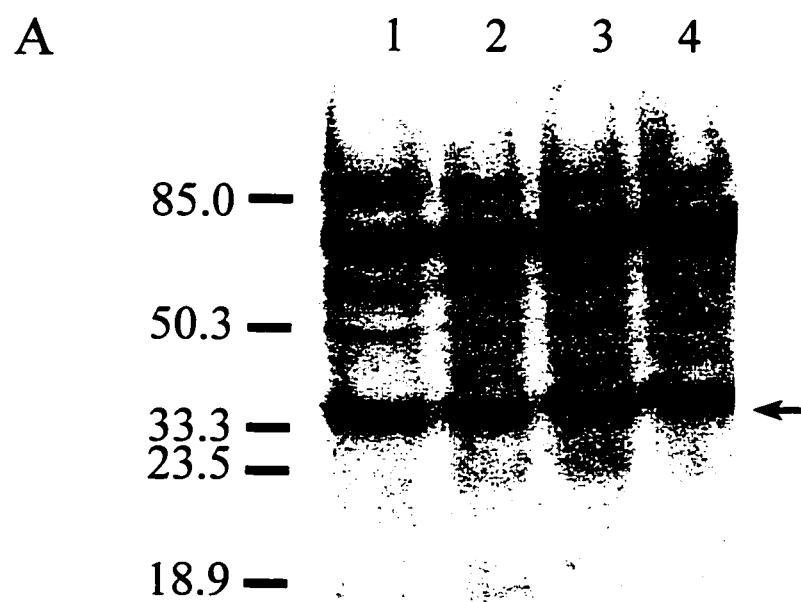


Figure 33. Subcellular Localization of Recombinant Epitope Tagged SLAP.

P19 cells were isolated that expressed the 6MYC epitope tagged SLAP (A, B) or the empty pcDNA3 expression vector (C, D). The cells were grown on glass coverslips, fixed and immunostained with the anti-MYC monoclonal antibody as described in material and methods. Panels A and C are Nomarski images of the fluorescence images in panels B and D respectively. Note the concentration of MYC staining in areas where cell membranes were juxtaposed, in contrast to the reduced staining in areas where the cell membranes were not abutted to another cell (B). No specific MYC staining was seen in the empty vector control (D). Scale bar equals 25 μm .

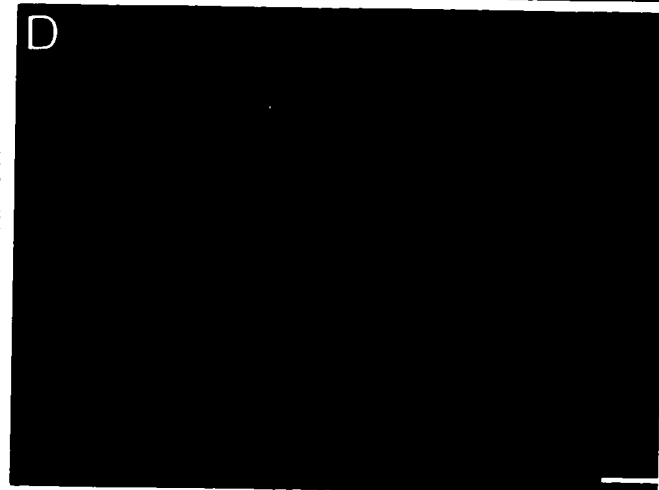
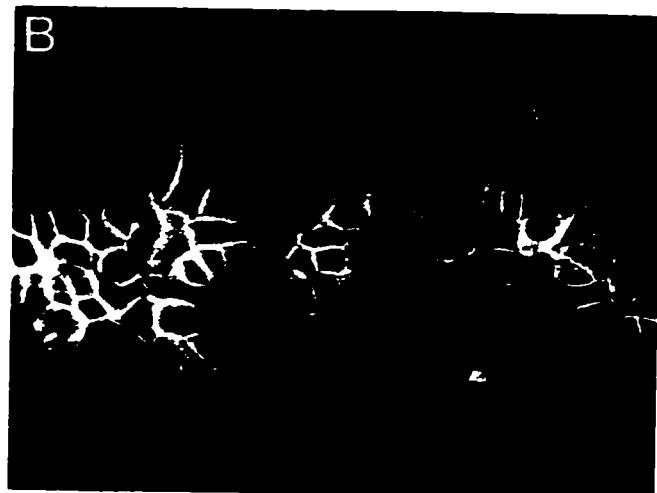
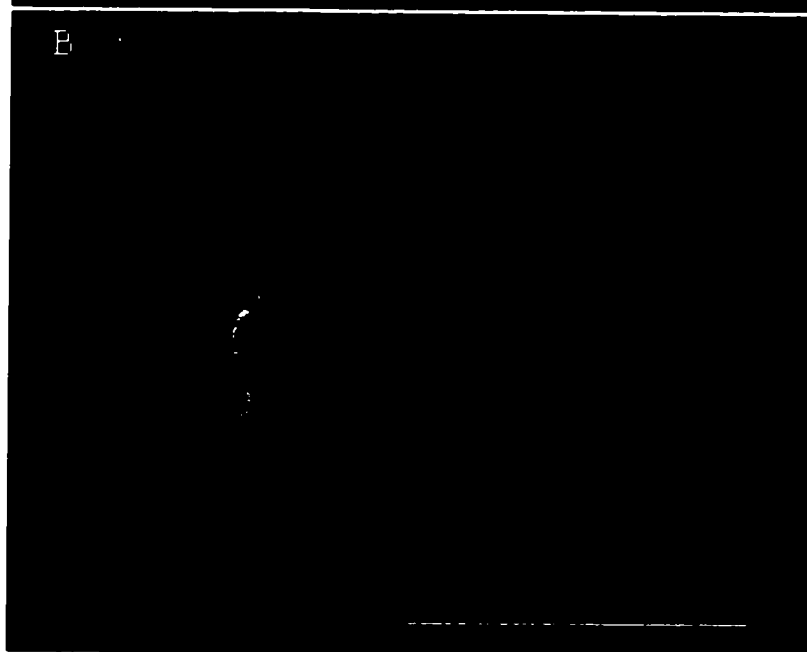


Figure 34. Subcellular Localization of SLAP Examined by Confocal Laser Microscopy.

Undifferentiated 6MYC-SLAP expressing P19 cells were cultured on coverslips and immunostained for MYC. When the immunostaining was examined using confocal laser microscopy, two populations of MYC positive cells were identified. Monolayers of P19 cells were observed where MYC staining was found to be concentrated at the interface between two adjacent plasma membranes (A). However, in P19 cells that were rounded and bipolar, SLAP appeared to be distributed uniformly in the plasma membrane and a subadjacent intracellular compartment. These images represent 1 μm optical sections from a confocal laser microscope. The scale bar equals 25 μm .



second smaller population of cells which had a rounded bipolar cell shape. SLAP was uniformly distributed in the membrane as well as an intracellular compartment (Fig. 34B). The rounded bipolar cell shape is characteristic of cells undergoing cleavage during mitosis. The chromosomes of cells undergoing mitosis are more condensed than those of cells at other stages of the cell cycle. In order to determine at what stage of the cell cycle the bipolar cells were at, transfected P19 cells were simultaneously stained for the MYC epitope and for DNA. In bipolar cells that were stained for SLAP, the chromosomes were condensed as well (Fig. 35). To further establish the topology of SLAP, immunofluorescence studies on live 6MYC-SLAP transfected P19 cells with either the rabbit anti-SLAP antiserum or the anti-MYC monoclonal antibody were performed in the presence or absence of detergent to permeabilize the cell membrane. If SLAP was a cell surface protein it could be stained in the absence of detergent, however, if it was oriented towards the cytoplasmic phase then it would only be stained when the cells were permeabilized with detergent. A clear immunofluorescence signal for both primary antibodies was seen only when the cells were permeabilized (Fig. 36).

The ability of the SLAP overexpressing P19 cells to differentiate along the mesodermal pathway following DMSO exposure was assayed both functionally, by the appearance of beating foci characteristic of muscle cells, and biochemically, by the induction of the expression of a cardiac specific form of actin. SLAP expression did not affect the appearance of beating foci of cells or the expression of the cardiac specific form of actin induced by DMSO (Fig. 37). Since the smaller (3.5 and 4.6 kb) SLAP transcripts were expressed highly in various developing and adult muscles, RNA from

Figure 35. DNA Condensation in Bipolar SLAP Positive Cells.

Undifferentiated 6MYC-SLAP expressing P19 cells were cultured on coverslips and immunostained for MYC as described in material and methods. The cells were then stained with Hoechst 33258 to label their DNA. The rounded P19 cells (indicated by arrowheads, A) were SLAP positive and had a similar morphology as those observed under confocal laser microscopy. Also, the chromosomes of the rounded, SLAP positive cells were condensed, which indicated the cells were undergoing mitosis (B). A phase contrast photograph of the cells clearly shows their morphology (C). Scale bar equals 25 μ m.

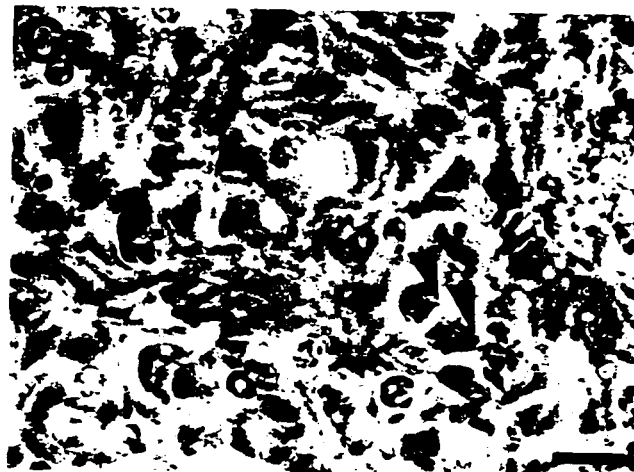
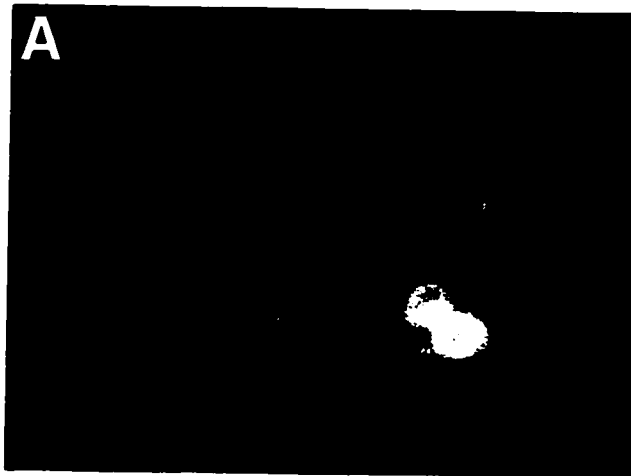


Figure 36. Staining For SLAP in Permeabilized and Unpermeabilized Cells.

Undifferentiated 6MYC-SLAP expressing P19 cells were cultured on coverslips and immunostained for SLAP (A) or MYC (B, C). Cells were either fixed, and primary antibodies were added in 0.3% TritonX-100 to permeabilize the membrane (A, B) or the primary antibodies were added directly to the culture medium after which the cells were fixed and permeabilized (C). SLAP and MYC staining was observed to be localized to the membrane in permeabilized cells (A, B). However, specific staining was not seen when the cells were unpermeabilized during the incubation with primary antibodies (C).

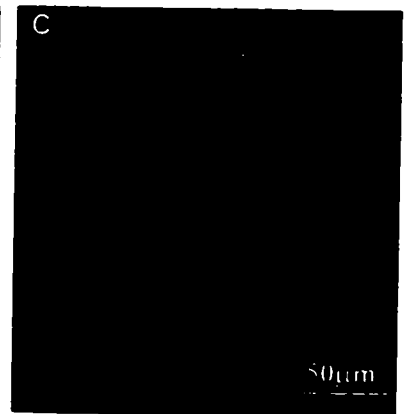
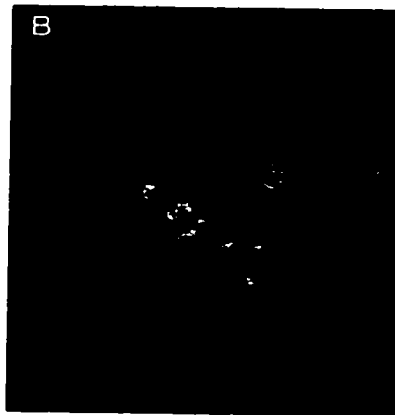
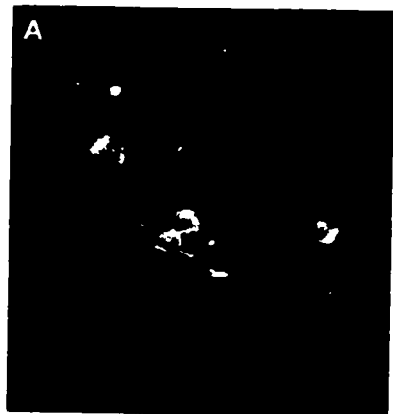
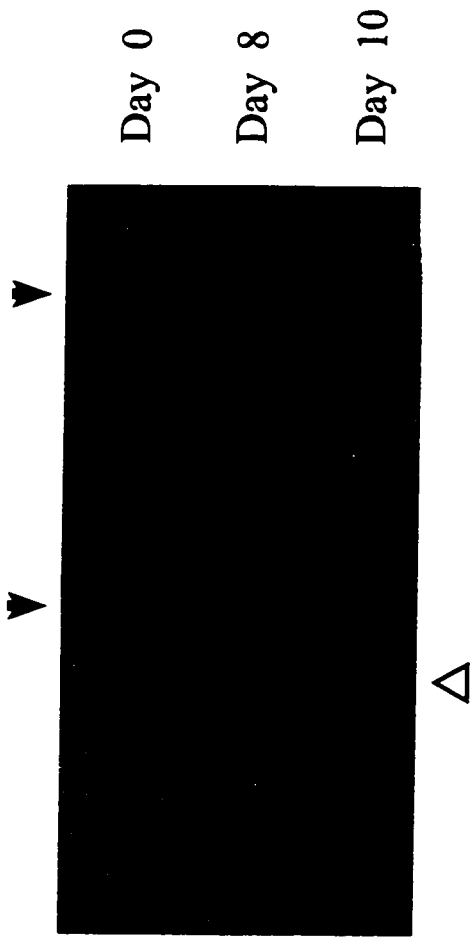


Figure 37. DMSO Induces Expression of Cardiac Actin in P19 cells.

Total RNA was isolated from SLAP expressing P19 cells that were undifferentiated (day 0) or differentiated with DMSO for various times (day 8 and day 10). The isolated RNA was subjected to northern blot analysis using a cDNA probe for cardiac actin. Two transcripts were seen in the undifferentiated cells but upon induction with DMSO a third transcript representing the cardiac specific form of actin was seen (denoted by open arrowhead). The arrows represent the mobility of the 28s and 18s rRNAs.



DMSO differentiated P19 cells was subjected to Northern blot analysis. Although the 5.9 kb transcript was found in both the undifferentiated and differentiated P19 cells, the 3.5 and 4.6 kb transcripts were not detected in either the undifferentiated or differentiated cells (Fig. 38). The possibility that small groups of DMSO differentiated P19 cells could express SLAP was examined immunohistochemically. Control and 6-MYC SLAP transfected P19 cells were differentiated with either RA or DMSO. In empty vector transfected P19 control cells differentiated with DMSO, spindle shaped SLAP positive cells were seen (Fig. 39A). These spindle-like SLAP cells resembled the morphology of neurons produced when P19 cells are differentiated with RA (Fig. 39D). However, these SLAP positive cells did not express the neuronal specific β -tubulin III marker (Fig. 40). SLAP positive cells were not seen in control cells differentiated with RA or when the SLAP pre-immune serum was used to stain DMSO differentiated cells (Figs. 39C and 39E). 6MCY-SLAP transfected P19 cells were examined immunohistochemically for SLAP expression after differentiation with DMSO or RA by staining with the anti-MYC monoclonal antibody. In both DMSO and RA cells, the ectopically expressed SLAP did not appear to be localized solely at the membrane but was distributed in an intracellular membrane compartment as well (Figs. 39B and 39D). Specific immunohistochemical staining was not seen when DMSO differentiated control P19 cells were stained with the anti-MYC monoclonal antibody (Fig. 38F).

Another good *in vitro* model system to study muscle differentiation is the C2C12 myoblast cell line (Yaffe and Saxel, 1977). The C2C12 cells are mononucleated myoblast cells that will proliferate indefinitely when propagated at sub-confluent densities and in

Figure 38. SLAP Transcript Expression in Differentiated P19 Cells.

Total RNA was isolated from undifferentiated P19 cells (day 0) or P19 cells differentiated with DMSO for various times in two separate experiments using two different stocks of P19 cells (P19A and P19B). Total RNA was also isolated from rabbit cardiac (heart) and fast twitch (fast) muscles as positive controls. The RNA was subjected to northern blot analysis with a radiolabelled cDNA probe derived from the 1.1 kb EcoRI/XbaI fragment of the LD1 clone. The 3.5 kb SLAP transcript was not detected in the differentiated or undifferentiated P19 cells. The mobility of the 5.9 kb SLAP transcript is indicated by the open arrowhead and the mobility of the 3.5 kb cardiac transcript is indicated by the closed arrowhead.

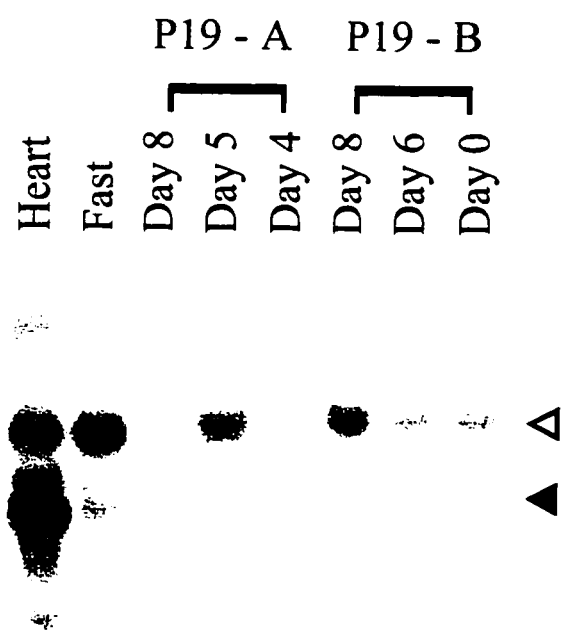
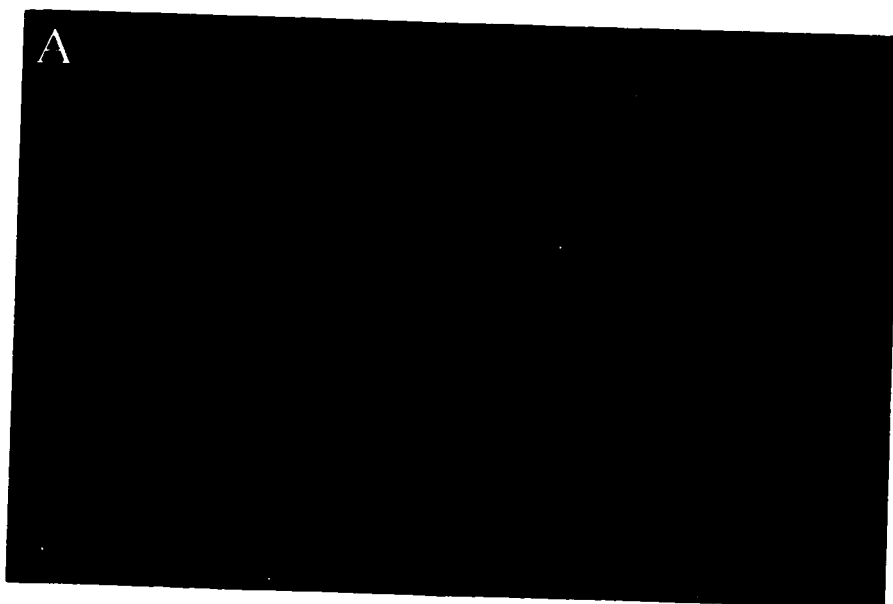


Figure 39. SLAP in Differentiated P19 Cell Cultures.

P19 cells were transfected with the empty pcDNA3 vector (A, C, E, F) or the vector encoding the 6MYC epitope tagged version of SLAP (B, D). These cells were either differentiated with DMSO (A, B, E, and F) or retinoic acid (C, D) for ten days. SLAP staining was seen in spindle-like cells in DMSO differentiated control cells (A). However, no SLAP staining was evident in the retinoic acid treated control cells (C) or when DMSO differentiated control cells were stained with the pre-immune serum (E). MYC staining was not solely localized to the plasma membrane in cells that were differentiated with DMSO (B) or RA (D). Specific staining was not seen when DMSO differentiated control cells were stained for MYC (F). Scale bar equals 25 μ m.

Figure 40. Double Immunofluorescence Analysis of Differentiated P19 cells.

P19 cells transfected with pcDNA3 expression vector were differentiated with DMSO for 10 days and were double immunohistochemically stained for SLAP and the neuronal specific marker β tubulin III as described in material and methods. Many spindle like SLAP positive cells were identified in the cultures (A) but these cells were not stained for β tubulin III (panel B). The scale bar equals 25 μ m.



medium containing 10-20% Fetal Bovine Serum (FBS). However, when the cells are cultured as confluent monolayers or in a medium supplemented with 10% horse serum, the myoblast cells will fuse to form multinucleated myotubes which express biochemical markers consistent with a differentiated skeletal muscle (Crescenzi et al., 1994). These cells were transfected with the 6MYC epitope tagged version of SLAP and four stable transfectants were isolated. Immunohistochemical staining of these clones with the anti-MYC monoclonal antibody or the rabbit anti-SLAP antiserum showed identical punctate staining for SLAP (Figs. 41A and 41B). A specific immunohistochemical signal was not seen when the parental C2C12 or empty vector transfected C2C12 were probed with the anti-MYC monoclonal antibody (Fig. 41D). When confocal image analysis was used to more closely examine the transfected cells, a clear reticular pattern of staining could be observed when either of the two antisera was used (Figs. 42A and 42B). When control C2C12 cells were induced to differentiate for 6 days the myoblasts were largely fused into large myotubes (Fig. 43A). Conversely, when SLAP overexpressing cells were induced to differentiate they did not form nearly as many myotubes (Figs. 43B). The fusion index is defined as the fraction of nuclei present in myotubes (cells with 3 or more nuclei) divided by the total number of nuclei in the field. Measurement of this fusion index allowed for the degree of myotube fusion between different clones to be analyzed. The fusion index of clone A was only 22% of the fusion index for the control cells (Fig. 44). The average fusion index for all four SLAP transfected clones was decreased by 60% when compared to control cells (Fig. 44). The SLAP cell lines were not fusing upon change to differentiation medium but perhaps they were still differentiating. A marker for

Figure 41. SLAP Localization in Myoblasts.

C2C12 myoblasts were transfected with a vector encoding the 6MYC epitope tagged version of SLAP (A, B) or the empty pcDNA3 vector (C, D). Cells were cultured on matrigel coated coverslips and stained for SLAP (A) or MYC (B, D). A similar pattern of staining was observed for both the SLAP and MYC staining for the 6MYC-SLAP transfected cells (A, B). Specific staining was not observed when the control cells were stained for MYC (D). Panel C is a phase contrast image of the cells in panel D. Scale bar equals 25 μm .

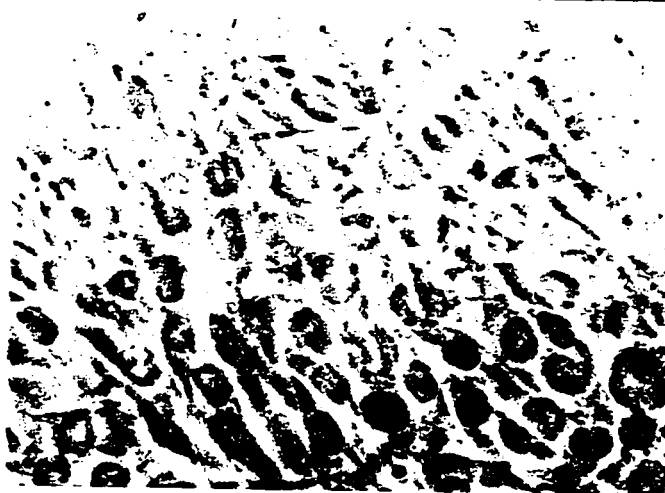
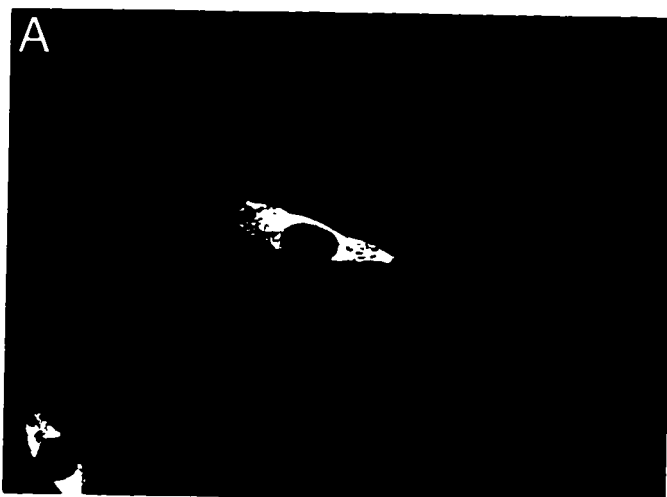
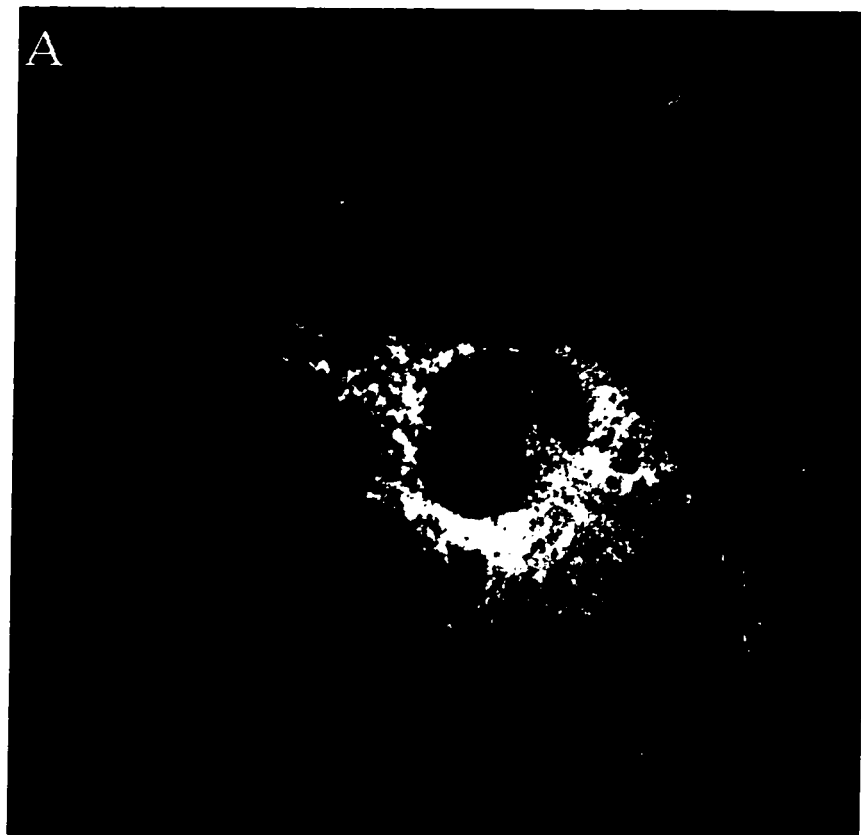


Figure 42. Localization of SLAP in C2C12 Cells.

C2C12 myoblasts were transfected with a vector encoding the 6MYC epitope tagged version of SLAP. These cells were cultured on matrigel coated coverslips and stained for MYC epitope (A) or SLAP (B). Both the SLAP and MYC staining was concentrated adjacent to the nucleus and in a clear reticular pattern near the periphery of the cell. These images represent 1 μm optical sections from a confocal laser microscope. The scale bar equals 25 μm .

A



B



Figure 43. Fusion of SLAP Transfected C2C12 Cells.

C2C12 cells transfected with a vector encoding the 6MYC epitope tagged version of SLAP (A) or the empty pcDNA3 vector (B). The clones were differentiated for 6 days in differentiation medium and then photographed. Many large multinucleated myotubes were seen in the control cultures but far fewer myotubes were seen in the SLAP expressing clones. Scale bar equals 50 μm .

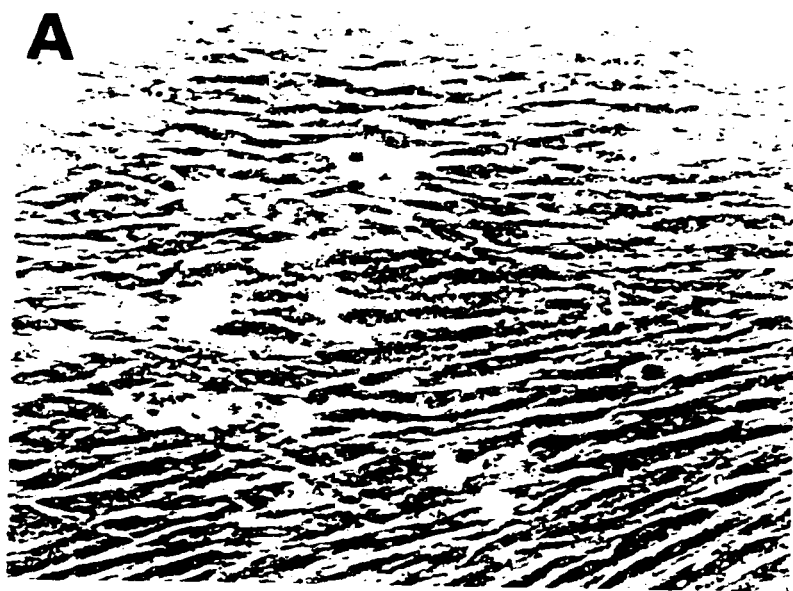
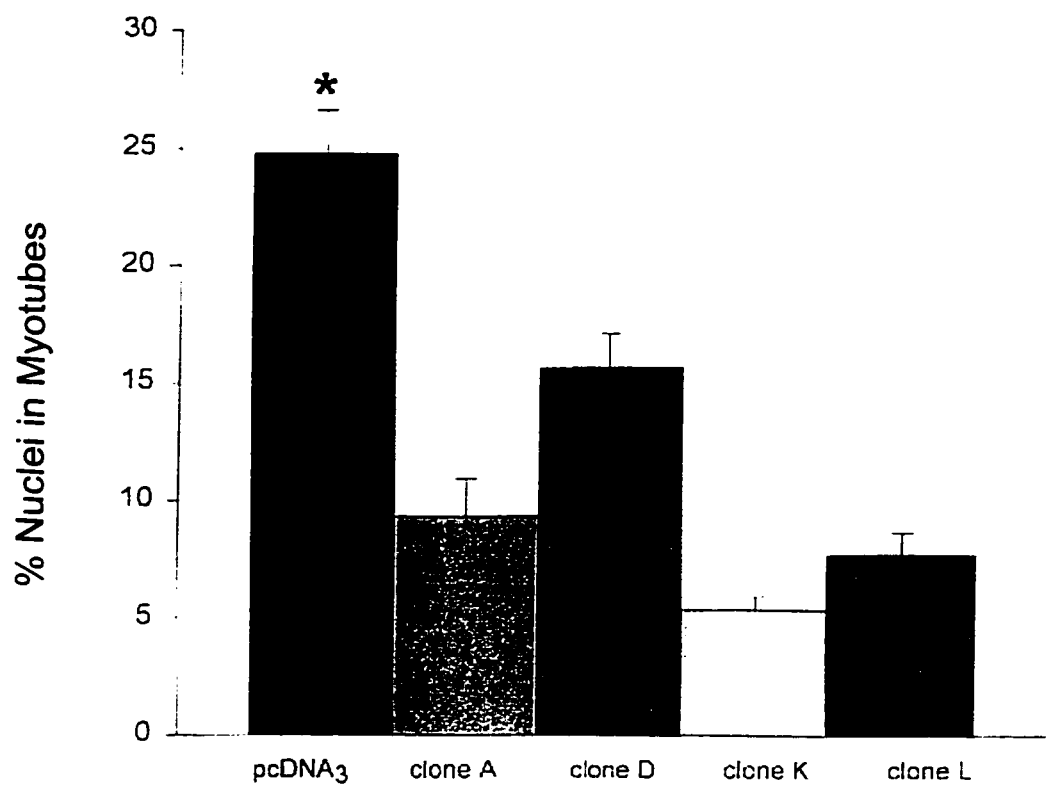


Figure 44. Fusion Index of SLAP Expressing Myoblasts.

C2C12 cultures were differentiated for 6 days in α -MEM supplemented with 10% horse serum. The cells were then fixed with 4% paraformaldehyde and stained with eosin and counterstained with Haematoxylin. Photographs were taken from at least 12 random fields in each culture. Total nuclei were counted and then nuclei in myotubes were counted for the same photograph. Myotubes were considered to be cells which contained three or more nuclei. The fusion index equals (the number of nuclei in myotubes/ total number of nuclei in the field)*100. The control pcDNA3 cultures were fused to a significantly (* $p < 0.005$, ANOVA) higher degree than were the SLAP expressing cultures.



muscle differentiation is the expression of the muscle transcription factor myogenin. Myogenin is not expressed in dividing myoblasts but is induced when the myoblasts differentiate into myocytes. Northern blot analysis showed that the induction of myogenin was similar between control and SLAP overexpressing cell lines (Fig. 45). Control C2C12 cells expressed low but detectable levels of the 5.9 kb SLAP transcript either when exposed to growth medium or differentiation medium for 6 days (Fig. 46). Interestingly, the levels of exogenously expressed SLAP transcript dramatically increased in C2C12 cells that were in differentiation medium compared to C2C12 cells in growth medium (Fig. 46).

Figure 45. Myogenin Expression in Differentiated C2C12 Cells.

Total RNA was isolated from differentiated (+) or undifferentiated (-) C2C12 clones. Clones D, K, and L were strong expressers, as assessed by western blot analysis, whereas clone J, was a weak expresser. The RNA was subjected to northern blot analysis using a cDNA probe specific to myogenin. After exposure to autoradiography the blot was stripped and re-probed with a GAPDH cDNA probe to control for loading variability (lower panel). The location of the myogenin transcript is indicated by the arrow on the left. The level of induction of myogenin expression upon differentiation was approximately the same for the various clones.

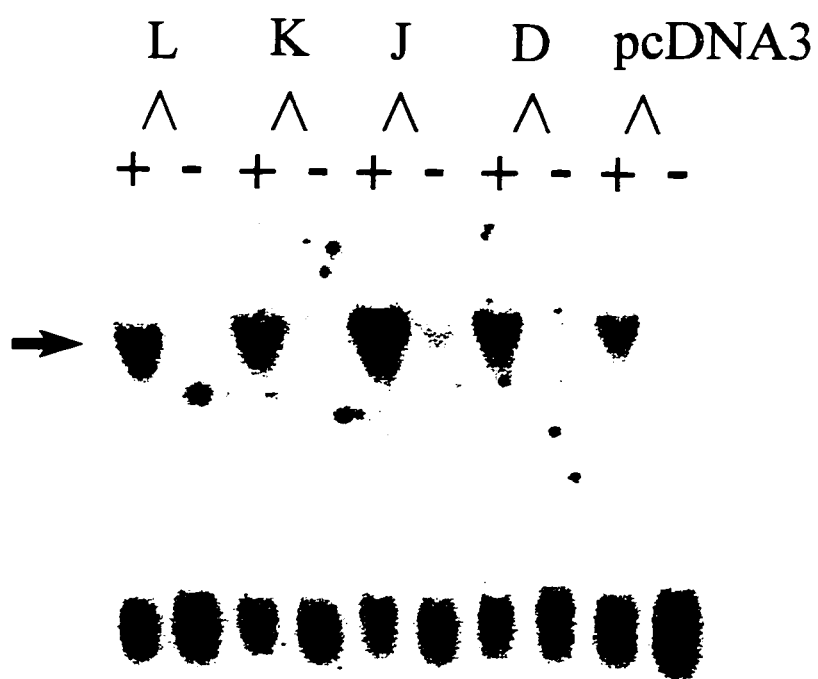
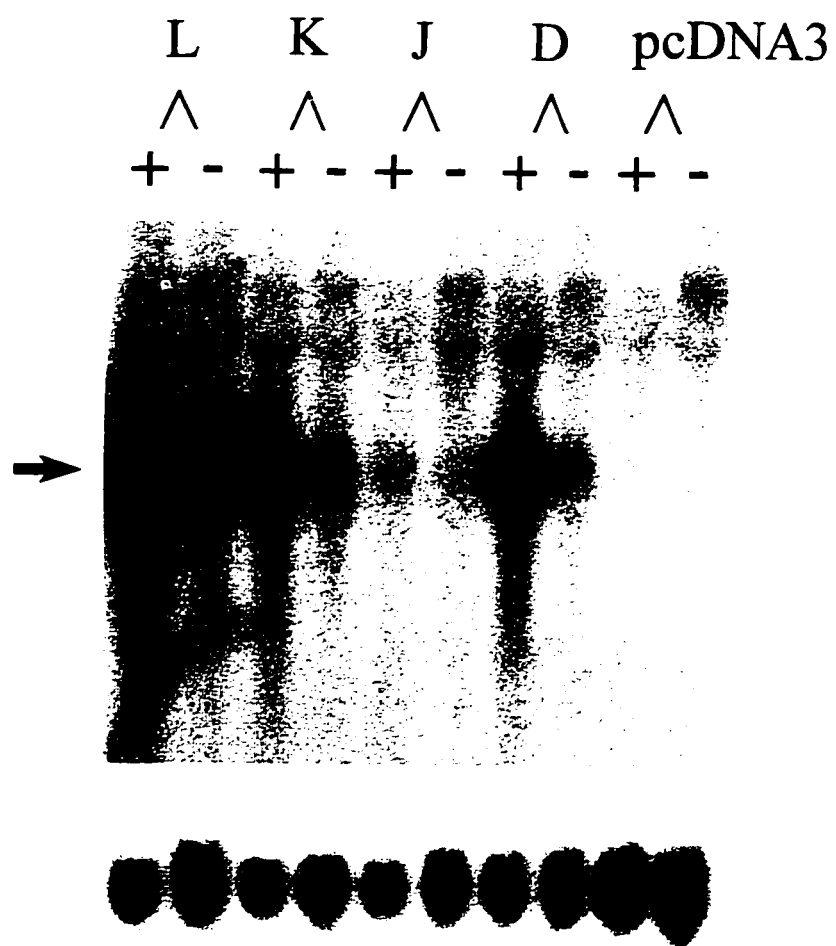


Figure 46. Expression of SLAP Transcripts in C2C12 Cells.

Total RNA was isolated from differentiated (+) or undifferentiated (-) C2C12 clones. The RNA was subjected to northern blot analysis using a cDNA probe specific for SLAP1 coding region. After exposure to autoradiography, the blot was stripped and re-probed with a GAPDH cDNA probe to control for loading variability (lower panel). The location of the exogenously expressed SLAP transcript is indicated by the arrow on the left. The level of the exogenously expressed SLAP transcripts was much higher after the clones were exposed to differentiation medium for 6 days.



DISCUSSION

Molecular components of the cardiac cell membrane play an instrumental role in determining cardiac cell function on a beat to beat basis. In particular, membrane proteins that regulate calcium levels in the cardiac cell appear to be critical for the precise control of the E-C coupling mechanism. In a previous study, two novel calcium binding polypeptides were identified in cardiac sarcolemma (Tuana et al., 1991) and two cDNAs that putatively encoded these polypeptides were isolated by immunoscreening of a cDNA expression library constructed from cardiac muscle (Demchyshyn, 1990). In this study, further analysis of these cDNAs resulted in the discovery of a novel gene that encodes a family of Sarcolemmal Associated Proteins designated SLAPs. Three full length cDNAs, SLAP1, SLAP2 and SLAP3, were isolated from myocardium that encoded coiled-coil tail-anchored membrane proteins. Biochemical and immunohistochemical studies indicated that the three SLAP polypeptides were integral membrane proteins. The expression of SLAP was tissue specific and developmentally regulated. Ectopic expression studies implied a potential role for SLAP in membrane fusion.

Two cDNAs encoding sarcolemmal associated protein (SLAP) were isolated by immunoscreening a rabbit heart cDNA expression library with antiserum raised to purified cardiac sarcolemmal proteins (Demchyshyn, 1990). These two SLAP cDNAs were polyadenylated at sites that were separated by 500 bp. Both polyadenylation sites were within 30 bp of potential consensus sites for polyadenylation. The results from the cDNA cloning experiments suggested that the

SLAP transcripts were alternatively polyadenylated. Three different SLAP transcripts (5.9 kb, 4.6 kb and 3.5 kb) were seen by Northern blot analysis in the heart. Significant levels of these three transcripts were expressed in all muscle types examined except for fast twitch muscle which expressed only the 5.9 kb transcript. The testes were the only non-muscle tissue to express significant amounts of the 3.5 kb transcript. The most abundant transcript in heart, as well as other muscle types such as smooth and soleus muscle, was the smallest 3.5 kb transcript. In the neonatal rat heart a large 12 kb transcript was also detected. Whether this larger SLAP transcript is an incompletely processed nuclear transcript or a novel SLAP transcript, has not yet been resolved. The larger 5.9 kb transcript was expressed, at varying levels, in all tissues examined including the brain. The smaller (3.5 and 4.6 kb transcripts) were expressed in a tissue specific manner whereas, the 5.9 transcript was expressed ubiquitously. Northern blot analysis with cDNA probes from different locations in the SLAP3 sequence demonstrated that the 3.5 and the 4.6 kb transcripts did not possess the 5' coding sequence of SLAP3. The molecular mechanism that generated this diversity of SLAP transcripts observed in cardiac RNA was investigated. Southern blot analysis of rabbit genomic DNA showed that SLAP hybridized in a simple pattern to genomic DNA restriction fragments. This finding was consistent with the idea of a single gene encoding the multiple transcripts rather than individual genes encoding each transcript. FISH analysis of the human SLAP orthologue also indicated the presence of a single gene for SLAP that localized to the p14.3-21.2 region of chromosome 3. These results suggest that

the different SLAP transcripts likely arise from either alternative promoter usage or by alternative mRNA splicing of a common precursor.

Three different potential 5' termini of SLAP were identified in heart RNA. The resulting cDNAs encoded putative polypeptides, which would have common carboxyl termini but unique amino termini. These putative polypeptides would have predicted molecular weights of 37 kDa (SLAP1), 46 kDa (SLAP2) and 74 kDa (SLAP3). The predicted molecular weights for the SLAP proteins encoded by the various cDNAs were similar to, but not identical with, the apparent molecular weights of 81, 45 and 35 kDa for the SLAPs in heart sarcolemmal fractions. For SLAP3 the identity of the translation start site was originally unclear since there were two inframe methionine codons. Experimental analysis of initiating methionine codons has revealed that the guanine nucleotide at the +4 position and the purine (adenine or guanine) nucleotide at the -3 position is required for effective translation of a cDNA sequence (Klein et al., 1985). The methionine codon at position 362 lacked these features whereas the methionine codon at position 758 had both required nucleotides. To determine which ATG could be utilized effectively, *in vitro* transcription/translation assays were performed using constructs with either both potential codons or only the second consensus codon. When the construct, which contained only the second consensus start site, was used in this assay, a polypeptide was efficiently produced that migrated with an apparent molecular weight of 85 kDa in SDS-PAGE. This predicted molecular weight was in good agreement with the predicted size of 81 kDa for the largest SLAP protein seen in

cardiac fractions. When the construct containing the first non-kozak site was used as a template in this assay a polypeptide of a deduced molecular weight of 95 kDa in SDS-PAGE was produced but much less effectively. Therefore, the second inframe methionine was considered to be the putative initiating methionine for SLAP3 because it was in good agreement with the Kozak start site and could be used efficiently as a translational start site in *in vitro* assays. Finally, the putative methionines for the other two SLAP isoforms were in good agreement with the Kozak consensus translation start sites.

The salient structural tendency predicted for SLAPs was an ability to form coiled-coils over most of their length which would allow for many potential protein-protein interactions. SLAPs have two predicted leucine zippers which together probably form an extended 11 heptad (77 amino acid) coiled-coil structure since the linker sequence between the two leucine zippers can assume a coiled-coil conformation as well. These large coiled-coil structures have been observed in other muscle proteins such as tropomyosin (Kohn et al., 1997). In the coiled-coil conformation, positions **a** and **d** of the heptad are usually occupied by hydrophobic residues which gives the alpha helix an amphipathic nature (Adamson et al., 1993). This hydrophobic face is important for protein-protein interactions such as those observed in transcription factor dimerization (Landschulz et al., 1988; Lehrer and Stafford, 3d, 1991). Five potential **i+3** and **i+4** intrahelical attractions were predicted that could stabilize the helical structure. The **e** and **g** positions of a coiled-coil are crucial for specificity of protein-protein interactions through ionic

interactions between dimerizing helices (O'Shea et al., 1992; Vinson et al., 1993). However, an equal number of interhelical repulsions and attractions would be predicted to form if SLAPs homodimerized. SLAP homodimerization does not appear to be electrostatically favoured through their leucine zippers but perhaps this association could be mediated by the other coiled-coil motifs in SLAP. In the leucine zipper region of the SLAPs, seven glutamate residues in a row align at the g position giving the helix an acidic or a negatively charged strip. Acidic amphipathic alpha helices have been shown to be important for the transactivation potential of various transcription factors (Hollenberg and Evans, 1988; Horikoshi et al., 1988; Cress and Triezenberg, 1991). By analogy, this negative strip in SLAP may function to determine the specificity of protein-protein interactions of this family of proteins by modulating the interactions between SLAPs and positively charged molecules.

Comparison of the putative SLAP3 protein sequence with a database containing consensus sites for protein motifs (PROSITE) revealed that SLAP3 contained 12 potential phosphorylation sites for protein kinase C, a single potential cAMP dependent protein kinase phosphorylation site and 20 potential casein kinase II phosphorylation sites. Phosphorylation could potentially alter SLAP function or localization although, whether SLAPs are indeed phosphorylated needs to be established. A PEST motif was found in the SLAP3 and SLAP2 protein sequence but not in SLAP1. The PEST motifs have been associated with proteins that are degraded rapidly and experimental evidence has recently supported the original empirical findings (Rogers et al., 1986; Rechsteiner and Rogers, 1996). Perhaps the

PEST motif allows the cell to finely regulate the level of SLAP through a post-translational mechanism.

The SLAPs were predicted to be largely hydrophilic with the exception of the carboxyl terminal 22 amino acids which was predicted to be hydrophobic. Several transmembrane predictor programs identified this region as being a potential transmembrane segment and this hydrophobic region would be long enough to traverse the lipid bilayer. Most transmembrane proteins have an amino terminal hydrophobic signal sequence that directs their insertion into the endoplasmic reticulum via the signal recognition particle. However, the SLAPs did not contain such a signal sequence. Thus in order to determine the nature of association of SLAPs with the cardiac membranes, biochemical fractionation and solubility studies were performed. Polyclonal antiserum raised to recombinant bacterially expressed SLAP fusion protein recognized a major polypeptide of 35 kDa and three less abundant polypeptides of 81, 63 and 45 kDa in cardiac sarcolemmal fractions. This data was consistent with the finding that the plaque purified anti-sarcolemmal antibodies used in the original cloning of SLAP recognized three polypeptides of 81, 63 and 35 kDa in cardiac sarcolemma from various species (Demchyshyn, 1990). Subcellular fractionation studies revealed that SLAP proteins were not present in the cytoplasmic subcellular fraction but were found in the sarcolemmal fraction. As well, SLAPs were enriched in the sarcolemma membrane when compared to starting homogenate. The findings that SLAP was enriched in the membrane fractions and not found in the cytoplasmic fraction was consistent with the idea of SLAP being

membrane associated but these results did not reveal the nature of this association (i.e. peripheral or integral). Peripheral membrane proteins can be extracted by various chaotropic buffers whereas integral membrane proteins can only be extracted by dissolving the membranes in detergents. Results of extraction experiments were consistent with SLAPs being integral membrane proteins of the sarcolemma since SLAPs could only be solubilized by detergents.

In situ localization of SLAP in cryosections of immature and adult hearts indicated a high level of immunostaining at each developmental stage examined. This protein data was consistent with mRNA results which had indicated that SLAP transcripts were abundantly expressed in the developing heart. SLAP staining was muscle specific since it was localized to the cardiomyocytes and was notably absent from non-muscle cells of the pericardium or endocardium. Staining of both heart cryosections and cultured cardiomyocytes illustrated that SLAP was concentrated in both longitudinal and transverse bands which suggested SLAP was likely associated with both the T-tubules and the peripheral sarcolemma. Dystrophin and SLAP staining co-localized at the peripheral sarcolemma but dystrophin was not present in the lattice-like network in the interior of the cardiocyte in cryosections of rat myocardium. The distribution of cardiac dystrophin in the T-tubules appears to be species dependent. In rat and mouse, dystrophin appears to be exclusively associated with the cardiac sarcolemma while in the rabbit and sheep it is found associated with the T-tubules as well (Byers et al., 1991; Frank et al., 1994). The dystrophin staining observed in the rat heart cryosections was consistent with

dystrophin being localized to the sarcolemma. The lattice-like distribution of SLAP also resembled the staining pattern observed for the SR Ca^{2+} ATPase. Double immunohistochemical staining indicated that the staining for SLAP and the Ca^{2+} ATPase significantly overlapped although regions were stained by SLAP that were not stained by the anti-ATPase antibody. Immunohistochemical studies showed that SLAP was associated with the peripheral cardiac sarcolemma as well as the T-tubules and the sarcoplasmic reticulum membrane systems. The question of which internal membrane system SLAP localized to was addressed by electron microscopy studies which can resolve the two closely apposed membrane systems. However, SLAP antisera did not show specific staining in sections of myocardium that had been prepared for electron microscopy perhaps reflecting the sensitivity of the epitopes recognized by the SLAP antisera to fixation conditions. In this respect, labile epitopes for other proteins have been characterized where the antibodies will stain cryosections clearly for immunohistofluorescence but do not specifically stain sections prepared for electron microscopy studies (Jorgensen et al., 1990).

Since SLAP was expressed in skeletal muscle, its localization was examined immunohistochemically in this striated muscle. The ultrastructure of the membrane systems in cardiac and skeletal muscle are very similar. In skeletal muscle T-tubules are found in close apposition to two junctional SR membranes in a structure called a triad. Whereas cardiac muscle T-tubules are found in apposition to only one junctional SR membrane in a structure called a diad. SLAP staining was localized to periodic transverse bands in longitudinal sections and in a lattice-like network in

transverse sections of skeletal muscle. This staining pattern was very reminiscent of SLAP staining in the cardiac sections as well as the staining reported for TS-28, an antigen found in skeletal muscle T-tubules (Jorgensen et al., 1990). SLAP stained tibialis anterior and extraocular muscle fibers in an apparent fiber specific manner. Double immunohistochemical staining of cryosections with SLAP antiserum and either monoclonal antibodies to the slow form of myosin (extraocular muscle) or the slow form of the Ca^{2+} ATPase (tibialis anterior) showed that all of the slow twitch muscle fibers stained positively for SLAP in these two muscle groups. However SLAP stained not only slow fibers but also another population of fibers. These fibers were shown to be the fast type IIA fibers by double immunostaining cryosections of extraocular muscle with SLAP and an antibody specific to the Type IIA myosin isoform. In fetal skeletal muscle, SLAP was largely localized in a punctate pattern at the peripheral sarcolemmal and was not present in the lattice-like network seen in adult muscle. As well, SLAP staining appeared to outline the nucleus of the fetal muscle cells. The DHPR, a protein found in the T-tubules and the sarcolemma, was localized to discrete foci at the periphery of the myocyte at fetal developmental stages but displayed a much wider intracellular distribution by early neo-natal developmental stages (Yuan et al., 1991). The localization of SLAP and the DHPR to the peripheral sarcolemma of fetal muscle fibers probably reflects the immature state of development of the T-tubular system (Yuan et al., 1990).

Another approach used to investigate the membrane association of SLAP was to ectopically express epitope tagged SLAP in cells in culture and study its

distribution. In clusters of transfected undifferentiated P19 cells, SLAP was seen to be concentrated at the cell membrane between adjoining cells and enriched at regions of apparent cell-cell contact. This observation is similar to that reported for the localization of various classes of cell-cell contact molecules such as the neural cell adhesion molecule (N-CAM) and the cadherins (Inuzuka et al., 1991; Pollerberg et al., 1985). Therefore, the possibility that SLAP could interact directly with neighbouring cells or the extracellular matrix was explored. SLAP immunofluorescence staining could only be observed when the cells had been permeabilized with detergent. As well, the ectopically expressed protein was resistant to treatment of live cells with trypsin protease. These two studies indicated that the SLAP epitopes were on the inner surface of the cell membrane. Consequently, SLAP is probably not oriented towards the extracellular space and likely does not directly interact with extracellular proteins. In this regard, the adhesive properties of P19 cells expressing SLAP to gelatin coated tissue culture dishes were not significantly different than control cells suggesting that SLAP expression did not alter the interactions of the cell with extracellular matrix. However, SLAP may still play a role in cell-cell contact but not via homodimerization.

Confocal microscopy revealed a population of SLAP positive P19 cells where SLAP was uniformly distributed in the membrane as well as having an intracellular localization. This group of cells had a rounded, bipolar morphology and condensed chromosomes. These characteristics are reminiscent of cells

undergoing mitosis. It appears that SLAP localization can change depending on the phase of the cell cycle. This observation is analogous to that reported for the tail-anchored membrane protein syntaxin which apparently redistributes to the cleavage furrow of cells undergoing mitosis (Burgess et al., 1997). Upon differentiation of the transfected P19 cells with either DMSO or RA, SLAP was found distributed intracellularly in a punctate pattern instead of the exclusive membrane localization of undifferentiated P19 cells. The retention of SLAP in an intracellular compartment in differentiated P19 cells may result from the expression of a receptor for SLAP that was not expressed in the undifferentiated P19 cells.

Homology searches of the Genbank database revealed that SLAP shared identity at the nucleotide and amino acid level with TOP_{AP}, a topographically graded antigen from the chick visual system (Savitt et al., 1995). SLAP1 had an overall 72% sequence identity with TOP_{AP} with gaps in homology near the amino terminus and the carboxyl hydrophobic tail. The acidic leucine zipper motif was conserved between the two proteins whereas the predicted transmembrane region was not conserved. Analysis of the TOP_{AP} cDNA revealed that it contained a sequence that was 85% conserved with the SLAP transmembrane region but this TOP_{AP} sequence was not translated because of an inframe stop codon. The possibility that the apparent differences between TOP_{AP} and SLAP in the carboxyl terminal hydrophobic segment arose from alternative splicing was examined by RT-PCR analysis (Wiesolwieski, Wigle and Tuana, manuscript in preparation). Two alternative cDNAs were amplified from rabbit heart that would encode for two

SLAP proteins with one or the other of the transmembrane domains. Thus, the SLAP family appears to be able to utilize different carboxyl terminal transmembrane domains by the alternative mRNA splicing. Interestingly, another membrane protein that shows this alternative usage of carboxyl terminal transmembrane domains is the coiled-coil tail-anchored membrane protein Syntaxin2 which can either have one of two transmembrane segments or no transmembrane segment at all (Bennett et al., 1993).

Sequence homology analysis suggested that SLAP was the vertebrate orthologue of the avian TOP_{AP} protein which was reported to be topographically graded in both the developing chick visual system and the optic tectum (Savitt et al., 1995). If SLAP and TOP_{AP} are indeed orthologues they should have similar functions. Since TOP_{AP} was postulated to play a role in the neuronal connections between the retina and the optic tectum, the expression of SLAP in the developing mammalian visual system was examined to determine if SLAP would demonstrate a similar pattern of expression. In the adult vertebrate visual system, the retinal ganglion cells (RGCs) are connected in a topographically precise manner to the optic tectum (Garritty and Zipursky, 1995). This precise order of connectivity arises from different developmental programs in lower vertebrates and mammalian species. The axons of chick RGCs project to the optic tectum in a very exact manner and gradients of molecules are proposed to determine this precision via chemotactic mechanisms (Sperry, 1963). In the developing rat visual system, the RGCs project axons to the superior colliculus (the mammalian equivalent of the optic tectum) in

a manner that initially appears to have very little topographic order (Simon and O'Leary, 1990). By the end of the second post-natal week, however, the axons of the RGCs have arborized within the topographically appropriate area of the superior colliculus and have retracted inappropriate branches (Simon and O'Leary, 1992). Apparently, the time course of appearance of SLAP in the developing rat retina was not consistent with an involvement in the pathfinding of axons of the retinal ganglion cells to their correct zones in the superior colliculus. At postnatal day 7 in the rat visual system, the connections between retina and the superior colliculus are becoming very localized and are approaching their final adult pattern (Simon and O'Leary, 1992) yet, SLAP staining was still very low in the developing retina. As well, there was no visible gradient of SLAP expression in the rat retina at any time point analyzed. Furthermore, SLAP expression appeared to be highly localized in the Mueller glial cells and was not evident in the RGCs or the optic nerve. In contrast, TOP_{AP} was reported to be expressed in a topographically graded manner during early embryonic development in the chick visual system and to be expressed in both glia and neurons (Savitt et al., 1995). In addition, TOP_{AP} was expressed in the avian tectum, while SLAP expression was not observed at any time point in the superior colliculus (the mammalian equivalent of the tectum). The original immunohistochemical characterization of the TOP_{AP} antigen was performed using a monoclonal antibody which was also used to isolate the TOP_{AP} cDNA from a retinal library. However, only one cDNA was isolated from the immunoscreening of the expression library and immunohistochemical results were not presented using

antibodies raised to a specific TOP_{AP} peptide or fusion protein. The monoclonal antibody recognized an epitope in the TOP_{AP} protein but perhaps TOP_{AP} does not truly represent the antigen recognized by the monoclonal antibody in sections of chick retina.

Subsequent to the initiation of these studies, evidence has been accumulated that the Eph tyrosine kinases and their ligands determine topographic order in the developing visual system (Tessier-Lavigne, 1995). The ELF-1 Eph receptor ligand is expressed in a gradient in the tectum and its receptor, Mek4, is expressed in a matching gradient in the retina (Cheng et al., 1995). RAGS, an Eph receptor ligand, has been shown to mediate repulsion of both nasal and temporal retinal ganglion cell axons in *in vitro* assays, whereas, ELF-1 specifically mediates the repulsion of only temporal axons (Monschau et al., 1997). The homeobox gene engrailed is a very early topographic marker in the developing tectum and its ectopic expression has been shown to alter the expression of both RAGS and ELF-1 (Logan et al., 1996). Neither SLAP nor TOP_{AP} proteins were related to the Eph receptors or their ligands. It is possible that there is a mammalian family member of SLAP/ TOP_{AP} that functions as a guidance molecule. However, in the rat visual system SLAP does not appear to be: topographically graded, expressed in the retinal ganglion cells or expressed early in development. Therefore, SLAP is not likely to function as a positional marker for RGCs in the developing mammalian visual system. Furthermore, the topology of SLAP also implied that it was not a cell surface molecule and as such would not serve a direct role in extracellular matrix

interactions.

While studying SLAP expression in the developing superior colliculus and other brain regions, SLAP expressing cells were seen in the neonatal hippocampus. The morphology and distribution of these cells in early neonatal animals was consistent with that of the pathways of migrating hippocampal granule cell neurons discovered by autoradiographic techniques (Schlessinger et al., 1975; Altman and Bayer, 1990). The hippocampus is one of the few areas in the neonatal mammalian brain that retains the capacity to undergo neurogenesis and migration (Altman and Das, 1965). The neuronal nature of some of these apparently migrating cells was shown by the double immunolabelling of a few of the cells with anti-NeuN (a neuronal specific nuclear protein) (Mullen et al., 1992) and SLAP antibodies. However, the majority of the migrating cells were negative for anti-NeuN immunoreactivity which may reflect their immature stage of development or their non-neuronal nature. The migrating neurons appear to be generated within the subventricular zone of the hippocampus and traverse several cell layers to reach the granule cell layer of the dentate gyrus. The expression of SLAP in the hippocampal neurons was developmentally regulated as no SLAP positive cells were seen in the mature hippocampus. These results imply that SLAP may serve a role in neuronal migration through mediating cell-cell interactions in this region of the brain. Another brain area that contains neonatal migratory neurons is the olfactory bulbs (Bayer, 1983; Luskin, 1993). This area contained many SLAP positive cells, however, they were not of the well characterized migratory population but were

(Bayer, 1983; Luskin, 1993). This area contained many SLAP positive cells, however, they were not of the well characterized migratory population but were most likely a type of glial-like support cell found in the olfactory nerve layer.

The studies of SLAP expression in the central nervous system were performed using antibodies raised against a recombinant fusion protein that was common to all three SLAP isoforms. Thus the immunohistochemical staining for SLAP represented the expression of all SLAP isoforms. Western blot analysis of proteins from microdissected regions would have resolved the question about which SLAP isoforms were being expressed. Another approach to this problem would have been to raise specific antibodies to each of the SLAP isoforms.

The SLAPs were not predicted to have a signal sequence although the biochemical properties of SLAPs were consistent with those of integral membrane proteins. In this respect, the SLAPs are related to a growing family of coiled-coil membrane proteins such as syntaxin, epimorphin and synaptobrevin which lack a signal sequence and are anchored in the membrane by a carboxyl hydrophobic segment (Spring et al., 1993; Kutay et al., 1993). Many of these tail anchored membrane proteins have been postulated to be involved in membrane fusion and docking in yeast (sec22p, sed5p) and mammals (syntaxin, synaptobrevin) (Sacher et al., 1997; Calakos et al., 1994). As well, SLAPs share 44% similarity with USO1, a yeast protein involved in transport of vesicles between the endoplasmic reticulum and the Golgi apparatus (Sapperstein et al., 1996). The proteins necessary for myoblast fusion are not yet well understood. Neutralizing antibody experiments

have indicated a role for N-cadherin in myoblast fusion but genetically N-Cadherin null myoblasts have been demonstrated to fuse with the same efficiency *in vitro* and *in vivo* as wild type control myoblasts (Charlton et al., 1997). Drosophila genetic studies have implicated two genes *blown fuse* and *rolling stone* genes in myoblast fusion (Doberstein et al., 1997; Paululat et al., 1997). The blown fuse gene product is a cytoplasmic protein whereas the rolling stone gene product contained putative transmembrane segments. The appearance of small vesicles immediately before fusion of myoblast cell membrane suggests a role for vesicle fusion in this process of myotube formation (Doberstein et al., 1997). The function of these vesicles in the fusion process is still unclear. Given the role of tail-anchored membrane proteins in membrane fusion in both yeast and mammals and the similarity of SLAP to a protein involved in vesicle transport, the effect of SLAP ectopic expression on membrane fusion during C2C12 myoblast differentiation was examined. Indeed, the fusion index for SLAP expressing C2C12 clones was reduced an average of 62% when compared to control myoblasts. SLAP overexpression appeared to inhibit the fusion process of myoblasts into myotubes. The isolated SLAP transfected C2C12 clones appeared to have a mosaic pattern of SLAP expression so if more uniform SLAP expressing clones were isolated by subcloning then the observed inhibition of the fusion index would probably be more dramatic. Although the SLAP clones did not fuse to the same degree as controls the induction of myogenin expression (a marker of muscle differentiation) was not altered between control and SLAP expressing clones. Intriguingly, SLAP expression apparently inhibited myoblast

fusion but not their differentiation into myocytes. Similarly, ectopic expression of either the oncogene or proto-oncogene form of myc prevented C2C12 fusion but did not prevent their differentiation as measured by the expression of myogenin and other myotube specific markers (Crescenzi et al., 1994). SLAP may inhibit fusion possibly by the sequestration of a protein (cytosolic or membrane associated) that are necessary for fusion of the myoblasts but this sequestration does not alter the differentiation process.

The level of exogenously expressed SLAP mRNA, which lacked both 5' and 3' UTRs, was dramatically elevated upon myoblast differentiation. The upregulation of ectopically expressed SLAP transcript levels upon differentiation of myoblasts indicate that the SLAP transcript turnover may be tightly regulated. The increased turnover of the myc transcript during differentiation of C2C12 cells has been demonstrated to be mediated by sequences in the coding region for the myc protein (Yeilding and Lee, 1997). The conservation of the SLAP 3'UTR between rabbits, mice and man was remarkable and actually better conserved than some sections of the coding region. This degree of conservation (98% over 340 nucleotides) suggests that the SLAP 3'UTR may function to regulate the rate of turnover of the SLAP transcript. Furthermore, the 3' UTR has been implicated in decreasing the stability of mRNA transcripts which have short half lives (Tsuchida et al., 1993; Geng et al., 1994).

Although the precise physiological role of SLAP is unknown, its localization in the sarcolemma, T-tubules and SR indicate a potential role in E-C coupling in

adult cardiac and other muscle types. The close apposition of sarcolemma to sarcoplasmic reticulum in muscle is generated through an undetermined mechanism and is critically important for effective signal transmission. Originally it had been proposed that direct physical interactions between the DHPR and the RYR mediated the formation of triads (skeletal muscle) or diads (cardiac muscle). However, analysis of dysgenic mice, which lack DHPRs, and dyspedic mice, which lack RyR, have shown that the docking of SR to sarcolemma occurs in the absence of these two major muscle proteins (Flucher et al., 1992; Takekura et al., 1995a). A SR protein called triadin was proposed to mediate the interaction between SR and SL but topological analysis has shown that it is not well suited to fulfill such a role since it primarily resides in the lumen of the SR (Knudson et al., 1993). A likely sarcolemmal protein to mediate the interaction has not yet been proposed but the potential candidate protein would be localized in the sarcolemma, T-tubules or the junctional SR. The E-C coupling in muscle cells is analogous to the well characterized excitation-secretion (E-S) coupling in nerve terminals. In E-S coupling, the excitation of the pre-synaptic membrane leads to calcium influx via the N-Type calcium channels which triggers neurotransmitter release. As in E-C coupling, efficient signal transmission in E-S coupling depends on the tight association of the plasma membrane (pre-synaptic membrane) with an internal membrane system (neurotransmitter containing vesicles). This arrangement of membrane systems is mediated by tail-anchored membrane proteins on both the synaptic vesicle and plasma membrane. Synaptic vesicles are docked at the pre-

synaptic membrane through interactions between vesicle proteins (synaptobrevin, synaptogamin) and plasma membrane proteins (syntaxin, ω conotoxin sensitive N-Type calcium channel, SNAP-25) (Calakos et al., 1994). This tight arrangement allows for the efficient utilization of extracellular calcium during exocytosis in the active zones by co-localizing the calcium influx machinery and the synaptic vesicles (Llinas et al., 1992). Both the E-C and E-S processes are exquisitely dependent on calcium and calcium binding proteins. In E-S coupling many of the protein-protein interactions are calcium dependent as well (Sheng et al., 1996; Sheng et al., 1997).

The major proteins involved in E-C coupling such as DHPR, RyR and the sodium calcium exchanger are calcium binding proteins. Originally, the SLAPs were identified by screening for potential calcium binding proteins of cardiac cell membrane. The sizes of the SLAPs do not correspond well with the sizes of the original calcium binding proteins detected in sarcolemma and SLAPs were not predicted to contain the EF hand calcium binding motif. However, proteins such as the sodium calcium exchanger do not contain an EF hand but can still bind calcium (Nicoll et al., 1990). By analogy, the SLAPs are acidic proteins and contain a localized negative strip of seven glutamates, which may bind calcium. Many of the protein-protein interaction involved in the synaptic vesicle docking complex are mediated through coiled-coil motifs of tail anchored membrane proteins such as those between syntaxin and the N-Type calcium channel (Sheng et al., 1994). The pre-synaptic membrane protein syntaxin plays a key role in E-S coupling by acting

as a scaffold to which both the synaptic vesicles and the N-type calcium channels can bind (Fig. 47A). Similarly, in cardiac muscle the close apposition of the junctional SR to the sarcolemma results in the influx of calcium through a single DHPR being sufficient to trigger the opening of a neighbouring RyR (calcium spark) (Opie, 1991; de Leon et al., 1995). As coiled-coil, tail-anchored membrane proteins found in the sarcolemma, T-tubules and the SR, SLAPs may potentially play a role in mediating interactions between the SR and the sarcolemma and thus regulate E-C coupling as well (Figure 47B). There is no clear evidence for the direct coupling of DHPRs and RyRs in cardiac and slow twitch muscle (Fabiato, 1985; Cullen et al., 1984). In fact there is evidence to the contrary for example the cardiac RyR is unable to interact with the skeletal or cardiac isoforms of the DHPR (Lu et al., 1994; Nakai et al., 1997). SLAPs could act as scaffold proteins to which the L-Type calcium channels or the RyR could bind.

Cardiac, smooth and a portion of the skeletal muscle E-C coupling is mediated via a calcium induced calcium release mechanism (Fabiato, 1985; Anderson and Meissner, 1995). Since the two smaller SLAPs are expressed in smooth and cardiac muscle, they may play a role in modulating E-C coupling that is dependent on external calcium.

Figure 47. Proposed Role of SLAPs in Modulating Excitation-Contraction Coupling.

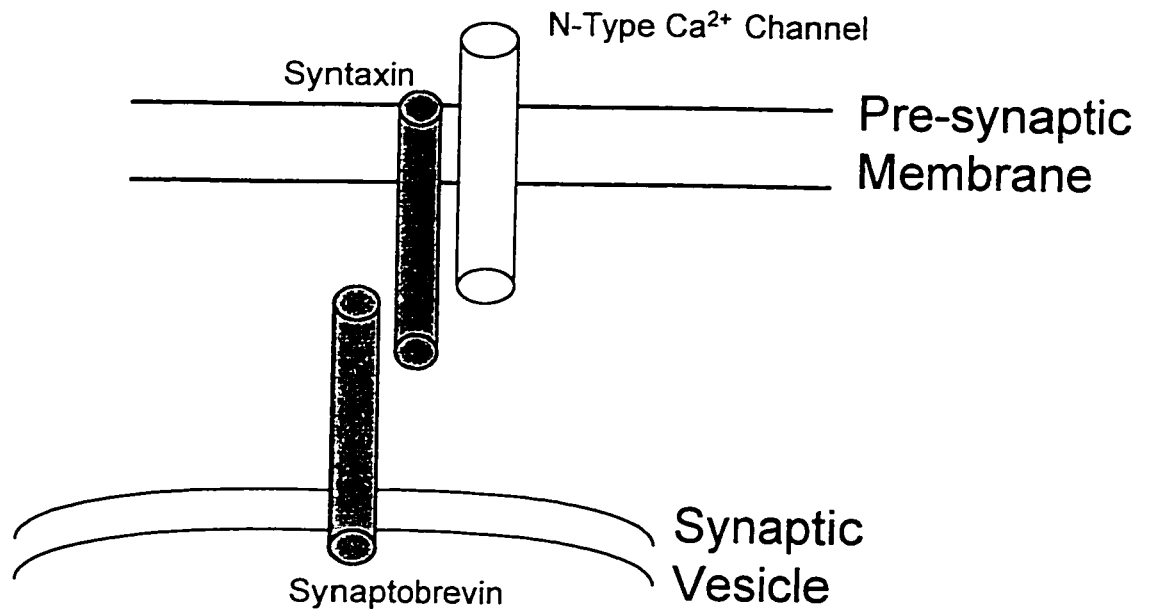
A) The role of syntaxin in Excitation-Secretion (E-S) Coupling.

Syntaxin is a coiled-coil tail anchored pre-synaptic membrane protein that binds several proteins involved in E-S coupling. Syntaxin binds plasma membrane proteins such as the N-Type Calcium channel as well as binding synaptic vesicle proteins such as synaptobrevin. Syntaxin fulfills a crucial role in excitation-secretion coupling by co-localizing the calcium channels and the synaptic vesicle membranes, which allows for the efficient use of extracellular calcium during secretion.

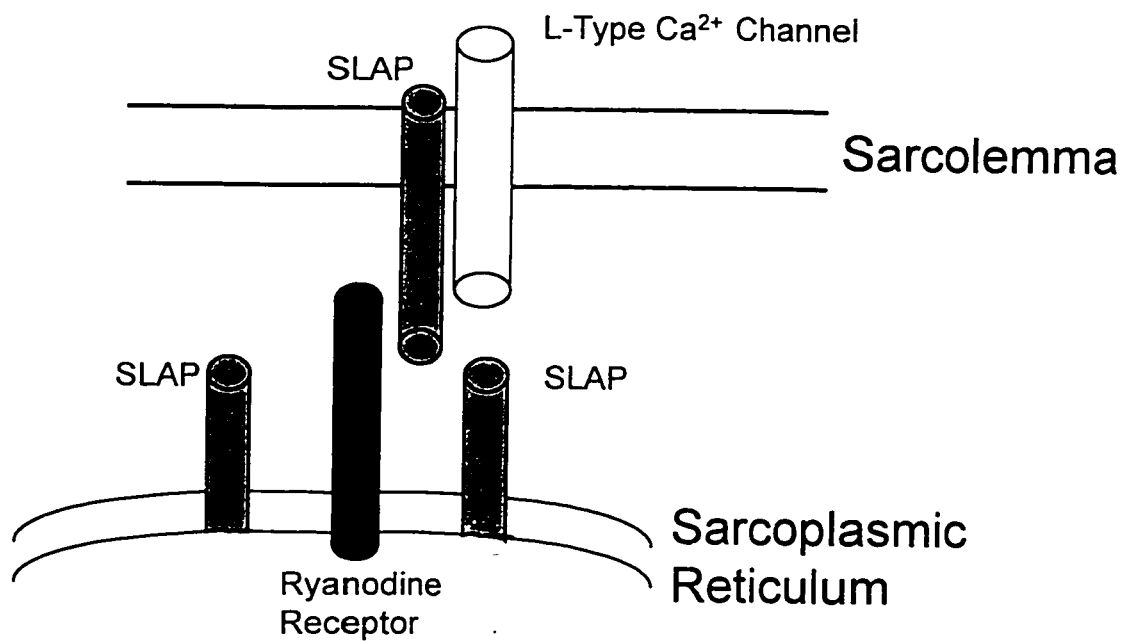
B) Putative role of SLAPs in Excitation-Contraction(E-C) Coupling.

SLAPs are coiled-coil tail anchored membrane proteins that are found in the sarcolemma and the T-tubules of the myocardium. By analogy with syntaxin, SLAPs could bind the L-type calcium channel and may also bind junctional SR proteins such as the ryanodine receptor or an unidentified receptor, which could be SLAP itself. These binding properties of SLAP would help to co-localize the calcium influx machinery and the calcium activated RyRs of the SR. This co-localization of calcium channels is required for efficient signal transduction during E-C coupling. As well, SLAP, by mediating interactions between the T-tubules and the SR, could help direct the formation of the diad structures during development. This mediation could occur through SLAP homodimerization since SLAPs are localized in the SR and the T-tubules.

A Excitation-Secretion Coupling



B Excitation-Contraction Coupling



The evidence of SLAP being expressed in the SR and the T-tubules raises the possibility that homodimerization between SLAPs may serve to modulate E-C coupling or the generation of E-C coupling units. The larger SLAP3 isoform was expressed in all muscle types and many other diverse tissues and thus may serve to modulate a broader range of membrane-membrane interactions. In addition to a possible role for SLAP in E-C coupling of adult muscle, studies in myoblast cells have elucidated a possible role for SLAP in membrane fusion during skeletal muscle development. The overexpression of SLAP in C2C12 myoblast cells significantly inhibited myotube formation but not the expression of a differentiated muscle marker, myogenin. Similarly, syntaxin also has two distinct roles in adult and developing tissues. In adult neurons, syntaxin play a crucial role in vesicle transport and fusion. Recently, however, a role for syntaxin in cell division has been proposed based on work in *Drosophila* embryos where syntaxin is required for the process of cellularization (Burgess et al., 1997). Cellularization is a specialized form of cell division that occurs when the multinucleated blastoderm is divided into individual cells. By analogy, the disruption of membrane fusion by SLAP overexpression would be consistent with the role of SLAP in membrane cleavage. In this regard, intracellular SLAP localization was noted in cells undergoing mitosis.

In conclusion, SLAP may fulfill distinct roles in different tissues or at different developmental stages. In muscle, SLAPs may be crucial for E-C coupling by either localizing the DHPRs and RyRs to microdomains of the diad or by modulating the formation of the diad itself. In neurons and glia, SLAP may be

involved in the trafficking of intracellular vesicles. The dynamic re-organization of SLAP during mitosis implicates it in the process of membrane cleavage as well. The role of SLAP in membrane turnover is further supported by the ectopic expression studies which demonstrated a correlation between SLAP expression and inhibition of myoblast fusion. SLAPs are a family of coiled-coil tail anchored membrane proteins that are involved in the regulation of diverse membrane systems.

The functional importance of SLAPs having two alternative transmembrane domains still needs to be addressed. The precise composition of the carboxyl terminal anchor does not appear to determine membrane insertion for tail-anchored membrane proteins since a chimeric synaptobrevin protein with an engineered tail of a minimum of 11 leucines is appropriately inserted into the endoplasmic reticulum (Whitley et al., 1996). However, the length of the transmembrane domain has been shown to determine which membrane system (ER, golgi or plasma membrane) a tail-anchored membrane protein would be localized (Yang et al., 1997). A first step in resolving the significance of the two alternative transmembrane domains would be to compare the subcellular localization of ectopically expressed SLAP which contained one or the other of the transmembrane domains. The expression and localization of SLAP with respect to T-tubular and SR markers could be examined by double immunostaining with SLAP and the DHPR or the RyR. The nature of the proteins that the SLAPs associate with in the diad/triad structure could be addressed by immunoprecipitation experiments using the SLAP antiserum or antibodies specific to T-tubular or junctional SR antigens. Another approach, to

identifying what proteins bind SLAP, would be to couple purified SLAP protein (purified from myocardium or bacterially expressed protein) to a matrix. Purified sarcolemmal or SR vesicles could then be loaded onto the SLAP affinity column and the SLAP binding proteins could be identified. The role of SLAP in the formation and maintenance of the triad/diad structures would be definitively addressed by the generation of SLAP null transgenic mice. If SLAP plays a role in the generation of these structures, either the number or spatial organization of the triads/diads should be greatly affected in the SLAP null mice. The transgenic mice may also allow for the determination of the role of SLAP in early development, particularly in terms of formation of the myotubes and myocardium.

REFERENCES

- Adamson, J.G., Zhou, N.E., and Hodges, R.S. (1993). Structure, function and application of the coiled-coil protein folding motif. *Curr. Opin. Biotechnol.* 4, 428-437.
- Altman, J. and Bayer, S.A. (1990). Mosaic organization of the hippocampal neuroepithelium and the multiple germinal sources of dentate granule cells. *J. Comp. Neurol.* 301, 325-342.
- Altman, J. and Das, G.D. (1965). Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *J. Comp. Neurol.* 124, 319-335.
- Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215, 403-410.
- Anderson, K., Cohn, A.H., and Meissner, G. (1994). High-affinity [3h]pn200-110 and [3h]ryanodine binding to rabbit and frog skeletal muscle. *Am J Physiol* 266, Pt 1:C462-6.
- Anderson, K. and Meissner, G. (1995). T-tubule depolarization-induced sr Ca²⁺ release is controlled by dihydropyridine receptor- and Ca(2+)-dependent mechanisms in cell homogenates from rabbit skeletal muscle. *J Gen Physiol* 105, 363-83.
- Appelt, D., Buenviaje, B., Champ, C., and Franzini-Armstrong, C. (1989). Quantitation of 'junctional feet' content in two types of muscle fiber from hind limb muscles of the rat. *Tissue Cell* 21, 783-94.
- Armstrong, C.M., Bezanilla, F.M., and Horowicz, P. (1972). Twitches in the presence of ethylene glycol bis(-aminoethyl ether)- N,N'-tetracetic acid. *Biochim. Biophys. Acta* 267, 605-608.
- Aviv, H. and Leder, P. (1972). Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. *Proc. Natl. Acad. Sci. U. S. A.* 69, 1408-1412.
- Bairoch, A., Bucher, P., and Hofmann, K. (1997). The PROSITE database, its status in 1997. *Nucleic. Acids. Res.* 25, 217-221.
- Barg, S., Copello, J.A., and Fleischer, S. (1997). Different interactions of cardiac

- and skeletal muscle ryanodine receptors with FK-506 binding protein isoforms. *Am J Physiol* 272, Pt 1:C1726-33.
- Barry, W.H. and Bridge, J.H. (1993). Intracellular calcium homeostasis in cardiac myocytes. *Circulation* 87, 1806-1815.
- Bayer, S.A. (1983). 3H-thymidine-radiographic studies of neurogenesis in the rat olfactory bulb. *Exp. Brain Res.* 50, 329-340.
- Bennett, M.K., Garcia-Ararras, J.E., Elferink, L.A., Peterson, K., Fleming, A.M., Hazuka, C.D., and Scheller, R.H. (1993). The syntaxin family of vesicular transport receptors. *Cell* 74, 863-873.
- Birnboim, H.C. and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic. Acids. Res.* 7, 1513-1523.
- Block, B.A., Imagawa, T., Campbell, K.P., and Franzini-Armstrong, C. (1988). Structural evidence for direct interaction between the molecular components of the transverse tubule/sarcoplasmic reticulum junction in skeletal muscle. *J. Cell Biol.* 107, 2587-2600.
- Burgess, R.W., Deitcher, D.L., and Schwarz, T.L. (1997). The synaptic protein syntaxin1 is required for cellularization of drosophila embryos. *J Cell Biol* 138, 861-75:
- Byers, T.J., Kunkel, L.M., and Watkins, S.C. (1991). The subcellular distribution of dystrophin in mouse skeletal, cardiac, and smooth muscle. *J Cell Biol* 115, 411-21:
- Caccamo, D., Katsetos, C.D., Herman, M.M., Frankfurter, A., Collins, V.P., and Rubinstein, L.J. (1989). Immunohistochemistry of a spontaneous murine ovarian teratoma with neuroepithelial differentiation. neuron-associated beta-tubulin as a marker for primitive neuroepithelium. *Lab Invest* 60, 390-8:
- Calakos, N., Bennett, M.K., Peterson, K.E., and Scheller, R.H. (1994). Protein-protein interactions contributing to the specificity of intracellular vesicular trafficking. *Science* 263, 1146-1149.
- Cannell, M.B. (1991). Contribution of sodium-calcium exchange to calcium regulation in cardiac muscle. *Ann N Y Acad Sci* 639, 428-43:
- Carl, S.L., Felix, K., Caswell, A.H., Brandt, N.R., Ball, W.J., Jr., Vaghy, P.L., Meissner, G., and Ferguson, D.G. (1995). Immunolocalization of sarcolemmal dihydropyridine receptor and sarcoplasmic reticular triadin and ryanodine receptor

in rabbit ventricle and atrium. *J. Cell Biol.* 129, 672-682.

Caswell, A.H., Brandt, N.R., Brunschwig, J.P., and Purkerson, S. (1991). Localization and partial characterization of the oligomeric disulfide-linked molecular weight 95,000 protein (triadin) which binds the ryanodine and dihydropyridine receptors in skeletal muscle triadic vesicles. *Biochemistry* 30, 7507-7513.

Catterall, W.A. (1991). Functional subunit structure of voltage-gated calcium channels. *Science* 253, 1499-1500.

Catterall, W.A. (1993). Structure and function of voltage-gated ion channels. *Trends Neurosci.* 16, 500-506.

Charlton, C.A., Mohler, W.A., Radice, G.L., Hynes, R.O., and Blau, H.M. (1997). Fusion Competence of Myoblasts Rendered Genetically Null for N-Cadherin in Culture. *J. Cell Biol.* 138, 331-336.

Chen, C. and Okayama, H. (1987). High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell Biol.* 7, 2745-2752.

Chen, S.R., Zhang, L., and MacLennan, D.H. (1992). Characterization of a Ca^{2+} binding and regulatory site in the Ca^{2+} release channel (ryanodine receptor) of rabbit skeletal muscle sarcoplasmic reticulum. *J Biol Chem* 267, 23318-26:

Cheng, H., Lederer, M.R., Xiao, R.P., Gomez, A.M., Zhou, Y.Y., Ziman, B., Spurgeon, H., Lakatta, E.G., and Lederer, W.J. (1996). Excitation-contraction coupling in heart: new insights from Ca^{2+} sparks. *Cell Calcium* 20, 129-140.

Cheng, H.J., Nakamoto, M., Bergemann, A.D., and Flanagan, J.G. (1995). Complementary gradients in expression and binding of ELF-1 and Mek4 in development of the topographic retinotectal projection map. *Cell* 82, 371-381.

Chien, A.J., Zhao, X., Shirokov, R.E., Puri, T.S., Chang, C.F., Sun, D., Rios, E., and Hosey, M.M. (1995). Roles of a membrane-localized beta subunit in the formation and targeting of functional L-type Ca^{2+} channels. *J. Biol. Chem.* 270, 30036-30044.

Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J., and Rutter, W.J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 18, 5294-5299.

Crescenzi, M., Crouch, D.H., and Tato, F. (1994). Transformation by myc prevents fusion but not biochemical differentiation of c2c12 myoblasts: mechanisms of phenotypic correction in mixed culture with normal cells. *J Cell Biol* 125, 1137-45:

- Cress, W.D. and Triezenberg, S.J. (1991). Critical structural elements of the VP16 transcriptional activation domain. *Science* 251, 87-90.
- Cullen, M.J., Hollingworth, S., and Marshall, M.W. (1984). A comparative study of the transverse tubular system of the rat extensor digitorum longus and soleus muscles. *J Anat* 138, 297-308.
- Damiani, E., Picello, E., Saggin, L., and Margreth, A. (1995). Identification of triadin and of histidine-rich Ca(2+)-binding protein as substrates of 60 kda calmodulin-dependent protein kinase in junctional terminal cisternae of sarcoplasmic reticulum of rabbit fast muscle. *Biochem Biophys Res Commun* 209, 457-65:
- De Jongh, K.S., Warner, C., and Catterall, W.A. (1990). Subunits of purified calcium channels. Alpha 2 and delta are encoded by the same gene. *J. Biol. Chem.* 265, 14738-14741.
- de Leon, M., Wang, Y., Jones, L., Perez-Reyes, E., Wei, X., Soong, T.W., Snutch, T.P., and Yue, D.T. (1995). Essential Ca(2+)-binding motif for Ca(2+)-sensitive inactivation of l- type Ca²⁺ channels. *Science* 270, 1502-6:
- Demchyshyn, L. (1990). Isolation of cDNAs for Heart Antigens. (Masters Thesis) University of Toronto.
- Doberstein, S.K., Fetter, R.D., Mehta, A.Y., and Goodman, C.S. (1997). Genetic analysis of myoblast fusion: blown fuse is required for progression beyond the prefusion complex. *J. Cell Biol.* 136, 1249-1261.
- Doucet, J.P., Murphy, B.J., and Tuana, B.S. (1990). Modification of a discontinuous and highly porous sodium dodecyl sulfate-polyacrylamide gel system for minigel electrophoresis. *Anal. Biochem.* 190, 209-211.
- Evan, G.I., Lewis, G.K., Ramsay, G., and Bishop, J.M. (1985). Isolation of monoclonal antibodies specific for human c-myc proto- oncogene product. *Mol. Cell Biol.* 5, 3610-3616.
- Fabiato, A. (1985). Simulated calcium current can both cause calcium loading in and trigger calcium release from the sarcoplasmic reticulum of a skinned canine cardiac Purkinje cell. *J. Gen. Physiol.* 85, 291-320.
- Fleischer, S. and Inui, M. (1989). Biochemistry and biophysics of excitation-contraction coupling. *Annu. Rev. Biophys. Biophys. Chem.* 18:333-64, 333-364.

- Flucher, B.E., Phillips, J.L., Powell, J.A., Andrews, S.B., and Daniels, M.P. (1992). Coordinated development of myofibrils, sarcoplasmic reticulum and transverse tubules in normal and dysgenic mouse skeletal muscle, in vivo and in vitro. *Dev. Biol.* 150, 266-280.
- Flucher, B.E. and Franzini-Armstrong, C. (1996). Formation of junctions involved in excitation-contraction coupling in skeletal and cardiac muscle. *Proc. Natl. Acad. Sci. U. S. A.* 93, 8101-8106.
- Frank, J.S., Rich, T.L., Beydler, S., and Kreman, M. (1982). Calcium depletion in rabbit myocardium. ultrastructure of the sarcolemma and correlation with the calcium paradox. *Circ Res* 51, 117-30:
- Frank, J.S., Mottino, G., Reid, D., Molday, R.S., and Philipson, K.D. (1992). Distribution of the Na(+)-Ca²⁺ exchange protein in mammalian cardiac myocytes: an immunofluorescence and immunocolloidal gold-labeling study. *J. Cell Biol.* 117, 337-345.
- Frank, J.S., Mottino, G., Chen, F., Peri, V., Holland, P., and Tuana, B.S. (1994). Subcellular distribution of dystrophin in isolated adult and neonatal cardiac myocytes. *Am J Physiol* 267, Pt 1):C1707-16.
- Franzini-Armstrong, C. and Protasi, F. (1997). Ryanodine receptors of striated muscles: a complex channel capable of multiple interactions. *Physiol. Rev.* 77, 699-729.
- Fujii, J., Maruyama, K., Tada, M., and MacLennan, D.H. (1989). Expression and site-specific mutagenesis of phospholamban. studies of residues involved in phosphorylation and pentamer formation. *J Biol Chem* 264, 12950-5:
- Gao, T., Yatani, A., Dell'Acqua, M.L., Sako, H., Green, S.A., Dascal, N., Scott, J.D., and Hosey, M.M. (1997). Camp-dependent regulation of cardiac l-type Ca²⁺ channels requires membrane targeting of pka and phosphorylation of channel subunits. *Neuron* 19, 185-96:
- Garrity, P.A. and Zipursky, S.L. (1995). Neuronal target recognition. *Cell* 83, 177-185.
- Gee, S.H., Montanaro, F., Lindenbaum, M.H., and Carbonetto, S. (1994). Dystroglycan-alpha, a dystrophin-associated glycoprotein, is a functional agrin receptor. *Cell* 77, 675-86:
- Geng, Y.J., Almqvist, M., and Hansson, G.K. (1994). cDNA cloning and expression of inducible nitric oxide synthase from rat vascular smooth muscle cells. *Biochim*

Biophys Acta 1218, 421-4:

Gregg, R.G., Messing, A., Strube, C., Beurg, M., Moss, R., Behan, M., Sukhareva, M., Haynes, S., Powell, J.A., Coronado, R., and Powers, P.A. (1996). Absence of the beta subunit (cchb1) of the skeletal muscle dihydropyridine receptor alters expression of the alpha 1 subunit and eliminates excitation-contraction coupling. Proc. Natl. Acad. Sci. U. S. A. 93, 13961-13966.

Gros, D.B. and Jongsma, H.J. (1996). Connexins in mammalian heart function. Bioessays 18, 719-30:

Guo, W. and Campbell, K.P. (1995). Association of triadin with the ryanodine receptor and calsequestrin in the lumen of the sarcoplasmic reticulum. J. Biol. Chem. 270, 9027-9030.

Hain, J., Onoue, H., Mayrleitner, M., Fleischer, S., and Schindler, H. (1995). Phosphorylation modulates the function of the calcium release channel of sarcoplasmic reticulum from cardiac muscle. J Biol Chem 270, 2074-81:

Heng, H.H., Squire, J., and Tsui, L.C. (1992). High-resolution mapping of mammalian genes by in situ hybridization to free chromatin. Proc. Natl. Acad. Sci. U. S. A. 89, 9509-9513.

Heng, H.H. and Tsui, L.C. (1993). Modes of DAPI banding and simultaneous in situ hybridization. Chromosoma 102, 325-332.

Hollenberg, S.M. and Evans, R.M. (1988). Multiple and cooperative trans-activation domains of the human glucocorticoid receptor. Cell 55, 899-906.

Horikoshi, M., Carey, M.F., Kakidani, H., and Roeder, R.G. (1988). Mechanism of action of a yeast activator: direct effect of GAL4 derivatives on mammalian TFIID-promoter interactions. Cell 54, 665-669.

Inui, M., Saito, A., and Fleischer, S. (1987a). Purification of the ryanodine receptor and identity with feet structures of junctional terminal cisternae of sarcoplasmic reticulum from fast skeletal muscle. J Biol Chem 262, 1740-7:

Inui, M., Saito, A., and Fleischer, S. (1987b). Isolation of the ryanodine receptor from cardiac sarcoplasmic reticulum and identity with the feet structures. J Biol Chem 262, 15637-42:

Inuzuka, H., Miyatani, S., and Takeichi, M. (1991). R-cadherin: a novel Ca(2+)-dependent cell-cell adhesion molecule expressed in the retina. Neuron 7, 69-79.

- Jacquemond, V., Csernoch, L., Klein, M.G., and Schneider, M.F. (1991). Voltage-gated and calcium-gated calcium release during depolarization of skeletal muscle fibers. *Biophys J* 60, 867-73:
- Jorgensen, A.O., Shen, A.C., Daly, P., and MacLennan, D.H. (1982). Localization of Ca^{2+} + Mg^{2+} -atpase of the sarcoplasmic reticulum in adult rat papillary muscle. *J Cell Biol* 93, 883-92:
- Jorgensen, A.O., Arnold, W., Pepper, D.R., Kahl, S.D., Mandel, F., and Campbell, K.P. (1988a). A monoclonal antibody to the Ca^{2+} -atpase of cardiac sarcoplasmic reticulum cross-reacts with slow type I but not with fast type II canine skeletal muscle fibers: an immunocytochemical and immunochemical study. *Cell Motil Cytoskeleton* 9, 164-74:
- Jorgensen, A.O., Broderick, R., Somlyo, A.P., and Somlyo, A.V. (1988b). Two structurally distinct calcium storage sites in rat cardiac sarcoplasmic reticulum: an electron microprobe analysis study. *Circ Res* 63, 1060-9:
- Jorgensen, A.O., Arnold, W., Shen, A.C., Yuan, S.H., Gaver, M., and Campbell, K.P. (1990). Identification of novel proteins unique to either transverse tubules (ts28) or the sarcolemma (sl50) in rabbit skeletal muscle. *Journal of Cell Biology* 110, 1173-1185.
- Jorgensen, A.O. and Jones, L.R. (1987). Immunoelectron microscopical localization of phospholamban in adult canine ventricular muscle. *J Cell Biol* 104, 1343-52:
- Kee, Y., Lin, R.C., Hsu, S.C., and Scheller, R.H. (1995). Distinct domains of syntaxin are required for synaptic vesicle fusion complex formation and dissociation. *Neuron* 14, 991-998.
- Klein, P., Kanehisa, M., and DeLisi, C. (1985). The detection and classification of membrane-spanning proteins. *Biochim. Biophys. Acta* 815, 468-476.
- Knudson, C.M., Stang, K.K., Moomaw, C.R., Slaughter, C.A., and Campbell, K.P. (1993). Primary structure and topological analysis of a skeletal muscle-specific junctional sarcoplasmic reticulum glycoprotein (triadin). *J. Biol. Chem.* 268, 12646-12654.
- Kohn, W.D., Mant, C.T., and Hodges, R.S. (1997). Alpha-helical protein assembly motifs. *J. Biol. Chem.* 272, 2583-2586.
- Kozak, M. (1991). An analysis of vertebrate mRNA sequences: intimations of translational control. *J. Cell Biol.* 115, 887-903.

- Kutay, U., Hartmann, E., and Rapoport, T.A. (1993). A class of membrane proteins with a C-terminal anchor. *Trends Cell Biol.* 2, 72-75.
- Kyselovic, J., Leddy, J.J., Ray, A., Wigle, J., and Tuana, B.S. (1994). Temporal differences in the induction of dihydropyridine receptor subunits and ryanodine receptors during skeletal muscle development. *J. Biol. Chem.* 269, 21770-21777.
- Kyte, J. and Doolittle, R.F. (1982). A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* 157, 105-132.
- Landschulz, W.H., Johnson, P.F., and McKnight, S.L. (1988). The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. *Science* 240, 1759-1764.
- Langer, G.A. (1987). The role of calcium at the sarcolemma in the control of myocardial contractility. *Can J Physiol Pharmacol* 65, 627-31.
- Lehrer, S.S. and Stafford, W.F., 3d (1991). Preferential assembly of the tropomyosin heterodimer: equilibrium studies. *Biochemistry* 30, 5682-5688.
- Li, Z., Nicoll, D.A., Collins, A., Hilgemann, D.W., Filoteo, A.G., Penniston, J.T., Weiss, J.N., Tomich, J.M., and Philipson, K.D. (1991). Identification of a peptide inhibitor of the cardiac sarcolemmal Na(+)-Ca²⁺ exchanger. *J Biol Chem* 266, 1014-20.
- Li, Z.P., Burke, E.P., Frank, J.S., Bennett, V., and Philipson, K.D. (1993). The cardiac Na⁺-Ca²⁺ exchanger binds to the cytoskeletal protein ankyrin. *J. Biol. Chem.* 268, 11489-11491.
- Llinas, R., Sugimori, M., and Silver, R.B. (1992). Microdomains of high calcium concentration in a presynaptic terminal. *Science* 256, 677-679.
- Logan, C., Wizenmann, A., Drescher, U., Monschau, B., Bonhoeffer, F., and Lumsden, A. (1996). Rostral optic tectum acquires caudal characteristics following ectopic engrailed expression. *Curr. Biol.* 6, 1006-1014.
- Lu, X., Xu, L., and Meissner, G. (1994). Activation of the skeletal muscle calcium release channel by a cytoplasmic loop of the dihydropyridine receptor. *J. Biol. Chem.* 269, 6511-6516.
- Lupas, A., Van Dyke, M., and Stock, J. (1991). Predicting coiled coils from protein sequences. *Science* 252, 1162-1164.
- Lupas, A. (1996). Coiled coils: new structures and new functions. *Trends. Biochem.*

Sci. 21, 375-382.

Luskin, M.B. (1993). Restricted proliferation and migration of postnatally generated neurons derived from the forebrain subventricular zone. *Neuron* 11, 173-189.

McBurney, M.W., Fournier, S., Jardine, K., and Sutherland, L. (1994). Intragenic regions of the murine pgk-1 locus enhance integration of transfected dnas into genomes of embryonal carcinoma cells. *Somat Cell Mol Genet* 20, 515-28:

McDonald, T.F., Pelzer, S., Trautwein, W., and Pelzer, D.J. (1994). Regulation and modulation of calcium channels in cardiac, skeletal, and smooth muscle cells. *Physiol Rev* 74, 365-507:

McPherson, P.S. and Campbell, K.P. (1993). The ryanodine receptor/Ca²⁺ release channel. *J. Biol. Chem.* 268, 13765-13768.

Melacini, P., Fanin, M., Danieli, G.A., Villanova, C., Martinello, F., Miorin, M., Freda, M.P., Miorelli, M., Mostacciuolo, M.L., Fasoli, G., Angelini, C., and Dalla Volta, S. (1996). Myocardial involvement is very frequent among patients affected with subclinical becker's muscular dystrophy. *Circulation* 94, 3168-75:

Meng, H., Leddy, J.J., Frank, J., Holland, P., and Tuana, B.S. (1996). The association of cardiac dystrophin with myofibrils/Z-disc regions in cardiac muscle suggests a novel role in the contractile apparatus. *J. Biol. Chem.* 271, 12364-12371.

Mikami, A., Imoto, K., Tanabe, T., Niidome, T., Mori, Y., Takeshima, H., Narumiya, S., and Numa, S. (1989). Primary structure and functional expression of the cardiac dihydropyridine-sensitive calcium channel. *Nature* 340, 230-233.

Mohana Rao, J.K. and Argos, P. (1986). A conformational preference parameter to predict helices in integral membrane proteins. *Biochim. Biophys. Acta* 869, 197-214.

Monschau, B., Kremoser, C., Ohta, K., Tanaka, H., Kaneko, T., Yamada, T., Handwerker, C., Hornberger, M.R., Loschinger, J., Pasquale, E.B., Siever, D.A., Verderame, M.F., Muller, B.K., Bonhoeffer, F., and Drescher, U. (1997). Shared and distinct functions of RAGS and ELF-1 in guiding retinal axons. *EMBO J.* 16, 1258-1267.

Mullen, R.J., Buck, C.R., and Smith, A.M. (1992). NeuN, a neuronal specific nuclear protein in vertebrates. *Development* 116, 201-211.

Nakai, J., Dirksen, R.T., Nguyen, H.T., Pessah, I.N., Beam, K.G., and Allen, P.D. (1996). Enhanced dihydropyridine receptor channel activity in the presence of

- ryanodine receptor. *Nature* 380, 72-75.
- Nakai, J., Ogura, T., Protasi, F., Franzini-Armstrong, C., Allen, P.D., and Beam, K.G. (1997). Functional nonequality of the cardiac and skeletal ryanodine receptors. *Proc Natl Acad Sci U S A* 94, 1019-22.
- Nakai, K. and Kanehisa, M. (1992). A knowledge base for predicting protein localization sites in eukaryotic cells. *Genomics* 14, 897-911.
- Nicoll, D.A., Longoni, S., and Philipson, K.D. (1990). Molecular cloning and functional expression of the cardiac sarcolemmal Na(+)-Ca²⁺ exchanger. *Science* 250, 562-5.
- Nielsen, H., Engelbrecht, J., Brunak, S., and von Heijne, G. (1997). Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng.* 10, 1-6.
- O'Shea, E.K., Rutkowski, R., and Kim, P.S. (1989). Evidence that the leucine zipper is a coiled coil. *Science* 243, 538-542.
- O'Shea, E.K., Klemm, J.D., Kim, P.S., and Alber, T. (1991). X-ray structure of the GCN4 leucine zipper, a two-stranded, parallel coiled coil. *Science* 254, 539-544.
- O'Shea, E.K., Rutkowski, R., and Kim, P.S. (1992). Mechanism of specificity in the Fos-Jun oncoprotein heterodimer. *Cell* 68, 699-708.
- Opie, L.H. (1991). *The Heart Physiology and Metabolism* (New York: Raven Press).
- Ortiz-Lopez, R., Li, H., Su, J., Goytia, V., and Towbin, J.A. (1997). Evidence for a dystrophin missense mutation as a cause of x-linked dilated cardiomyopathy [see comments]. *Circulation* 95, 2434-40.
- Page, E. and McCallister, L.P. (1973). Quantitative electron microscopic description of heart muscle cells. application to normal, hypertrophied and thyroxin-stimulated hearts. *Am J Cardiol* 31, 172-181.
- Paululat, A., Goubeaud, A., Damm, C., Knirr, S., Burchard, S., and Renkawitz-Pohl, R. (1997). The Mesodermal Expression *rolling stone (rost)* is Essential for Myoblast Fusion in *Drosophila* and Encodes a Potential Transmembrane Protein. *J. Cell Biol.* 138, 337-348.
- Pollerberg, E.G., Sadoul, R., Goridis, C., and Schachner, M. (1985). Selective expression of the 180-kD component of the neural cell adhesion molecule N-CAM during development. *J. Cell Biol.* 101, 1921-1929.

- Powell, J.A., Petherbridge, L., and Flucher, B.E. (1996). Formation of triads without the dihydropyridine receptor alpha subunits in cell lines from dysgenic skeletal muscle. *J Cell Biol* 134, 375-87.
- Pragnell, M., De Waard, M., Mori, Y., Tanabe, T., Snutch, T.P., and Campbell, K.P. (1994). Calcium channel beta-subunit binds to a conserved motif in the I-II cytoplasmic linker of the alpha 1-subunit [see comments]. *Nature* 368, 67-70.
- Pratt, M.A., Kralova, J., and McBurney, M.W. (1990). A dominant negative mutation of the alpha retinoic acid receptor gene in a retinoic acid-nonresponsive embryonal carcinoma cell. *Mol. Cell Biol.* 10, 6445-6453.
- Protasi, F., Franzini-Armstrong, C., and Flucher, B.E. (1997). Coordinated incorporation of skeletal muscle dihydropyridine receptors and ryanodine receptors in peripheral couplings of BC3H1 cells. *J. Cell Biol.* 137, 859-870.
- Rechsteiner, M. and Rogers, S.W. (1996). PEST sequences and regulation by proteolysis. *Trends Biochem. Sci.* 21, 267-271.
- Reeves, J.P. and Hale, C.C. (1984). The stoichiometry of the cardiac sodium-calcium exchange system. *J Biol Chem* 259, 7733-9.
- Rogers, S., Wells, R., and Rechsteiner, M. (1986). Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. *Science* 234, 364-368.
- Roth, M.B., Zahler, A.M., and Stolk, J.A. (1991). A conserved family of nuclear phosphoproteins localized to sites of polymerase II transcription. *J. Cell Biol.* 115, 587-596.
- Rudnicki, M.A. and McBurney, M.W. (1987). Cell culture methods and induction of differentiation of embryonal carcinoma cell lines. In *Teratocarcinomas and embryonic stem cells*. E.J. Robertson, ed. (Oxford, England: IRL Press), pp. 19-49.
- Sacher, M., Stone, S., and Ferro-Novick, S. (1997). The synaptobrevin-related domains of bos1p and sec22p bind to the syntaxin-like region of sed5p. *J Biol Chem* 272, 17134-8.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).
- Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. U. S. A.* 74, 5463-5467.
- Sapperstein, S.K., Lupashin, V.V., Schmitt, H.D., and Waters, M.G. (1996).

- Assembly of the ER to golgi snare complex requires usolp. *J Cell Biol* 132, 755-67:
- Sasaki, T., Inui, M., Kimura, Y., Kuzuya, T., and Tada, M. (1992). Molecular mechanism of regulation of Ca^{2+} pump atpase by phospholamban in cardiac sarcoplasmic reticulum. effects of synthetic phospholamban peptides on Ca^{2+} pump atpase. *J Biol Chem* 267, 1674-9:
- Sather, W.A., Yang, J., Tsien, R.W., Tang, S., Mikala, G., Bahinski, A., Yatani, A., Varadi, G., and Schwartz, A. (1993). Structural basis of ion channel permeation and selectivity Molecular localization of ion selectivity sites within the pore of a human l-type cardiac calcium channel. *Curr Opin Neurobiol* 268, 13026-9:
- Savitt, J.M., Trisler, D., and Hilt, D.C. (1995). Molecular cloning of TOPAP: a topographically graded protein in the developing chick visual system. *Neuron* 14, 253-261.
- Schlessinger, A.R., Cowan, W.M., and Gottlieb, D.I. (1975). An autoradiographic study of the time of origin and the pattern of granule cell migration in the dentate gyrus of the rat. *J. Comp. Neurol.* 159, 149-175.
- Schwartz, L.M., McCleskey, E.W., and Almers, W. (1985). Dihydropyridine receptors in muscle are voltage-dependent but most are not functional calcium channels. *Nature* 314, 747-751.
- Sheng, Z.H., Rettig, J., Takahashi, M., and Catterall, W.A. (1994). Identification of a syntaxin-binding site on N-type calcium channels. *Neuron* 13, 1303-1313.
- Sheng, Z.H., Rettig, J., Cook, T., and Catterall, W.A. (1996). Calcium-dependent interaction of N-type calcium channels with the synaptic core complex. *Nature* 379, 451-454.
- Sheng, Z.H., Yokoyama, C.T., and Catterall, W.A. (1997). Interaction of the synprint site of N-type Ca^{2+} channels with the C2B domain of synaptotagmin I. *Proc. Natl. Acad. Sci. U. S. A.* 94, 5405-5410.
- Sheu, S.S. and Fozzard, H.A. (1982). Transmembrane Na^{+} and Ca^{2+} electrochemical gradients in cardiac muscle and their relationship to force development. *J Gen Physiol* 80, 325-51:
- Simon, D.K. and O'Leary, D.D. (1990). Limited topographic specificity in the targeting and branching of mammalian retinal axons. *Dev. Biol.* 137, 125-134.
- Simon, D.K. and O'Leary, D.D. (1992). Development of topographic order in the mammalian retinocollicular projection. *J. Neurosci.* 12, 1212-1232.

- Smith, T.W. (1988). Digitalis. mechanisms of action and clinical use. *N Engl J Med* 318, 358-65:
- Sperry, R.W. (1963). Chemoaffinity in the orderly growth of nerve fiber patterns and connections. *Proc. Natl. Acad. Sci. U. S. A.* 50, 735-743.
- Spring, J., Kato, M., and Bernfield, M. (1993). Epimorphin is related to a new class of neuronal and yeast vesicle targeting proteins. *Trends. Biochem. Sci.* 18, 124-125.
- Striessnig, J., Glossmann, H., and Catterall, W.A. (1990). Identification of a phenylalkylamine binding region within the alpha 1 subunit of skeletal muscle Ca²⁺ channels. *Proc. Natl. Acad. Sci. U. S. A.* 87, 9108-9112.
- Sun, X.H., Protasi, F., Takahashi, M., Takeshima, H., Ferguson, D.G., and Franzini-Armstrong, C. (1995). Molecular architecture of membranes involved in excitation-contraction coupling of cardiac muscle. *J. Cell Biol.* 129, 659-671.
- Takekura, H., Nishi, M., Noda, T., Takeshima, H., and Franzini-Armstrong, C. (1995a). Abnormal junctions between surface membrane and sarcoplasmic reticulum in skeletal muscle with a mutation targeted to the ryanodine receptor. *Proc. Natl. Acad. Sci. U. S. A.* 92, 3381-3385.
- Takekura, H., Takeshima, H., Nishimura, S., Takahashi, M., Tanabe, T., Flockerzi, V., Hofmann, F., and Franzini-Armstrong, C. (1995b). Co-expression in CHO cells of two muscle proteins involved in excitation-contraction coupling. *J Muscle Res Cell Motil* 16, 465-80:
- Tanabe, T., Beam, K.G., Powell, J.A., and Numa, S. (1988). Restoration of excitation-contraction coupling and slow calcium current in dysgenic muscle by dihydropyridine receptor complementary DNA. *Nature* 336, 134-139.
- Tanabe, T., Beam, K.G., Adams, B.A., Niidome, T., and Numa, S. (1990a). Regions of the skeletal muscle dihydropyridine receptor critical for excitation-contraction coupling. *Nature* 346, 567-569.
- Tanabe, T., Mikami, A., Numa, S., and Beam, K.G. (1990b). Cardiac-type excitation-contraction coupling in dysgenic skeletal muscle injected with cardiac dihydropyridine receptor cDNA. *Nature* 344, 451-453.
- Tessier-Lavigne, M. (1995). Eph receptor tyrosine kinases, axon repulsion, and the development of topographic maps. *Cell* 82, 345-348.
- Timerman, A.P., Onoue, H., Xin, H.B., Barg, S., Copello, J., Wiederrecht, G., and Fleischer, S. (1996). Selective binding of FKBP12.6 by the cardiac ryanodine

receptor. *J Biol Chem* 271, 20385-91:

Titus, J.A., Haugland, R., Sharrow, S.O., and Segal, D.M. (1982). Texas red, a hydrophilic, red-emitting fluorophore for use with fluorescein in dual parameter flow microfluorometric and fluorescence microscopic studies. *J Immunol Methods* 50, 193-204:

Towbin, H., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U. S. A.* 76, 4350-4354.

Tsuchida, M., Takahara, H., Minami, N., Arai, T., Kobayashi, Y., Tsujimoto, H., Fukazawa, C., and Sugawara, K. (1993). cDNA nucleotide sequence and primary structure of mouse uterine peptidylarginine deaminase. detection of a 3'-untranslated nucleotide sequence common to the mRNA of transiently expressed genes and rapid turnover of this enzyme's mRNA in the estrous cycle. *Eur J Biochem* 215, 677-85:

Tuana, B.S., Murphy, B.J., and Schwarzkopf, C. (1987a). A calmodulin dependent protein kinase activity associated with rabbit heart sarcolemma. *Mol Cell Biochem* 78, 47-54:

Tuana, B.S., Murphy, B.J., and Yi, Q. (1987b). Subcellular distribution and isolation of the Ca²⁺ antagonist receptor associated with the voltage regulated Ca²⁺ channel from rabbit heart muscle. *Mol. Cell Biochem.* 76, 173-184.

Tuana, B.S., Sunahara, R.K., Murphy, B.J., and Pierce, G.N. (1991). Two major antigens of heart sarcolemma are Ca²⁺(+)-binding glycoproteins that copurify with the dihydropyridine receptor. *Biochim. Biophys. Acta* 1091, 236-241.

Vinson, C.R., Hai, T., and Boyd, S.M. (1993). Dimerization specificity of the leucine zipper-containing bZIP motif on DNA binding: prediction and rational design. *Genes Dev.* 7, 1047-1058.

Wang, J. and Best, P.M. (1992). Inactivation of the sarcoplasmic reticulum calcium channel by protein kinase. *Nature* 359, 739-41:

Whitley, P., Grahn, E., Kutay, U., Rapoport, T.A., and von Heijne, G. (1996). A 12-residue-long polyleucine tail is sufficient to anchor synaptobrevin to the endoplasmic reticulum membrane. *J. Biol. Chem.* 271, 7583-7586.

Yaffe, D. and Saxel, O. (1977). Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. *Nature* 270, 725-7:

Yang, M., Ellenberg, J., Bonifacino, J.S., and Weissman, A.M. (1997). The

transmembrane domain of a carboxyl-terminal anchored protein determines localization to the endoplasmic reticulum. *J. Biol. Chem.* 272, 1970-1975.

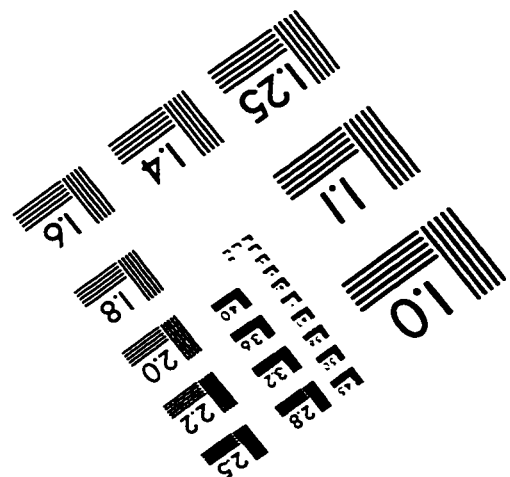
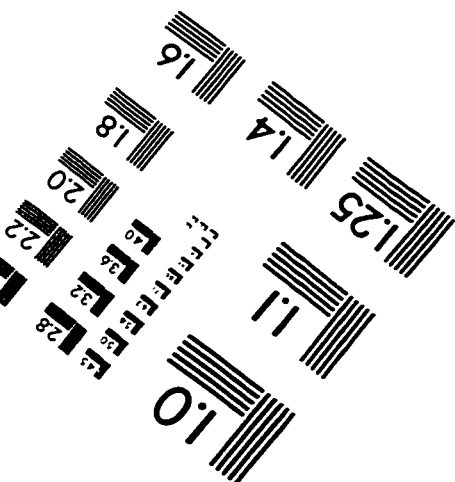
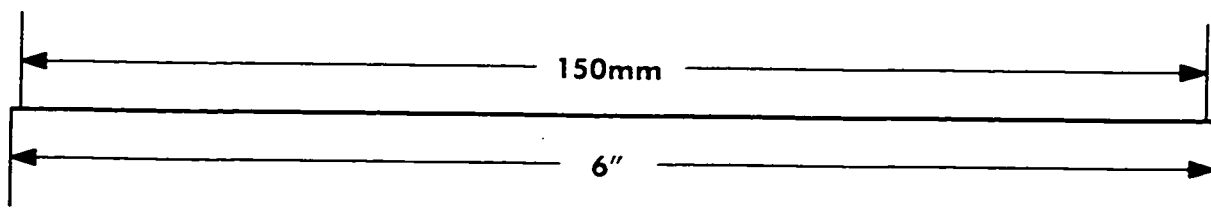
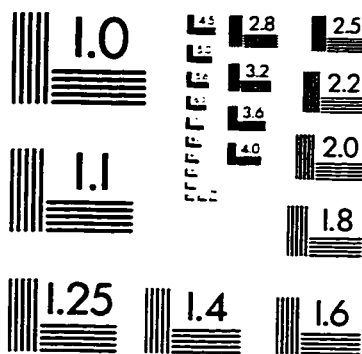
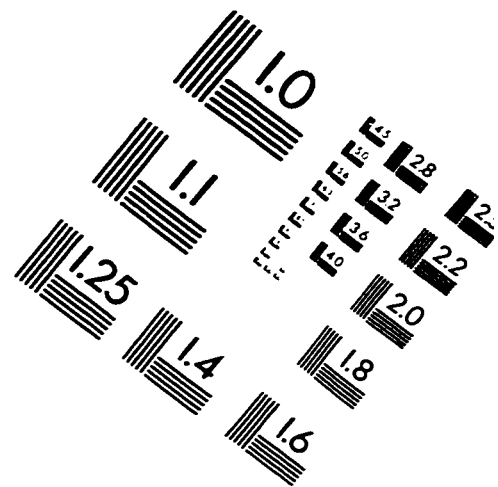
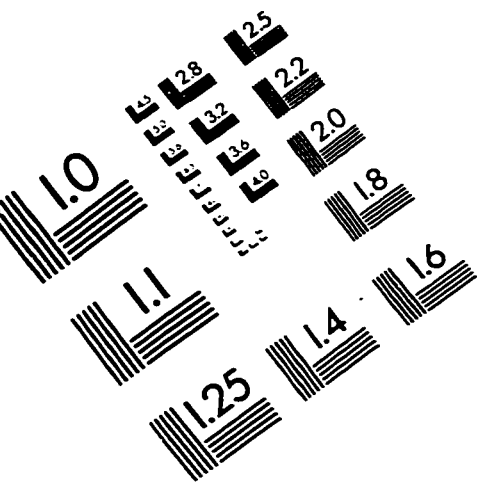
Yeilding, N.M. and Lee, W.M. (1997). Coding elements in exons 2 and 3 target c-myc mrna downregulation during myogenic differentiation. *Mol Cell Biol* 17, 2698-707.

Yuan, S., Arnold, W., and Jorgensen, A.O. (1990). Biogenesis of transverse tubules: immunocytochemical localization of a transverse tubular protein (ts28) and a sarcolemmal protein (sl50) in rabbit skeletal muscle developing in situ. *Journal of Cell Biology* 110, 1187-1198.

Yuan, S.H., Arnold, W., and Jorgensen, A.O. (1991). Biogenesis of transverse tubules and triads: immunolocalization of the 1,4-dihydropyridine receptor, ts28, and the ryanodine receptor in rabbit skeletal muscle developing in situ. *Journal of Cell Biology* 112, 289-301.

Zhang, L., Kelley, J., Schmeisser, G., Kobayashi, Y.M., and Jones, L.R. (1997). Complex formation between junctin, triadin, calsequestrin, and the ryanodine receptor. proteins of the cardiac junctional sarcoplasmic reticulum membrane. *J Biol Chem* 272, 23389-97.

IMAGE EVALUATION TEST TARGET (QA-3)



APPLIED IMAGE, Inc.
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989

© 1993, Applied Image, Inc., All Rights Reserved