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THE CLONING AND CHARACTERIZATION OF THE TIK KINASE

Pamela L. Icely

A thesis
presented to the University of Ottawa
in partial fulfillment of the
requirements for the degree of
Master of Science

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ABSTRACT:

Protein phosphorylation is a mechanism of modulating protein activity which plays a central role in a wide variety of cellular functions. The ready reversibility of the phosphotransfer reaction makes it well-suited for rapid cellular responses, such as the responses to signals from extracellular stimuli. Members of the protein kinase family have been identified as components of cellular signal transduction networks, and in fact many protein kinases mediate signals controlling cell growth, differentiation, and development.

The work carried out in this thesis describes the cloning and characterization of the TIK kinase, a novel member of the protein kinase family. The TIK kinase was isolated and cloned from murine pre-B cells on the basis of its immunoreactivity with antibodies to phosphotyrosine, and was therefore originally thought to be a protein tyrosine kinase. Characterization of the *in vitro* and *in vivo* activity of this kinase, however, revealed that the TIK kinase possesses only serine and threonine phosphorylating ability. A kinase deficient TIK protein, constructed by site-directed mutagenesis, is not immunoreactive with the antiphosphotyrosine antibodies. These observations indicate that phosphoserine and/or phosphothreonine residues may be assuming a conformation within the context of this protein which mimicks the epitope(s) recognized by antiphosphotyrosine antibodies.

The gene encoding the TIK kinase is transcribed to produce three distinct messenger RNA (mRNA) transcripts in all murine tissues examined, and in a number of murine leukemic cell lines. In the murine lymphocytic leukemia line L1210,

however, three truncated mRNA molecules are produced in addition to the three normal transcripts detected in other tissues. Evidence is presented here that one allele of the TIK gene has undergone a genomic rearrangement in this cell line, and appears to be giving rise to the abnormal pattern of mRNA expression observed. A cDNA corresponding to the smallest truncated mRNA transcript encoding the TIK kinase in the L1210 cell line has been obtained, and this cDNA encodes a mutant TIK protein lacking kinase activity. I postulate that the TIK kinase plays some role in the control of growth and differentiation during lymphocyte development, and that the mutation of the TIK gene has contributed to the initiation or maintenance of transformation in the L1210 cell line.

ACKNOWLEDGEMENTS:

I would like to express my gratitude and to allow recognition of some of the individuals who have contributed to the completion of this work:

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- My co-workers not mentioned above, E.M.J. Douville, B.W. Howell, T. Marshall, and D.E.H. Afar, for frequent help and advice.

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Chapter One

INTRODUCTION:

In multicellular organisms, strict control must exist over the processes of cellular proliferation and differentiation. Thus complex networks of regulatory molecules have evolved to detect and transmit signals within and between cells. Currently, attempts are being made to dissect the pathways that transduce signals controlling cellular growth and development. The identification of enzymes which are mediators of such signals has been facilitated by the study of the protein products of certain oncogenes. This work has clearly implicated the protein kinase family of enzymes in the control of cell growth and development.

The work carried out in this thesis was directed toward the cloning and characterization of a novel member of the protein kinase family. The information obtained from this project will contribute to the existing knowledge of this family of enzymes, and will aid in the understanding of the role these proteins play in the process of cellular growth, differentiation, and development.

Outlined in the following is a brief description of the protein kinase family of enzymes, and a summary of what is known about the involvement of these enzymes in the control of cellular growth and development. Following this is an outline of the evidence implicating protein kinases in the control of hematopoiesis and hematopoietic development.

1.1 Properties of Protein Kinases:

The protein kinases make up a diverse group of enzymes characterized by a

highly conserved catalytic domain. This domain contains regions of short sequence motifs which are required for ATP binding and substrate phosphorylation (1). Protein kinases can be broadly classified by their amino acid substrate specificty. Although the catalytic domains of the protein serine/threonine kinases (PSKs) and protein tyrosine kinases (PTKs) are highly homologous, they can be distinguished by certain primary sequence features.

Since the identification of the first kinase in 1959 (2), over one hundred kinases have been identified, and it has been estimated that the mammalian genome may encode as many as a thousand different protein kinases (3). Although these molecules have multiple cellular functions in processes such as metabolism, their central role in cellular growth and differentiation control became apparent with the observation that almost half of all known oncogene products encode kinases (4,5).

The receptor mediated recognition of growth factors and hormones initiates cellular signals which commonly proceed through the activation of intracellular protein kinases. In order for these signals to elicit pleiotropic responses, the primary signal-transducing kinase must activate several pathways. This can be effected by the activation of other protein kinases through phosphorylation. In this way, the signal is propagated and amplified by a network of kinases (6). The identification of members of the PTK family as transmembrane molecules or membrane-associated cytoplasmic molecules indicated that these kinases were well positioned to act as primary signal transducers. PSKs are predominantly cytoplasmic proteins, possibly acting to serve as the link between received extracellular signals and alterations in

cellular processes (7). Further complexity has been added to this picture with the recent identification of kinases capable of phosphorylating all three hydroxyamino acids.

1.1.1 Protein Tyrosine Kinases:

Transmembrane PTKs:

Receptors for a number of growth factors and hormones are transmembrane PTKs, each having a ligand binding extracellular domain, a transmembrane domain, and an intracellular catalytic domain (see figure 1). The receptor PTKs generally exist in the cell in a down-regulated state, and ligand binding results in an increase in tyrosine kinase activity. It has been shown that PTK activity is essential for the mitogenic signals transduced by these receptors. Examples of this type of PTK are the epidermal growth factor (EGF) and platelet derived growth factor (PDGF) receptors (for a review, see 8). Ligand binding is thought to induce dimerization of the receptor molecules, allowing transphosphorylation between paired kinases. Good evidence for this model has been obtained for the EGF and PDGF receptors (9-11). All receptor PTKs undergo autophosphorylation in response to ligand binding, and this appears to correlate with an increase in activity toward other substrates (12). It has been suggested that the autophosphorylation sites act to block access of the catalytic domain to other substrates until phosphorylated. In fact, truncation of the EGF receptor to remove the autophosphorylation sites enhances the transforming ability of this kinase in erythroid cells (13). Activity of the receptor PTKs can also

Figure 1: A comparison of the receptor PTK and cytoplasmic PTK molecules
Receptor PTKs contain an extracellular ligand binding domain, a transmembrane domain, and a catalytic domain. This catalytic domain is highly conserved among all protein kinases. The non-receptor type PTKs contain SH2 domains, and members of the src family also contain an SH3 domain, and a glycine residue which allows them to associate with the membrane through a myristylation event (shown here as MYR).

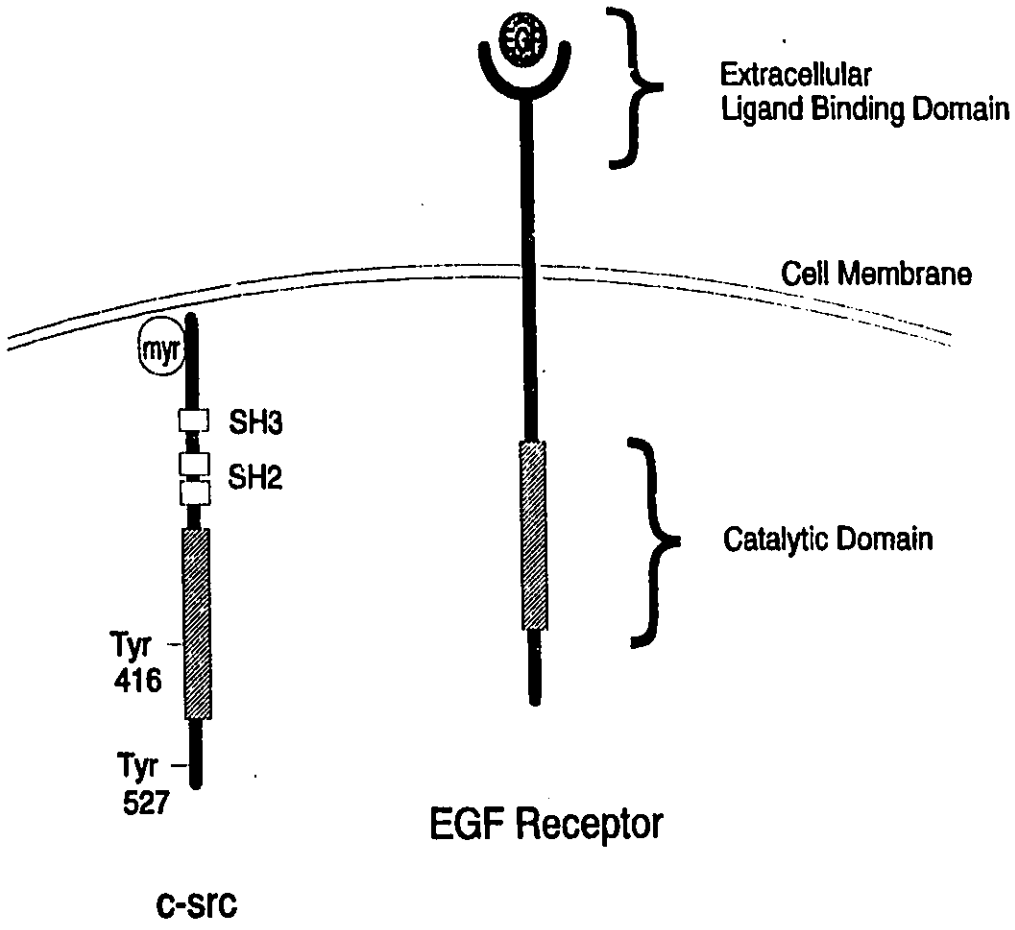

Extracellular
Ligand Binding Domain
Cell Membrane
myr
SH3
SH2
Catalytic Domain
Tyr
416
Tyr
527
EGF Receptor
c-src

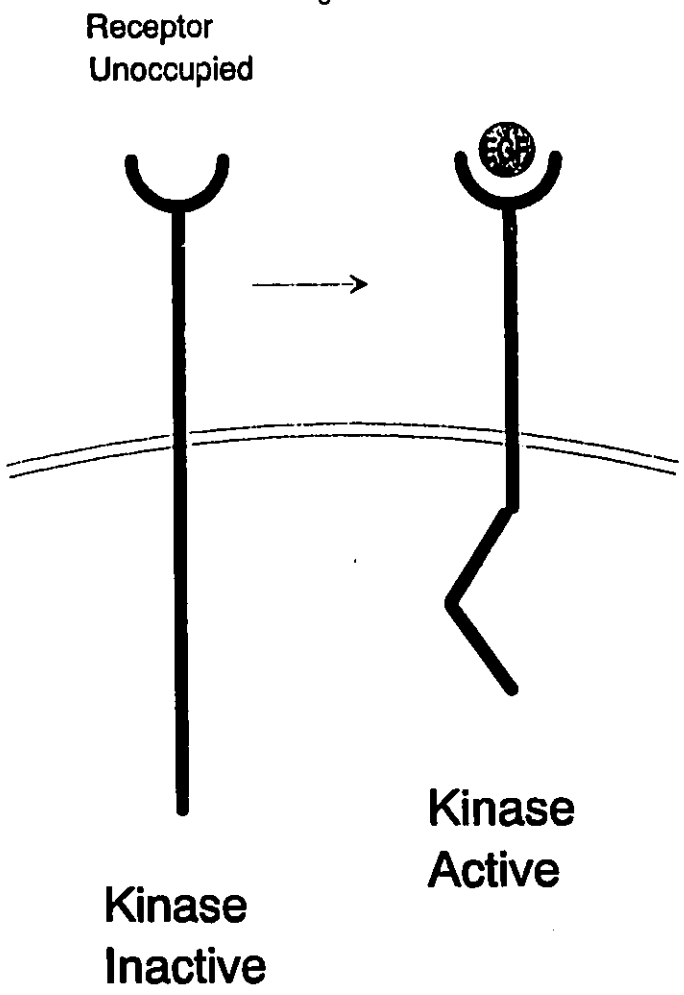
Figure 1

be regulated through phosphorylation by other kinases. Phosphorylation of the EGF receptor by the PSK protein kinase C results in a decrease in its activity (14).

The relationship of certain PTK oncogene products to growth factor receptors indicates that the constitutive activity of receptor PTKs, which are normally activated by binding growth factors, can play a part in neoplastic transformation. Consistent with this idea is the observation that growth factors themselves can act as oncogenes (15). Comparisons of structure and function have determined the molecular alterations converting normal growth factor receptors to oncogenes inducing cell transformation, and provided evidence for the link between PTK activity and cell proliferation.

Characterization of the product of the erbB oncogene of avian erythroblastosis virus revealed that the erbB protein is a truncated form of the EGF receptor from which the extracellular EGF binding domain has been deleted (16). The erbB protein also contains other mutations, including a point mutation in the kinase domain and deletion of a portion of the carboxy-terminal region, but the amino-terminal deletion is essential for its transforming ability (17,18). The biochemical consequence of this deletion is to generate an enzyme which is a constitutively active PTK. As discussed above, the kinase activity of the normal EGF receptor is expressed only subsequent to EGF binding (figure 2). Deletion of the EGF binding region from the erbB molecule has by-passed this normal regulatory system, and in the absence of negative regulation, this truncated kinase is responsible for abnormal cell proliferation. Similar mutations involving deletion of the amino-terminal ligand

Figure 2: Oncogenic activation of the erb B kinase
Tyrosine kinase activity of the EGF receptor (the erb B proto-oncogene) is regulated by ligand binding. The erb B oncogene product is deregulated and constitutively active.


Receptor
Unoccupied
Kinase
Active
Kinase
Inactive

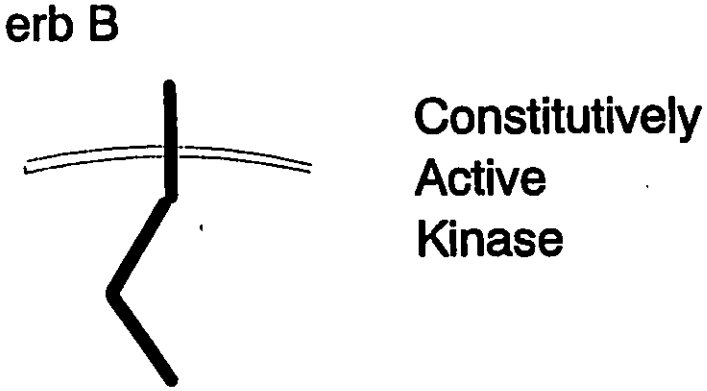

erb B
Constitutively
Active
Kinase

Figure 2

binding domains of other growth factor receptor PTKs such as the ros and trk proto-oncogene products have been found to activate the transforming potential of these kinases (19,20). As in the case of erbB, these mutations generate a protein with constitutive unregulated PTK activity. Other mutations unmasking the transforming potential of receptor PTKs also lead to elevated PTK activity. The neu oncogene product contains a single point mutation in the transmembrane domain which is essential for the transforming ability of this mutant PTK. This mutation is believed to cause aggregation of the receptors in the absence of ligand, mimicking ligand-incuded aggregation and activating the PTK activity of the neu enzymes (21). It appears that elevated or deregulated PTK activity, normally only expressed in response to the binding of growth factors or hormones, is responsible for the transforming ability of oncogenes generated through mutation of receptor PTKs.

Cytoplasmic PTKs:

The non-receptor PTKs are subdivided into the src, fps, and abl families. Although these kinases are not transmembrane proteins, many of them are associated with membranes. The src family PTKs are attached to the inner face of cytoplasmic membranes via an amino-terminal myristyl group. This membrane attachment is essential for the function of these kinases. In addition to the cytoplasmic PTKs encoded by the src and fps families, the type IV c-abl protein has been found to be localized in the nucleus (22). Like the receptor PTKs, nonreceptor PTKs generally exist in the cell in an inactive state. The exact role of the nonreceptor PTKs in

signal transduction has not been identified, but it is thought that they may associate with surface receptors which lack their own protein kinase domains. This idea followed the discovery that the PTK lck, which is a member of the src family, is associated with the cell surface proteins CD4 and CD8 in T cells (23) Stimulation of T lymphocytes through their antigen receptor requires the interaction of either the CD8 or CD4 molecules with MHC 1 or MHC II molecules on the antigen presenting cell. This interaction results in the activation of lck, and this increase in lck PTK activity is associated with the rapid phosphorylation of one of the subunits of the T cell receptor on tyrosine residues (figure 3) (24). These results suggest that CD4 can function as a signal transducer and that tyrosine phosphorylation events may be important in CD4-mediated signalling. A direct link between other cytoplasmic PTKs and transmembrane receptor molecules has not been identified, but there is evidence that cytoplasmic PTKs are activated by growth factors and other cellular activators. In platelets, thrombin acitivates a number of the src family member PTKs (25).

The src family PTKs are regulated by their phosphorylation state. Phosphorylation of a carboxy-terminal tyrosyl residue lying just downstream of the catalytic domain negatively regulates PTK activity (for a review, see 26). In the c-src molecule this is Tyr 527. Under some conditions, the src kinase is capable of autophosphorylating this residue (27), suggesting that negative feedback can occur. Studies of a kinase-inactive mutant src molecule, however, show that the mutant molecule is phosphorylated at Tyr 527 *in vivo*, indicating the involvement of another PTK (28). Dephosphorylation of Tyr 527 by a phosphotyrosine phosphatase would

Figure 3: Signal transduction by the PTK lck

T cell activation by antigen-presenting cells involves an interaction between the T cell receptor and the antigen presented in the context of Major Histocompatability Complex (MHC) class I or class II molecules. The CD4 or CD8 molecules interact with either the MHC class II or class I molecules, respectively, and the phosphotyrosine phosphatase CD45 presumably interacts with a ligand which is yet to be identified. Dephosphorylation of Tyr 505 of the lck kinase by CD45 leads to an increase in its kinase activity, and phosphorylation of one of the subunits of the T cell receptor.

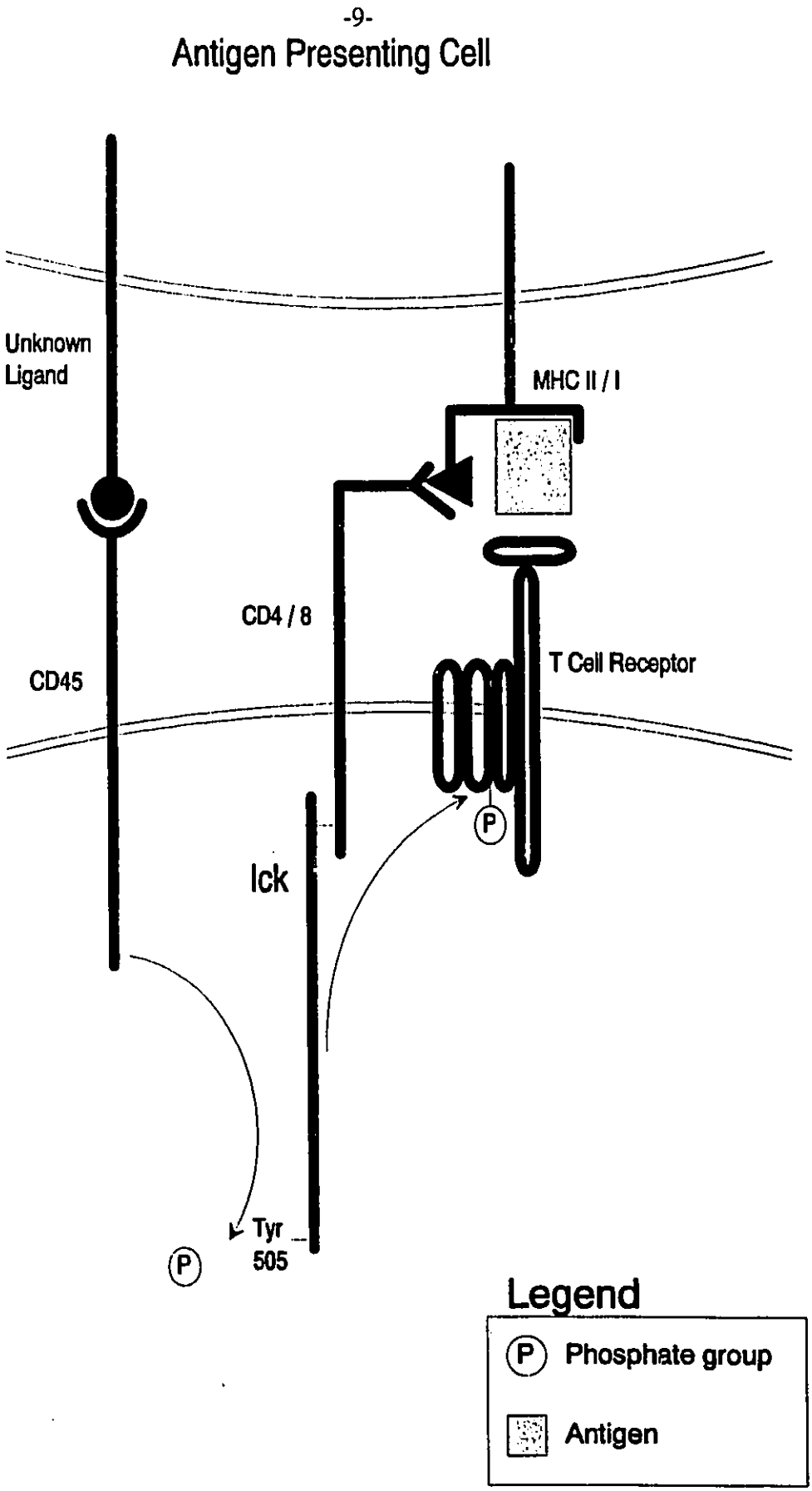

Antigen Presenting Cell
Unknown Ligand
MHC II / I
CD4 / 8
CD45
T Cell Receptor
P
lck
Tyr 505
P
Legend
P Phosphate group
Antigen

Figure 3

result in activation of the src kinase. The lck kinase is negatively regulated by phosphorylation of Tyr 505. *In vitro* studies carried out by Mustelin *et al* (29) have shown that the lck Tyr 505 residue is dephosphorylated by the transmembrane T cell phosphotyrosine phosphatase CD45 (figure 3). It is possible that all the cytoplasmic PTKs are r ... eceptor phosphotyrosine phosphatases like CD45. Another phosphorylation ... regulating the activity of c-src occurs on Tyr 416, a conserved residue lying in the catalytic domain. Although this residue in c-src is not detectably phosphorylated *in vivo*, it is the major *in vitro* autophosphorylation site (30). It is also the major phosphorylation site of the virally-encoded transforming v-src kinase, and site-directed mutagenesis of this residue to phenylalanine reduces the transforming ability of the kinase (31). This result indicates that phosphorylation of Tyr 416 could be a mechanism of autoactivation, as it is for receptor PTKs.

Although activation of the oncogenic potential of the cytoplasmic PTKs occurs through a number of different mechanisms, the end result in each case is to generate a transforming protein with deregulated PTK activity. The v-src oncogene, encoded by Rous s...rcoma virus, encodes an altered src protein lacking nineteen amino acids of the normal src carboxy termi... domain. The tyrosine residue at amino acid 527 is deleted from the v-src molecule, so the activity of this kinase can not be down-regulated by phosphorylation of this residue (figure 4). This deregulating mutation activates the transforming ability of src (32). The abl and fes oncogenes encode proteins whose transforming potential is activated through increased PTK activity. Mutation of the c-abl proto-oncogene to the transforming abl oncogene is discussed

Figure 4: Regulation of src kinase activity by its phosphorylation state
The c-src molecule is down-regulated by phosphorylation on Tyr 527, and activated when phosphorylated on Tyr 416. The Tyr 527 residue has been deleted from the v-src oncogene product, which is activated as a kinase and autophosphorylates on Tyr 416.

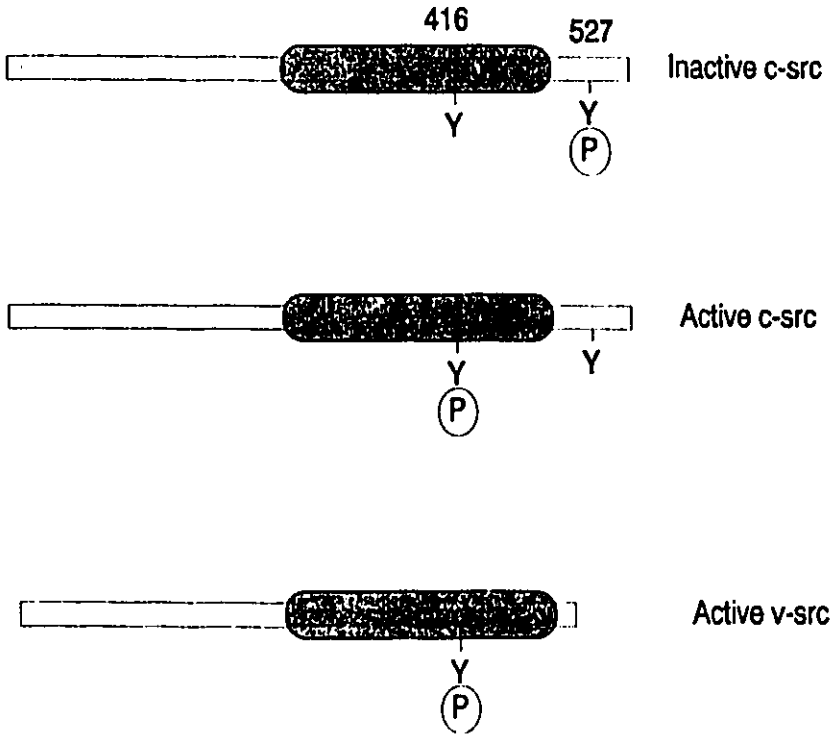


Figure 4

in detail later. The viral fes oncogene is expressed as a fusion protein with the viral *gag* protein, and this mutation alone appears to be sufficient to activate transforming ability and kinase activity (33).

In contrast to the receptor PTKs, all cytoplasmic PTKs share a domain called SH2, for src homology region 2 (34) (see figure 1). Members of the src and abl families also contain a second region termed SH3. The SH2 domain was originally identified in the v-fps and v-src kinases because of its effects on the catalytic activity and substrate specificity of these kinases (34). Mutation of the SH2 domains of these kinases either reduces kinase activity and transforming ability, or renders transforming ability host-dependent (35,36). The observation that SH2 mutation introduced host-range dependency to transforming ability implicated the SH2 domain in protein-protein interactions. These domains are in fact involved in the recognition and binding of sequences encompassing tyrosine phosphorylation sites (37). The function of the SH2 domain in cellular signal transduction will be outlined in the following section. The SH3 domain is a motif of approximately 45 residues (38). Although the function of this domain remains unclear at present, it has been determined that mutation of the SH3 domain of the c-src kinase results in oncogenic activation, suggesting that SH3 normally negatively regulates PTK activity (39). SH3 domains have also been identified in a number of proteins which make up or associate with the cellular cytoskeleton (40), possibly suggesting a role for this domain in subcellular localization.

1.1.2 Protein Serine/Threonine Kinases:

In mammalian cells, most of the protein kinase activity results in the phosphorylation of serine and threonine residues, rather than tyrosine residues. At least fifty PSKs have been identified, and these enzymes regulate a wide variety of metabolic processes. Many members of the PSK family are cytoplasmic kinases which are activated by second messengers, and these kinases play a role in intracellular transmission of the signal from such molecules (41). These types of enzymes are generally classified by their mode of regulation. The activity of the cyclic nucleotide-regulated PSKs is increased through elevation of the levels of cAMP or cGMP in the cell. An example of signal transduction via the cAMP system is provided by the control of glycogen metabolism in the liver (figure 5). In this system, the PSKs cAMP-dependent protein kinase and phosphorylase kinase act in series to couple the hormone-stimulated production of cAMP to a metabolic response (production of glucose from glycogen). The binding of hormone results in G-protein mediated activation of adenylate cyclase, which increases the cellular cAMP levels and leads to the activation of cAMP-dependent kinase. In the down-regulated state, cAMP-dependent kinase exists as multimer made up of two regulatory (R) and two catalytic (C) subunits. Two cAMP molecules bind to each regulatory subunit, resulting in dissociation and activation of the catalytic sunbunits. The catalytic subunit is now constitutively active rather than cAMP dependent. The activity of phosphorylase kinase is increased following phosphorylation by the catalytic subunit of cAMP-dependent kinase (42), and once activated this kinase phosphorylates

Figure 5: Regulation of glycogen metabolism by a protein phosphorylation cascade Hormone binding results in G-protein mediated activation of adenylate cyclase, which increases the intracellular levels of cAMP, activating cAMP dependent kinase. The dissociated catalytic subunits of cAMP dependent kinase phosphorylate and activate phosphorylase kinase, which in turn phosphorylates and activates glycogen phosphorylase.

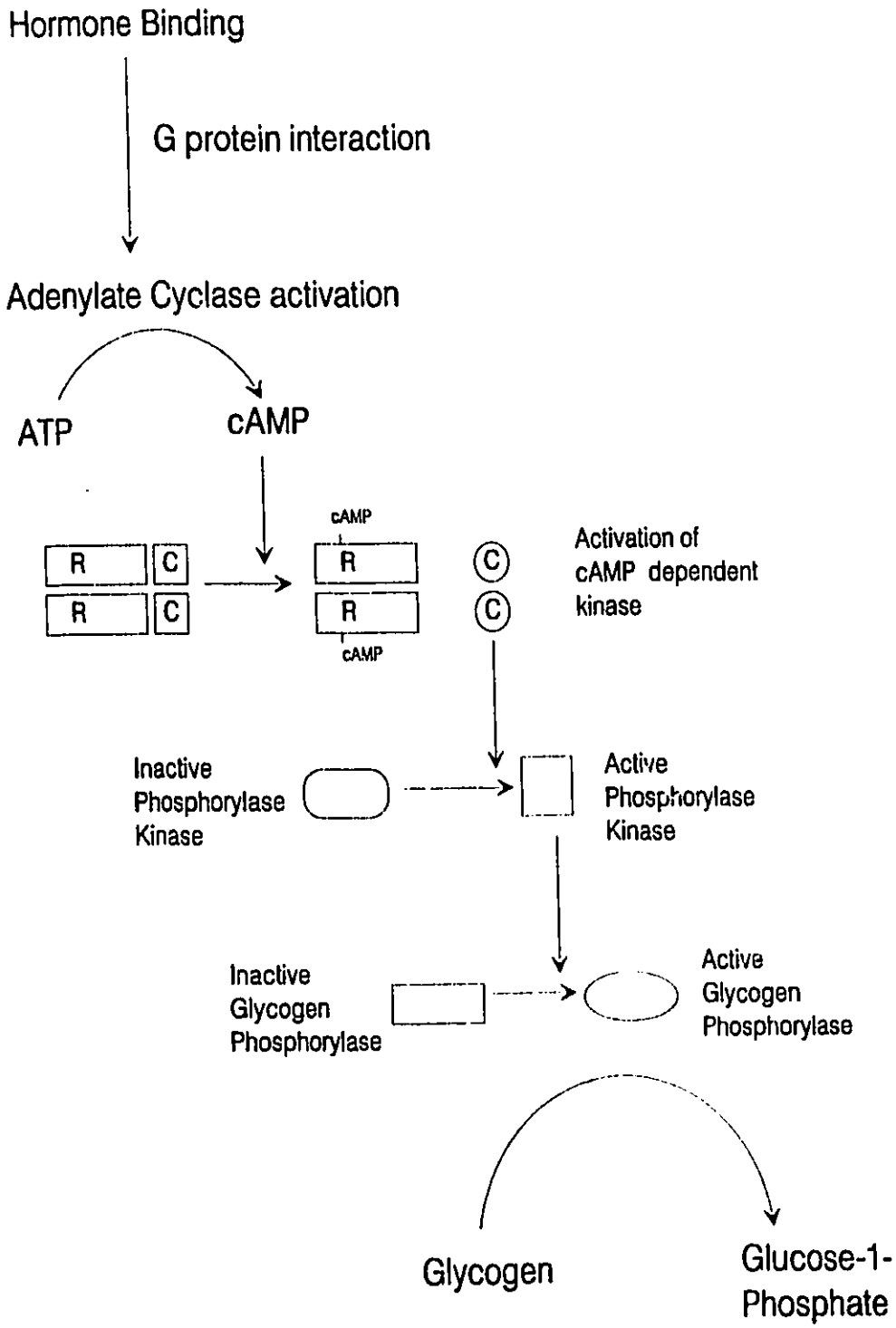

Hormone Binding
G protein interaction
Adenylate Cyclase activation
ATP
cAMP
R
C
R
C
cAMP
R
R
cAMP
C
C
Activation of cAMP dependent kinase
Inactive Phosphorylase Kinase
Active Phosphorylase Kinase
Inactive Glycogen Phosphorylase
Active Glycogen Phosphorylase
Glycogen
Glucose-1-Phosphate

Figure 5

glycogen phosphorylase, which catalyzes the conversion of glycogen to glucose-1-phosphate.

The second messengers generated by hydrolysis of inositol phospholipids also activate PSKs. Members of the protein kinase C family of kinases are stimulated by diacylglycerol, which is produced through turnover of phosphoinositides in response to growth factors or hormones. The release of calcium from intracellular stores results in the activation of calcium/calmodulin dependent PSKs. The activity of PSKs, as well as PTKs, is clearly implicated in the regulation of cell physiology, including the control of cell proliferation, and some oncogenes have been found to encode proteins having PSK activity.

The raf oncogenes:

The c-raf-1 gene was originally identified as the cellular homologue of the transforming v-raf gene of a murine sarcoma virus (43), and members of the raf family have since been identified as oncogenes activated by DNA rearrangements during gene transfer experiments (44). Although the c-raf-1 gene is expressed in all tissues examined to date, the related genes A-raf and B-raf are expressed in a tissue-specific manner. The raf genes encodes PSKs having a two domain structure, with a carboxy terminal kinase domain and an amino terminal regulatory domain. The molecular alterations converting normal raf proto-oncogenes to active oncogenes involve deletion of the amino-terminal regulatory sequences, presumably resulting in constitutive deregulated activity of the kinase catalytic domain. The truncated raf

genes generated by these deletions are expressed as fusion proteins, either with viral gag sequences in the case of raf genes transduced by retroviruses, or with other cellular coding sequences in the case of raf genes activated during gene transfer (45,46).

Protein Kinase C:

The PSK protein kinase C (PKC) is thought to have a role in the control of cell proliferation as a result of two separate lines of investigation. It was first identified as an effector enzyme of the inositol phospholipid second messenger pathway, and subsequently as the intracellular target activated by tumour-promoting phorbol esters (for a review, see 47). The catalytic activity of PKC is dependent upon the presence of calcium and phospholipid. Once it has bound diacylglycerol, PKC is activated at physiological calcium concentrations. Tumour-promoting phorbol esters mimic the effect of diacylglycerol in enzyme activation. PKC is also activated by proteolysis. Limited proteolytic treatment of PKC generates an amino terminal fragment having phospholipid binding ability, and a carboxy terminal fragment containing the catalytic domain. Once liberated from the amino terminal region, the catalytic domain is active independent of calcium and phospholipid (48). This observation indicates that PKC consists of two domains: a catalytic domain, and regulatory domain which apparently contains a binding site for phospholipid, and possibly for calcium. This regulatory region would presumably function as a negative regulator, and the down-regulating signal would be abrogated by diacylglycerol and calcium binding. The

structure and regulation of PKC seems analogous to that of the raf kinases, although the specific factors which bind and activate members of the raf family are not yet identified.

The mos oncogene:

The mos oncogene was originally identified as the transforming gene of Moloney murine sarcoma virus (49). Like the raf kinases and PKC, the mos enzyme is a PSK primarily localized in the cytoplasm. However, this kinase is a much smaller protein than either the raf or PKC family members. The mos protein encompasses little more than the consensus catalytic domain required for enzyme activity. As outlined above, the amino terminal regions of the raf and PKC kinases impose regulatory effects on the catalytic domains of these kinases. The absence of such a domain from the mos kinase may suggest that its activity is regulated in another fashion. In fact, activation of mos to an oncogene requires only its enhanced expression, and not changes in coding sequence. Expression of the mos proto-oncogene in normal cells is restricted to the germ cells of both sexes (50). Taken together, these pieces of evidence may suggest that the mos kinase is constitutively active, like the truncated raf or PKC kinases, and that expression of the mos kinase is controlled at the level of gene expression. Similarly, the pim-1 PSK also lacks an amino terminal regulatory region (51), and activation of the pim-1 proto-oncogene in murine lymphomas is a result of abnormally high levels of expression.

Transmembrane PSKs:

Although most transmembrane receptor kinases have PTK activity, there have been, to date, three transmembrane receptor PSKs identified. These molecules include the daf-1 gene product of *Caenorhabditis elegans* (52), the Zmpk1 protein from *Zea mays* (53), and the murine activin receptor (54). These enzymes have only recently been identified and their activities have not been well characterized, however, there is evidence to suggest that both the daf-1 gene product and the activin receptor may play some role in the control of cell growth and development. The daf-1 gene product is involved in the control of *C. elegans* larval development, and the activin growth factors have been implicated in many biological processes, including cellular proliferation and embryonic development.

1.1.3 Protein Kinases and Their Associated Signal Transduction Networks:

While it is clear that some cell surface receptors possess intrinsic tyrosine kinase activity or associate with tyrosine kinases, implicating PTKs as primary signal transducers, only very recently has there been much progress in the elucidation of the signalling pathways acting downstream of these enzymes. Extensive studies carried out by Lewis T. Williams and his group have identified the components of the signal transduction network which are phosphorylated by, and presumably act downstream of, the PDGF receptor. Other work has begun to decipher the networks controlling the eukaryotic cell cycle, implicating members of both the PSK and PTK family as interplaying elements. Some of the data obtained from these studies will be outlined

in brief here, and illustrated in figure 6.

Signal Transduction by the PDGF receptor:

The binding of PDGF by the PDGF receptor induces autophosphorylation of the receptor itself, and the physical association of the intracellular portion of this kinase with a number of proteins, including phospholipase C-γ1 (PLC) (55), phosphatidylinositol 3-kinase (PI3K) (56), ras GTPase-activating protein (GAP) (57), the PSK raf (58), and the src kinase and other members of the src family (59). With the exception of the raf kinase, these associating molecules all contain SH2 domains, which bind tyrosine phosphorylated peptides and presumably mediate the interaction of these proteins with the autophosphorylated PDGF receptor. PLC-γ 1 cleaves the phosopholipid phosphatidylinositol 4,5-bisphosphate (PI), generating the second messengers diacylglycerol (DAG) and inositol trisphophate (IP3), which in turn stimulates PKC and raises intracellular calcium levels (60). There is evidence suggesting that this enzyme may be the link between PDGF stimulation and PIP2 breakdown (55). The PI3K phosphorylates the inositol ring of PI (61), and the accumulation of PI-3,4-P2 and PI-3,4,5-P3 is induced by PDGF stimulation (62). The ras protein is active in its GTP-bound state, and inactive once it has hydrolyzed GTP to GDP. GAP stimulates the GTPase activity of ras, acting as a negative regulator (63). It has been previously suggested that the ras protein plays a part in PDGF-induced mitogenic signalling from the work of Mulcahy *et al* (64), who observed that microinjection of anti-ras antibodies blocks the mitogenic effects of PDGF and serum

Figure 6: Signal Transduction by the PDGF receptor

The PDGF receptor undergoes ligand-induced dimerization, resulting in cross-phosphorylation of the subunits on tyrosine residues. The phosphotyrosine residues provide binding sites for enzymes containing SH2 domains. Once associated, these proteins are substrates for the PTK activity of the PDGF receptor. Tyrosine phosphorylation of these proteins bay modulate their activity (see text). Recruitment to the membrane alone may be important, as the substrates for some of these signal transducing molecules are found at the plasma membrane. The c-raf-1 kinase is also activated following PDGF treatment of cells, however, it has no SH2 domain, and it is not clear whether this protein directly interacts with the PDGF receptor or not.

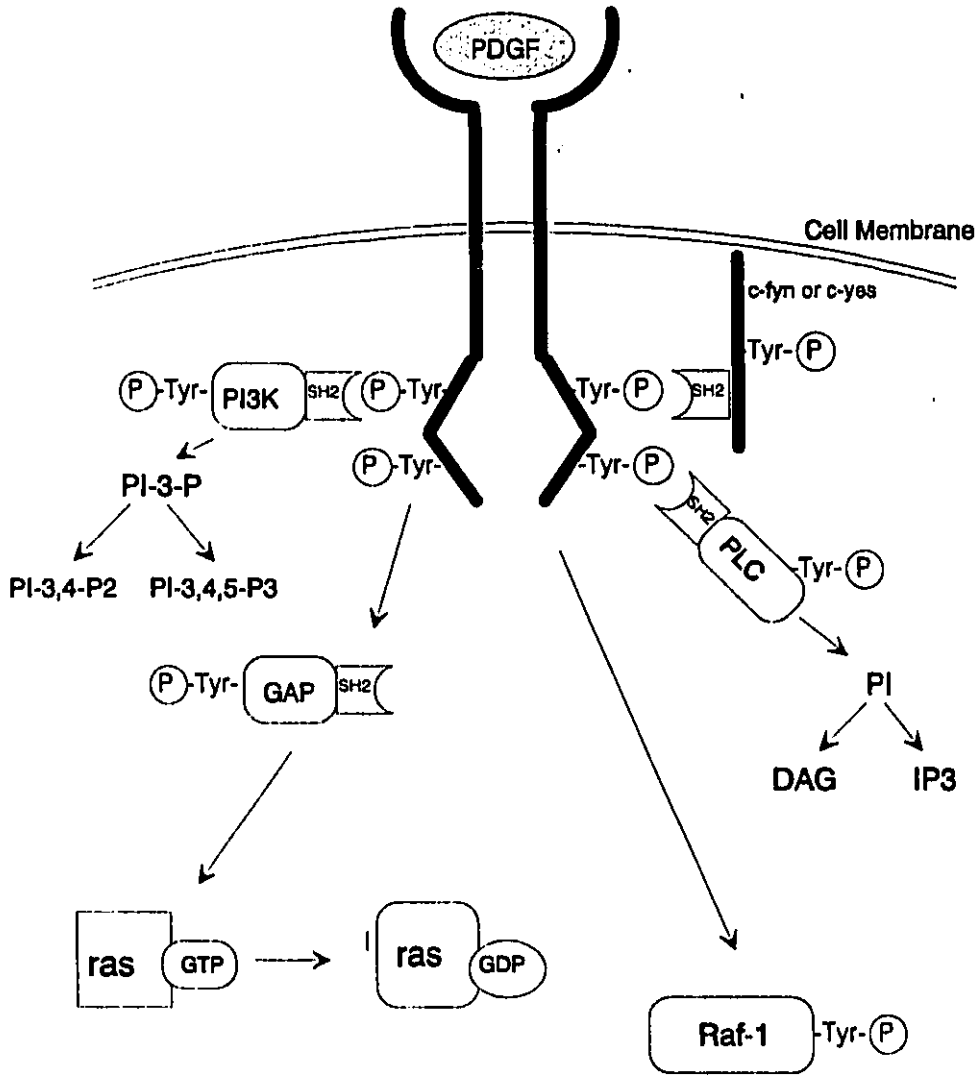

PDGF
Cell Membrane
c-fyn or c-yes
Tyr-P
P-Tyr-
PI3K
SH2
PI-3-P
PI-3,4-P2
PI-3,4,5-P3
GAP
PLC
PI
DAG
IP3
ras
GTP
GDP
Raf-1

Figure 6

in swiss mouse NIH-3T3 cells. Modulation of the GAP protein may provide the biochemical link between PDGF stimulation and alteration of ras activity. Ligand-induced activation of the PDGF receptor results in tyrosine phophorylation of the PSK raf, which activates its PSK activity (65). It is thought that the raf kinase interacts directly with the activated PDGF receptor, and while there is some evidence for this model, it has also been suggested that the activating signal may be transduced by an indirect route (66). The PDGF receptor has been shown to interact with the src kinase and other members of the src family, specifically fyn and yes, and these kinases all show elevated activity following PDGF treatment of quiescent fibroblasts (59). The interactions outlined above may represent common associations between activated transmembrane enzymes and the molecules listed here. The raf kinase has been implicated in signal transduction mediated by other growth factor receptors and membrane bound oncogene products (58). The EGF receptor has also been shown to associate with PLC-γ 1, GAP, and the PI3 kinase following EGF-stimulated activation. It has been sugested that an activated PTK in fact represents a binding site or gathering place for cytosolic proteins which are signal transducers such as those proteins listed here, and recruitment of these enzymes to the membrane alone may be contributing to their activation (67).

Protein Phosphorylation and Cell Cycle Control:

In all eukaryotic cells examined to date, the M phase of mitosis and meiosis is initiated by activation of pp34, a PSK identified as the product of the cdc2 gene of

Scizosaccharomyces pombe and the cdc28 gene of *Saccharomyces cerevisiae.* This kinase is the catalytic subunit of maturation promoting factor (MPF) (68), which is made up of a pp34-cyclin complex. The phosphorylation state of pp34, which appears to control its activity, is altered throughout the cell cycle. In late interphase, pp34 is phosphorylated on tyrosine and threonine residues (69), and the activation of MPF occurs with the dephosphorylation of these residues in a variety of eukaryotic cell types (70,71). Direct evidence for the regulation of pp34 by tyrosine phosphorylation comes from studies carried out in *S. pombe* (72). The tyrosine residue at position 15 of pp34 is the single known site of tyrosine phosphorylation of this kinase. Site-directed mutagenesis of this residue to phenylalanine prematurely advances *S. pombe* cells into mitosis. The activation of pp34 is a multistep process, and several other proteins are known to be involved in both inhibitory and stimulatory regulation of this kinase (73-75). The cdc25 gene product was originally identified as an inducer in mitotic control in *S. pombe*, and has in fact been found to be a protein phophatase which directly dephosphorylates and activates pp34 (76). The signal transduced by the tyrosine phosphorylation of pp34 is one which suppresses cell division, indicating that the PTK phosphorylating pp34 may have a growth-suppressing role. This contrasts with the role of other PTKs whose activity transduces growth-stimulating signals. It has also been suggested that, in addition to its PSK activity, the pp34 kinase may have tyrosine phosphorylating ability (77), as it has homology to the protein serine/threonine/tyrosine kinases ERK-1 and ERK-2, described below.

1.1.4 Protein serine/threonine/tyrosine kinases:

The idea that protein kinases fall into two classes, containing those which phosphorylate tyrosyl residues and those which phosphorylate serine and threonine residues, has recently been challenged by the identification of kinases which are capable of phosphorylating all three hydroxyamino acids. This group includes two yeast kinases, MCK1 (78) and SPK1 (79), and the mammalian enzymes STY (80), ERK-1, and ERK-2 (77). All these kinases appear to be localized in the cytoplasm. By inspection of the amino acid sequences of the conserved regions in the catalytic domains of these kinases, they would originally have been classified PSKs by the conventions outlined by Quinn *et al* (1). Although not much is currently known about the regulation of this family of enzymes, at least one of these kinases, ERK-1, is activated in response to growth factor or hormone treatment of certain cell types.

The activity of the ERK-1 kinase (formerly termed MAP2 kinase) is controlled by phosphorylation on tyrosine and threonine residues, and tyrosine phosphorylation is required to achieve maximum activity of the enzyme (81). ERK-1 kinase activity is stimulated in murine cells in culture by a variety of extracellular factors, including insulin (82), EGF (83), and phorbol esters (84). Studies carried out with ERK-1 have shown that the ribosomal S6 kinase is an *in vitro* substrate for this kinase, and phosphorylation of the S6 kinase causes an increase in its activity (81,85,86). It was originally thought that the ERK-1 kinase occupied a pivotal position in kinase cascades, transducing the signal from tyrosine phosphorylation events into serine/threonine phosphorylation events (87). In this model the ERK-1 kinase

activator is a tyrosine kinase. However, the finding that ERK-1 is capable of tyrosine autophosphorylation indicates that the ERK-1 kinase activator need not be a kinase itself.

1.1.5 Protein Kinases and Development:

The identification of protein kinases as oncogenes or growth factor receoptors led to the idea that these enzymes are associated with growth control. While this is true, it has become evident that these enzymes are also involved in the control of developmental programs. A study of the homeotic mutation sevenless in Drosophila melanogaster has demonstrated that a PTK is responsible for the differentiation of a specific set of optic cells (88). Other studies in Drosophila have shown that the PSKs zeste-white 3, fused, and D-raf are essential for determination of cell fate during embryonic development (for a review, see 7).

1.1.6 Protein Tyrosine Kinases and Hematopoietic Development:

The regulated expression of specific PTKs during hematopoiesis is crucial to normal hematopoietic development. This became evident with the mapping of the PTK c-kit to the white locus (W) of the mouse (89). Mutations at the W locus lead to pleiotropic developmental defects including sterility, coat colour abnormalities, severe macrocytic anemia, and mast cell deficiency. These defects reflect an inablility of primordial germ cells, melanoblasts, and hematopoietic stem cells to proliferate and migrate during embryogenesis. All mutant W alleles investigated to

date have been found to represent loss-of-function mutations resulting in a decrease in the level of c-kit tyrosine kinase activity (90,91), indicating that kinase activity is the relevant biochemical activity which is affected in W mutations. Some W mutations represent regulatory mutations which cause a decrease in the level of c-kit expression, while other mutations are structural, leading to the expression of an altered kit protein. In the heterozygous state, structural W mutations tend to be strongly dominant, and the affected mice show anemia, reduced fertility and extensive coat spotting (90) In contrast, regulatory W mutations in the heterozygous state generate a much milder phenotype in which the mice exhibit only coat spotting and no other abnormalities. These observations suggest that in these systems the structural kit mutant protein may be interfering with the signal transducing ability of the normal kit kinase (90), as illustrated in figure 7.

1.1.7 Protein Kinases and Hematopoietic Malignancies:

PTKs have been found to play a part in controlling normal hematopoietic development, and furthermore, mutation of specific PTKs can contribute to the loss of growth control in certain hematopoietic cell types.

The murine leukemia viruses are replication-competent retroviruses which lack transforming genes, but cause lymphomas after a latency period, presumably through the activation of cellular genes by integrated proviruses (92). Berns *et al* have shown that the gene encoding the PSK pim-1 can be activated to its full oncogenic potential by proviral insertion in murine leukemia virus induced T cell lymphomas (51).

Figure 7: Signal transduction by the PTK kit

The binding of steel, the ligand for the kit receptor, results in dimerization and cross-phosphorylation of receptor molecules, and ultimately in signal transduction. Dimerization of a normal kit receptor molecule with a mutant kit kinase which still retains ligand binding activity, but has no kinase activity, leads to abrogation of signal transduction.

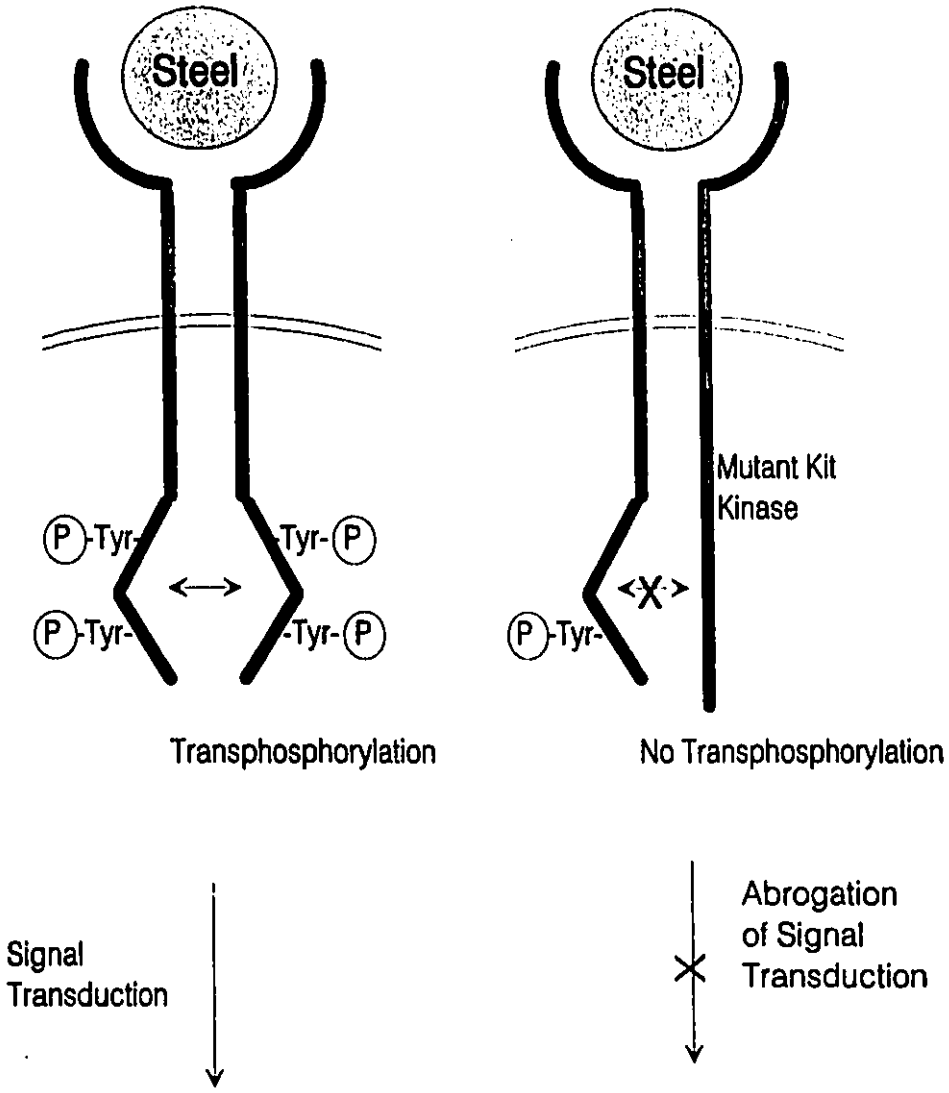

Steel
Steel
Mutant Kit
Kinase
P-Tyr-
-Tyr-P
P-Tyr-
-Tyr-P
P-Tyr-
Transphosphorylation
No Transphosphorylation
Signal
Transduction
Abrogation
of Signal
Transduction

Figure 7

Identification of the abl proto-oncogene followed the discovery of the transforming gene (v-abl) of Abelson Murine leukemia virus, which commonly induces B cell lymphomas (93). This transforming gene was found to encode a fusion protein of the viral *gag* protein sequence and the normal cellular gene c-abl. The resultant *gag*-abl fusion protein was found to have tyrosine kinase activity, and this activity is required for its transforming ability (94).

Activation of abl has also been detected in human leukemias characterized by a chromosomal abnormality called the Philadelphia chromosome (95). This chromosomal abberation, which is found in over 90% of chronic myelogenous leukemias (CML), and approximately 25% of acute lymphocytic leukemias (ALL), is generated by a translocation that fuses sequences from the abl locus on chromosome 9 with sequences from the bcr locus on chromosome 22. The result of this fusion is the replacement of amino terminal abl sequences with bcr sequences, giving rise to a gene product having abl tyrosine kinase activity. The chronic phase of CML is characterized by the clonal dominance of Philadelphia chromosome bearing stem cells. The production of this bcr-abl protein apparently represents only one step toward the generation of malignancy, and further events that trigger transition from the chronic to blast crisis phase of the disease remain unclear (96). In clinical settings, it appears that the presence of the Philadelphia chromosome in leukemic blast cells of a patient is correlated with a poorer prognosis than a patient whose blast cells do not contain this chromosomal rearrangement (97).

1.2 Area of Investigation:

The protein kinases are implicated as mediators of cellular growth, differentiation, and development in cells of the hematopoietic 'ineage. The goal of the work carried out in this thesis was to identify a novel kinase which may play a role in the process of hematopoiesis and/or hematopoietic development.

The work described here is presented in three chapters. Chapter two outlines the method by which cDNAs encoding active kinases were obtained from a mammalian cDNA expression library, along with cDNA sequence analysis and studies showing the expression of mRNA corresponding to these cDNAs. Chapter three deals with the determination of the kinase activity for the TIK kinase, the protein encoded by one of the cDNAs isolated as outlined in Chapter two. Finally, Chapter four describes our observation that one allele of the gene encoding the TIK kinase has undergone a rearrangement in a murine lymphocytic leukemia line, apparently giving rise to an aberrant pattern of expression of TIK mRNA transcripts.

Chapter Two

The Cloning and Characterization of cDNAs encoding kinases from the Murine pre-B cell line 70Z/3

2.1 Introduction:

Members of the protein tyrosine kinase (PTK) gene family have been identified in both vertebrate and invertebrate tissues, and while these genes appear to have been widely elaborated in multicellular organisms, apparently no PTKs are encoded by unicellular organisms such as bacteria. Bacteria therefore provide an ideal cloning system for mammalian PTKs. Functional PTKs undergo autophosphorylation, allowing their detection with antibodies to phosphotyrosine. Such antibodies have been successfully used in a variety of systems to screen cDNA expression libraries for cDNAs encoding functional kinases (98-100).

The involvement of certain kinases in the process of hematopoiesis suggests that PTKs play critical roles mediating cell growth and differentiation in the B cell lineage. We have used antiphosphotyrosine antibodies to clone PTKs from a cDNA expression library generated from the murine pre-B cell line 70Z/3. Using this method, a total of eight distinct cDNAs encoding kinases were isolated from this expression library. Through cDNA sequence analysis, one of these clones was identified to encode the murine PTK c-lyn, and two other clones were found to encode novel kinases.

2.2 Materials and Methods

2.2.1 Isolation of cDNA clones:

cDNAs encoding antiphosphotyrosine immunoreactive proteins were isolated from a λgt11 cDNA expression library generated from poly-A^+ selected RNA from the 70Z/3 cell line. 5 X 10^5 plaques were screened by infecting the bacterial strain Y1090 with the bacteriophage and incubating at 42℃ for 4-6 hr, overlaying with filters soaked in 10mM isopropyl-ß-D-thiogalactopyranoside and incubating for an additional 6 hr at 37℃. Filters were then treated with polyclonal murine antiphosphotyrosine antibody and anti-mouse antibody coupled to alkaline phophatase. Positive clones were visualized through reaction with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate. These clones were rescreened twice to homogeneity.

Positive clones were cross hybridized to identify distinct clones by the following method: 1 μl of phage in solution at 10 pfu was spotted onto a plated bacterial lawn of Y1090, allowed to adsorb for 20 min at 22℃, and incubated for 8-12 hr at 37℃. Nitrocellulose filters (Millipore) were overlayed for 4 min, denatured in 1.5 M NaCl, 0.5 M NaOH for 2 min, neutralized in 1.5 M NaCl, 0.5 M Tris-HCl, pH 8, for 5 min, and rinsed in 0.2 M Tris-HCl, pH 7.5, 2 X SSC for 30 sec. Filters were baked at 80℃ under vacuum for 2 hr. Filters were probed with DNA probes generated by random primer extension labelling of PCR-amplified cDNA inserts. Prehybridizations were carried out with 6 X SSC, 5 X Denhardt's Solution, 0.5% SDS, and denatured salmon sperm DNA at 0.1 mg/ml at 65℃ for 6 hr.

Hybridizations were performed using radioactively labelled probe at 1-4 X 10^5 cpm/ml for 12 hr. Filters were washed in 2 X SSC at 22℃ for 5 min and once in 0.1 X SSC, 0.1% SDS at 22℃ for 15 min, then autoradiographed by exposure to Kodak XAR-5 X-ray film.

2.2.2 Sequencing of cDNA clones:

The cDNA inserts were cloned into the EcoR1 site of the bacteriophage M13 (Pharmacia) and single stranded sequence analysis was carried out using the Sanger dideoxy chain termination method (101). Predicted amino acid sequences were compared with the proteins of the NBRF protein data bank using the RUNFASTP program.

2.2.3 RNA isolation and Northern blot analysis:

Total RNA was prepared from cells or tissues by the method of Auffray and Rougeon (102). Poly A^+ selection was carried out by passage of total RNA over oligo-d(T) cellulose, following the method of Jacobson (103). Aliquots of 4 μg of poly A^+ RNA were electrophoresed in 1% agarose gels containing 2.2 M Formaldehyde, 20 mM 3-[N-Morpholino]propane-sulfonic acid (pH 6.8), 1 mM EDTA, and 5 mM sodium acetate, and the electrophoretically separated RNAs were transferred to Hybond-N membrane (Amersham). Filters were baked for 2 hr at 80℃ and treated with ultraviolet light for 5 min. Prehybridization was carried out 12-24hr in 5 X SSPE, 50% formamide, 2 mM sodium phosphate (pH 6.8), 5 X Denhardt's solution, 5% dextran sulfate, 0.5% SDS, and 250 μg/ml denatured non-homologous DNA, at 42℃. Hybridizations were performed using a random-primed ^{32}P-labelled DNA

insert at 1-2 X 10^6 cpm/ml at 42℃ for 12-24 hr. Filters were washed to a final stringency of 0.1 X SSC, 0.1% SDS at 22℃, and exposed to Kodak XAR-5 X-ray film.

2.3 Results

2.3.1 Isolation of cDNA clones:

Antibodies to phosphotyrosine were used to screen a λgt11 cDNA expression library prepared with mRNA isolated from the pre-B cell line 70Z/3 (104). From the screening of 5 X 10^5 recombinant plaques, 32 clones encoding fusion proteins reacting with a polyclonal antiphosphotyrosine antibody were isolated. These clones were tested for their ability to cross-hybridize to one another using Southern blot analysis techniques. This procedure revealed that the 32 independently isolated λgt11 phage clones in fact represented 8 distinct and unique clones. Through cDNA sequence analysis, one cDNA was identified to encode the tyrosine kinase c-lyn (105). The mRNA sizes for the remaining 7 clones were determined by Northern blot analysis using poly-A^+ selected RNA from the 70Z/3 line (figure 8). It has been shown previously that the tyrosine kinase c-abl gene is expressed in 70Z/3 cells (104). None of our clones, however, were found to hybridize with a c-abl probe.

2.3.2. Characterization of the TIK kinase:

One novel kinase which we designated TIK, for antiphospho *t*yrosine *i*mmunoreactive *k*inase, was selected for further study. A 2.1 kb TIK cDNA

Figure 8: Northern blot analysis showing mRNA sizes corresponding to the seven distinct cDNAs isolated from the 70Z/3 cell line

5 μg of poly(A)-selected RNA isolated from the 70Z/3 cell line was used for each lane of the gel and hybridized to a cDNA probe generated for each cDNA. The 18 and 28s rRNAs are 1869 and 4712 nucleotides, respectively.

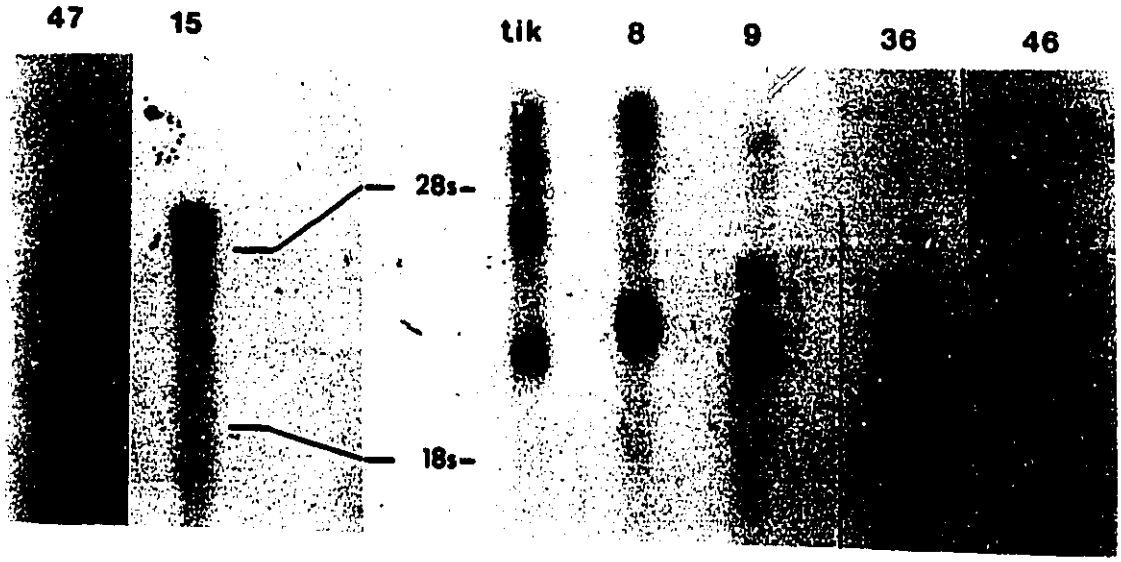


Figure 8

hybridizes to three mRNAs of 6, 4, and 2.5 kb in the 70Z/3 line (figure 8). Transcripts hybridizing to the TIK cDNA are also detected in murine heart, lung, brain, kidney, testes, thymus, and bone marrow (figure 9). High stringency hybridization of the complete TIK cDNA genomic 70Z/3 DNA digested with a number of restriction endonucleases yielded a very simple pattern of bands, indicating that the three mRNAs originate from a single gene (data not shown).

The nucleotide sequence of the TIK cDNA is shown in figure 10. The sequence contains an open reading frame of 517 amino acids ending at position 1,701. The putative initiating methionine residue at nucleotide position 147 is preceded upstream by one termination codon in the same reading frame, and the sequence surrounding this ATG site conforms to the consensus predicited by Kozak's rules for ribosome binding (106). The TIK protein predicted from the cDNA sequence would have a molecular mass of 58,573 Da, with an isoelectric point of 8.38.

While the amino acid sequence of the TIK kinase contains the consensus sequences thought to be essential for kinase activity, the sequence shows greater similarity to the serine/threonine family of kinases than to the tyrosine kinase family (see figure 11).

2.3.3 Analysis and Identification of Other cDNAs:

As mentioned previously, one of the cDNAs isolated from the 70Z/3 cDNA expression library was identified through cDNA sequence analysis to encode the murine c-lyn PTK (see figure 12). This partial murine c-lyn sequence encompasses

Figure 9: Northern blot analysis of TIK mRNA in a variety of adult mouse tissues Each lane contains approximately 5 μg of poly(A)-selected RNA isolated from each mouse tissue. The blot was hybridized to a TIK cDNA and murine ß-actin probe. The 18 and 28s rRNAs are 1896 and 4712 nucleotides, respectively.

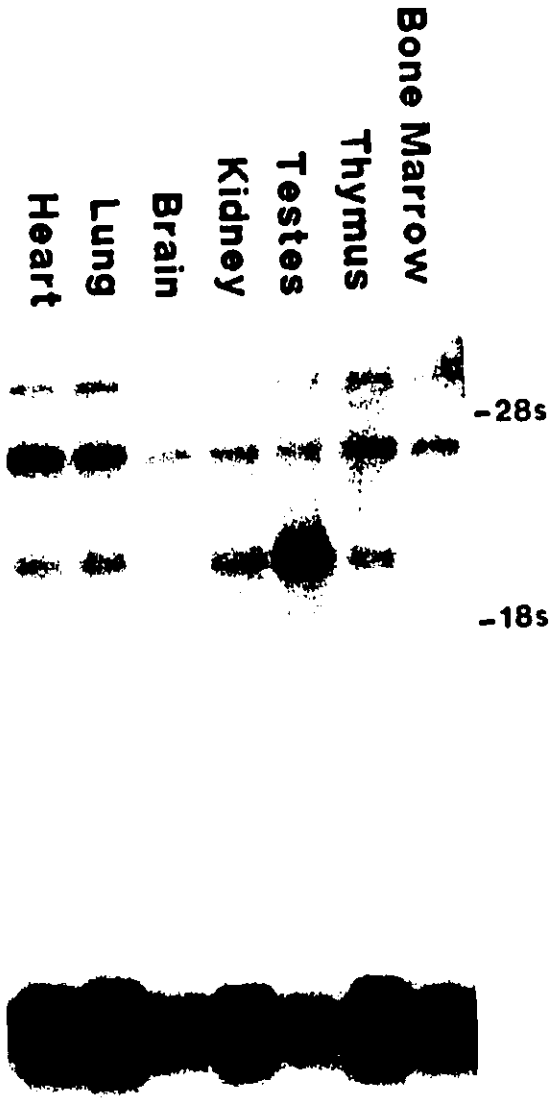


Figure 9

Figure 10: The nucleotide and predicted amino acid sequence of the TIK cDNA The cdc2 phosphorylation consensus sequence is boxed, and the residues making up the catalytic domain are underlined.

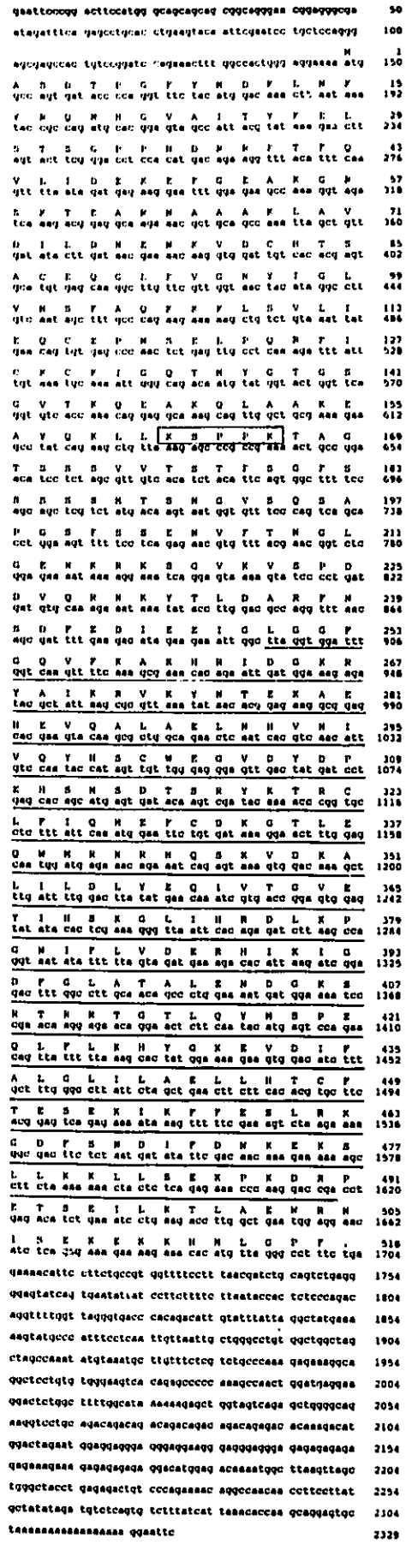


Figure 11: A schematic representation of the TIK kinase
Serine/threonine and tyrosine kinase subdomain consensus sequences are indicated by Roman numerals. X represents any amino acid. Boxed amino acids represent conserved residues.

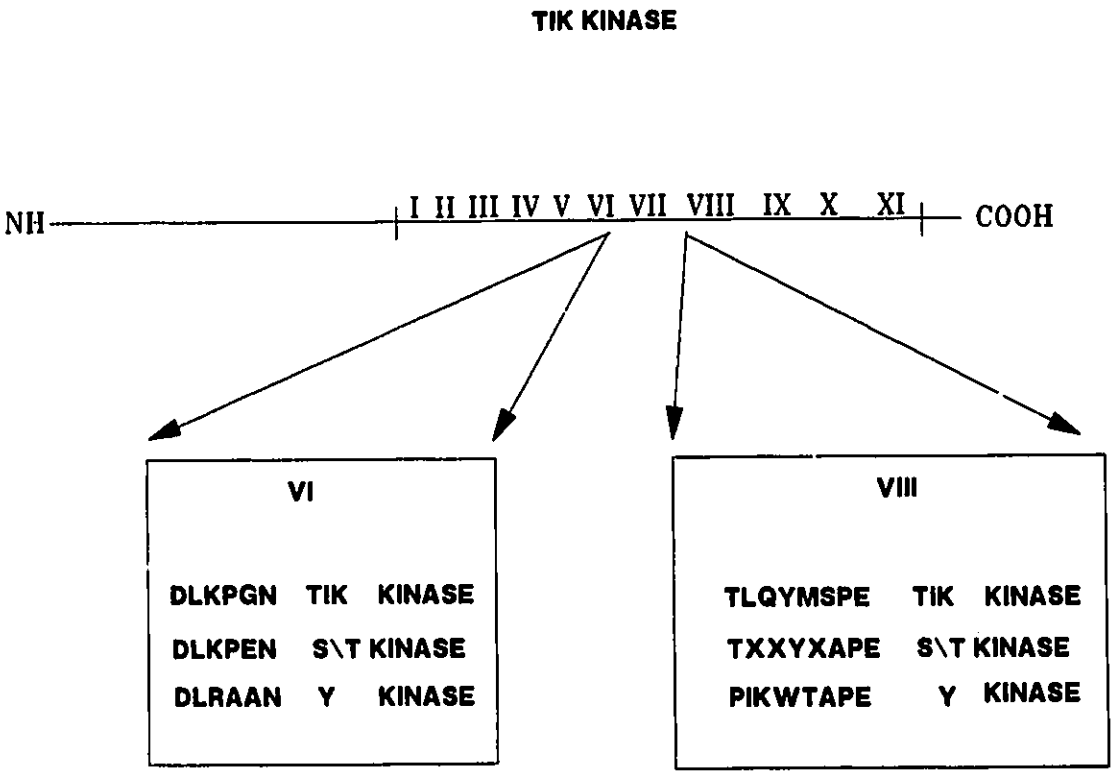

TIK KINASE
NH
I II III IV V VI VII VIII IX X XI
COOH
VI
DLKPGN TIK KINASE
DLKPEN S\T KINASE
DLRAAN Y KINASE
VIII
TLQYMSPE TIK KINASE
TXXYXAPE S\T KINASE
PIKWTAPE Y KINASE

Figure 11

Figure 12: The nucleotide and predicted amino acid sequence of the murine c-lyn cDNA

The amino acid residues differing between the human and murine c-lyn peptide sequences are marked with an asterik (*).

```
P   K   P   Q   K   P   W   D   K   D   A   W     12
ccc aaa cct cag aag cca tgg gat aaa gat gcc tgg
                                            *
E   I   P   R   E   S   I   K   L   V   K   K     24
gag atc ccc cgg gag tcc att aag ttg gtg aaa aag

L   G   A   G   Q   F   G   E   V   W   M   G     36
ctt ggc gca ggg cag ttt ggg gaa gtc tgg atg ggt

Y   Y   N   N   S   T   K   V   A   V   K   T     48
tac tat aac aac agc aca aag gtg gct gtg aag acc

L   K   P   G   T   M   S   V   Q   A   F   L     60
ctc aag ccc ggc acc atg tct gtg cag gca ttc ctg

E   E   A   N   L   M   K   T   L   Q   H   D     72
gaa gag gcc aac ctc atg aag acc ttg caa cat gac
                                        *
K   L   V   R   L   Y   A   V   V   T   K   E     84
aag cta gtg cgg ctg tac gct gtg gtc acc aag gag
                                    *
E   P   I   Y   I   I   T   E   F   M   A   K     96
gag ccc atc tac atc atc acc gag ttc atg gct aag

G   S   L   L   D   F   L   K   S   D   E   G    108
ggt agt ttg ctg gat ttc ctc aag agt gat gaa ggt

G   K   V   L   L   P   K   L   I   D   F   S    120
ggc aag gtg ctg ctg ccc aag ctc att gac ttc tcg

A   Q   I   A   E   G   M   A   Y   I   E   R    132
gcc cag att gca gaa ggc atg gcg tac atc gag cgg

K   N   Y   I   H   R   D   L   R   A   A   N    144
aag aac tac atc cac cgt gat ctg cga gct gct aac

V   L   V   S   E   S   L   M   C   K   I   A    156
gtc ctg gtc tct gag tca ctc atg tgc aag att gca

D   F   G   L   A   R   V   I   E   D   N   E    168
gac ttt ggc ctc gcg aga gtc atc gaa gat aac gag

Y   T   A   R   E   G   A   K   F   P   I   K    180
tac aca gca agg gaa ggt gcg aag ttc cct atc aag

W   T   A   P   E   A   I   N   F   G   C   F    192
tgg aca gct cca gag gcn atc aac ttc ggc tgc ttc

T   I   K   S   D   V   W                        204
act atc aaa tct gac gtg tgg
```

Figure 13: The nucleotide and predicted amino acid sequence of the clone 8 cDNA The consensus sequences for catalytic subdomains VI and VIII are underlined.

```
P   T   A   F   T   V   H   S   A   L   L   S     12
cct acg gcc ttc acg gtc cat tcg gcc ctt ctt tct
A   T   G   R   H   G   A   A   A   G   S   K     24
gcc acc ggc cgc cat gga gca gcc gcc ggg tcc aag
S   K   L   K   K   L   S   E   D   S   L   T     36
agt aag cta aaa aag ctg agt gaa gac agt ttg act
K   Q   P   E   E   V   F   D   V   L   E   K     48
aag cag cct gaa gaa gtt ttt gat gta ctg gag aag
L   G   E   G   S   Y   G   S   V   F   K   A     60
ctt gga gaa ggg tct tat gga agt gtt ttt aaa gca
I   H   K   E   S   G   Q   V   V   A   I   K     72
ata cat aag gaa tct ggt caa gtg gtt gca att aag
Q   V   P   V   E   S   D   L   Q   E   I   I     84
caa gta cct gtt gag tca gat ctt cag gaa ata atc
K   E   I   S   I   M   Q   Q   C   D   S   P     96
aaa gaa att tcc ata atg caa caa tgt gac agt cca
Y   V   V   K   Y   Y   G   S   Y   F   K   N    108
tat gtt gtg aag tac tat ggc agt tac ttt aag aac
T   D   L   W   I   V   M   E   Y   C   G   A    120
aca gac ctc tgg att gtt atg gag tac tgt gga gcg
G   S   V   S   D   I   I   R   L   R   N   K    132
ggg tcc gtt tca gac ata att aga ttg cga aac aag
T   L   T   E   D   E   I   A   T   I   L   K    144
aca tta aca gaa gat gaa att gca act att cta aaa
S   T   L   K   G   L   Q   Y   L   H   F   M    156
tcc aca ttg aaa gga tta caa tat ttg cat ttt atg
R   K   I   H   R   D   I   K   A   G   N   I    168
agg aaa ata cac aga gat ata aaa gcc ggg aat att
L   L   N   T   E   G   H   A   K   L   A   D    180
ctc ctc aat aca gaa gga cat gca aag ctt gca gat
F   G   V   A   G   Q   L   T   D   R   M   A    192
ttt gga gtg gct ggc cag tta aca gat aga atg gca
K   R   N   T   V   I   G   T   P   F   W   M    204
aaa cgc aac act gta ata gga acc cca ttt tgg atg
A   P   E   V   I   Q   E   I   G   Y   N   C    216
gct cct gag gta att caa gaa ata ggt tac aac tgt
V   A   D   I   W   S   L   G   I   T   S   I    228
gtg gct gac atc tgg tcc ctt ggc att act tct ata
E   M   A   E   G   K   P   P   Y   A   D   I    240
gaa atg gca gaa gga aaa cct cct tat gct gat ata
H   P   M   T   R   I   F   M   I   P   T   N    252
cat ccg atg acc cgt att ttt atg atc cct aca aac
P   P   P   T   F   R   K   P   E   L   W   S    264
cca cca cca aca ttc agg aaa cct gaa ctt tgg tct
D   D   F   T   D   F   V   K   K   C   L   E    276
gat gac ttc acc gat ttt gtg aag aag tgc ttg gag
K   S   P   E   Q   R   A   T                    284
aag agt cct gag cag aga gcc act
```

Figure 14: Northern blot analysis of clone 8 mRNA in a variety of adult mouse tissues and murine leukemic cell lines

Each lane contains approximately 5 μg of poly(A)-selected RNA isolated from each mouse tissue or cell line. The blot was hybridized with a clone 8 cDNA probe. The 18 and 28s rRNAs are 1869 and 4712 nucleotides, respectively. BM=Bone Marrow. A description of the cell lines P388, SP10, 70Z, and L1210 can be found in appendix II.

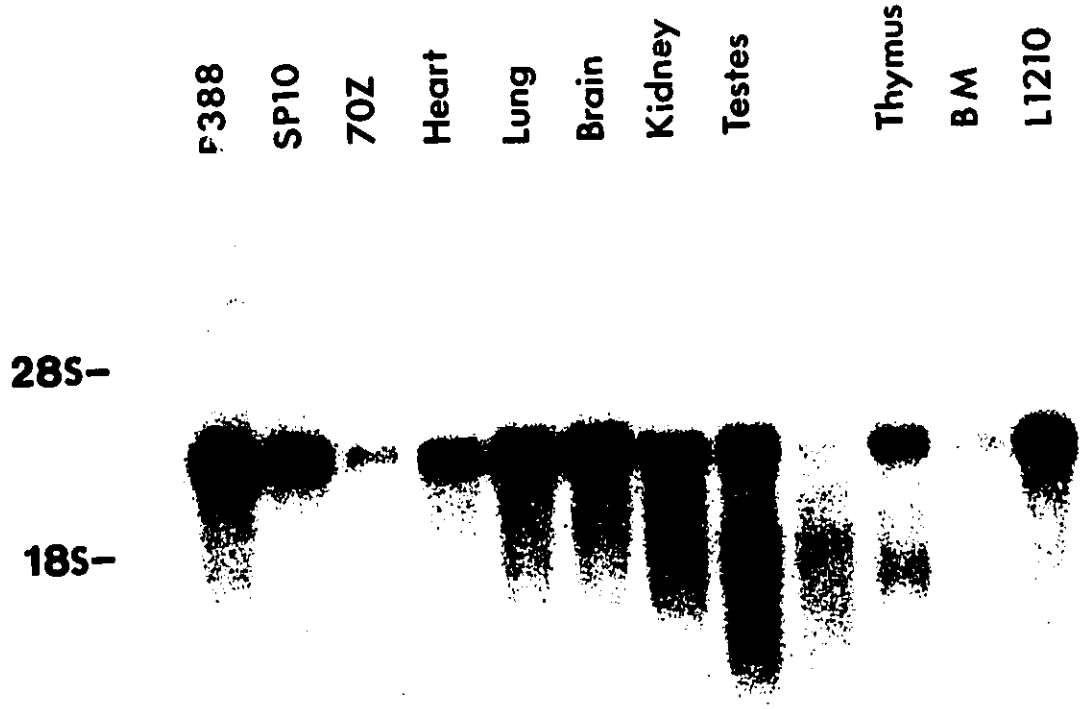

F388
SP10
70Z
Heart
Lung
Brain
Kidney
Testes
Thymus
BM
L1210
28S–
18S–

Figure 14

a portion of the catalytic domain. The murine amino acid sequence differs from the human c-lyn sequence by 3 residues in this region. Another cDNA, designated clone 8, was also characterized using cDNA sequence analysis (see figure 13), and found to encode a novel kinase. The kinase encoded by this cDNA appears to be novel, as the partial sequence determined does not correspond to that of any kinases identified to date. A 2.6 Kb clone 8 cDNA probe hybridizes to a single mRNA transcript of 3.2 Kb in the 70Z/3 line, and in a variety of murine tissues and cell lines analyzed (see figure 14).

2.4 Discussion:

Antiphosphotyrosine antibodies have been successfully used to isolate cDNAs encoding functional PTKs from a variety of mammalian cDNA expression systems (98-100). The use of such antibodies to screen a cDNA expression library prepared from the murine pre-B cell line 70Z/3 (104) detected 8 distinct cDNAs. One of these cDNAs was identified to encode the previously identified PTK c-lyn, verifying the efficacy of this screening procedure. Northern blot analysis using the remaining 7 cDNAs as probes is presented in figure 8. In agreement with their designation as distinct gene products, these cDNAs hybridize to mRNAs of varying abundance and transcript size.

One cDNA, designated the TIK cDNA for antiphosphotyrosine immunoreactive kinase-encoding cDNA, was chosen for further study. This cDNA detects three mRNA transcripts of 6, 4, and 2.5 Kb expressed by the 70Z/3 cell line, and also

hybridizes to three mRNA transcripts in all mouse tissues analyzed (figure 9). There are tissue-specific differences in the ratios of the three TIK transcripts; for example in both lung and heart tissue the 4 kb mRNA is predominant over the 6 and 2.5 kb mRNAs, and the 2.5 Kb mRNA is expressed at high levels in testes tissue. Other single copy kinase genes have been shown to give rise to multiple mRNAs through alternative splicing of a single mRNA species or differential promoter usage (107-112). These additional transcripts encode proteins differing in specific domains. While the size difference of the TIK transcripts could represent varying untranslated regions, it is possible that these mRNAs give rise to three isoforms of this enzyme. The ubiquitous expression of the TIK kinase suggests that it may play an important role in cell physiology.

The presumed catalytic region of the TIK enzyme is contained within amino acid residues 252-505. When compared to the NBRF-PIR(r) data base using the Lipman and Pearson algorithm (113), TIK was found to share a high degree of amino acid sequence identity with serine/threonine kinases. The highest degree of identity was found with pp34, the PSK encoded by the cdc2 gene (27% identity in a 185 amino acid overlap). In general, the catalytic domain of protein kinases contains conserved regions designated by Hanks *et al* (1) as subdomains I-XI. As the TIK kinase was cloned by virtue of its immunoreactivity with the antiphosphotyrosine antibody, its conserved subdomains would be expected to most closely resemble those of the tyrosine kinase family. The primary sequences of subdomains VI and VIII are thought to be indicative of the hydroxyamino acid specificity of the kinase. As

illustrated in figure 11, these regions in TIK match more closely the serine/threonine than the tyrosine kinase consensus sequence. In addition, the TIK kinase does not contain a tyrosine residue between catalytic subdomains VII and VIII, as do all other known members of the PTK family. This residue is thought to be an autophosphorylation site.

Other features of the TIK kinase which may be of significance lie amino terminal to the catalytic domain. This region of the predicted TIK polypeptide (residues 1-252) shares little homology with previously identified serine/threonine or tyrosine kinases. The primary sequence does not provide a clear indication of the subcellular localization of the TIK enzyme. The kinase has no apparent transmembrane domain, nor does it have a myristylation site at residue 2, which is typically found in tyrosine kinases targetted to membrane surfaces (114). The TIK protein does not have an SH2 domain (37), which is found in non-receptor type tyrosine kinases and other enzymes of the signal transduction network.

The kinase recognition sequence for pp34 phosphorylation, S/T-P-X-R/K (115), is found at residues 157-161 of the TIK protein (figure 10). This consensus sequence is followed by several serine and threonine residues, in particular two clusters of serine residues. This region may contain phosphorylation sites for other kinases which act to regulate the activity of TIK. The presence of a phosphorylation site for pp34 may indicate that the TIK enzyme is involved in some way in cell cycle control. Consistent with this speculation is our observation that mRNA encoding TIK is ubiquitously expressed in murine cells. The pp34 kinase is also ubiquitously

expressed in eukaryotic cells, and its activity is regulated throughout the cell cycle by its phosphorylation state (69-72).

The region carboxy terminal to the catalytic domain of PTKs often contains tyrosine residues which represent phosphorylation sites. In some kinases, such as the EGF receptor, these residues represent autophosphorylation sites (8), and in other kinases, such as src, these sites are thought to be phosphorylated by other kinases (28). In both cases phosphorylation of these sites acts to regulate the activity of the kinase. The TIK protein does not contain any tyrosine residues in its carboxy terminal tail, however, a few serine and threonine residues are present in this region. These residues may represent phosphorylation sites which regulate the activity of this kinase.

Another cDNA, designated clone 8, was also found to encode a novel kinase. The cDNA sequence and predicted peptide sequence of the catalytic domain of this kinase is presented in figure 13. Like the TIK kinase, the kinase encoded by the clone 8 cDNA apparently lacks some of the features thought to be present in all PTKs. The sequence of catalytic subdomain VI, at residues 162-169, is D-I-K-A-G-N, where the sequence in this region of all known PTKs is D-L/I-R-A-A-N or D-L/I/V-A-A-R-N, and in known PSKs the sequence consensus is D-L/I/V-K-X-X-N (X represents any amino acid). The kinase encoded by the clone 8 cDNA also lacks a tyrosine residue between catalytic subdomains VII and VIII. All other PTKs identified to date contain a tyrosine residue in this region. The clone 8 cDNA detects one mRNA transcript of 3.2 Kb in the 70Z/3 cell line and in a variety of

murine tissues and cell lines analyzed (figure 14).

One of the isolated cDNAs was found to encode the PTK c-lyn (105). Partial cDNA sequence from this clone is shown in figure 11. The predicted amino acid sequence from this cDNA differs from the previously identified human c-lyn sequence by 3 residues (see figure 12). These differences represent conserved amino acid substitutions. Two lysine residues, Lys 23 and Lys 82, correspond to two Arg residues of the human protein, and Phe 93 corresponds to a Tyr residue of human c-lyn. The high degree of similarity found between the murine and human c-lyn primary sequences suggests that the amino acid sequence in this region is important for the catalytic activity or substrate specificity of this enzyme.

Chapter Three

Determination of the Substrate Specificity of the TIK Kinase

3.1 Introduction:

As described in the previous chapter, a cDNA encoding the novel kinase TIK was isolated from a murine pre-B cell expression cDNA library through detection with an antibody to phosphotyrosine. This screening procedure identifies kinases capable of undergoing tyrosine autophosphorylation. The amino acid sequence of the protein encoded by the TIK cDNA, however, revealed an apparent paradox. Although the TIK kinase was presumably identified by its ability to phosphorylate tyrosine residues, the catalytic domain of this protein resembled most closely the protein serine/threonine kinase (PSK) family of enzymes. This observation prompted an investigation of the hydroxyamino acid substrate specificity of the TIK kinase.

This chapter describes a series of experiments designed to determine the enzymatic activity of the TIK kinase. Evidence is presented here that although this enzyme specifically reacts with a variety of monoclonal, and one polyclonal, antiphosphotyrosine antibody, the TIK kinase in fact autophosphorylates only on serine and threonine residues. The biochemical basis of the immunoreactivity of the TIK protein with this antibody is undetermined, however, site-directed mutagenesis of the TIK kinase to generate an enzyme incapable of autophosphorylation abolishes its antiphosphotyrosine immunoreactivity.

3.2 Materials and Methods:

3.2.1 Bacterial expression of the TIK cDNA:

The entire coding region of the TIK cDNA from the BamH1 site at position 116 (21 base pairs upstream of the putative initiating ATG codon) to position 1849 (146 base pairs 3-prime to the stop codon) was subcloned into the BamH1 site of the pET11c expression vector (116,117). This expression system places the TIK cDNA under the control of the T7 promoter. The T7 translation signals are supplied by the T7 gene 10 translation start (S_{10}), and the T7 gene 10 reading frame continues to codon 11, so the TIK cDNA is expressed as a fusion protein when T7 RNA polymerase is present. This construct is shown in figure 15. The RNA polymerase is supplied by the host bacterial cells, *E. coli* BL21. This host is a lysogen of bacteriophage lambda DE3, which contains the polymerase gene under the control of the inducible *lac*UV5 promoter. Addition of (isopropyl-ß-D-thiogalactopyranoside) to a culture induces the polymerase, which in turn transcribes the DNA encoding the fusion protein. The pETll vectors also contain a *lac* operator sequence just downstream of the T7 promoter, and the promoter and coding sequence for the *lac* repressor upstream and oriented in the opposite direction. This repressor acts both to repress the transcription of T7 RNA polymerase and to block transcription of the TIK cDNA by any T7 RNA polymerase that may be produced in the absence of IPTG. The *E. coli* BL21 host also contains pLysS, a plasmid encoding T7 lysozyme, an inhibitor of T7 RNA polymerase (118). The presence of this plasmid produces a low level of T7 lysozyme, which is sufficient to inhibit any T7 polymerase which

Figure 15: The pET11c/TIK expression construct
The features of the pET vector shown are the Ampicillin resistance gene (Amp r), the lac I gene, which produces lac repressor, the lac operator (lac O) which binds the lac repressor, and the T7 promoter which drives the expression of the TIK cDNA in the presence of T7 RNA ploymerase. Refer to text for further details.

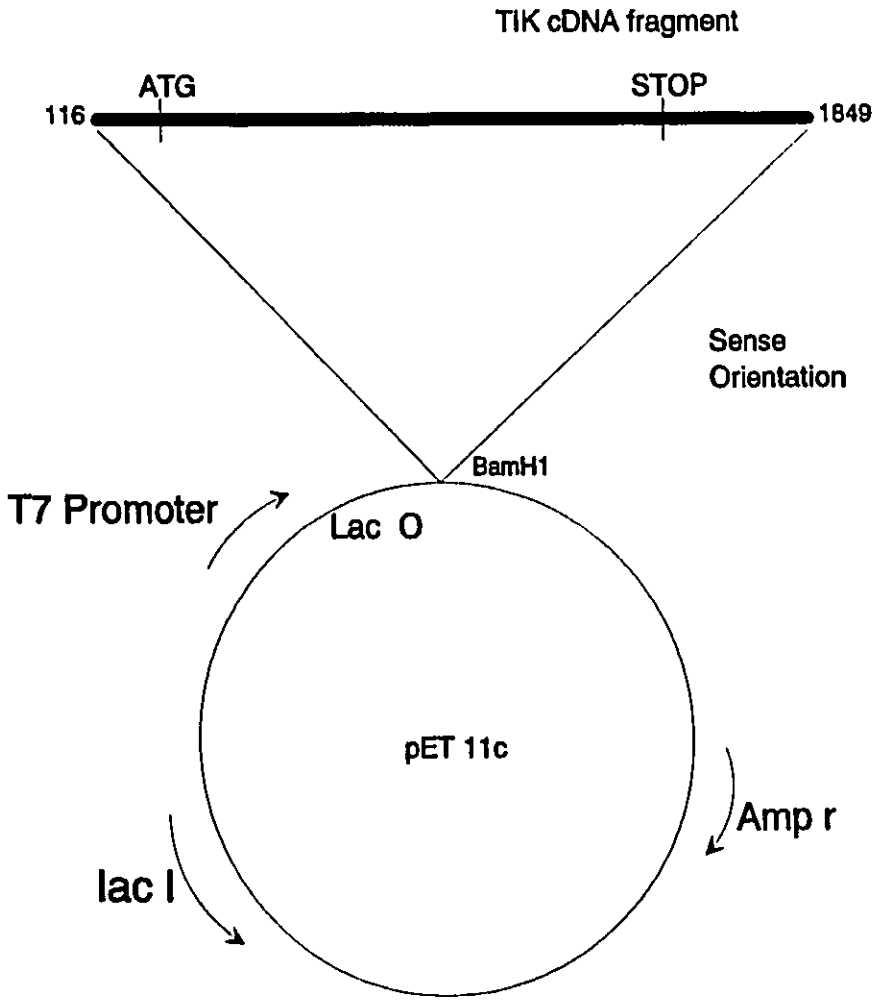

TIK cDNA fragment
ATG
STOP
116
1849
Sense
Orientation
BamH1
T7 Promoter
Lac O
pET 11c
Amp r
lac I

Figure 15

may be present prior to induction with IPTG. The T7 lysozyme also compromises the integrity of the *E. coli* cell wall by breaking a bond in the peptidoglycan layer of the cell wall, allowing lysis of the *E. coli* (pLysS) BL21 bacteria simply by a freeze-thawing technique. *E. coli* BL21 containing the pET/TIK construct were typically induced to express the T7 gene 10 product-TIK fusion protein by growing the bacteria to an OD_{600} of 0.6 in LB medium containing 50 μg/ml ampicillin, and adding 0.4 mM IPTG to the bacterial medium and growing for 2 hours.

Protein and Phosphoamino acid analysis:

in vitro labelling experiments:

E. coli BL21 bacteria containing pLysS and the TIK sense or antisense pET11c construct were induced (as described above) to express the T7 gene 10 product-TIK fusion protein and lysed by freeze-thawing. Lysates were cleared by centrifugation, and the supernatants were immunoprecipitated with either PY20 (from ICN Biochemicals), IgG2bk (from Upstate Biotechnology, Inc.), MA1G2 (generous gift of Dr. A. Raymond Frackelton, Jr., Brown University), or a polyclonal rabbit antiphosphotyrosine antibody, in 10 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1 % Triton X-100, 2 mM NaF, 2 mM sodium pyrophosphate, 500 μM ammonium vanadate, and 200 μg of phenylmethylsulfonyl fluoride per ml. The immunoprecipitates were assayed for kinase activity in 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) pH 7.1 and 10 mM $MnCl_2$ with 0.1 mCi/ml [γ-^{32}P]ATP for 30 min at 22℃. The reaction products were resolved by 10 % sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and the dried

gels were exposed to Kodak XAR-5 film. Phosphoproteins were electroeluted for phosphoamino acid analysis by the method of Edwards *et al* (119).

in vivo labelling experiments:

Bacteria harbouring the TIK sense or antisense expression construct were grown to an OD_{600}, spun down, and resuspended in 50 mM Tris (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$, and 10 mM $MnCl_2$. Either [^{35}S]methionine or [^{32}P]orthophosphate were added to 10 μCi/ml or 2mCi/ml, respectively. The bacteria were then induced (as described above) and incubated at 37℃ with shaking for 1-2 hr, lysed, immunoprecipitated, and the products analyzed as described above.

Kinase Renaturation Assay:

This procedure was carried out essentially as described in Ferrell and Martin (120). Whole cell lysates from induced bacteria were resolved by 10% SDS-PAGE and transferred to Immobilon P membranes. Blotted proteins were denatured in 6 M guanidinium chloride, 50 mM Tris, 50 mM dithiothreitol, and 2 mM EDTA, and then renatured overnight at 4℃ in 100 mM NaCl, 50 mM Tris, 2 mM dithiothreitol, 2 mM EDTA, and 0.1% (w/v) Nonidet P-40 (ph 7.5). Blots were then blocked with 5% (w/v) albumin in 30 mM Tris (pH 7.5) at room temperature for 1 hr. The kinase assay was performed by incubating blocked blots in 30 mM Tris, 10 mM $MgCl_2$, and 10 mM $MnCl_2$ (pH 7.5), with 50 μCi/ml [γ-^{32}P]ATP at room temperature for 30 min. Blots were washed twice with 30 mM Tris (pH 7.5), followed by 30 mM Tris (pH 7.5) and 0.05% Nonidet P-40 for 10 minutes at room temperature prior to autoradiography.

3.2.2 Site-Directed Mutagenesis of the TIK Kinase:

The site-directed mutagenesis technique followed is described in Hemsley *et al* (121). Synthetic oligonucleotide primers complementary to the TIK cDNA were generated using an Applied Biosystems 391 DNA Synthesizer. Primers were constructed so as to lie 'back-to-back' on the TIK cDNA, oriented for extension in opposite directions around the pET 11c vector (see figure 16) Primer 1 contains a 1 base pair mismatch with the TIK cDNA sequence, and primer 2 contains a 2 base pair mismatch. These introduced mutations change two amino acids of the TIK peptide sequence from I K to N I, and also generate an Sspl restriction enzyme site in the mutated plasmid. The lysine residue which has been mutated is known to be essential for kinase activity, so the mutant TIK protein produced from the altered cDNA will be kinase deficient.

The template pET11c/TIK DNA used for the polymerase chain reaction (PCR) was prepared by a standard alkaline lysis mini-prep method (122). The PCR reactions were carried out using 1 ng of template DNA in 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, and 0.1 mg/ml bovine serum albumin (BSA) with 0.2 mM dATP, dTTP, dCTP and dGTP, 20 pmoles of each primer, and 1-2 units of Vent polymerase (Vent Polymerase). These reaction mixtures were incubated in a programmable thermal cycler (MJ Research, Inc.) through 31 cycles of denaturation at 95℃ for 40 sec, annealing at 55℃ for 1 min, and primer extension at 72℃ for 2 min.

The double stranded DNA produced from the PCR was separated from the

Figure 16: Site-directed mutagenesis of the TIK cDNA
Schematic of the site-directed mutagenesis technique using the polymerase chain reaction. The expanded sequence shows the positions of the two primers with respect to each other and the sites of the introduced mutations in the TIK coding sequence (*).

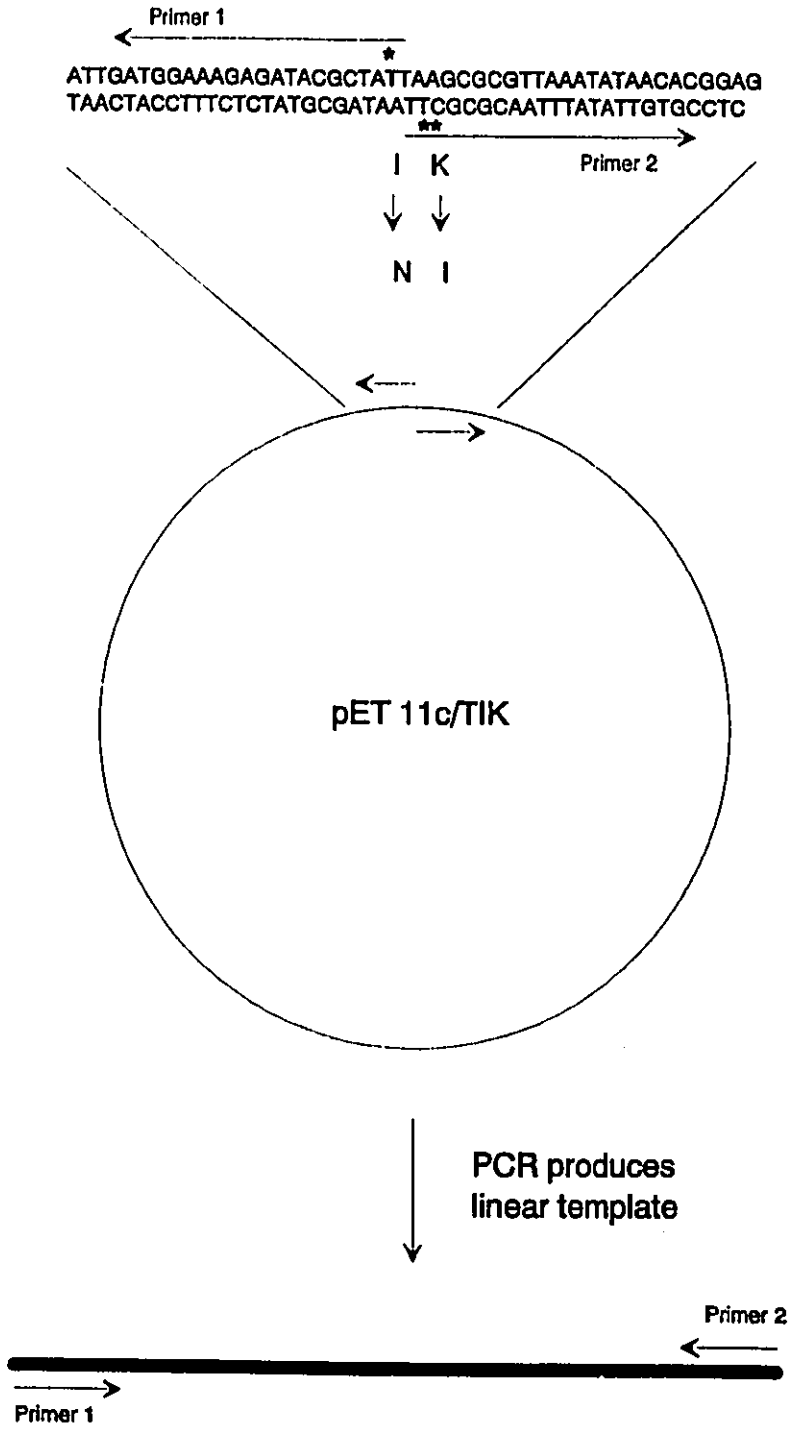

Primer 1
ATTGATGGAAAGAGATACGCTATTAAGCGCGTTAAATATAACACGGAG
TAACTACCTTTCTCTATGCGATAATTCGCGCAATTTATATTGTGCCTC
Primer 2
I K
N I
pET 11c/TIK
PCR produces
linear template
Primer 2
Primer 1

Figure 16

primers in the reaction mix by agarose gel electrophoresis, and isolated from the gel with Geneclean (Geneclean ™, BIO 101, Inc.), using the standard protocol for DNA isolation. The DNA was then phosphorylated at the 5' ends using T4 polynucleotide kinase (Pharmacia) in 66 mM Tris-HCl, pH 7.6, 1 mM ATP, 1 mM Spermidine-HCl, 10 mM $MgCl_2$, 10 mM DTT, and 0.2 mg/ml BSA.

The phosphorylated DNA sample was then ligated with T4 DNA ligase overnight at 4℃, ethanol precipitated, and used to transform competent *E. Coli* BL21 bacteria by electroporation using a Bio-Rad Gene Pulser.

Bacterial Expression of the (ATP-)TIK cDNA:

E. coli BL21 bacteria containing the (ATP-)TIK cDNA pET11c construct were induced (as described above) to express the T7 gene 10 product-(ATP-)TIK fusion protein, spun down, resuspended in sonication buffer (20 mM Tris-HCl pH 8, 1 mM EDTA, 100 mM NaCl, 1 mM DTT), and sonicated three times for 30 seconds to lyse the cells. The insoluble portion of these whole cell lysates was then collected by centrifugation, and resuspended in SDS sample buffer (62.5 mM Tris-HCl pH 6.8, 10% glycerol, 2%(w/v) SDS, 5% ß-mercaptoethanol, and 0.00125% bromophenol blue). The insoluble proteins were resolved by 10% SDS-PAGE and stained in the gel matrix using a solution of 40% methanol, 10% acetic acid, and 1% coomassie blue. Destaining was then carried out for 1-2 hours in 40% methanol and 10% acetic acid.

3.3.3 Antiphosphotyrosine Immunoblotting:

E. coli BL21 bacteria containing the (ATP-)TIK cDNA pET11c construct were

induced (as described above) to express the T7 gene 10 product-(ATP-)TIK fusion protein, spun down, resuspended in SDS sample buffer (see above), and sonicated three times for 30 seconds to lyse the cells. The whole cell lysates were then heated for one minute to 100℃ and resolved by 10% SDS-PAGE. The proteins were then transferred to nitrocellulose membranes by electroblotting for 1 hr at 0.7 Amps in 25 mM Tris, 192 mM Glycine, and 20% Methanol. The membranes were then blocked for 1 hr by treatment with TBS (10 mM Tris-HCl, pH 8, and 150 mM NaCl) and 20% fetal calf serum. The antiphosphotyrosine antibody, either PY20 (from ICN Biochemicals), IgG2bk (from Upstate Biotechnology, Inc.), MA1G2 (generous gift of Dr. A. Raymond Frackelton, Jr., Brown University), or a polyclonal rabbit antiphosphotyrosine antibody, was added in TBS and 20% fetal calf serum for 1 hr, and the membranes were then washed three times in TBST (TBS with 0.05% Tween 20) for 10 min each time. The secondary antibody, conjugated to alkaline phosphatase or [^{125}I], was added in TBST and 20% fetal calf serum for 1 hr, and the membranes were washed as above. Membranes treated with secondary antibodies coupled to alkaline phosphatase were then treated with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate to visualize immunoreactive bands. Membranes treated with secondary antibodies coupled to [^{125}I] were autoradiographed by exposure to Kodak XAR-5 X-ray film.

3.3 Results:

3.3.1 Kinase activity of TIK:

The TIK kinase was expressed as a fusion protein with the T7 gene 10 product using the pET vector system (see figure 15). This system was selected because previous attempts to express the TIK protein in bacteria using less tightly controlled expression systems were unsuccessful, suggesting that overexpression of the TIK protein is toxic to bacterial cells. *In vitro* transcription and translation of this TIK fusion protein produced a polypeptide of approximately 67 kDa (data not shown).

When intact bacteria expressing the TIK fusion protein were metabolically labelled with [^{35}S]methionine, a 67 kDa polypeptide was produced that can be immunoprecipitated with antibodies to phosphotyrosine (figure 17). Bacteria expressing an identical TIK antisense construct did not produce this polypeptide, confirming the identity of this 67 kDa polypeptide with the TIK fusion protein uproduced by *in vitro* transcription and translation. The immunoprecipitated 67 kDa protein was found to have an associated kinase activity when incubated with [γ-^{32}P]ATP in an *in vitro* kinase assay (figure 18A). Phosphoamino acid analysis shows that this protein contains phosphoserine and phosphothreonine, but not phosphotyrosine (figure 18B). In an experiment in which bacteria expressing the TIK fusion protein were labelled *in vivo* with [^{32}P]orthophosphate, a labelled 67 kDa protein was immunoprecipitated with antiphosphotyrosine antibodies (see figure 18C). Phosphoamino acid analysis of this 67 kDa band showed phosphoserine and phosphothreonine, but no phosphotyrosine (see figure 18D).

Figure 17: In vivo labelling of the TIK kinase

Lysates of *E. Coli* BL21 expressing the TIK fusion protein in either the sense (A) or antisense (B) orientation in the presence of [^{35}S]methionine. Lane 1 [^{35}S]methionine added at the time of induction and bacteria harvested after 1.5 h. Lane 2 [^{35}S]methionine added 0.5 h post induction and bacteria harvested after 1 h.

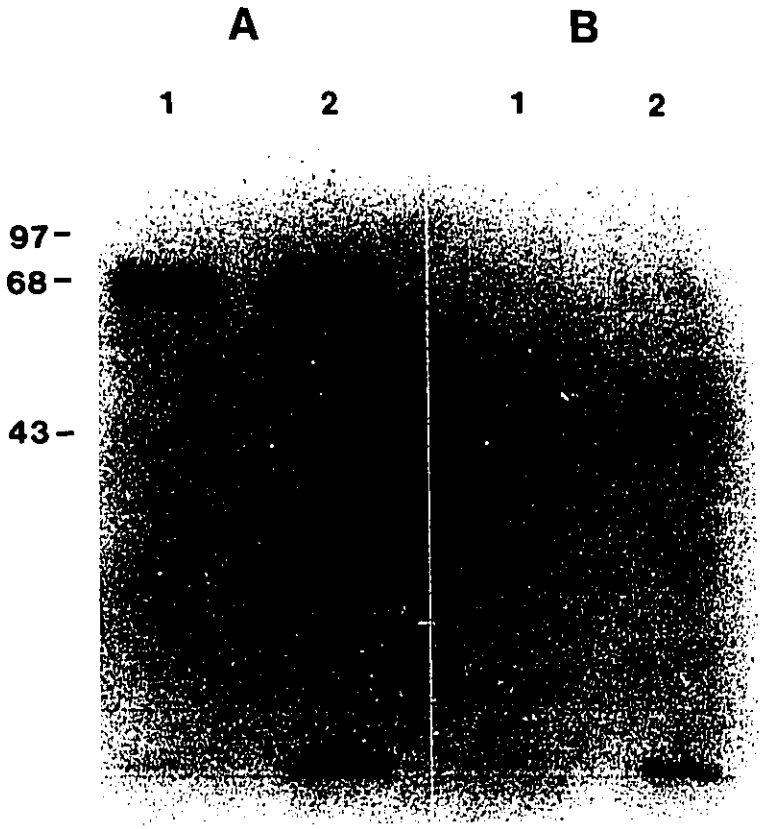
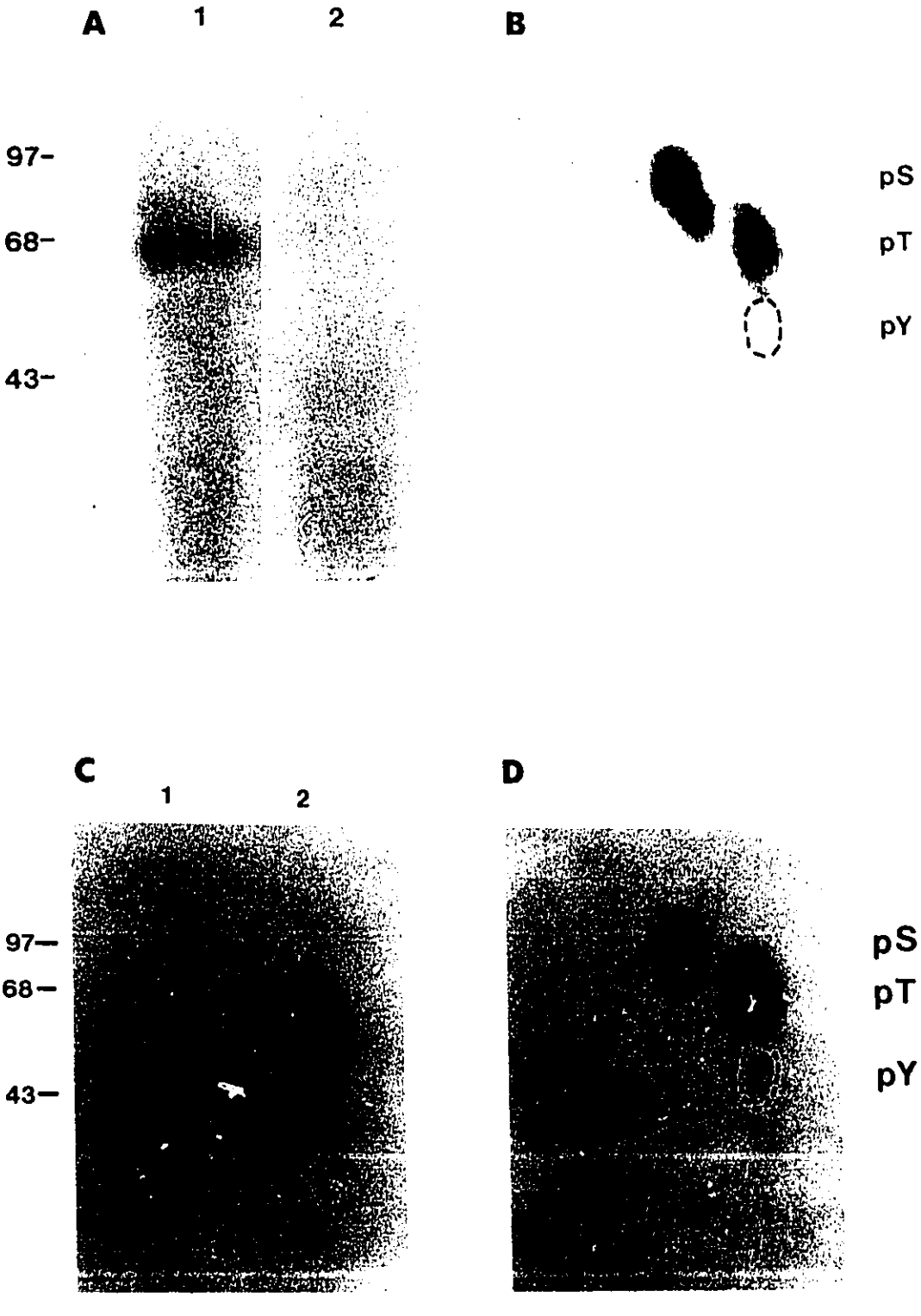


Figure 17

Figure 18: **A:** *In vitro* kinase reactions of the TIK fusion protein in bacterial lysates. Lysates of *E.Coli* BL21 expressing the TIK fusion protein in either the sense (lane 2) or antisense (lane 1) orientation were immunoprecipitated with an antiphosphotyrosine antibody and assayed for kinase activity in the presence of [γ-^{32}P]ATP prior to SDS-polyacrylamide gel electrophoresis. **B:** Phosphoamino acid analysis of electroeluted products from panel A. The positions of the phosphoamino acid standards are indicated: pS-phosphoserine, pT-phosphothreonine, and pY-phosphotyrosine. **C:** *In vivo* labelling of the TIK fusion protein. *E. Coli* BL21 expressing the TIK fusion protein in either the antisense (lane 1) or sense (lane 2) orientation were grown in the presence of [^{32}P]orthophosphate. Lysates from these bacteria were resolved on SDS-polyacrylamide gel electrophoresis and autoradiographed. **D:** Phosphoamino acid analysis of electroeluted products from panel C. (Please see following page) **E:** Kinase renaturation assay of the TIK kinase. Lysates of *E Coli* BL21 expressing the TIK fusion protein in either the sense (lane 1) or antisense (lane 2) orientation were resolved by SDS-PAGE, and transferred to an Immobilon P membrane. The blotted proteins were denatured and renatured as described in Materials and Methods and assayed for kinase activity in the presence of [γ-^{32}P]ATP. The blot was then washed and autoradiographed. **F:** Phosphoamino acid analysis of the protein blotted onto Immobilon P shown in panel E.


A
1
2
97-
68-
43-
B
pS
pT
pY
C
1
2
97-
68-
43-
D
pS
pT
pY

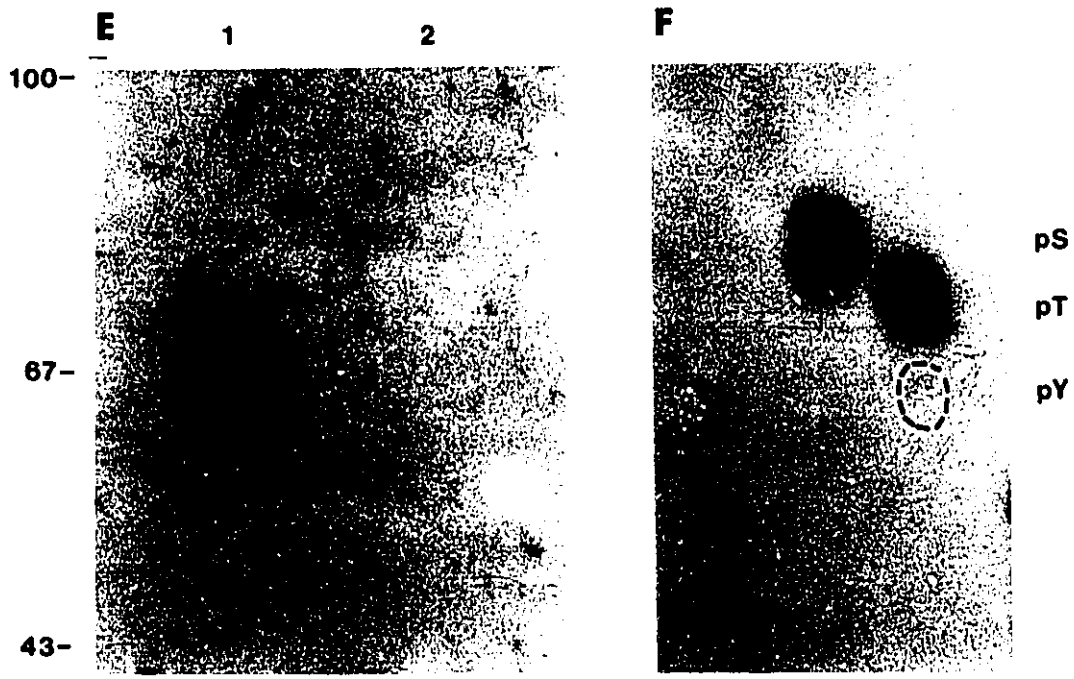


Figure 18

Although these experiments strongly suggest that the 67 kDa TIK fusion protein has intrinsic serine/threonine kinase activity, it remained formally possible that this activity is attributable to an associated bacterial kinase. To rule out this possibility, a kinase renaturation assay was performed. In this procedure, lysates from bacteria expressing the TIK fusion protein were separated by gel electrophoresis, blotted to an Immobilon P membrane, induced to renature as described by Ferrell and Martin (120), and incubated with [γ-^{32}P]ATP. The renatured 67 kDa TIK fusion protein incorporates radioactively labelled phosphate (figure 18E). Lysates from bacteria expressing the TIK antisense construct did not show this activity. Phosphoamino acid analysis of the renatured TIK kinase showed that it contains phosphoserine and phosphothreonine (see figure 18F), confirming that the TIK protein has intrinsic serine/threonine kinase activity.

3.3.2 The TIK Kinase is Immunoreactive with Antibodies to Phosphotyrosine

The determination that the TIK kinase is in fact a PSK, and has no tyrosine phosphorylating ability, begs the question of the biochemical basis of its antiphosphotyrosine immunoreactivity. To investigate the possible involvement of phosphorylated serine and threonine residues in this immunoreactivity, a mutant TIK protein, (ATP-)TIK, lacking kinase activity was produced by site-directed mutagenesis (figure 16). The mutant kinase produced lacks a lysine residue which is conserved among all members of the kinase family and is essential for kinase activity (123). Bacterial expression of the T7 gene 10-(ATP-)TIK fusion protein produces a band on a Coomassie-stained polyacrylamide gel of approximately 60 kD (figure 19). This

Figure 19: Expression of the (ATP-)TIK protein
Coomassie blue stained gel showing lysates of *E. coli* BL21 bacteria expressing the (ATP-)TIK fusion protein. The control lane is a lysate of *E. coli* BL21 bacteria alone. The arrow indicates the enriched band at 60 kD.

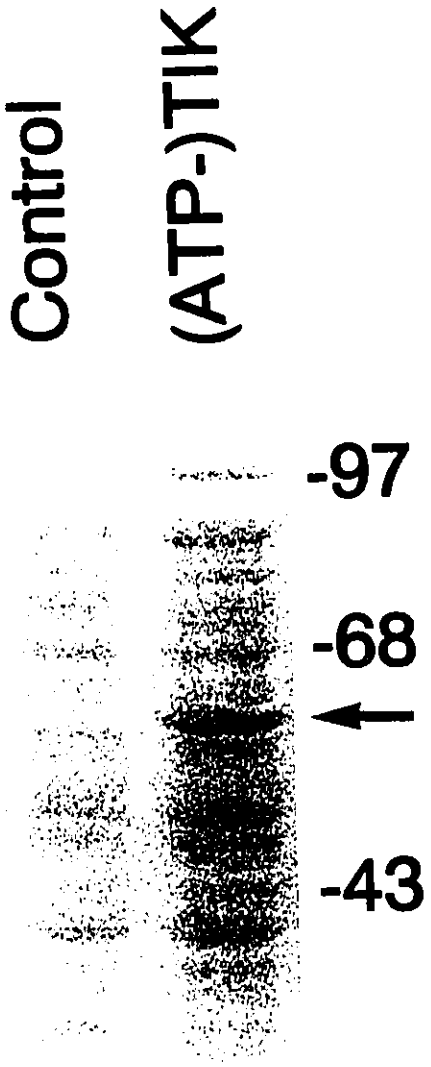


Figure 19

(ATP-)TIK fusion protein does not bind the antiphosphotyrosine antibody on an immunoblot (figure 20).

3.4 Discussion:

The TIK kinase was expressed in bacteria as a fusion protein from a cDNA construct in which the coding region for the first 11 amino acids of the T7 gene 10 product is fused in frame with the 5-prime end of the entire TIK coding region (see figure 15). This fusion protein migrates at approximately 67 kDa in an SDS-PAGE system (figure 17). The expected molecular mass of the TIK fusion protein is approximately 60 kDa, so it appears to be migrating anomolously in the SDS-PAGE system. It is possible that the presence of negatively charged phosphate groups on the TIK protein affect the ability of SDS molecules to bind the protein, and this may affect its migration in this system. Other phosphoproteins also migrate to unexpected apparent molecular masses in SDS-PAGE systems, for example the E1A protein of adenovirus (124) and the NS protein of vesicular stomatitus virus (125). Consistent with this notion is the observation that the kinase inactive mutant TIK protein, (ATP-)TIK, which differs from the TIK protein only in the substitution of two amino acids (figure 16), migrates to the expected molecular mass of 60 kDa in an SDS-PAGE system (figure 19).

The immunoreactivity of the TIK kinase with antibodies to phosphotyrosine suggests that this enzyme is capable of tyrosine autophosphorylation. Unexpectedly, the 67 kDa T7 gene 10-TIK fusion protein was found to have only serine and

Figure 20: Antiphosphotyrosine immunoreactivity of the TIK and (ATP-)TIK proteins

Antiphosphotyrosine immunoblot of lysates of *E. coli* BL21 expressing the TIK or (ATP-)TIK proteins.

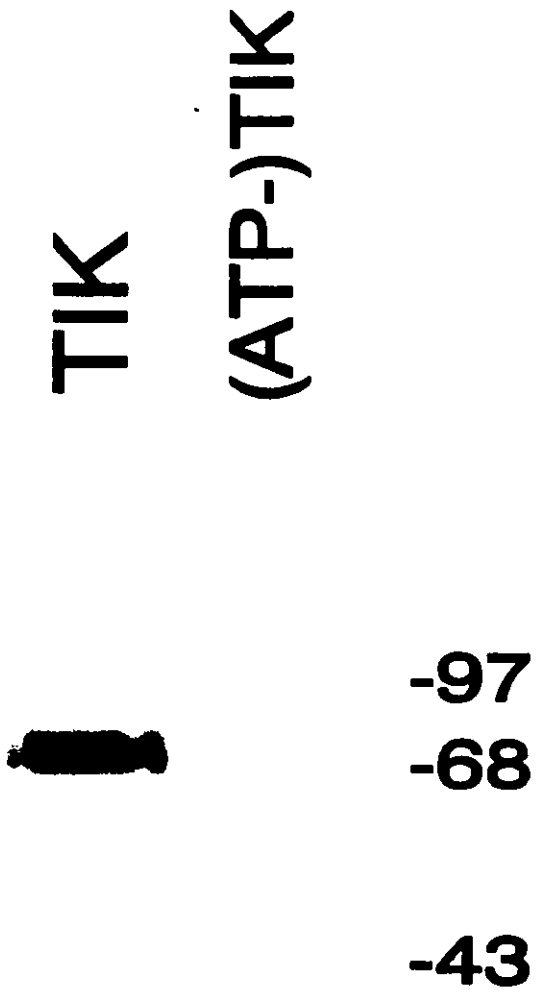


Figure 20

threonine kinase activity in an *in vitro* kinase assay (figure 18A and 18B). This experiment may indicate that the *in vitro* activity of the kinase results in serine and threonine phosphorylation, and that tyrosine phosphorylation occurs only *in vivo*. However, the TIK fusion protein immunoprecipitated with antiphosphotyrosine from bacteria labelled *in vivo* with [^{32}P]orthophosphate was found to have incorporated the radioactively labelled phosphate onto only serine and threonine residues (figure 18C and 18D). Finally, the TIK fusion protein was shown to autophosphorylate on serine and threonine residues in a kinase renaturation assay (figure 18E and 18F). These experiments confirm that this kinase has only intrinsic PSK activity.

Despite its lack of phosphotyrosyl residues, the TIK protein can be detected and immunoprecipitated with three different monoclonal antiphosphotyrosine antibodies and a polyclonal antiphosphotyrosine antibody. A possible explanation for this observation is that these antibodies are cross reacting with phosphoserine or phosphothreonine. However, detection of the 67 kDa TIK fusion protein band in antiphosphotyrosine immunoblots of lysates from TIK expressing cells was blocked by competition with 5 mM phosphotyrosine, but not with phosphoserine or phosphothreonine (data not shown). This evidence suggests that the TIK protein may contain an epitope resembling phosphotyrosine. To determine the possible involvement of phosphorylation on serine and/or threonine residues in antiphosphotyrosine immunoreactivity, a kinase inactive mutant TIK protein was generated by site-directed mutagenesis. As illustrated in figure 16, this mutant protein was produced by substitution of an isoleucine residue for a lysine residue

which is highly conserved in all kinases, and known to be essential for the phosphotransfer reaction (123). This mutant has been designated (ATP-)TIK due to its inability to effect phosphotransfer from the ATP molecule. This kinase deficient TIK protein is not immunoreactive with antibodies to phosphotyrosine (figure 20), indicating that phosphoserine and/or phosphothreonine residues may be assuming a conformation mimicking the epitope(s) to which these antibodies bind.

If indeed such a phosphotyrosine-mimicking epitope does exist it may play a role in the regulation of the biochemical and biological properties of the TIK kinase. The TIK epitope detected by the antiphosphotyrosine antibodies may represent a binding site for molecules which specifically recognize phosphotyrosyl residues. As outlined in the introduction, cytoplasmic PTKs and other molecules of the signal transducing network contain SH2 domains, which specifically bind phosphotyrosine-containing peptides. The phosphotyrosine-resembling epitope of the TIK kinase may represent an SH2 binding site, and could therefore allow the TIK protein to gain access to signal transduction molecules which normally communicate via phosphotyrosine:SH2 interactions. In support of this idea, the TIK kinase has been found to bind the SH2 domain of phospholipase C-γ (data not shown).

It is also possible that the TIK protein may contain a small amount of phosphotyrosine, sufficient for detection and immunoprecipitation with the antibody, but undetectable by phosphoamino acid analysis. This, however, seems unlikely, as the method of phosphoamino acid analysis employed here is extremely sensitive.

Use of the antiphosphotyrosine antibody has been considered a specific screen

for functional tyrosine kinases (98-100). The observation that the TIK kinase reacts with such antibodies but does not possess tyrosine phosphorylating ability indicates that this screening procedure also unexpectedly detects novel serine/threonine kinases.

Chapter Four

One Allele of the Gene Encoding TIK has been rearranged in the Murine Lymphocytic Leukemia Line L1210

4.1 Introduction:

A large body of evidence suggests that members of the kinase family play roles controlling growth and development of cells of the hematopoietic lineage. Hoping to obtain clues to the role the novel TIK kinase may be playing in these processes, I determined the pattern of expression of mRNA transcripts encoding TIK in a variety of hematopoietic cells and cell lines. The cDNA encoding TIK detects three mRNA transcripts in all murine tissues investigated and in some murine leukemia cell lines. The murine lymphocytic leukemia cell line L1210, however, expresses not only these three mRNAs, but also three transcripts which are each smaller in size than the normal mRNAs. Evidence is presented here that one allele of the gene encoding the TIK kinase has undergone a rearrangement in the L1210 line, and transcription of this mutant allele appears to give rise to the abberant pattern of TIK mRNA expression observed. Presuming that the TIK kinase is involved in some way in the control of growth and differentiation during lymphocyte development, mutation of the TIK kinase may have contributed to the process of initiation or maintenance of transformation in the L1210 cell line. A cDNA corresponding to the smallest truncated TIK mRNA expressed in the L1210 line was isolated. Outlined here is evidence that this mRNA encodes a mutant TIK enzyme lacking kinase activity.

4.2 Materials and Methods:

4.2.1 Genomic DNA isolation and Southern blot analysis:

Cells in culture were grown to a density of approximately 10^5 cells per ml, harvested, and resuspended into 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, 10mM NaCl, and 0.5% SDS. Proteinase K was added to a final concentration of 50 μg/ml and the sample was incubated 30 min at 55°C. Following this incubation, the sample was extracted twice with phenol (pH 8.0), once with chloroform/isoamylalcohol (24:1), and finally dialyzed overnight against three changes of 10 mM Tris-HCl pH 7.5, 1 mM EDTA. Digestion of genomic DNA was typically carried out overnight in the appropriate restriction enzyme buffer using 10 units of enzyme per μg of genomic DNA. Aliquots of 10 μg of DNA were electrophoresed in 1% agarose gels containing 0.04 M Tris-acetate and 0.001 M EDTA, and the gels were treated in a denaturing solution of 1.5 M NaCl, 0.5 M NaOH for 45 min, and neutralized in 1.5 M NaCl, 0.5 M Tris-HCl, pH 8, for 45 min. The electrophoretically separated DNAs were transferred to nylon-backed nitrocellulose membranes (Micron Separations, Inc), and these filters were baked at 80°C under vacuum for 2 hr. Filters were then probed with DNA probes generated by random primer extension labelling of cDNA fragments. Prehybridizations were carried out in 6 X SSC, 5 X Denhardt's Solution, 0.5% SDS, and denatured salmon sperm DNA at 0.1 mg/ml at 65°C for 6 hr. Hybridizations were performed using radioactively labelled probe at 1-4 X 10^5 cpm/ml for 12 hr. Filters were washed in 2 X SSC at 65°C for 5 min and once in 0.1 X SSC, 0.1% SDS at 65°C for 30 min, then autoradiographed by exposure to Kodak

XAR-5 X-ray film.

4.2.2 Isolation of cDNA corresponding to the truncated TIK mRNA transcripts:

Total RNA was prepared from the L1210 cell line by the method of Auffray and Rougeon (102). Poly A^+ selection was carried out by passage of total RNA over oligo-d(T) cellulose, following the method of Jacobson (103). 45 μg of poly-A^+-selected L1210 mRNA was then added to a 5-20% sucrose gradient in 70% formamide. The sample was spun at 36K for 20 hrs, and fractions were collected in 160 μl aliquots. The RNA in these samples was ethanol precipitated and resuspended in a small volume of water, and a third of each sample was carried through Northern blot analysis (please refer to Materials and Methods, chapter 1). The Northern blot was probed with a random-primed ^{32}P-labelled TIK cDNA probe, and fractions containing 0.5 to 2.0 Kb mRNA hybridizing to the TIK cDNA were selected for cDNA library construction. This mRNA was used as a template to generate double stranded cDNA using the BRL cDNA synthesis system. The blunt cDNA produced was ligated to Not 1/Eco R1 linker molecules, and then to λgt10 phage which had been digested with Eco R1. The recombinant phage DNA was packaged into λgt10 phage heads, and this phage was used to infect *E. Coli* C600 hfl bacteria in order to propagate the recombinant phage.

The L1210 partial cDNA library produced was screened to detect TIK cDNAs by the following method: Recombinant λgt10 phage was allowed to adsorb with *E. Coli* C600 bacteria for 15 min at 37℃, plated, and incubated for 8-12 hr at 37℃. Nitrocellulose filters (Du Pont) were overlayed on the plates for 1 min, and then

treated in a denaturing solution of 1.5 M NaCl, 0.5 M NaOH for 5 min, neutralized in 1.5 M NaCl, 0.5 M Tris-HCl, pH 8, for 5 min, and rinsed in 0.2 M Tris-HCl pH 7.5, 2 X SSC for 30 sec. Filters were baked at 80℃ under vacuum for 2 hr. Filters were then probed with a TIK cDNA probe generated by random primer extension labelling. Prehybridizations were carried out in 6 X SSC, 5 X Denhardt's Solution, 0.5% SDS, and denatured salmon sperm DNA at 0.1 mg/ml at 65℃ for 6 hr. Hybridizations were performed using radioactively labelled probe at 1-4 X 10^5 cpm/ml for 12 hr. Filters were washed in 2 X SSC at 65℃ for 5 min and once in 0.1 X SSC, 0.1% SDS at 65℃ for 30 min, then autoradiographed by exposure to Kodak XAR-5 X-ray film. Positive clones were rescreened twice to homogeneity. The cDNA isolated from positive phage clones was analyzed by cDNA sequence analysis using the Sanger dideoxy chain-termination method (101).

Nothern blot analysis was performed as described in Chapter One.

4.3 Results:

4.3.1 TIK mRNA Transcripts are Aberrantly Expressed in the L1210 cell line:

Through Northern blot analysis using poly-A selected RNA isolated from the 70Z/3 cell line, the TIK cDNA was found to hybridize to 3 distinct mRNAs of 6, 4, and 2.5 Kb (figure 8). In the murine lymphoctic leukemia line L1210, however, three mRNAs of approximately 5, 3, and 1.5 Kb were detected in addition to the expected 6, 4, and 2.5 Kb mRNAs (see figure 21). The lymphocytic leukemia line P388 expresses predominantly the 6 Kb TIK mRNA transcript.

Figure 21: Northern blot analysis of TIK mRNA in adult mouse tissues and murine leukemic cell lines

Each lane contains approximately 5 μg of poly(A)-selected RNA isolated from each mouse tissue. The blot was hybridized to a TIK cDNA probe. The 18 and 28s rRNAs are 1896 and 4712 nucleotides, respectively. Please refer to appendix II for a description of the cell lines P388 and L1210.

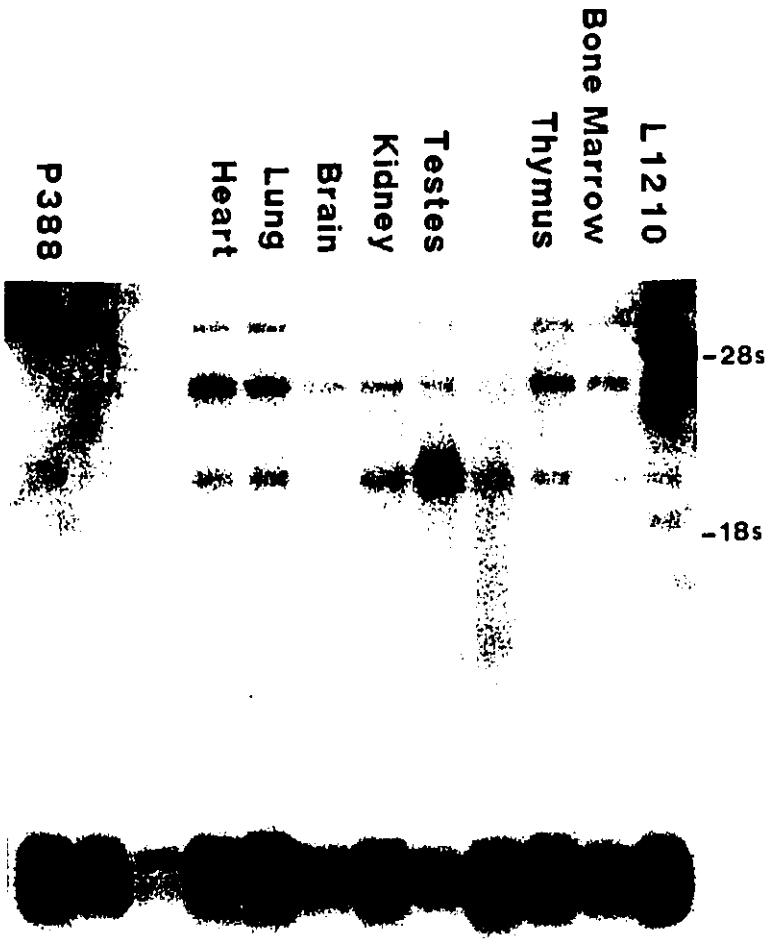

P388
Heart
Lung
Brain
Kidney
Testes
Thymus
Bone Marrow
L1210
-28s
-18s

Figure 21

4.3.2 One allele of the TIK gene has undergone a rearrangement in the L1210 line:

Southern blot analysis of genomic DNA isolated from the 70Z/3 line and the L1210 line is shown in figure 22. The radioactively labelled cDNA probe used for hybridization to this blot was produced using the entire 2.1 Kb TIK cDNA. Eco R1 digestion of DNA from the 70Z/3 line generates 4 bands of 7.5, 6.0, 3.5, and 1.7 Kb. Eco R1 Digestion of DNA isolated from the L1210 line generates these expected bands, and also an additional band of 3.0 Kb. Similarly, digestion of DNA from the 70Z/3 line with Kpn 1 shows a pattern of bands of 12, 8.5, 6.5, and 1.5 Kb, and digestion of L1210 DNA with this enzyme shows the same pattern, along with 2 additional bands of 9.0 and 4.0 Kb. These data indicate that one allele of the gene encoding the TIK kinase has undergone a rearrangement in this cell line, giving rise to the novel bands visualized using Southern blot analysis.

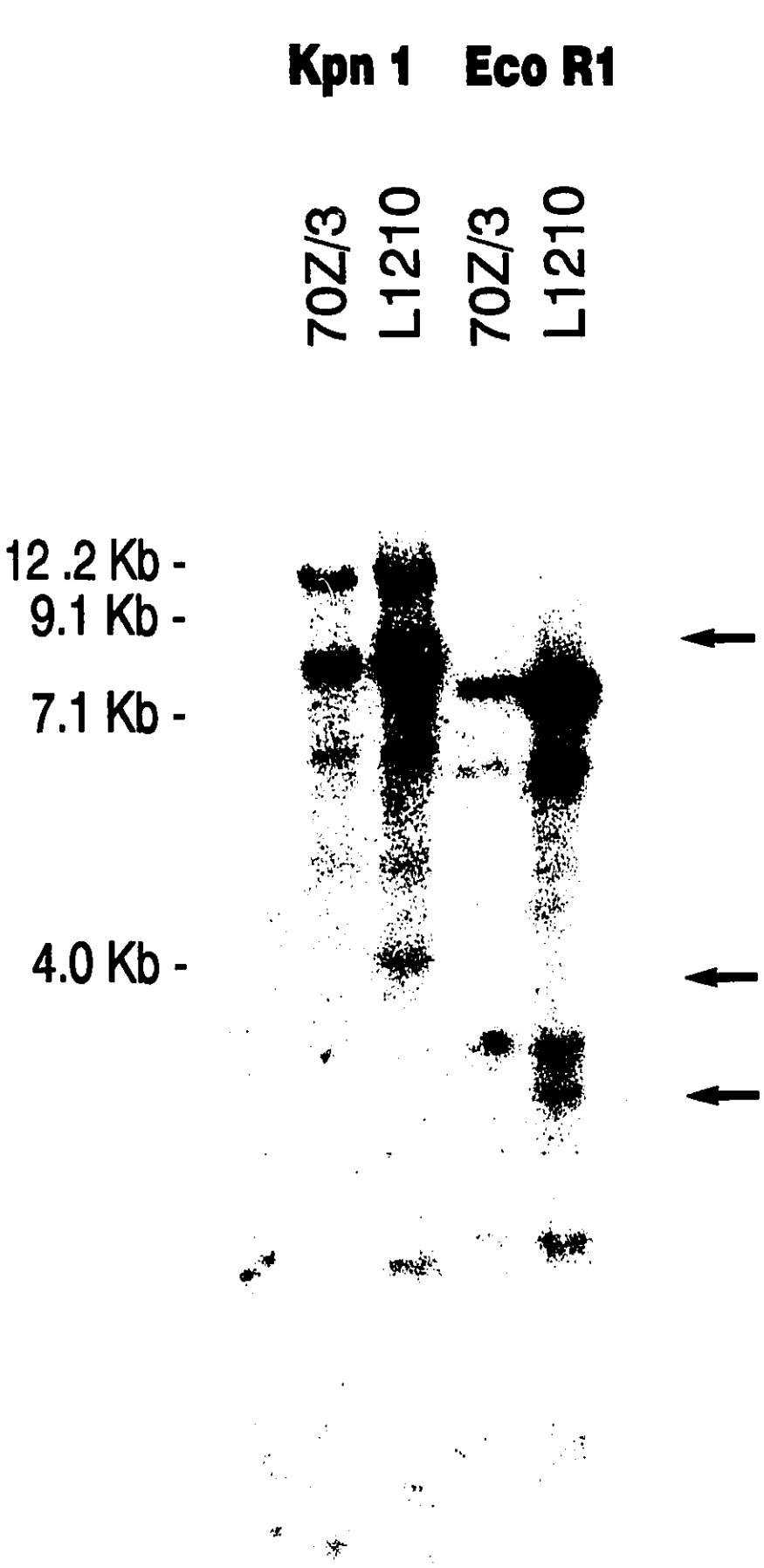
Southern blot analysis using genomic DNA isolated from DBA/2 mice, the parental strain of the L1210 cell line, digested with the restriction enzymes Eco R1 and Kpn 1 shows a pattern of bands identical to those seen with the 70Z/3 cell line (data not shown).

4.3.3 Isolation and Characterization of a Mutant TIK cDNA from the L1210 line:

Poly-A+ selected L1210 mRNA was size fractionated on a sucrose gradient, and a small sample of each fraction was used for Northern blot analysis, and probed with a riboprobe generated from the 2.1 Kb TIK cDNA. A partial λgt10 cDNA library was constructed from the fraction which hybridized to the smallest TIK mRNA

Figure 22: Southern blot analysis of genomic DNA from the 70Z/3 and L1210 cell lines

Each lane contains approximately 10 μg of genomic DNA isolated from the 70Z/3 or L1210 cell lines digested with the restriction enzymes Kpn1 or Eco R1. The blot was hybridized to a TIK cDNA probe. Arrows indicate the bands only present in DNA isolated from the L1210 cell line.


Kpn 1
Eco R1
70Z/3
L1210
70Z/3
L1210
12 .2 Kb -
9.1 Kb -
7.1 Kb -
4.0 Kb -

transcript. This partial cDNA library was then probed with a cDNA probe made from the 2.1 Kb TIK cDNA, and one λgt10 clone was isolated from the screening of 40,000 phage clones. The cDNA sequence of this clone showed complete identity with the sequence of the previously identified 2.1 Kb TIK cDNA, however, the putative mutant cDNA was found to have an internal in-frame deletion of 582 base pairs corresponding to nucleotides 522-1104 of the TIK sequence. This cDNA has been designated T-TIK. The T-TIK cDNA contains 254 nucleotides at the extreme 5-prime end which are not present in the TIK cDNA. Although this region contains a 160 base pair open reading frame, no initiating ATG codon is present. The 3-prime end of this cDNA does not contain a poly-A tail, and the final nucleotide at the 3-prime end of the T-TIK cDNA corresponds to nucleotide 1662 of the TIK cDNA. A linear representation of the TIK cDNA compared to the T-TIK cDNA and the proteins they encode may be found in figure 23.

In order to verify that the T-TIK cDNA isolated from the L1210 line actually corresponded to the smallest mutant mRNA transcript and did not merely represent an artifact of cloning, the 582 base pair fragment deleted from this cDNA was used as a probe in Northern blot analysis of mRNA from the L1210 line and normal mouse tissues. As shown in figure 24, the 582 base pair fragment hybridzes only to the normal TIK mRNAs of 6, 4, and 2.5 Kb and not to the mutant truncated mRNAs expressed in the L1210 line.

Figure 23: A schematic representation of the TIK and T-TIK cDNAs
The kinase catalytic subdomain sequences are indicated by Roman numerals. Arabic numbers represent nucleotides of the TIK cDNA.

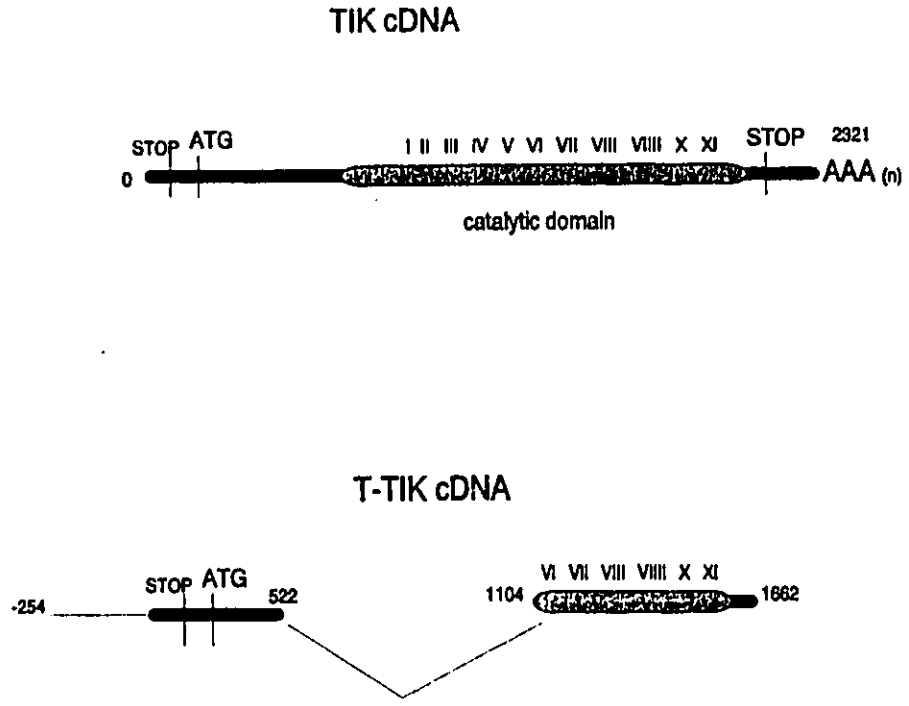
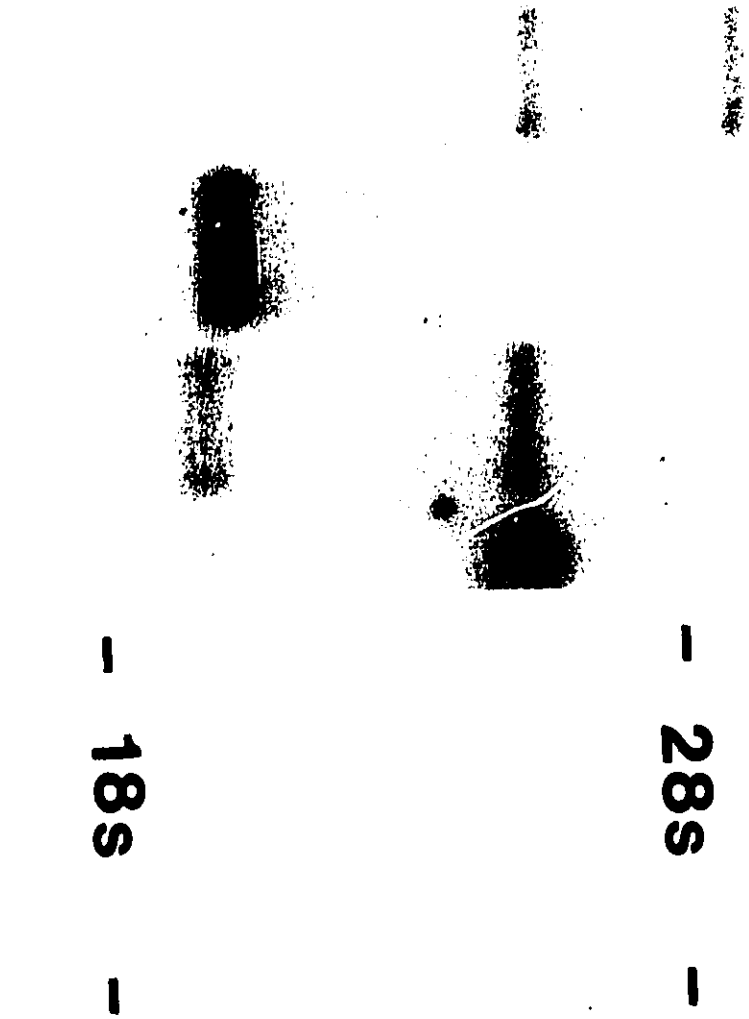

TIK cDNA
STOP
ATG
I II III IV V VI VII VIII VIII X XI
STOP
2321
0
AAA (n)
catalytic domain
T-TIK cDNA
-254
STOP
ATG
522
VI VII VIII VIII X XI
1104
1662

Figure 23

Figure 24: Northern blot analysis showing the 582 base pair fragment deleted from T-TIK does not detect the truncated L1210 mRNAs

Each lane contains approximately 5 μg of poly(A)-selected RNA isolated from each mouse tissue. Panel A: The blot was hybridized to the 582 base pair cDNA probe. Panel B: The blot was hybridized to a TIK cDNA probe. The 18 and 28s rRNAs are 1896 and 4712 nucleotides, respectively.

A



B

Figure 25: Genomic Southern blot analysis showing that the 582 base pair fragment deleted from T-TIK detects the unique L1210 genomic fragments

Each lane contains approximately 10 μg of genomic DNA isolated from the 70Z/3 or L1210 cell lines digested with the restriction enzymes Kpn1 or Eco R1. The blot was hybridized to the 582 base pair cDNA probe. Arrows indicate the bands present only in DNA isolated from the L1210 cell line.

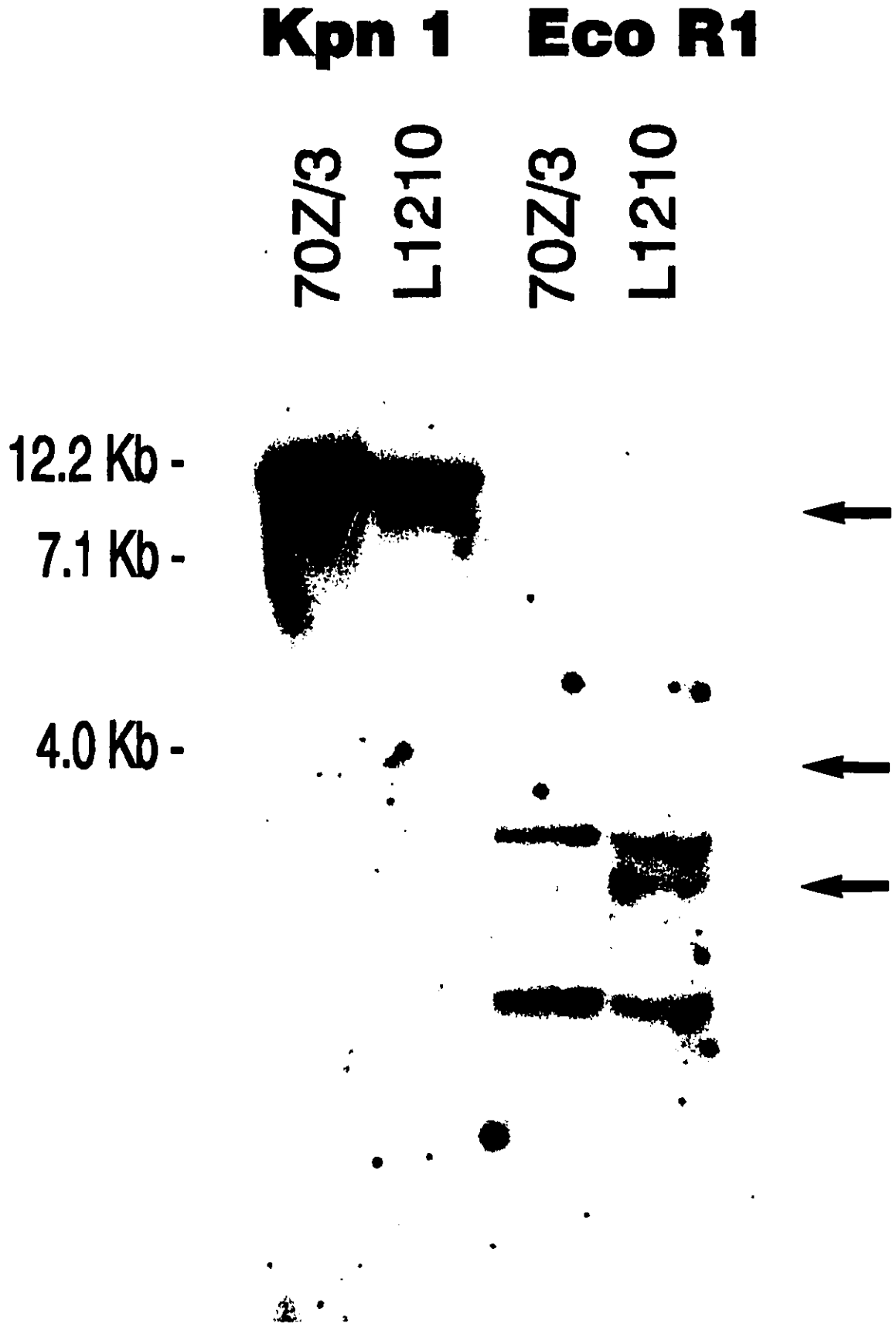
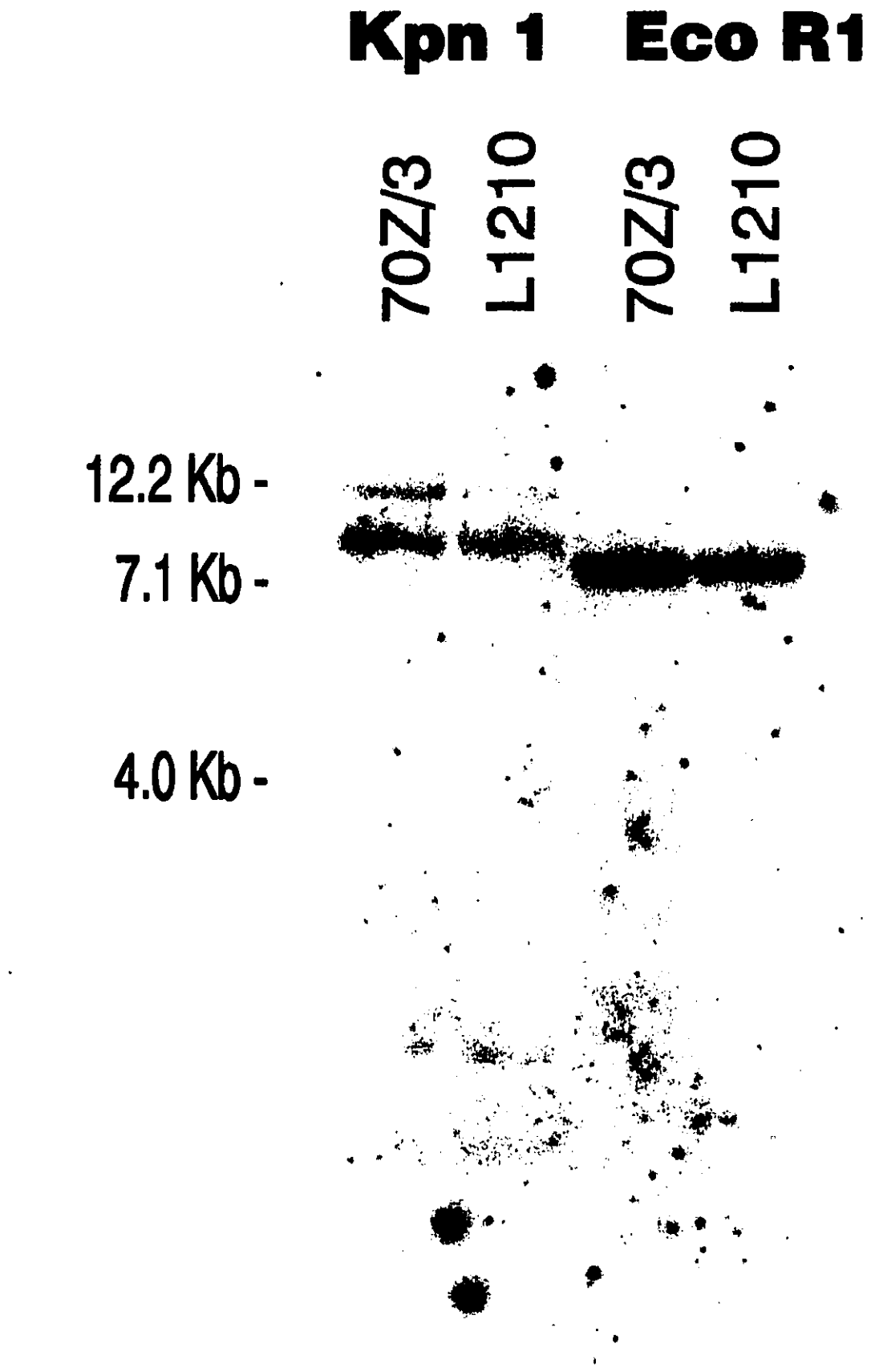

Kpn 1
Eco R1
70Z/3
L1210
70Z/3
L1210
12.2 Kb -
7.1 Kb -
4.0 Kb -

Figure 26: Genomic Southern blot analysis showing that a cDNA fragment common to both the TIK and T-TIK cDNAs does not detect the unique L1210 genomic fragments

Each lane contains approximately 10 μg of genomic DNA isolated from the 70Z/3 or L1210 cell lines digested with the restriction enzymes Kpn1 or Eco R1. The blot was hybridized to the cDNA probe common to both the TIK and (ATP-)TIK cDNAs.


Kpn 1
Eco R1
70Z/3
L1210
70Z/3
L1210
12.2 Kb -
7.1 Kb -
4.0 Kb -

Partial Characterization of the Genomic Rearrangement in the L1210 line:

The 582 base pair fragment absent from the T-TIK cDNA was used to probe genomic Southern blots of L1210 and 70Z/3 DNA. As shown in figure 25, this probe hybridizes to the 12 Kb Kpn 1 band, and the 3.5 and 1.7 Kb Eco R1 bands, in additon to the unique bands detected only in digestion of the L1210 DNA. A second cDNA fragment probe was used on genomic Southern blots of L1210 and 70Z/3 DNA. This probe represents nucleotides 1132-1662 of the TIK cDNA, a region encoding the portion of the catalytic domain of the TIK kinase which is also encoded by the T-TIK cDNA (see figure 26). This cDNA probe does not hybridize to these unique L1210 DNA fragments, but detects only the 12 and 8.5 Kb Kpn1 bands and the 7.5 Kb Eco R1 band (see figure 22).

4.4 Discussion:

In all normal murine tissues examined, the gene encoding the novel TIK kinase is transcribed to produce three TIK mRNA transcripts. The murine lymphocytic leukemia line L1210, however, expresses six TIK mRNA transcripts, three of which are the same size as those produced in other tissues, and three which are each approximately one kilobase smaller in size than the normal mRNAs (figure 21). These three truncated mRNA transcripts appear to originate from one allele of the TIK gene in L1210 cells which has undergone a genomic rearrangement. The other allele encoding TIK apparently remains intact, and is transcribed to produce TIK mRNAs of the expected sizes (figure 22).

We have isolated a cDNA corresponding to the smallest truncated TIK mRNA transcript expressed in the L1210 line. As shown in figure 23, this cDNA contains an in-frame internal 582 base pair deletion of a portion of the TIK coding region. The altered TIK protein encoded by this cDNA would be lacking the kinase catatlytic subdomains I, II, III, and IV. Subdomains I and II contain the ATP-binding site and a conserved lysine residue known to be essential for kinase activity, respectively, and this protein would therefore lack kinase activity. This cDNA has been designated T-TIK, for truncated TIK cDNA. The T-TIK cDNA also contains an additional 254 base pairs of sequence at the extreme 5-prime end which is not found in the normal TIK cDNA. Although this region contains a 160 base pair open reading frame, it does not contain an ATG translation initiation codon. When compared with the DNA sequences stored of the GenBank/EMBL data bank, this 254 base pair sequence showed the greatest homology with untranslated regions of other mammalian cDNAs. These pieces of evidence indicate that it is unlikely that the small open reading frame identified is actually transcribed and translated. This 254 base pair fragment may represent a portion of the 5-prime untranslated region (UTR) which is present in both the normal and truncated TIK mRNA transcripts, or it may be present only in the truncated TIK mRNA. Its absence from the normal TIK cDNA may merely indicate that this cDNA is not full-length.

The 3-prime end of the T-TIK cDNA does not extend to the "stop" codon of the normal TIK cDNA, and does not contain a poly-A tail. As outlined in Materials and Methods, this cDNA was produced using the BRL cDNA synthesis system, which

uses an oligo-dT primer for first strand cDNA synthesis. The absence of poly-A sequences suggests that second strand cDNA synthesis did not proceed the entire length of the first strand synthesis, and the 3-prime end sequences were lost.

The 582 base pairs of sequence deleted from the T-TIK cDNA are also deleted from all three of the truncated TIK mRNA transcripts in the L1210 line (figure 24). This suggests that not only the 1.5 Kb, but also the 3 and 5 Kb mRNAs are likely to encode kinase deficient proteins. Since the 3-prime end of the T-TIK cDNA is incomplete, it remains to be determined whether the carboxy terminus of the T-TIK protein is altered or resembles the normal protein.

The observation that the T-TIK cDNA lacks a portion of the TIK-encoding region present in the TIK cDNA could suggest that the genomic rearrangement of the TIK gene in the L1210 line may involve deletion of the exon(s) containing these sequences. To investigate this possibility, the deleted 582 base pair fragment was used to probe genomic Southern blots of DNA from the 70Z/3 cell line and the L1210 cell line. This fragment hybridized to the 3.0 Kb EcoR1 band and the 9.0 and 4.0 Kb Kpn1 bands present only in the L1210 DNA, indicating that the genomic rearrangment has not resulted in deletion of the exon(s) encoding these sequences, but that the exons reside in a region directly affected by the rearrangement. It is possible that the genomic mutation alters the splicing of the primary RNA transcript produced from the TIK gene, and the 582 base pair sequence is not included in the mature mRNA molecule.

Another fragment of the TIK coding region of the cDNA was used as a probe

for genomic Southern blot analysis. This fragment, nucleotides 1132 to 1662 of the TIK cDNA, is contained in both the TIK and T-TIK cDNAs. As shown in figure 26, this probe does not hybridize to the bands present only in the L1210 DNA, indicating that the exon(s) containing these sequences are not involved in the genomic rearrangement.

Members of the protein kinase family have been implicated in oncogenesis in a variety of tissues, including hematopoietic tissues. The role that these kinases play is generally dependent upon their kinase activity, and the mutations unveiling the oncogenic potential of kinases represent dominant gain-of-function mutations which stimulate kinase activity. The observations described here portray a genomic rearrangement which has led to the generation of an inactive kinase. It is possible that the TIK kinase plays a role in the control of cellular growth or development in the hematopoietic lineage. If this role were to transduce a growth-suppressing signal as opposed to a growth-stimulatory signal, abrogation of this signal could represent an oncogenic lesion contributing to the generation of a malignancy. As outlined previously, structural mutations of the kit kinase represent dominant mutations and it is thought that in the heterozygous state, the mutant kit proteins associate with the normal kit enzymes and interfere with transduction of the normal kit signal (figure 7). An analogous situation could exist in the L1210 cell line, where one allele of the TIK gene appears to be normal, and one allele has undergone a genomic rearrangement, generating a heterozygous TIK/- genotype. The TIK structural mutant encoded by one, or all three of the truncated TIK mRNAs expressed in the

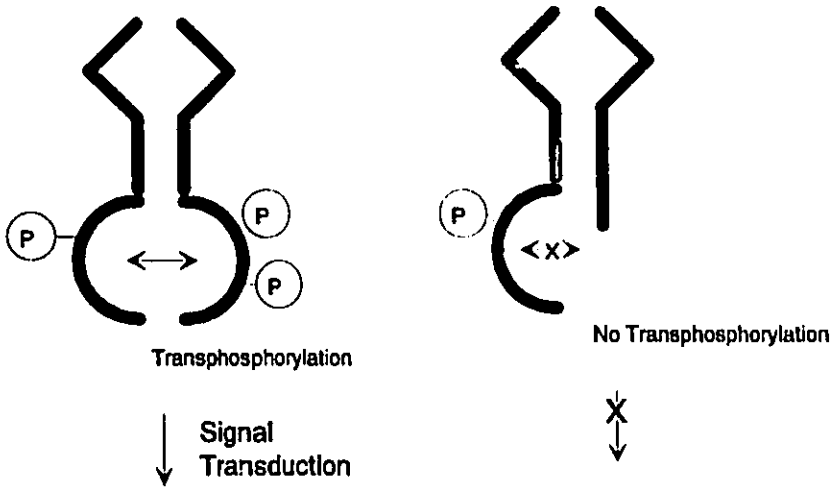
L1210 line could be associating with the normal TIK proteins and disrupting their signal transducing ability, compromising the growth suppressing signal, and contributing to the generation or maintenance of the transformed state of the L1210 cell line. Alternatively, mutation of the TIK enzyme may affect its interaction with one or more of its substrates. The normal function of TIK may be to phosphorylate and regulate the activity of a molecule or molecules having a growth-suppressing function. The TIK kinase may transiently bind these molecules, and then release them subsequent to their phosphorylation. The mutant TIK enzyme, unable to phosphorylate these substrates, may bind substrates and be unable to release them, effectively sequestering these molecules away. These trapped molecules would then be unable to interact with the normal TIK enzyme and to fulfill their growth suppressing role. Conversly, the TIK kinase may play a growth stimulating role, and the mutant kinase may be sequestering a downregulating molecule. In this model, the normal TIK kinase exists in an active state unless it is downregulated through an interaction with another molecule. The mutant kinase stably interacts with this molecule, decreasing the amounts of it available to downregulate the normal kinase, leading to abnormal levels of TIK kinase activity. This deregulation of the signal transduced by the TIK kinase may contribute to a loss of growth control. These models are depicted in figure 27. Although these arguments are purely speculative, it seems reasonable to suggest that the mutation of the TIK gene in this system, which is stably maintained throughout passage of this cell line in culture, is involved in some way in the loss of growth control.

Figure 27: The kinase deficient TIK protein as a dominant negative mutant
Model 1: The mutant TIK kinase interacts with the normal TIK kinase and abrogates signal transduction
Model 2: The mutant TIK kinase stably interacts with and sequesters a substrate molecule.
Model 3: (Please see following page) The TIK kinase as a growth stimulating molecule. The mutant TIK kinase stably interacts with and sequesters a downregulating molecule, leading to deregulation of the activity of the normal kinase.
A: Regulation of the TIK kinase in normal cellular equilibrium B: Regulation of the TIK kinase in the presence of the mutant TIK molecule

Model 1:

Interaction between two TIK molecules

Interaction between the normal and mutant TIK molecules



Model 2:

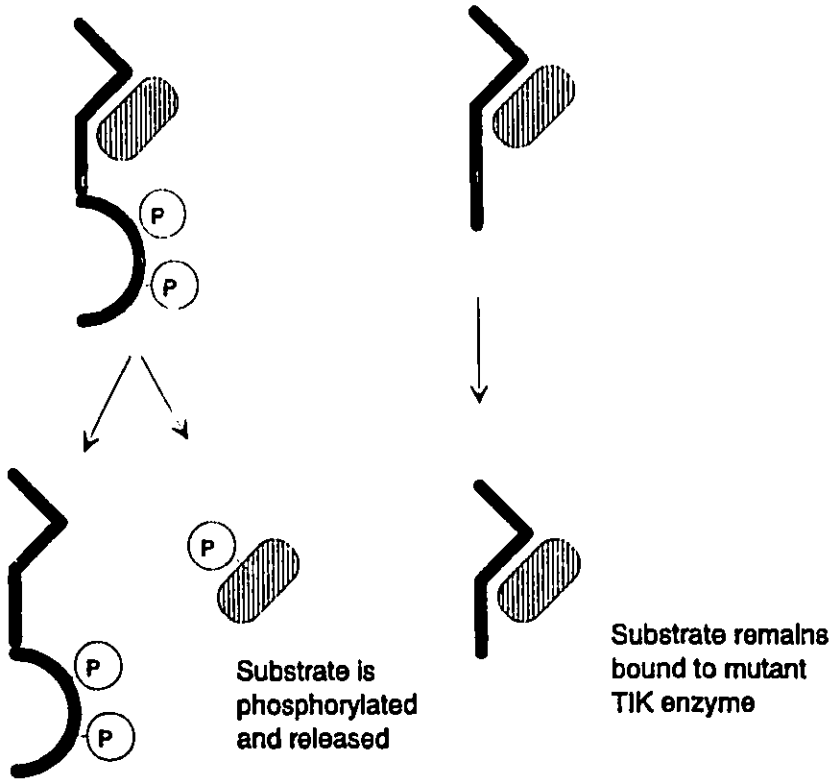
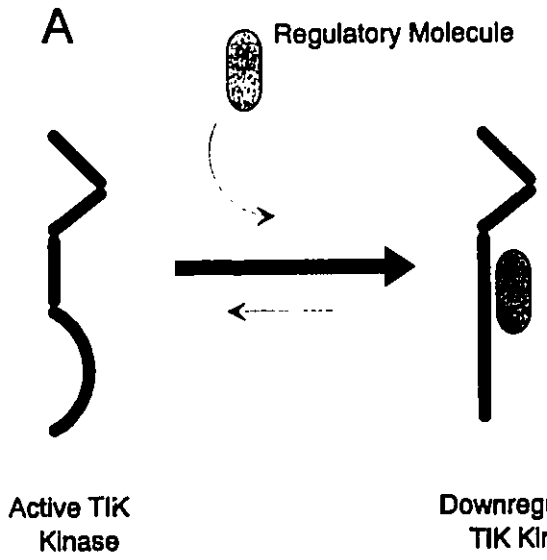


Figure 27



A large proportion of the TIK molecules exist in an inactive state

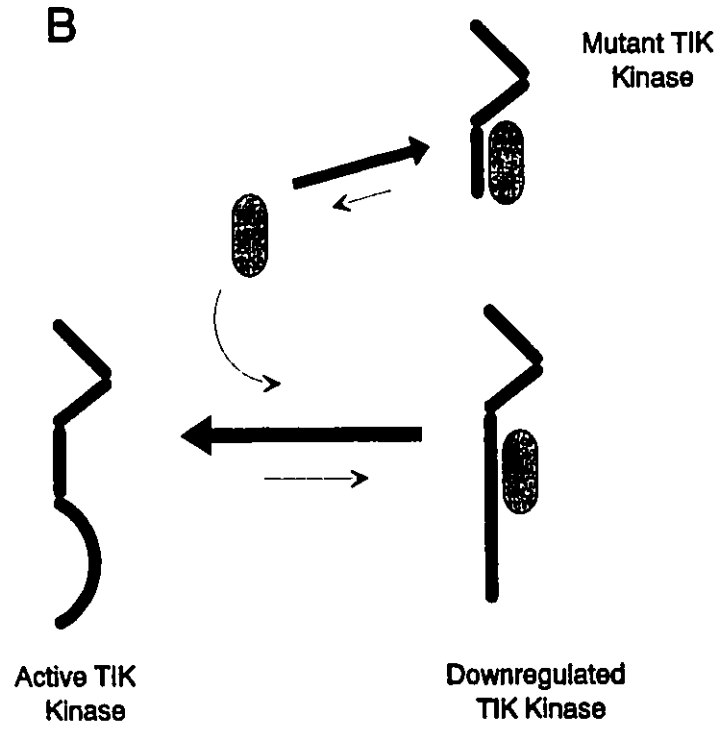


Figure 27

The mutant kinase sequesters the regulatory molecules and shifts the normal cellular equilibrium such that a large proportion of the TIK molecules exist in an active state

Chapter 5

5.1 Conclusions:

The work carried out in this thesis identified a novel member of the protein kinase family, the TIK kinase. Although this kinase was isolated on the basis of its immunoreactivity with antibodies to phosphotyrosine, it was found to have only serine and threonine phosphorylating ability, and no tyrosine phosphorylating ability. The biochemical basis of the immunoreactivity of the TIK kinase with antiphosphotyrosine antibodies remains unclear, however, a kinase deficient TIK enzyme does not bind these antibodies, suggesting that phosphorylated serine and/or threonine residues may be assuming a conformation which mimicks the epitope(s) to which these antibodies bind. Antiphosphotyrosine antibodies have been previously used to detect novel PTKs (98-100). The observations reported here have implications for the future use of these antibodies for such a procedure.

One allele of the gene encoding the TIK kinase has undergone a genomic rearrangement in the murine lymphocytic leukemia line L1210, apparently generating an abberant pattern of TIK mRNA expression. The isolation and characterization of cDNA corresponding to the smallest truncated TIK mRNA transcript expressed in the L1210 line revealed that it encoded a mutant TIK enzyme lacking kinase activity. Three possible models by which this kinase deficient TIK protein may be contributing to the generation or maintenance of the leukemic phenotype of the L1210 cells are depicted in figure 27.

5.2 Future Directions:

The continuation of this project should involve an investigation of the biological significance of the rearrangement of the TIK gene in the L1210 cell line. Some experiments designed to determine this are outlined below. These studies would necessitate further characterization of the truncated mRNA transcripts expressed in the L1210 cell line. As discussed in chapter three, the cDNA corresponding to the smallest mRNA expressed in the L1210 cell line did not extend to the extreme 3-prime end of the mRNA. The sequences in this region should be obtained in order to determine what the carboxy terminal end of the mutant TIK protein is.

The mutation of genes encoding enzymes of the cellular signal transduction network can lead to the activation of the oncogenic potential of these genes. Such mutations can constitute one step in the generation of a malignancy. The observation that the L1210 cell line expresses mRNA encoding a mutant TIK kinase may suggest that the genomic rearrangement of the TIK gene is a mutation which has contributed to the generation or maintenance of the leukemic phenotype of this cell line. The transforming potential of the mutant TIK protein could be assessed through its expression in cells in culture. Recombinant retroviruses encoding the normal TIK protein, and the kinase deficient mutant encoded by the smallest mRNA transcript in L1210 cells could be constructed and used in a transformation assay using murine NIH 3T3 cells. Although partially transformed, these cells lack the ability to form colonies in soft agar, and their growth is contact inhibited. NIH 3T3 cells infected with retroviruses encoding the normal or mutant TIK proteins would

be assessed for their ability to from colonies in soft agar, and grown to confluency to determine if they exhibit contact inhibition of growth. If the TIK mutation characterized in the L1210 cells has contributed to the generation or maintenance of the leukemic phenotype of these cells, it would be expected that expression of the kinase negative mutant TIK proteins in NIH 3T3 cells would lead to complete transformation.

Further experiments could also be carried out to determine the nature of the genomic rearrangement in the L1210 cell line.

These studies should lead to a better understanding of the biological consequences of mutation of the TIK gene in the L1210 cell line, and may perhaps indicate the role that the normal kinase plays in mammalian cell physiology.

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Appendix I
Cell Lines

70Z/3:
This is a murine pre-B lymphocyte line derived from a methyl nitrosourea-induced tumor in a (C57BL/6 X DBA/2) F_1 mouse. Reference: J. Immunol. 121, 641-644, 1978.

L1210:
This is a lymphocytic mouse leukemia derived from a tumour arising in a DBA/2 mouse following skin paintings with 0.2% methylcholanthrene in ethyl ether. Reference: G. Moore *et al* (1966) J. Nat. Cancer Inst. 36, 405-421.

P388D_1:
This is a methylcholanthrene-induced lymphoid neoplasm which originated in a DBA/2 mouse. Reference: Dawe, C.J. and Potter, M. (1957) Amer. J. Path. 33, 603-606.

Appendix II
Solutions

Solutions were made up as follows:

Denhardt's Solution :

50 X Solution	
Ficoll	5g
polyvinylpyrrolidine	5g
BSA	5g
water	to 500 ml

Filter-sterilized, and stored at -20℃

SSC:

20 X Solution	
NaCl	175.3g
sodium citrate	88.2g
water	to 1 l

pH adjusted to 7.0 with NaOH, sterilized by autoclaving

SSPE:

20 X Solution	
NaCl	174g
$NaH_2PO_4.H_2O$	27.6g
EDTA	7.4g
water	to 1 l

pH adjusted to 7.4 with NaOH, sterilized by autoclaving

PAMELA ICELY
414 Riverdale Ave.
Ottawa, Ont.
K1S 1S2
(613) 738-0888

DATE OF BIRTH: 22nd February, 1966
PLACE OF BIRTH: Montreal, Quebec

EDUCATION:

1984-1988	B.Sc. (Hons) Biochemistry, University of Western Ontario
1988-present	M.Sc. Biochemistry, University of Ottawa

EXPERIENCE:

Summer 1988	Summer Student for Dr. Larry Moran, Department of Biochemistry, University of Toronto, Toronto, Ontario
Sept. 1987	Student (research project) in the lab of Dr. Bishnu D. Sanwal, Department of Biochemistry, University of Western Ontario, London, Ontario

PUBLICATIONS AND ABSTRACTS:

Full Papers:

Icely, P.L., Gros, P., Bergeron, J.J.M., Devault, A., Afar, D.E.H., and Bell, J.C. (1991) J. Biol. Chem. **266**(24), 16073-16077

Howell, B.W., Afar, D.E.H., Lew, J., Douville, E.M.J., Icely, P.L., Gray, D.A., and Bell, J.C. (1991) Mol. Cell. Biol. **11**, 568-572

Abstracts:

Icely, P.L. and Bell, J.C. The Gene Encoding the Novel Kinase TIK has Undergone a Rearrangement in the Murine Lymphocytic Leukemia Line L1210 (Abstract of poster presented at the American Association for Cancer Reasearch Meeting on Chromosomal and Growth Factor Abnormalities in Leukemia, Chatham, Mass. USA, October, 1990)