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Src Family Kinases in the Yeast Two-Hybrid Assay

by

Sherrie L. Hans

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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GRADUATE DIVISION

of the

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San Francisco

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To David C. Bowen, I owe today and the future.

Conventional knowledge is death
to our souls, and it is not really ours.

We must become ignorant
of what we've been taught,
and be, instead, bewildered.

Run from what's profitable and comfortable.
If you drink those liqueurs, you'll spill
the springwater of your real life.

Forget safety.
Live where you fear to live.
Destroy your reputation.
Be notorious.

I have tried prudent planning
long enough. From now
on, I'll be mad.

Rumi
A Spider Playing in the House

(in *Feeling the Shoulder of the Lion: Poetry and Teaching Stories of Rumi*
Putney, VT: Threshold Books, 1991.)

ABSTRACT

Src Family Kinases in the Yeast Two-Hybrid Assay

by Sherrie L. Hans

We were interested in the regulation and activity of the Src family kinase Hck. Our aim was to identify cellular partners of Hck and thus begin to place Hck in a functional context in hemopoietic cells. I will describe our efforts to identify proteins that interact with Hck. We used the two-hybrid genetic system to approach this problem.

We utilized the well characterized interaction of CD4 with Lck to test the utility of the yeast two-hybrid assay for the study of Src family kinases. We were able to show that CD4 and Lck interact in this in vivo assay. Furthermore, we were able to identify a potential ionic stabilization motif that plays a role in the interaction of CD4 with Lck that was overlooked with less sensitive assay systems. In addition to being sensitive, however, this system also gave a background level of transcriptional activity when expressing Src family kinases that has the potential to interfere with efforts to identify novel proteins that interact with these target molecules.

A more stringent two-hybrid assay was sought to use in a library screen for proteins that interact with the unique domain of the Src family kinase Hck. This version of the two-hybrid assay was employed to survey a B cell library for potential protein partners of Hck. We have tentatively identified three candidate Hck interacting proteins; two of unknown function and the cytoskeletal protein actin.

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Chapter I

INTRODUCTION

Historical Foundation: Peyton Rous and Retroviral Oncogenes

In the opening decade of this century, Peyton Rous was the first researcher to clearly demonstrate that a virus could act as a causative agent in tumorigenesis. He was able to show this by inoculating naive chickens with a highly filtered extract that had been derived from chicken sarcomas. This procedure resulted in the formation of sarcomas in the newly inoculated birds. His experiments mark the beginning of the profitable exploitation of Rous Sarcoma Virus (RSV) in the study of oncogenesis (Stephenson, 1980; Weiss *et al.*, 1982).

By the latter part of this century, a number of tumor biologists were studying RSV. They reasoned that if they could understand how RSV managed to transform a cell, they might gain valuable insights into the mechanism(s) underlying tumorigenesis in humans (Bishop, 1985; Emmelot and Bentvelzen, 1972). The relevance of viral tumor models to human cancer was controversial, however, since evidence existed that supported a genetic basis for cancer rather than an infectious one. For instance, it had long been observed by medical geneticists that heritable traits existed which predispose an individual to develop cancer. Some experimental cancer models in animals also reinforced the idea that susceptibility to tumor formation was inherited in a mendelian fashion (Bishop, 1983; Bishop, 1987).

It wasn't until the mid-1970's that evidence was presented which helped to reconcile these two models (Bishop, 1996). Researchers showed that the viral oncogene present in RSV was also present in normal avian and mammalian cells (Spector *et al.*, 1978; Stehelin *et al.*, 1976). The hypothesis was put forward that all cells contain genes (proto-oncogenes) which, if mutated, can lead to cancer. Retroviruses like RSV had simply captured one of these cellular sequences sometime in the past.

With the recognition that retroviral oncogenes were slightly altered versions of normal cellular genes, the argument to study tumor viruses became even more compelling. Researchers were now interested in studying RSV not only as a model of tumorigenesis but also to understand the normal physiological role of these cellular genes. The question remained, however, of why normal cells harbored these potentially detrimental sequences. Since many proto-oncogenes are evolutionarily conserved, researchers postulated that these proteins might play an important role in essential life processes. It is now obvious that the products of proto-oncogenes are often pivotal regulators of cell growth and differentiation (Bishop, 1987; Jahner and Hunter, 1991; Karin and Smeal, 1992; Pawson, 1991; Taylor and Shalloway, 1996). By studying the products of both viral and cellular oncogenes, we have gained important insights into the regulation of these key cellular events.

Cell Growth, Transformation and Src.

The viral oncogene responsible for the transforming activity of Avian Sarcoma Virus, v-*src*, and its cellular counterpart, c-*src*, have

been studied extensively over the past 20 years (Cooper, 1989; Taylor and Shalloway, 1996) Initial experiments identified the product of the *src* gene as a 60 Kd phosphoprotein (pp60$^{v\text{-}src}$ or vSrc) with associated kinase activity that could promote transformation when localized to the plasma membrane (Brugge and Erikson, 1977; Collett and Erikson, 1978; Krueger *et al.*, 1980; Levinson *et al.*, 1978; Purchio *et al.*, 1978). These observations were of interest since early changes in the cell following mitogenic stimulation were known to occur at the cell periphery and included the activation of kinases such as PKC. By analogy, therefore, researchers speculated that vSrc might be involved in a pathway that controlled cell growth and division. We now know that the products of many proto-oncogenes such as *ras*, *raf*, *erb b-1* and *myc* are central regulatory molecules in signaling cascades. These cascades relay mitogenic signals from the exterior of the cell to interior targets such as the nucleus or the cytoskeleton (reviewed in (Karin and Smeal, 1992; Pawson, 1991; Treisman, 1992). Viral versions of these proteins tend to be constitutively active copies of their cellular counterparts. This constitutive activity is responsible for the inappropriate stimulation of these signaling cascades, resulting in growth stimulation when the cell should remain quiescent (Cantley *et al.*, 1991).

The Src Family of Protein Tyrosine Kinases

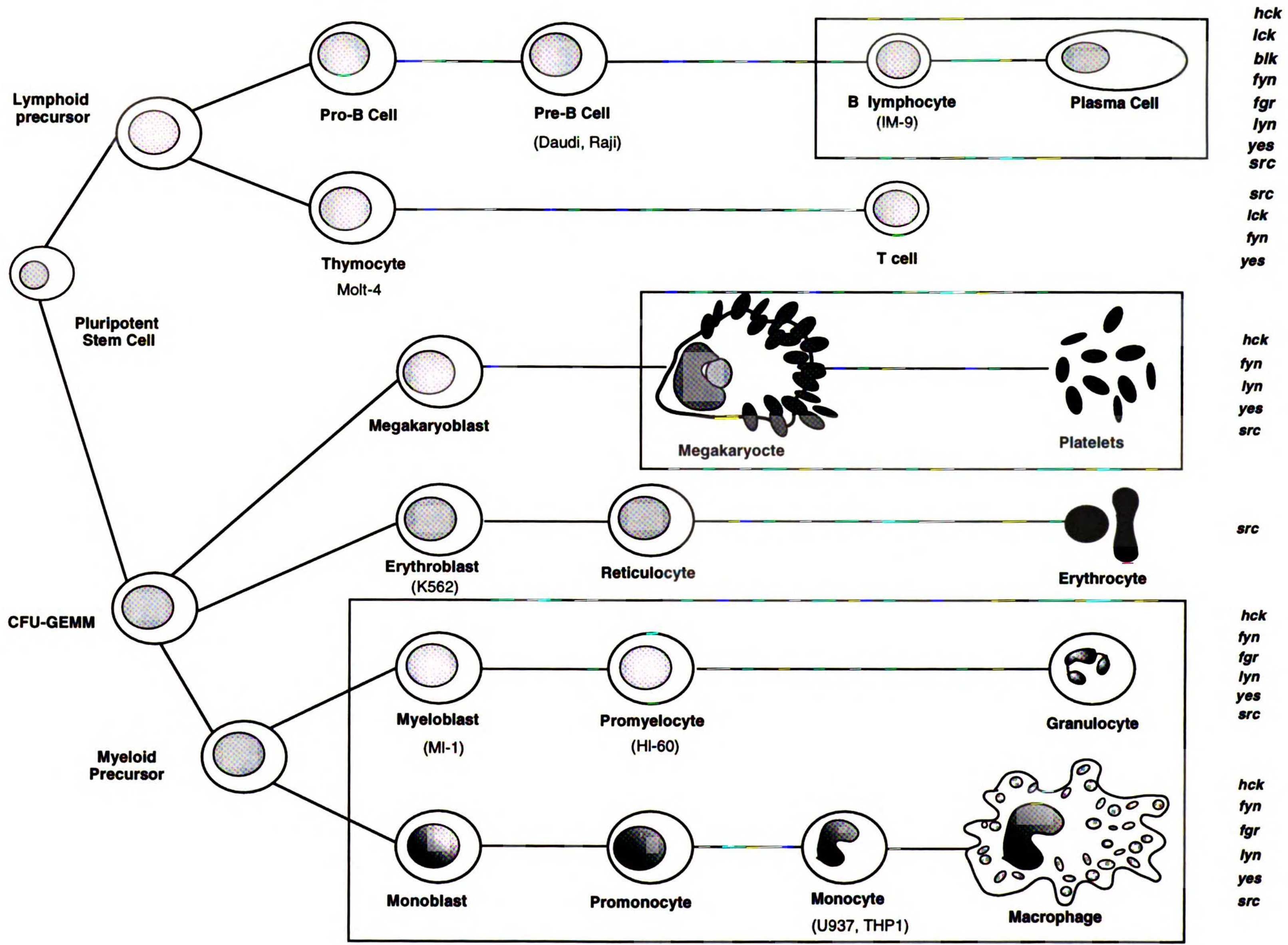
Src is the prototype for a family of related protein tyrosine kinases. Currently, there are nine members of this family recognized in mammalian cells; Src, Fyn, Yes, Lyn, Hck, Fgr, Blk, Lck and Yrk

(Kefalas *et al.*, 1995). Some members, like Src and Yes, are ubiquitously expressed in all cell types examined. Other members, like Hck and Lck, are more limited in their expression, being seen only in certain hemopoietic lineages (See Figure 1.1) (Mustelin and Burn, 1993; Rudd *et al.*, 1993). Homologues have been identified from insects to mammals. Mice express all of the same family members identified in humans, including the many isoforms that arise through alternative splicing or alternative translational mechanisms. All of the proteins are similar at the amino acid level throughout the catalytic domain and the Src homology domains (SH2 and SH3; See below) (Rudd *et al.*, 1993).

When investigators realized that a large family of these kinases existed, they speculated that each individual family member must serve a different purpose in the cell in order to have been conserved during evolution. When the first embryonic knock out mouse of this family of kinases was produced, however, researchers were surprised to hear that the phenotype resulting from the complete absence of Src was confined to the system controlling bone morphogenesis (Soriano *et al.*, 1991). The ubiquitous expression of Src and its postulated role in the regulation of cell growth had led many to believe its presence would be essential for cell growth during development. The results obtained with the Src deficient mouse meant that either Src did not play an essential function in most cell types in which it was expressed or, other Src family members are similar enough to functionally compensate for the loss of Src.

Figure 1.1: Hemopoietic Differentiation.

A schematic outlining hemopoietic cell differentiation is shown. Cells expressing Hck are boxed. The Src family members expressed in each mature cell type is noted at right. Human cell lines corresponding to each stage of differentiation are shown in parenthesis. (Eiseman and Bolen, 1990; Miyajima *et al.*, 1992; Mustelin and Burn, 1993).



Lymphoid precursor
Pro-B Cell
Pre-B Cell
(Daudi, Raji)
B lymphocyte
(IM-9)
Plasma Cell
hck
lck
blk
fyn
fgr
lyn
yes
src
Thymocyte
Molt-4
T cell
src
lck
fyn
yes
Pluripotent Stem Cell
Megakaryoblast
Megakaryocte
Platelets
hck
fyn
lyn
yes
src
Erythroblast
(K562)
Reticulocyte
Erythrocyte
src
CFU-GEMM
Myeloblast
(MI-1)
Promyelocyte
(HI-60)
Granulocyte
hck
fyn
fgr
lyn
yes
src
Myeloid Precursor
Monoblast
Promonocyte
Monocyte
(U937, THP1)
Macrophage
hck
fyn
fgr
lyn
yes
src

Much of the work produced in this field over the past five years, including the work discussed here, have addressed the question of which activities are shared by all Src family members and which, if any, are unique?

Structure and Regulation of Src

Despite many years of study, the precise mechanism by which vSrc transforms cells and the normal physiological role of cSrc are still not entirely clear today (Cooper, 1989). One approach that was taken to understand the function of Src was to dissect the protein through mutational analysis and attempt to correlate particular mutations with known activities of the protein. (I.E.. transforming ability, kinase activity, subcellular localization.) Results from these mutational studies have led to the following elementary structural model that is assumed to roughly apply to all Src family members.

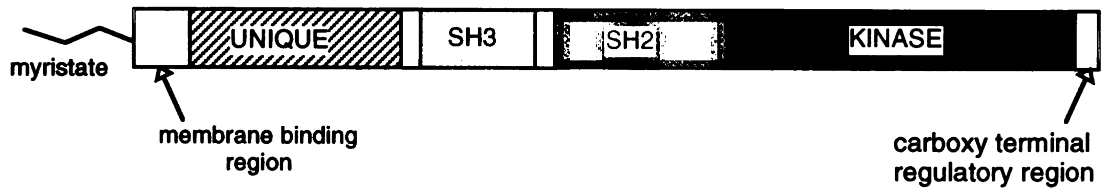
Kinase Domain

Src is a 60 Kd phosphoprotein characterized by carboxy terminal catalytic sequences and amino terminal regulatory features (See Figure 1.2). The carboxy terminal half of the protein consists of approximately 240 amino acids that form a highly conserved kinase consensus domain. The kinase domain contains a prototypical ATP binding subdomain and a substrate binding region. The substrate binding region includes both an activation loop near the catalytic site, and a sequence characteristic of kinases that recognize the hydroxy amino acid tyrosine (Hanks *et al.*, 1988; Kemp and Pearson, 1990; Superti and Courtneidge, 1995). The activation loop contains the site

Figure 1.2: Conserved Primary Structure of Src.

Src is the prototype of a conserved family of protein tyrosine kinases. This family of kinases share homology in their kinase domains (260 amino acids), SH2 (100 amino acids), and SH3 (60 amino acids) domains. The carboxy terminal regulatory region and the amino terminal membrane binding region are also conserved. The unique region is the least conserved domain.

Src Primary Structure



of Src autophosphorylation. This site is one of two regulatory tyrosine phosphorylations seen in the carboxy terminal half of Src. Phosphorylation of this site typically occurs upon stimulation of the kinase, resulting in a modest increase in kinase activity (Kmiecik and Shalloway, 1987).

The second site of tyrosine phosphorylation observed in the carboxy terminal half of Src, is the negative regulatory phosphorylation seen on tyrosine 527 (Y^{527}), which lies just outside the kinase domain (Figure 1.2) (Kmiecik and Shalloway, 1987). Src is constitutively phosphorylated at this site by a family of tyrosine kinases that are structurally related to Src (Chow and Veillette, 1995). Removal of this phosphorylation site, as is seen in vSrc, or the substitution of this tyrosine for another amino acid, causes the activation of Src and is sufficient to give a transforming allele (Cooper and Howell, 1993; Kmiecik and Shalloway, 1987).

The catalytic domain of Src was traditionally thought to contribute minimally to the specificity of the enzyme. In vitro, Src is a fairly indiscriminate protein tyrosine kinase, phosphorylating a wide variety of substrate sites. This is also true for other members of the Src family of protein tyrosine kinases. They display a similar promiscuous bent and will often phosphorylate the same in vitro substrates as Src with equal efficiency (Cheng *et al.*, 1992; Kemp and Pearson, 1990). This being the case, if Src and other Src family members are to have exclusive functions as kinases, then the regulation of substrate choice must involve more than the specificity inherent in substrate binding and the phosphotransfer reaction.

Researchers have therefore looked to the amino terminal half of Src to discern how specificity is achieved with these kinases.

Regulatory Domains: SH2 and SH3

The regulatory half of Src contains an SH2 domain, an SH3 domain, the unique region and an amino terminal membrane binding domain (Superti and Courtneidge, 1995). The SH2 and SH3 (Src Homology) domains of Src perform complex regulatory functions. They are responsible for regulating the activity of the kinase domain as well as facilitating interactions with other proteins that are potential regulators or substrates of Src (Superti and Courtneidge, 1995).

The SH2 domain is a 100 amino acid protein module that specifically binds to phosphotyrosine containing peptides (Superti and Courtneidge, 1995). Within Src itself, the SH2 domain has been shown to bind to the tyrosine phosphorylated negative regulatory site at Y^{527}. This intramolecular reaction is thought to cause the amino terminus of Src to wrap around the kinase domain, occluding substrates from the catalytic site (Cooper and Howell, 1993; Koch *et al.*, 1991).. Like mutations at Y^{527}, mutations within the SH2 domain that interfere with this intramolecular interaction result in temperature sensitive or frankly transforming alleles (Hirai and Varmus, 1990; Koch *et al.*, 1991; Margolis, 1992; O'Brien *et al.*, 1990; Sadowski *et al.*, 1986; Seidel-Dugan *et al.*, 1992; Veillette *et al.*, 1992). The SH2 domain is also thought to modulate phosphotyrosine dependent interactions with other molecules. High affinity sites on other proteins compete with the binding of phosphorylated Y^{527}

(pY^{527}), leading to an open conformation, a structure that promotes the activity of this kinase (Bibbins *et al.*, 1993; Roussel *et al.*, 1991).

The SH3 domain is a 60 amino acid protein module that specifically binds to proline rich sequences (Ren *et al.*, 1993; Superti and Courtneidge, 1995; Yu *et al.*, 1992). Like the SH2 domain, the SH3 domain is responsible for both intra and intermolecular interactions. Mutations in the SH3 domain are seen to activate the transforming potential of Src (Potts *et al.*, 1988; Seidel-Dugan *et al.*, 1992) It's thought that these mutations destabilize the SH2-pY^{527} interaction, although the precise molecular characteristics of SH3's putative interaction with the other two domains is not entirely clear (Murphy *et al.*, 1993; Okada *et al.*, 1993; Seidel-Dugan *et al.*, 1992; Superti-Furga *et al.*, 1993). The SH3 domain also facilitates interactions with other molecular targets in the cell and may contribute to the specificity of this enzyme by directing Src to particular substrates or specific subcellular locations (Kaplan *et al.*, 1990; Kaplan *et al.*, 1994; Weng *et al.*, 1993).

SH2 and SH3 domains are highly conserved among the different Src family members (Cooper, 1989). Before much was known about the details of SH2 and SH3 mediated interactions, many people speculated that, due to this conservation, Src family members might all recognize the same set of binding partners. As with the lack of discrimination seen with the catalytic domain, this presumed overlap of binding specificities once again raises the specter of how these kinases achieve distinct functions.

Regulatory Domains: Unique Region and Membrane Binding Domain

The most pronounced differences among the sequences of the Src family members lie within the first 80 amino acids of each protein (Bolen *et al.*, 1991). This region contains the membrane binding site and the unique domain of Src. It has been postulated that functions exclusive to each particular family member may be mediated through these domains (Bolen *et al.*, 1991). Some evidence exists to uphold this hypothesis and there are many other intriguing bits of data which suggest it might be true.

Early studies of vSrc indicated that it was associated with the plasma membrane (Courtneidge *et al.*, 1980). Subsequently, cSrc was shown to be localized to additional membrane populations including perinuclear membranes, endosomal membranes and secretory vesicles in chromaffin cells and platelets (Kaplan *et al.*, 1992; Parsons and Creutz, 1986; Rendu *et al.*, 1989; Resh and Erikson, 1985). Mutational analysis and proteolytic digestion of the mature protein indicated that the membrane binding function was contained in the amino terminus of the molecule and that membrane binding was necessary for transformation to occur (Cross *et al.*, 1984; Krueger *et al.*, 1982; Levinson *et al.*, 1981). Researchers determined that Src was co-translationally modified with myristic acid that was attached through a conserved amino terminal glycine residue (Superti and Courtneidge, 1995). This fatty acid modification is generally*

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*Some mutant versions of Src have been described that are deficient in myristoylation but which still bind membranes weakly (Garber and Hanafusa, 1987; Krueger *et al.*, 1982).

considered to be necessary for membrane localization but alone is not sufficient to mediate membrane binding (Buss *et al.*, 1984; Superti and Courtneidge, 1995). This observation suggested to many researchers that membrane binding might also require protein/protein interactions in addition to the lipid/lipid interaction provided by the myristic acid moiety. Evidence for the involvement of a membrane protein in the attachment of Src to membranes was provided by reconstitution studies of purified Src into membrane vesicles (Resh, 1988). Saturable, high affinity (75μM) binding that was inhibited by heat or trypsin treatment was observed (Goddard *et al.*, 1989; Resh, 1988; Resh, 1989). This binding was localized to the first 15 amino acids of cSrc and was shown to require the myristoyl modification in order to occur (Feder and Bishop, 1991; Goddard *et al.*, 1989). A number of candidate Src interacting proteins have been described that can be crosslinked to the N-myristoyl Src (15) peptide in various membrane preparations. (Feder and Bishop, 1991; Resh and Ling, 1990). In platelet membranes, the Src peptide has been shown to crosslink to the platelet integrin $GPII_bIII_a$ and to bind and crosslink to the cytoskeletal protein actin (Feder and Bishop, 1991; Feder and Bishop, 1996). Although a 32Kd Src interacting protein has been observed in both platelet membranes and fibroblast membranes, no physiologically relevent Src binding protein has been unequivecally identified in this size range (Feder, 1993; Feder and Bishop, 1991; Feder and Bishop, 1996; Sigal and Resh, 1993).

In addition to this 15 amino acid membrane binding domain, evidence exists that hints at other possible functions for the unique

region of Src. For instance, deletion of the entire unique domain (AA 15-81) of vSrc causes this allele to be "somewhat defective in tumor induction" in vivo and gives a fusiform morphology to cells in culture (Cross *et al.*, 1984). There are also a number of serine phosphorylations seen in the unique region that are mediated by PKA and PKC (Collett *et al.*, 1979; Gentry *et al.*, 1986; Gould *et al.*, 1985; Purchio *et al.*, 1985; Roth *et al.*, 1983; Tamura *et al.*, 1984). These phosphorylations have no known effects on the activity of Src in vitro and the effects of these modifications in vivo were enigmatic at the time this study was begun. One thought was that these phosphorylations could either mediate or disrupt interactions with other protiens in vivo.

Studies carried out in this lab to determine the regions of Src responsible for the subcellular membrane distribution described earlier also point to an additional function for the unique domain. A construct containing only the first 54 amino acids of Src was seen to associate with the plasma membrane, perinuclear region and cytoplasmic granules. The first 14 amino acids alone, however, display a simple granular distribution (Kaplan, 1990). This implies that additional membrane targeting informatin lies within the first 54 amino acids of the unique region of Src.

A Model System for the Function of the Unique Domain: Lck and CD4

The most striking evidence in support of the idea that the unique domain lends specificity to this family of kinases comes from studies of the Src family member Lck. Lck is predominantly

expressed in T cells and plays an important functional role in this cell type. Lck is cued to perform its signaling function by an interaction with CD4 and CD8 that occurs through its unique amino terminal domain (Chan *et al.*, 1994; Weiss, 1993).

CD4 and CD8 are structurally related cell surface molecules that are expressed in different subsets of T cells. CD4 and CD8 play an important role as co-receptors to the T cell receptor during both T cell development and in the activation of mature T cells. Both of these processes are dependent on functional signaling through the T cell receptor complex. In turn, functional signaling depends on the activation of tyrosine kinases in the T cell undergoing selection or activation (Luo and Sefton, 1990; Veillette *et al.*, 1989a; Veillette *et al.*, 1989b; Veillette *et al.*, 1989c). The intracellular tyrosine kinase found in association with CD4 and CD8 is Lck (Rudd *et al.*, 1988; Veillette *et al.*, 1988; Veillette *et al.*, 1989b). This association was demonstrated through co-immunoprecipitation of Lck with CD4 and the reconstitution of the Lck-CD4 interaction in a heterologous system (Simpson *et al.*, 1989). The interaction was thought to be functionally important since Lck is strongly expressed in mature T cells and its expression is regulated during their maturation (Wildin *et al.*, 1991). The importance of Lck in T cells was substantiated by functional gene knock outs of Lck in mice which were shown to cause a profound defect in T cell development (Molina *et al.*, 1992). Genetic studies carried out in cultured T cells definitively showed that the elimination of Lck expression prevents signaling through the T cell receptor complex (Straus and Weiss, 1992).

Once the interaction of CD4 with Lck had been identified, the molecular nature of this interaction was investigated. Expression of a fusion protein with the extracellular and transmembrane domain of VSVG and the cytoplasmic region of CD4 showed that CD4 bound to Lck through its cytoplasmic domain (Shaw *et al.*, 1989). This interaction is independent of other T cell specific proteins as it could be reconstituted in other cell types (Shaw *et al.*, 1989). It was later shown that the cytoplasmic domain of CD8 also mediates an interaction with Lck, but to a much lower level than that seen with CD4 (Turner *et al.*, 1990). Comparison of the cytoplasmic domains of CD4 and CD8 delineated a motif (+ - + - X-cys-X-cys-Pro) that is conserved between the two proteins and between the human and mouse homologues of these molecules. Mutational analysis demonstrated that the two cysteine residues were necessary for the interaction of CD4 and CD8 with Lck (Shaw *et al.*, 1990; Turner *et al.*, 1990).

Deletion analysis and domain swapping with Src showed that amino acids 10-32 of the unique domain of Lck were necessary for the interaction with CD4 (Shaw *et al.*, 1989). Furthermore, by creating a fusion protein with these 22 amino acids fused to CAT, they were able to show that this region was also sufficient to mediate interactions with CD4 (Shaw *et al.*, 1989). Finally the amino terminal myristoylation of Lck was shown not to be absolutely necessary for the interaction with CD4 (Shaw *et al.*, 1990). Despite the fact that unmyristoylated Lck is not localized to membranes when expressed on its own, it was brought to the membrane when co-expressed with CD4, presumably through its interaction with this protein.

The Src Family Member Hck: Characteristics and Expression

Hck is one member of the Src family of non receptor tyrosine kinases. In humans, Hck is expressed exclusively in cells of hemopoietic origin (Holtzman *et al.*, 1987; Quintrell *et al.*, 1987; Ziegler *et al.*, 1987). Highest expression is observed in mature B cells and terminally differentiated myeloid cells (See Figure 1.1) (Willman *et al.*, 1991; Ziegler *et al.*, 1988). Because of its limited tissue distribution and elevated expression in post-mitotic cells, it has been suggested that Hck may play a role in a pathway specific to the function of these mature cells. Initial analysis of Hck deficient mice, however, did not provide any clues about the nature of this pathway (Lowell *et al.*, 1994). Hck^- mice display no gross developmental or hemopoietic cell defects or deficiencies. Macrophages from these mice exhibited normal responses in a variety of in vitro functional assays such as a PMA triggered repiratory burst, tumor cell cytotoxicity, or production of TNF-α, IL-1α, IL-6 or NO_2 in response to stimulants. The only deficit seen with the Hck- macrophages was a slight deficiency in the uptake of latex beads which was not Ig dependant. Unlike the single mutant animal, Hck- Fgr- double mutant animals did show an increased sensitivity to infection with the microbe *Listeria monocytogenes.* No molecular basis for this sensitivity was immediately apparent since all of the in vitro assays of macrophage function from these doubly mutant mice were normal (Lowell *et al.*, 1994).

Hck was identified in the Bishop lab through low stringency hybridization of the *src* kinase sequence to human retinal and

placental cDNA libraries (Quintrell *et al.*, 1987) Like the other members of the Src family, Hck has a conserved primary structure, consisting of a carboxy terminal kinase domain and amino terminal regulatory sequences (Hirai and Varmus, 1990; Liebl *et al.*, 1992; Sadowski *et al.*, 1986; Veillette *et al.*, 1992). Also like several other members of the Src family, the *hck* gene encodes two different forms of the Hck protein. The two forms differ from one another by 21 amino acids in their amino terminal unique region. Unlike other Src family members, however, these two forms are generated through alternative translational initiation start sites rather than the alternative splicing seen with many of the other family members. One site is a consensus ATG while the alternative site is 63 nucleotides upstream at a non canonical CTG initiation codon. Both proteins are made in vivo and are myristoylated at their amino termini (Lock *et al.*, 1991; Robbins *et al.*, 1995).

We felt that the existence of these two forms of Hck was provocative in light of the hypothesis that the unique region may mediate specific interactions of these kinases with other components of a signaling cascade. Following the logic of this model, the existence of two Hck unique domain isoforms means that Hck could potentially be involved in two different signaling cascades. Hck is not the only Src family member to display two unique domain isoforms. Lyn also exists with two different forms of this domain. In the case of Lyn, this is a 21 amino acid insertion just after the 23rd amino acid seen in the smaller of the two proteins (Stanley *et al.*, 1991; Yi *et al.*, 1991). Studies performed before this project was initiated provided evidence that the two forms of Lyn display different kinetics of

down regulation in response to IgM receptor signaling (Yamanashi *et al.*, 1991). No data were presented however, to indicate how Lyn was interfacing with the IgM receptor complex.

Despite detailed knowledge of the structural features of Hck, characterization of its expression pattern and the generation of mice lacking functional Hck, at the time this study was begun the role of this kinase in myeloid cells was not understood and little was known about the proteins to which it might bind. Therefore, we initiated this project to identify proteins that bind to the unique amino terminal domain of Hck. We had hoped that the identification of such proteins might point to a signaling cascade that involved Hck, thereby indicating a potential role for Hck in hemopoietic cells. Because many biochemical approaches to this question had already been applied to Src and yielded limited information, we undertook to apply a newly emergent (pseudo) genetic approach to this problem. This technique is described below.

Placing Hck in a Signal Transduction Cascade: The Two-Hybrid Assay

In 1989 Fields and Song published a paper demonstrating that it was possible to create a reporter system that would allow for the recognition of protein-protein interactions in vivo (Fields and Song, 1989). Although their application of the technology was new, the ideas underlying the two-hybrid system had been laid down in the transcription field during the mid 1980's. Once specific transcription factors had been identified in eukaryotes, researchers began to ask whether discrete portions of these proteins contained the necessary

information to carry out specific tasks. Through mutagenesis studies and the creation of chimaeric molecules, it was possible to conclude that there were at least two discrete functional domains in most transcription factors. One domain is responsible for the specific DNA binding function of the factor and a second domain seems to interact with the polymerase to promote transcription (Hope and Struhl, 1986; Keegan *et al.*, 1986).

The studies performed with chimaeric molecules indicated that the rules governing transcriptional activation were flexible. It was possible to pair yeast DNA binding domains with viral transcriptional activation domains or bacterial sequences that coded for acidic protein domains and observe the activation of transcription (Brent and Ptashne, 1985; Ma and Ptashne, 1987; Ruden *et al.*, 1991). It was also shown that the DNA binding domain and the transcriptional activation domain need not reside in the same protein for transcriptional activation to occur. The only strict requirement was that these domains be brought together in a complex near the promoter (Hope and Struhl, 1986; Keegan *et al.*, 1986). For example, in the absence of galactose, gal 80 acts as a transcriptional repressor of the full length gal4. Gal80 inhibits transcription by binding to the DNA bound gal4 and preventing its acidic activation domain from contacting the polymerase. If an acidic domain is then added to gal80 this gal4/gal80 complex could be converted from a repressor into a transcriptional activator (Keegan *et al.*, 1986). It was a small step from this observation to postulate that any protein-protein interaction would suffice to bring together a DNA binding domain with a transcriptional activation domain to form a functional complex

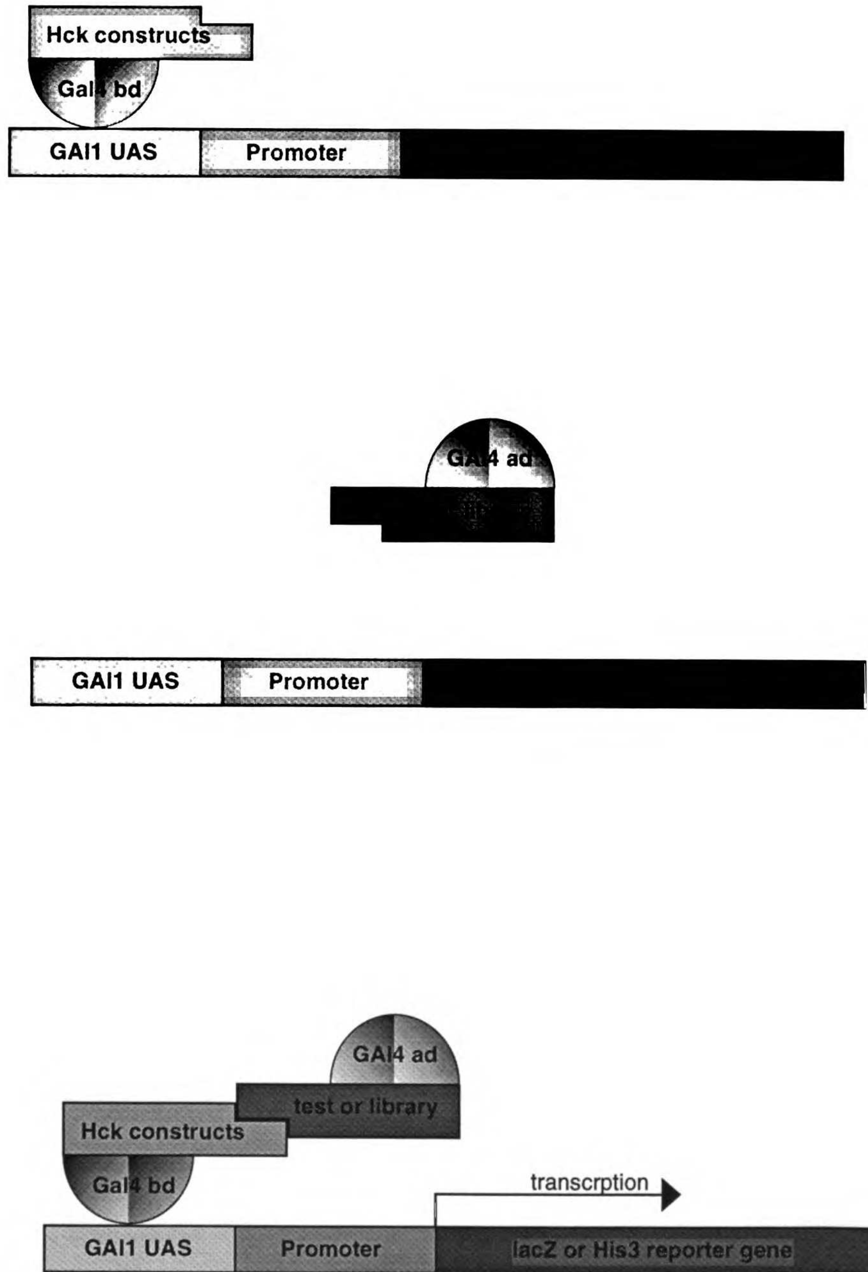
(See figure 1.3). Fields and Song demonstrated the concept by showing that a SNF4-acidic domain fusion protein would activate transcription in the presence of a DNA binding domain-SNF1 fusion protein in vivo (Fields and Song, 1989). (Fields and Song, 1989)(SNF4 and SNF1 were known to bind one another and neither protein contains DNA binding or transcriptional activation activity on their own (Celenza and Carlson, 1989). They and others went on to use this system to test proposed protein-protein interactions, to map these interactions through deletion analysis and to identify novel protein-protein interactions (Chevray and Nathans, 1992; Chien *et al.*, 1991; Dalton and Treisman, 1992; Hardy *et al.*, 1992; Luban *et al.*, 1992; Yang *et al.*, 1992).

We used the two-hybrid System to screen a library for proteins that interact with the unique domain of Hck. Prior to this screen, however, we tested whether we could use this system to detect the known unique domain interaction seen between Lck and CD4. The results of these studies are discussed in the following chapters.

Figure 1.3: Two-Hybrid System

The Two-Hybrid system depends on protein-protein interactions to build a functional transcriptional activation complex. In this example, protein-protein interactions between Hck and an unknown library protein bring the Gal4 DNA binding domain (gal4DB) and the Gal4 activation domain (gal4AD) together to create a functional complex.

Two-hybrid System



Chapter II

LCK AND CD4 IN THE TWO-HYBRID SYSTEM

Introduction

Protein-Protein Interactions

Study of the Src family of tyrosine kinases has provided years of challenging research for a great many scientists. With the recognition that Src was a protein kinase, some researchers may have anticipated a quick resolution to the question of Src's cellular function. As Src readily displays kinase activity in vitro, all that was necessary, it seemed, was to identify the substrates phosphorylated by this kinase and the nature of Src's cellular function would be fully understood. With the perspective we have now, it is apparent that Src is a complex protein whose enzymatic activity is but a single facet of the multidimensional role it plays in the cell. The picture we see emerging today paints the subcellular localization and the association of Src with other molecules as phenomena which are critically important to the physiological role of this protein. Thus, understanding the function of any Src family member is dependent on understanding its cellular context and identifying the proteins with which it interacts. In this chapter, I describe an experimental method for probing protein-protein interactions which I applied to the Src family kinases Lck and Hck.

Biochemical Methods

A number of laboratory techniques can be used to identify protein-protein interactions. Biochemical approaches include coimmunoprecipitation, protein cross-linking and protein affinity chromatography (Phizicky and Fields, 1995). Experiments performed in this lab in the past, using these techniques, indicated that Src is not easily manipulated in vitro (Feder, 1993; Kaplan, 1990). Src is relatively difficult to solubilize and purified Src readily self aggregates under conditions that favor interactions with other proteins (Feder, 1993).

Coimmunoprecipitation studies are also hampered by the fact that members of the Src family are found in association with many different proteins in the same cell. The interaction of a Src family kinase with any single molecule may only account for 0.5-1% of the total pool of the kinase present in a particular cell (Hatakeyama *et al.*, 1991; Kypta *et al.*, 1990; Samelson *et al.*, 1990; Twamley *et al.*, 1992). This means that often a mixture of interacting proteins, each present at very low concentrations, is present in a precipitate of a Src family kinase, making it difficult to distinguish a particular protein partner. Despite these difficulties, at the time this study was begun, researchers in the lab were in the process of applying these techniques, or had plans in the immediate future to apply these techniques, to Hck and Src (Feder and Bishop, 1991; Robbins, 1995).

Genetic Methods

Several pseudo-genetic methods were developed in 1991 for exploring protein-protein interactions (Chien *et al.*, 1991; Fearon *et al.*, 1992; Vasavada *et al.*, 1991). These are the yeast two-hybrid assay, the karyoplasmic interaction selection strategy (KISS) and the contingent replication assay. All three techniques are based on the reconstitution of a functional transcriptional complex at the promoter of a reporter gene, as described in Chapter 1 for the yeast two-hybrid assay. In the yeast two-hybrid assay, positive clones are identified through a combination of growth selection and screening for β-gal activity that is performed in *S. cerevisiae.* KISS is nearly identical to the yeast two-hybrid assay, except that it is carried out in cultured animal cells and relies on FACS sorting rather than growth selection to identify clones expressing the reporter gene. In the contigent replication assay reconstitution of a functional transcription complex also takes place in cultered animal cells, leading to the replication of the test plasmids. Selection of positive clones, however, requires processing through *E. coli* where the plasmids replicated in the previous step are positively selected and recovered.

All of these techniques have certain advantages over in vitro biochemical methods but also have their own intrinsic limitations (Guarente, 1993; Phizicky and Fields, 1995). The two-hybrid system, KISS and the contingent replication assay all have the advantage that clones are available as soon as an interaction is identified. There is no need for biochemical purification, protein sequencing or additional cloning strategies. Another advantage is that unlike in vitro methods

where the experimental conditions are empirically derived, these three methods assay interactions in vivo. This is generally thought to provide a normal physiological environment for the protein being assayed. This is certainly true when assaying nuclear proteins, however this premise may not hold for proteins normally found in the cytoplasm, at the membrane or in some other specialized cellular compartment. Local ion concentrations, redox potential and the hydrophobic environment of the membrane may all contribute to protein-protein interactions and these specific conditions may not be readily replicated in these genetic assays (Buser *et al.*, 1995; Narayanaswami *et al.*, 1993; Rodgers and Glaser, 1991; Rodgers and Glaser, 1993; Swierczynski and Blackshear, 1995; Yang and Glaser, 1995).

These three genetic approaches may also allow for the identification of low affinity and transient interactions (Durfee *et al.*,; Li and Fields, 1993; Van Aelst *et al.*, 1993). This is a distinct advantage over coimmunoprecipitation studies and phage display methods and may rival protein affinity chromatography in sensitivity (Formosa *et al.*, 1991). Unlike protein affinity chromatography, however, these genetic methods also allow a wide range of binding affinities to be surveyed, since each potential interacting protein is tested in the absence of other competing fusion proteins (Phizicky and Fields, 1995). This could also be viewed as a disadvantage, however, since competition on protein affinity columns may help to eliminate spurious interactions (Formosa *et al.*, 1991).

Compared with the other two in vivo systems, the yeast two-hybrid method is the most practical screening technique. The KISS

assay, since it is carried out in cultured animal cells, is more likely to support post translational modifications specific to higher eukaryotes, such as tyrosine phosphorylation. This system might, therefore, provide an advantage if an interaction is dependent on such post-translational modifications. As a library screening method, however, this system has the disadvantage of being limited by the transfection efficiency in animal cells. Not only is transfection into animal cells less efficient than transfection into yeast, but animal cells grow more slowly than yeast and it is far more difficult and costly to produce animal cells in large quantities (Guthrie and Fink, 1991; Kriegler, 1990). The contingent replication assay has the same inherent drawback as KISS, since it is also performed in animal cells. In addition, the contingent replication assay is an enrichment process rather than a strict genetic selection. The assay results in only a 100X enrichment of positive clones in the library pool every cycle and would therefore require multiple rounds of enrichment before single clones could be analyzed.

Specific Concerns with Hck in the Two-Hybrid System

Although working with yeast has many advantages over other cell systems, we had specific concerns about screening for Hck interacting proteins in the yeast two-hybrid system. One concern was that the expression of Hck might affect cell growth. This concern arose from observations that the expression of vSrc in *Saccharomyces cerevisiae* is lethal and the expression of cSrc is growth inhibitory (Brugge *et al.*, 1987; Kornbluth *et al.*, 1987). Since

the two-hybrid test consists of growth of colonies on selective media, growth inhibition mediated by Hck could interfere with this assay.

A second concern was that appropriate post-translational modifications would not be made to Hck in the yeast two-hybrid system. We know that Src and Hck are myristoylated on their amino termini. For vSrc, this modification is necessary for membrane association and transformation activity (Courtneidge *et al.*, 1980; Cross *et al.*, 1984; Krueger *et al.*, 1982). Although myristoylation can occur in yeast (Gordon *et al.*, 1991), we would predict that the constructs used in the two-hybrid system would not be modified in this way. The myristoylation signal on Src and Hck must be at the amino terminus to be recognized for modification (Resh, 1994). In the fusion protein for this system, the amino terminus of Hck falls in the middle of the fusion protein and therefore would not be modified (Figure 2.1). In addition to this post-translational fatty acid modification, we also recognized that the phosphorylation of proteins on tyrosine was unlikely to occur in yeast. *Saccharomyces cerevisiae* has no tyrosine kinase analogs. As a result, the only tyrosine phosphorylation seen in these cells is provided by a limited number of dual specificity kinases (Lindberg *et al.*, 1992). Since most SH2 dependent interactions require phosphorylated tyrosine residues*, we assumed that we would be unable to identify phosphotyrosine dependent interactions mediated by the SH2 domain of Hck. As interactions with the unique domain were our primary focus,

* One example of a tyrosine independent interaction with an SH2 domain has been reported (Muller *et al.*, 1992).

however, this issue did not influence our choice of the two-hybrid system as a screening method.

Finally, our last concern specific to Hck in the two-hybrid system was whether transmembrane or membrane associated proteins would function properly in this assay. The best characterized unique domain interaction among Src family members is that seen between Lck and CD4. By analogy to that system, we might expect to identify a transmembrane molecule in a screen for proteins that interact with the unique domain of Hck. The two-hybrid system does not provide a directed mechanism for a hydrophobic domain to become buried in a lipid bilayer. A hydrophobic domain on a nuclear transcription factor might cause misfolding, aggregation or lead to spurious interactions with other hydrophobic proteins. To address all of these concerns, we analyzed the interaction of Lck and CD4 using the two-hybrid system. We reasoned that if this interaction could be seen using the yeast two-hybrid assay then we should be able to use this assay to identify novel protein-protein interactions involving the Src family kinase, Hck.

Results and Discussion

Testing the Basic Constructs in the Two-Hybrid System

Constructs for this test of the two-hybrid system were made in vectors provided by Roger Brent as described in Appendix A, Materials and Methods (Table A-1 and Table A-2) (Zervos *et al.*, 1993). The first 31 amino acids of murine Lck were fused in frame

to the DNA binding domain of LexA (Figure 2.1). Initially, two constructs of CD4 were made: CD4Small contains the entire cytoplasmic domain of murine CD4 fused to the transcriptional activator B42. CD4Large contains the entire cytoplasmic domain, the transmembrane domain and half of the extracellular domain of murine CD4 fused to the transcriptional activator B42 (Figure 2.2).

To test whether the expression of Lck or CD4 fusion proteins had any effect on cell growth, EGY.048 cells, which contain an integrated Leu2 gene downstream of six LexA binding sites (Gyuris *et al.*, 1993), were transfected with the constructs indicated in Figure 2.3. Cells were grown on media that selected for the presence of the two vectors. Under these conditions, cells carrying the vectors for Lck, CD4Small or CD4Large all exhibited normal growth when compared to the positive control pma41, a LexA-Gal4 fusion protein (Figure 2,3, left panel). This indicates that there is no obvious growth inhibition in *S. cerevisiae* expressing either the unique domain of a Src family kinase or the transmembrane domain of CD4 in the context of a nuclear protein.

The lack of growth inhibition observed with the Lck fusion protein is not surprising in light of recently published experiments. These show that both the kinase domain and the SH2 domain of Src are necessary to observe complete growth inhibition in *S. cerevisiae* (Boschelli *et al.*, 1993; Florio *et al.*, 1994). It is interesting to note, however, that even full length, kinase active Src, in the context of the two-hybrid system, does not display any ability to inhibit growth in *S. cerevisiae* (S. Fields and G.S. Martin, pers. comm.). A trivial explanation for this result is that the fusion protein is no longer

Figure 2.1: LexA-Hck and LexA-Lck Constructs.

A diagrammatic representation of the products of the constructs used in this study. The left hand column gives the names of the proteins as referred to in the text. The constructs were made as described in Table A2 in Materials and Methods.

LexA - Hck and LexA - Lck Constructs

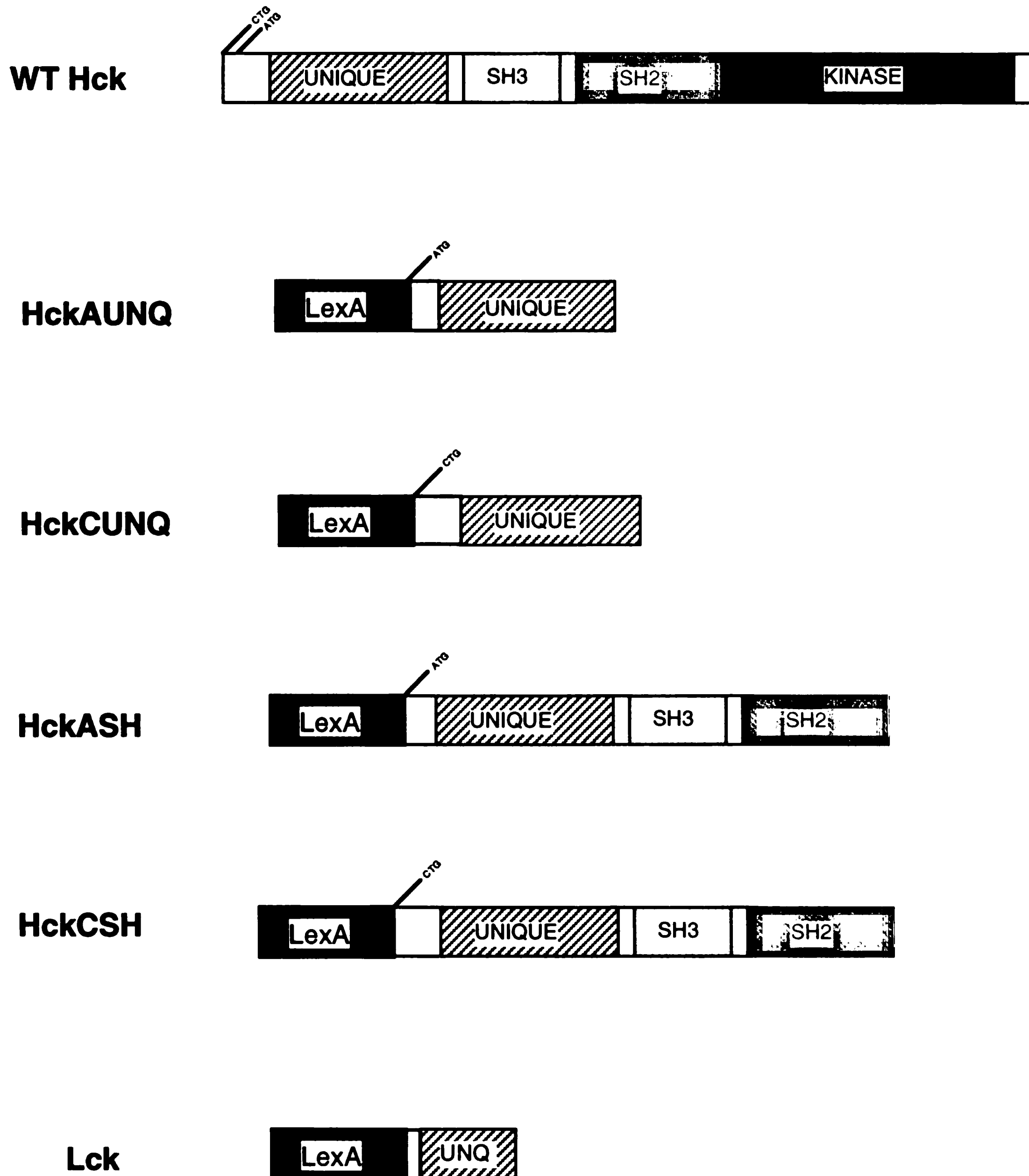
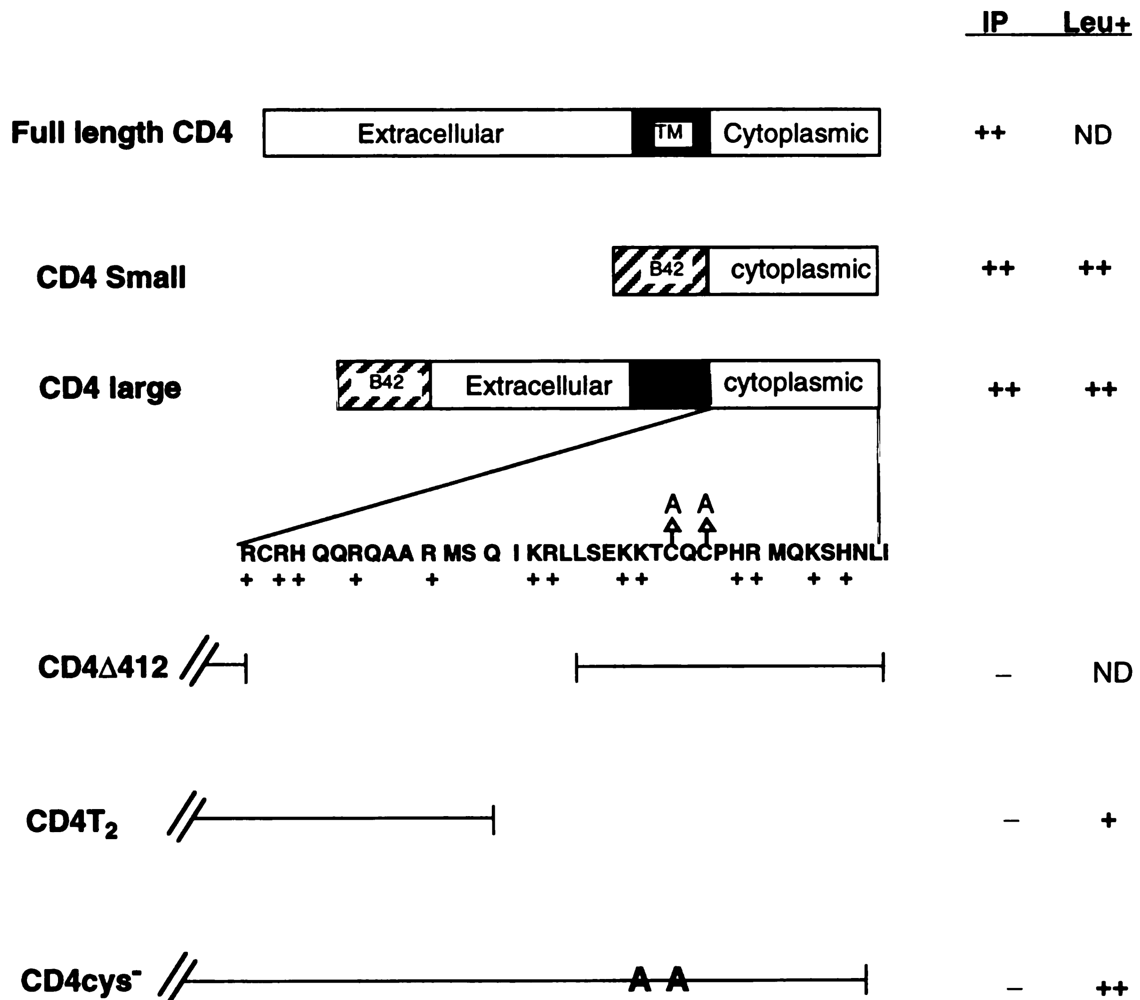


Figure 2.2: CD4 Transcriptional Activation Domain Fusions

Diagrammatic representation of the products of the transcriptional activation domain constructs used in this study. The left hand column gives the names of the proteins as referred to in the text. The right hand columns shows the results of coimmunoprecipitations (IP) performed by (Turner *et al.*, 1990) and the results of yeast growth assays performed in this study (Leu+).

CD4Small contains the cytoplasmic domain of CD4 fused to the bacterial sequences, B42, which behaves as a transcriptional activator in eukaryotes. CD4Large contains the cytoplasmic, transmembrane and half of the extracellular domain of CD4 fused to B42. CD4Δ412 is a construct made by Turner et. al. containing a deletion in the cytoplasmic domain of CD4, as indicated. This construct was not tested in the two-hybrid assay. $CD4T_2$ contains a carboxy terminal truncation of the cytoplasmic domain of CD4 fused to B42. $CD4cys^-$ is analogous to CD4Small except that the two critical cysteine residues are changed to alanine in $CD4cys^-$. ND means not done.

CD4 - Transcriptional Activation Domain Fusions



active toward the relevant targets. Another possible explanation is that the critical targets for growth inhibition are not accessible to a Src construct that has been targeted to the nucleus. These two possibilities could be explored by comparing the tyrosine phosphorylated proteins present in intact yeast cells transformed with Src and Gal4-Src with the substrates phosphorylated in an in vitro kinase assay cell and nuclear lysis. If the second explaination is correct, it could be significant for future studies of Src family kinases in the two-hybrid system. Although many studies have been carried out with isolated Src homology domains, evidence has accumulated to support the idea that some interactions require a completely intact molecule to be properly regulated (see the discussion in Chapter 3). The observation that full length, kinase active Src does not interfere with cell growth in the two-hybrid system, means that these kinds of interactions may be amenable to study in the yeast two-hybrid assay.

To test whether Lck or CD4 fusion proteins displayed transcriptional activity on their own, cells were grown on media that selected for both vectors as well as for active transcription from the leu2 gene. Under these conditions, no growth was seen in cells carrying a single specific fusion protein, compared to that seen with the positive control pma41 (Figure 2.3, right panel). This result indicates that under triple selection conditions, the LexA-Lck fusion protein does not significantly activate transcription. This suggests that Lck cannot interact with B42 in the absence of CD4 sequences. This result also suggests that neither CD4Small nor CD4large are able

Figure 2.3: Specificity of Lck-CD4 Interactions.

EGY.048 cells carrying the indicated plasmids were grown overnight in raffinose $Trp^{-}His^{-}$ media. The cultures were normalized to an OD=0.9 and serial four fold dilutions were made in adjacent wells, proceeding from left to right. The resulting cultures were spotted onto selective galactose plates as indicated. After 2.5 days at $30^{o}C$, the plates were photographed.

pma41 is a LexA DNA binding domain-Gal4 transcriptional activation domain fusion protein. This construct indicates the growth that can be achieved when an active transcriptional complex is present. B42 is a bacterial sequence which acts as a transcriptional activator in eukaryotic cells. Here, B42 represents the parent vector alone with no target specific inserts. LexA is a construct containing the DNA binding domain of LexA. Here, LexA represents the parent vector alone with no target specific inserts. See Figures 2.1 and 2.2 for explanations of Lck and CD4 constructs.

trp$^-$ his$^-$ | trp$^-$ his$^-$ leu$^-$

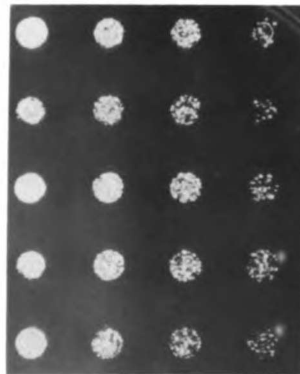
pma 41

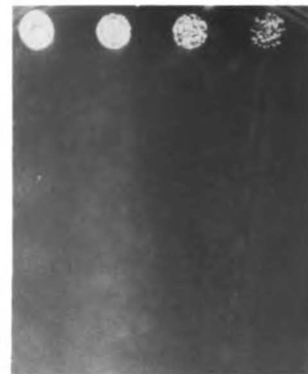
lck, B42

lexA, CD4 small

lexA, CD4 large

lexA, CD4 T_2





to contact LexA in the absence of Lck sequences. I concluded that the presence of the hydrophobic transmembrane domain of CD4Large does not result in spurious interactions with LexA.

CD4 and Lck Interact in the Yeast Two-Hybrid System

To test whether an interaction between the unique domain of Lck and the cytoplasmic domain of CD4 could be detected using the two-hybrid system, EGY.048 cells containing the integrated reporter gene Leu2, were cotransfected with either Lck and CD4Small or Lck and CD4Large. Under growth conditions that select for transcriptional activation of the reporter gene, Lck, in combination with either CD4 construct, was able to promote growth of these cells (Figure 2.4, right panel). This was specific for the Lck and CD4 portions of the fusion proteins as neither Lck in combination with B42 nor LexA in combination with either CD4 construct were sufficient to support growth under leucine selection (Figure 2.4, right panel). This result demonstrates that the two-hybrid system can be used to recapitulate the CD4-Lck interaction previously identified in coimmunoprecipitation studies (Rudd *et al.*, 1988; Veillette *et al.*, 1988). In addition, the interaction of Lck with the larger version of CD4 in this system indicates that there is no detrimental effect of including the transmembrane domain of CD4 in this construct. The presence of this transmembrane domain does not seem to lead to nonspecific interactions as neither LexA alone nor a LexA construct containing the unique domain of the Src family member Hck[†] show

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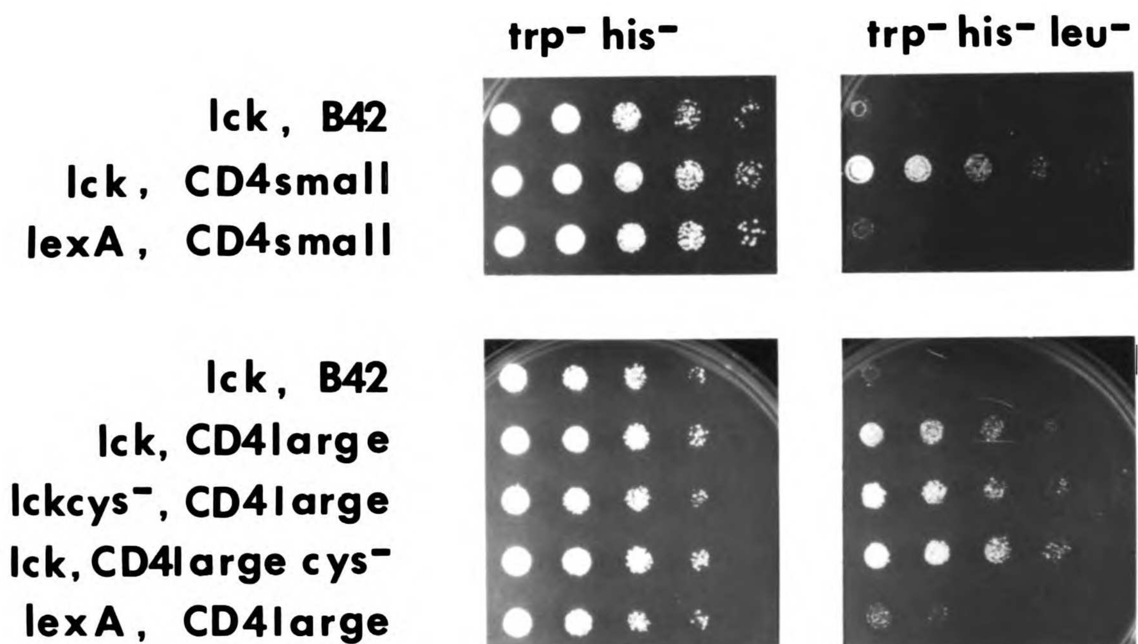
[†] This may not be significant as human Hck was tested against murine CD4. Conservation does exist in the CD4-Lck system, however, as human CD4 readily associates with murine Lck (Turner *et al.*, 1990).

Figure 2.4: Lck and CD4 Interact in the Two-Hybrid System.

EGY.048 cells carrying the indicated plasmids were grown overnight in raffinose Trp^-His^- media. The cultures were normalized to an OD=1 and serial five fold dilutions were made in adjacent wells, proceeding from left to right. The resulting cultures were spotted onto selective galactose plates as indicated. After 2 days at 30°C, the plates were photographed.

Lck and CD4 interact in the Two-Hybrid System



any ability to activate transcription in the presence of CD4large (Figure 2,4, right panel; Figure 2.6, right panel). We can also conclude that conditions in the nucleus are able to support an interaction that normally takes place on the cytoplasmic face of the plasma membrane.

The interaction of CD4 with Lck in this system suggests that myristoylation is not necessary for this interaction to occur. This observation is consistent with results from coimmunoprecipitation studies where myristoylation minus Lck was still seen to interact with CD4, albeit much less efficiently than that seen with wild type Lck (Shaw *et al.*, 1990). It is thought that the presence of a myristoyl group brings Lck to the membrane where it can then find CD4 in a two dimensional search in the plane of the membrane, rather than the three dimensional search necessary for a cytoplasmically located Lck. The interaction of CD4 with Lck in the absence of myristoylation was also independently confirmed in the KISS assay where similar Lck and CD4 constructs were shown to interact in that assay system (Fearon *et al.*, 1992; Zhang *et al.*, 1995).

Point Mutations and Deletions in CD4 Uncover an Ionic Stabilization Domain

Coimmunoprecipitation studies of Lck and CD4 showed that two cysteine residues present in both the cytoplasmic domain of CD4 and the unique domain of Lck were responsible for the ability of CD4 to bind to Lck (Figure 2.2) (Shaw *et al.*, 1990; Turner *et al.*, 1990). In order to test whether this result could be recapitulated in the two-hybrid system, a CD4LargeCys$^-$ construct, where both critical

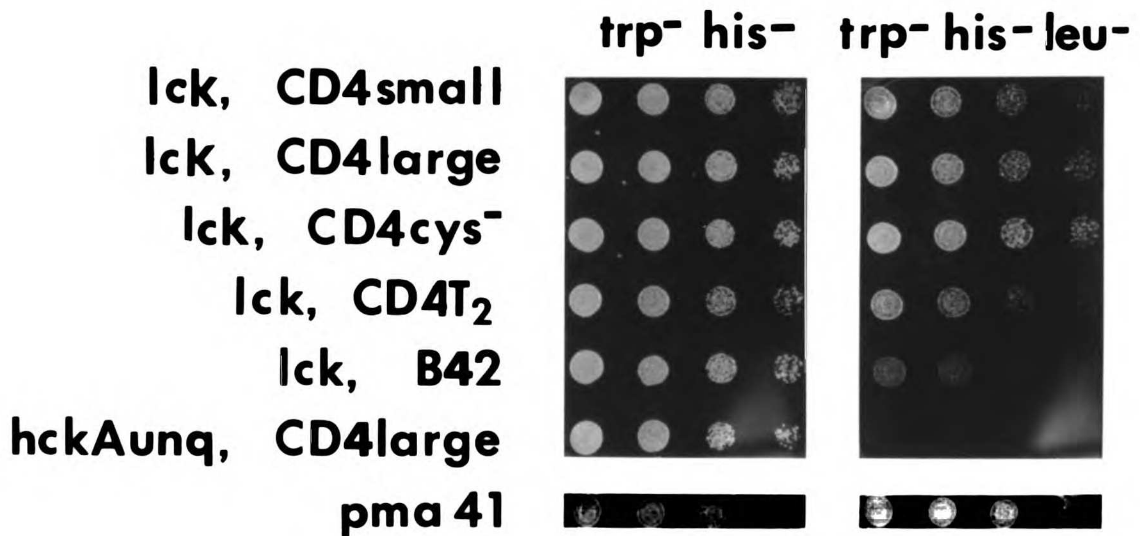
cysteines were mutated to alanine, was prepared (Figure 2.2). This construct was transfected into EGY.048 cells carrying Lck and the standard Leucine dependent growth assay was performed. In this experiment, the combination of Lck with CD4LargeCys- gave the same robust growth seen with wild type CD4Large (Figure 2.3, right panel; Figure 2.5, right panel). This result directly contradicts the findings of earlier coimmunoprecipitation studies.

A possible explanation for this discrepancy may be that the substitution of alanine for cysteine in this context retained enough of the structural features of wild-type CD4 for the interaction with Lck to be maintained under the mild, in vivo conditions of the two-hybrid assay. We considered this hypothesis since the two sets of cysteines residues on CD4 and Lck are known not to form inter or intra-molecular sulfhydryl bonds (Shaw *et al.*, 1989; Shaw *et al.*, 1990; Turner *et al.*, 1990). It has been suggested that these cysteines might stabilize structural features of the complex through the coordination of a metal ion, although no experimental evidence exists to support this model (Turner *et al.*, 1990). Our hypothesis was further buttressed by the demonstration that the two-hybrid assay can detect protein-protein interactions that are too weak to be seen by coimmunoprecipitation (Li and Fields, 1993). Since our goal was to recapitulate the results obtained in coimmunoprecipitation studies, we went on to make a truncation mutant that completely removes the cysteine containing motif in CD4 ($CD4T_2$, Figure 2.2) in an attempt to completely eliminate the interaction betweeen these two proteins. This construct was transfected into EGY.048 cells carrying Lck and the standard Leucine dependent growth assay was

Figure 2.5: Truncated CD4 Still Shows an Interaction with Lck

EGY.048 cells carrying the indicated plasmids were grown overnight in YPD. The cultures were normalized to an OD=1.3 and serial five fold dilutions were made in adjacent wells, proceeding from left to right. The resulting cultures were spotted onto selective galactose plates as indicated. After 2.5 days at 30°C, the plates were photographed.

Truncated CD4 Still Shows an Interaction with Lck



performed. In this experiment, growth was still evident in the cells carrying Lck and the CD4 truncation, $CD4T_2$ (Figure 2.5). In the experiment shown here, it looks as if $CD4T_2$ was unable to support the same vigorous growth as the other CD4 constructs, however, in repetitions of this assay, $CD4T_2$ gave results comparable to the other CD4 constructs (data not shown). We conclude that $CD4T_2$ is still able to interact with Lck in the two-hybrid system, despite the complete removal of the cysteine motif reported to be responsible for the interaction of CD4 with Lck.

This result prompted us to reconsider the model of how CD4 interacts with Lck. In the original coimmunoprecipitation studies performed by Turner et al, one deletion mutant, CD4-Δ412 (Figure 2.2), failed to interact with Lck. CD4-Δ412 retains the cysteine motif but lacks a juxtamembrane segment of CD4. This result indicates that the cysteine motif alone is not sufficient to mediate the interaction of CD4 with Lck and suggests that an additional mechanism may also contribute to this interaction.

Close inspection of the amino acids deleted in CD4-Δ412 reveals the presence of six basic amino acids in the region deleted. This cluster of basic residues is conserved in both human and murine versions of CD4 and CD8 (Figure 2.6) and were retained in our truncation mutant $CD4T_2$. Examination of Lck shows that this molecule contains a complementary set of six acidic residues in the portion of the unique domain employed in this study (Figure 2.6). This observation and our results in the two-hybrid assay suggest the possibility that the interaction between CD4 and Lck may be stabilized through an electrostatic interaction in addition to the

Charged Residues in CD4, CD8 and Lck

Murine CD4	RCRHQQRQAARMSQIKRLLSEKKTCQCPHRMQKSHNLI
Human CD4	RCRHRRRQAARMSQIKRLLSEKKTCQCPHRMQKSHNLI
Murine CD8α	RSRKRVCKCPRPLVRQEGKPRPSEKIV
Human CD8α	HRNRRRVCKCPRPVVKSGDKPSLSHRYV
Human CD8 β1	RRRRARLRFMKQFYK
Human CD8 β2	RRRRARLRFMKQLRLHPLEKCSRMDY
VSVG-CD8	HTKKRVCKCPR
Murine Lck	MGCVCSSNPEDDWMENIDVCENCHYPIVPL

Figure 2.6: Charged Residues in CD4, CD8 and Lck

The amino acid sequences for the cytoplasmic tails of CD4 and CD8 are shown. The charged residues are indicated in bold and the cysteine residues critical for interactions with Lck are underlined. The first 30 amino acids of murine Lck are also shown with the charged residues indicated by bold lettering.

critical cysteine mediated association. This possibility was suggested by Turner, et al, to explain their result with the CD4-Δ412 mutant.

In coimmunoprecipitation studies, this electrostatic stabilization would be said to be necessary but not sufficient for the interaction of CD4 with Lck. Researchers did attempt to show that the cysteine motifs alone were sufficient to support the interaction of CD4 with Lck. They attempted too demonstrate this by transferring the cysteine motifs of CD4 or CD8 to an unrelated protein and then testing to see if these chimeric molecules bound to Lck. When the cysteine motif from CD4 was fused to the heterologous transmembrane protein VSVG, no association with Lck was observed. In contrast, however, when the cysteine motif from CD8 was fused to VSVG, Lck was able to bind this chimera (Shaw *et al.*, 1990). Interpretation of this result is clouded, however, by the fact that VSVG includes a group of basic amino acids in its juxtamembrane region like that seen in CD8 (Figure 2.6). Therefore, this CD8-VSVG chimera recapitulates the dual motif and thereby causes this set of experiments to fall short of proving that the cysteine motif alone is sufficient to mediate an interaction of CD4 or CD8 with Lck.

If our observation that two different motifs contribute to the interaction of Lck with CD4 is borne out by further experiments, this would not be the first example of a Src family member utilizing a dual signal to localize to the plasma membrane. In the case of Src, both myristoylation and a polybasic sequence contribute to the binding of Src to acidic phospholipids (Buser *et al.*, 1994; Kwong and Lublin, 1995; Sigal *et al.*, 1994; Silverman and Resh, 1992; Silverman *et al.*, 1993). The myristoyl moiety alone binds to phospholipid

vesicles with an apparent Kd of 10^{-4} M and a nonmyristoylated version of the first 15 amino acids of Src (net charge +5) bind to phospholipid vesicles containing the acidic phospholipid phosphotidylserine with an apparent Kd of 10^{-3} M (Sigal *et al.*, 1994). The presence of a single charged residue in an amino terminal peptide of Src (myr Src^{15} (NKN)) was not sufficient to increase the affinity of this peptide for acidic phospholipids over that observed with myristoylation alone (Buser *et al.*, 1994). In the binding of Src to plasma membrane preparations, however, addition of a single charged amino acid followed by the addition of another caused incremental increases in the affinity of the myristoylated Src peptide for membranes (Silverman and Resh, 1992). Although the presence of dual binding motifs in Lck and Src are not entirely analogous, we would argue that our observation of a potential electrostatic interaction between Lck and CD4 is significant in comparison with Src since Lck is the only Src family member to completely lack positively charged residues in its amino terminus (See Figure 2.6) (Silverman and Resh, 1992; Silverman *et al.*, 1993). In addition, other than the longer version of Hck (p61Hck), Lck is also the only Src family member to display a net negative charge in its amino terminus (see Figure 2.6) (Silverman and Resh, 1992; Silverman *et al.*, 1993).

We presume that the hypothesized electrostatic stabilization is sufficient for us to observe an interaction between CD4 and Lck in our system because the two-hybrid assay is sensitive enough to record associations that are too weak to be seen by coimmunoprecipitation (Li and Fields, 1993). If true, the discovery of this electrostatic interaction would demonstrate the advantage of

the yeast two-hybrid assay, that of uncovering subtle features of protein-protein interactions. Some of the sensitivity we see may be contributed by the stabilization achieved through the interaction of LexA with DNA and B42 with the transcriptional machinery (Fields and Sternglanz, 1994). In this way, our results may be somewhat artifactual in that this electrostatic interaction is probably not sufficient, on its own, to facilitate an interaction between CD4 and Lck at the plasma membrane, even if a technique existed to detect such a low affinity association. This supposition is supported by results where no activation of Lck kinase activity was observed after CD4 crosslinking in CD4 mutants that contained the polybasic domain in the absence of the cysteine motif, indicating that either Lck does not associate with a cys- version of CD4 at the membrane or Lck can not be activated when in association with a cys- version of CD4 (Levin *et al.*, 1993).

The contribution of these ionic motifs to the interaction of CD4 with Lck needs to be examined further through additional experiments. The result of mutating all of the basic amino acids in CD4 to neutral residues should be evaluated in both the two-hybrid assay and in coimmunoprecipitation studies. From the results obtained with CD4-Δ412 (Turner *et al.*, 1990), we would predict that no interaction would be seen in the biochemical study, while the two-hybrid assay might show that the cysteine motifs alone would be sufficient to observe this interaction. The polybasic domain should also be eliminated through the substitution of neutral residues in the context of $CD4T_2$ to confirm that it was these charged amino acids that were responsible for maintaining the interaction

observed in this study. Mutation of one residue at a time would allow the minimal number of charged residues necessary to maintain the interaction of CD4 with Lck to be determined. In previous coimmunoprecipitation studies, three of the acidic residues in Lck were mutated without affecting the association of the two molecules. The mutant used, however, still retained three of the six charged residues in this region of Lck (Shaw *et al.*, 1990).

Potential Problems with the Two-Hybrid System

In previous studies, cysteine containing motifs on both CD4 and Lck had been identified as being important for the interaction of Lck with CD4. In a further effort to test the bounds of the two-hybrid system, we prepared a Lck fusion protein whose critical cysteine residues had been replaced with alanine. When this construct was tested in the two-hybrid assay for its ability to interact with CD4Large, it showed robust growth under triple selection conditions (Figure 2.4, right panel). In light of the vigorous growth observed with CD4LargeCys-, this result was not surprising. However, LckCys- displayed additional peculiar characteristics which led us to discount the positive signal we observed in the two-hybrid assay with this construct.

The two-hybrid assay utilized in the experiments discussed thus far in Chapter Two were an early version of the two-hybrid system developed in the laboratory of Roger Brent. In this version of the system, the transcriptional activation domain fusion proteins, (all of the CD4 constructs in this study) were under the control of the Gal1 promoter. In the strain utilized in this study, gal80 was intact

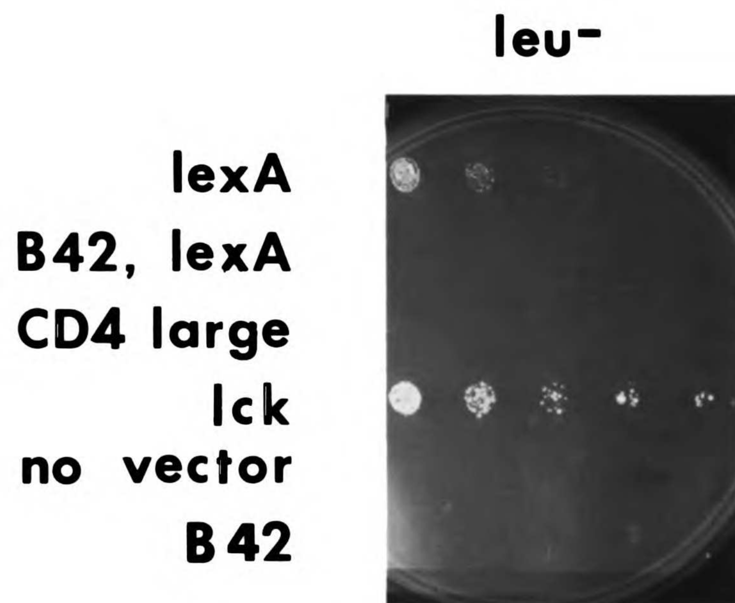
and therefore repressed transcription from the Gal1 promoter in the presence of glucose. Therefore, in the presence of glucose the CD4 constructs should not be expressed and any transcriptional activity observed from the Leu2 reporter gene should be due solely to the DNA binding domain -Lck fusion proteins which are expressed constitutively from the ADH promoter. Under triple selection conditions on glucose containing plates, LckCys- was obviously prototrophic for leucine (data not shown). Under the same conditions, the wild type Lck construct also displayed some activity although well below that seen with the LckCys- construct (data not shown). In all of the growth assays discussed thus far, selection on Leucine was carried out concurrent with selection for Tryptophan and Histidine to retain the plasmids expressing the fusion proteins. Such triple selection can be stressful to the cells, generally slowing cell growth and causing the test condition, leucine selection, to appear more stringent than it actually is. When the Lck-LexA containing cells were assayed for background transcriptional activity under Leucine selection alone, considerable basal transcriptional activity was now observed with this construct (Figure 2.7).

The observation that Lck-LexA can activate transcription, to some degree, when expressed alone, leads us to be cautious about the conclusions drawn from our Lck-CD4 interaction studies. In the experiments we performed (Figure 2.4 and 2.5), strong transcriptional activity did seem to depend on the presence of CD4 sequences. Perhaps the high level of basal transcriptional activity seen with Lck made it easier to detect the very weak ionic stabilization we observed between CD4 and Lck. Due to this

Figure 2.7: Basal Activity of LexA and B42 Fusion Proteins.

EGY.048 cells carrying the indicated plasmids were grown overnight in YPD. The cultures were normalized to an OD=0.9 and serial five fold dilutions were made in adjacent wells, proceeding from left to right. The resulting cultures were spotted onto selective galactose plates as indicated. After 2.5 days at 30°C, the plates were photographed.

Basal Activity of LexA and B42 Fusion Proteins



background problem, however, it is impossible to make a definitive statement concerning the importance of the proposed ionic stabilization motif. This series of experiments, along with the additional experiments discussed in the previous section, need to be performed in a two-hybrid system where a Lck-LexA fusion protein displays no basal transcriptional activity on its own. This may be difficult to achieve since the acidic residues present in the unique domain of Lck alleged to interact with the basic residues in CD4 may also allow this domain of Lck to act as a weak transcriptional activator.

In light of the difficulties encountered with Lck in the two-hybrid system, we wanted to fully explore the background transcriptional activity of Hck in the two-hybrid system. Under our standard assay conditions we saw very little growth with the LexA-Hck constructs (data not shown). We were interested, however, in the level of background growth we might observe under the plating conditions to be used during the library screen (see Materials and Methods). We carried out a set of mock library platings in which B42 alone was substituted in place of the library and tallied the number of colonies observed for each the LexA-Hck constructs (Table 2-I). These data indicated that using a growth assay alone in a library screen with the LexA-Hck constructs would result in only a 1000X enrichment rather than a stringent selection. At the time, the EGY.048 cells we had been using for the two-hybrid assays contained only one integrated reporter, the Leu2 gene. A second reporter, β-galactasidase, was available on a high copy number plasmid. When

Expected Background with LexA and Gal4 Hck Constructs

CONSTRUCT	CELLS PLATED[a]	COLONIES	FALSE (+) for $2x10^6$ CELLS PLATED
LexA-hckAUNQ	$3\ x10^3$	5	$3x10^3$
LexA-hckCUNQ	$3\ x10^3$	4	$3x10^3$
LexA-hckCKIN	$3\ x10^3$	2	$1x10^3$
Gal4-hckCUNQ	$1\ x10^3$	0	0
	$1\ x10^4$	0	0
Gal4-hckAKin	$3\ x10^3$	6	$4x10^3$
	$3\ x10^4$	28	$2x10^3$

a For LexA constructs this number was estimated from OD_{600} of $1.5=1x10^8$cells/ml. For Gal4 constructs this number was derived from matched samples plated on nonselective media. For Gal4 constructs, the estimated and derived numbers are within the same order of magnitude.

Table 2-I: Expected Background with LexA and Gal4 Hck Constructs.

EGY.048 cells containing the LexA constructs listed and Y153 cells carrying the Gal4 constructs listed were plated at the indicated densities. Colonies containing LexA constructs were scored after 5 days at 30°C on Trp-His-Leu- plates. Colonies expressing Gal4 constructs were scored after three days on trp-his-leu- 50mm 3 amino triazole plates. The number of colonies indicated is the average of two experiments. See Figure 2.1 and Figure 3.1 in the next chapter for explainations of the Hck constructs.

this reporter was tested, however, all of the Hck-DNA binding domain constructs gave a high β-gal signal (data not shown). We suspected that this high background was due to the combination of a leaky DNA binding domain fusion construct with the large number of target sites present when using a high copy number plasmid.

At about this time, we became aware of another yeast strain and set of plasmids for the two-hybrid system that had been designed by Steve Elledge (Durfee *et al.*, 1994). This strain, Y153, has two integrated reporter genes, Gal1-His3 and Gal1-LacZ. In addition to the convenience of the second integrated reporter, the use of His3 as a selectable marker also allows for the use of the Histidine antimetabolite, 3-aminotriazole, to increase the stringency of the Histidine selection (Kishone and Shah, 1988). This allows for the stringency of the selection to be titered to the basal transcriptional activity of each individual DNA binding domain construct.

Three Hck-Gal4 DNA binding domain constructs were made in the new system (See next chapter, Figure 3.1). The level of basal transcriptional activation seen with these constructs was tested and the concentration of 3-aminotriazole necessary to reduce this background was determined. A mock library plating using the new Hck-Gal4 constructs was performed in strain Y153 and the number of colonies obtained in the presence of 50mm 3-aminotriazole was determined (Table 2-I). The larger Gal4-HckSH constructs that contain the SH2 and SH3 domains of Hck in addition to the unique domain gave a background comparable to the LexA constructs. The smaller fusion protein expressing only the unique domain of Hck in

this system, however, had considerably lower background growth. We decided to move forward with a screen using the Gal4-HckCUnq construct in the Y153 based yeast two-hybrid system.

Summary

We used the well characterized interaction of CD4 with Lck to test the utility of the yeast two-hybrid system for the study of Src family kinases. We were able to show that CD4 and Lck interact in this in vivo assay. Furthermore, we were able to identify a potential ionic stabilization motif that plays a role in the interaction of CD4 with Lck that was overlooked with less sensitive assay systems. In addition to being sensitive, however, this system also gave a background level of transcriptional activity when expressing Src family kinases that could interfere with efforts to identify novel proteins that interact with these target molecules. A more stringent two-hybrid assay was sought to use in a library screen for proteins that interact with the unique domain of the Src family kinase, Hck.

Chapter III

SCREENING FOR HCK INTERACTING PROTEINS IN THE YEAST TWO-HYBRID SYSTEM.

Introduction

The physiological role of the Src family kinase, Hck, is not currently understood. We wished to identify upstream event(s) responsible for the activation of Hck in hemopoietic cells. Knowledge of this initiating signal and the ability to control this event, would provide an experimental system that could be used to dissect the regulation and activity of Hck in a physiologically relevant context. Consideration of the signaling events known to trigger the activation of other Src family kinases (Taniguchi, 1995) led this lab to carry out experiments to test a cadre of signaling molecules relevant to hemopoietic cell function for their ability to activate Hck. The factors tested included IL-6, LIF, Oncostatin M, G-CSF, GM-CSF, CSF-1, met-leu-phe, TNF, LPS and crosslinking of the Fcγ receptors. Of these, only LPS caused even a marginal change in Hck's kinase activity (Robbins, 1995). This gave us little indication of what experimental conditions to pursue in our investigations of this enzyme. Furthermore, experiments using cells from Hck null mice also failed to provide a clear research path toward the elucidation of the function of this kinase since they lacked any clear functional deficits in known signaling systems (Lowell *et al.*, 1994). Therefore, we chose an experimental approach that would theoretically allow us to survey all of the possible interactions of the unique domain of Hck

with other molecules in a given hemopoietic cell type without the necessity of prior knowledge of Hck's role in these cells.

We were particularly interested in the potential partners that recognize the unique domain of Hck since precedent with the Src family member Lck had shown this region to be important in the recognition of Lck by its upstream signaling partner CD4 (Shaw *et al.*, 1989; Shaw *et al.*, 1990; Turner *et al.*, 1990). Our interest in the unique domain was further heightened by the observation that Hck is found as two isoforms, p59 and p61 in human cells. When it is compared to p59Hck, p61Hck is seen to contain an additional 21 amino acids in its unique amino terminal domain (Lock *et al.*, 1991; Robbins *et al.*, 1995). We wondered if this additional sequence allows p61Hck to bind partners not recognized by the smaller p59Hck protein.

We used the unique domain of Hck to survey a B cell library for potential protein partners using the yeast two-hybrid system. We identified three candidate Hck interacting proteins; two of unknown function and the cytoskeletal protein actin.

Results

Constructs and the Library

Previous experiments with Src had indicated that the unique domain mediates interactions with other cellular components (Feder and Bishop, 1991; Goddard *et al.*, 1989; Kaplan *et al.*, 1990). In addition, it is well established that SH2 and SH3 domains are responsible for the binding of Src and other Src family members to a

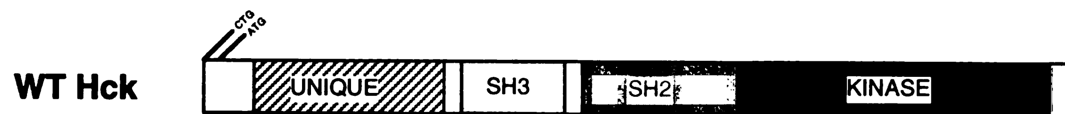
variety of signaling molecules (da *et al.*, 1993; Koch *et al.*, 1991; Malek and Desiderio, 1993; Panchamoorthy *et al.*, 1994; Pleiman *et al.*, 1993; Ramos-Morales *et al.*, 1994; Sudol *et al.*, 1993; Swope and Huganir, 1994). In order to identify such interactions involving the Src family kinase Hck, we prepared fusion proteins for use in the yeast two-hybrid assay. The chimaeric proteins consisted of the DNA binding domain of yeast Gal4 fused either to the unique domain of Hck (AA 1-84) or the entire amino terminal portion of Hck (AA 1-248 or AA 22-248) in the pAS1 vector (Figure 3.1; Table A-2 in Materials and Methods). The two larger constructs, HckASH and HckCSH that contain SH2, SH3 and unique sequences, were designed to differ from each other at the junction between the Gal4 DNA binding domain and the Hck containing portion of the chimaeric protein. The Hck coding portion of HckASH begins with the ATG start codon that is used to produce the p59Hck isoform. In contrast, the Hck coding region of HckCSH begins at the upstream CTG, adding an additional 21 amino acids to this construct compared to that seen with HckASH. This makes HckCSH analogous to the p61Hck isoform observed in human cells (Robbins *et al.*, 1995). The smaller construct, HckCUNQ also begins with this upstream CTG.

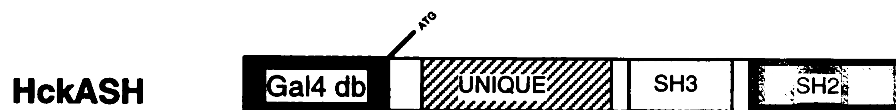
From experiments described in Chapter 2, we know that the constructs containing the SH2 and SH3 domains of Hck exhibited some basal level of activity in the two-hybrid system when growth on Trp-His-Leu- 50mM 3AT was used as an assay (Table 2-I). Since we would be using both the growth assay and the β-galactasidase assay to screen a library in this system, we needed to determine if

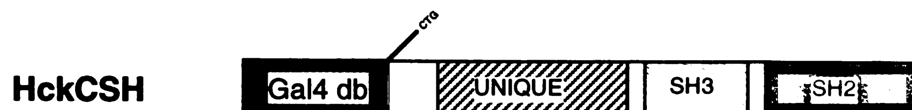
Figure 3.1: Gal4-Hck Constructs.

A diagrammatic representation of the products of the Gal4 DNA binding domain constructs used in this study. (Gal4db=Gal4 DNA binding domain.) The constructs were made as described in Table A2 in Materials and Methods.

Gal4-Hck Constructs







HckASH and HckCSH exhibited a similar level of basal activity with the β -gal filter assay (See Materials and Methods).

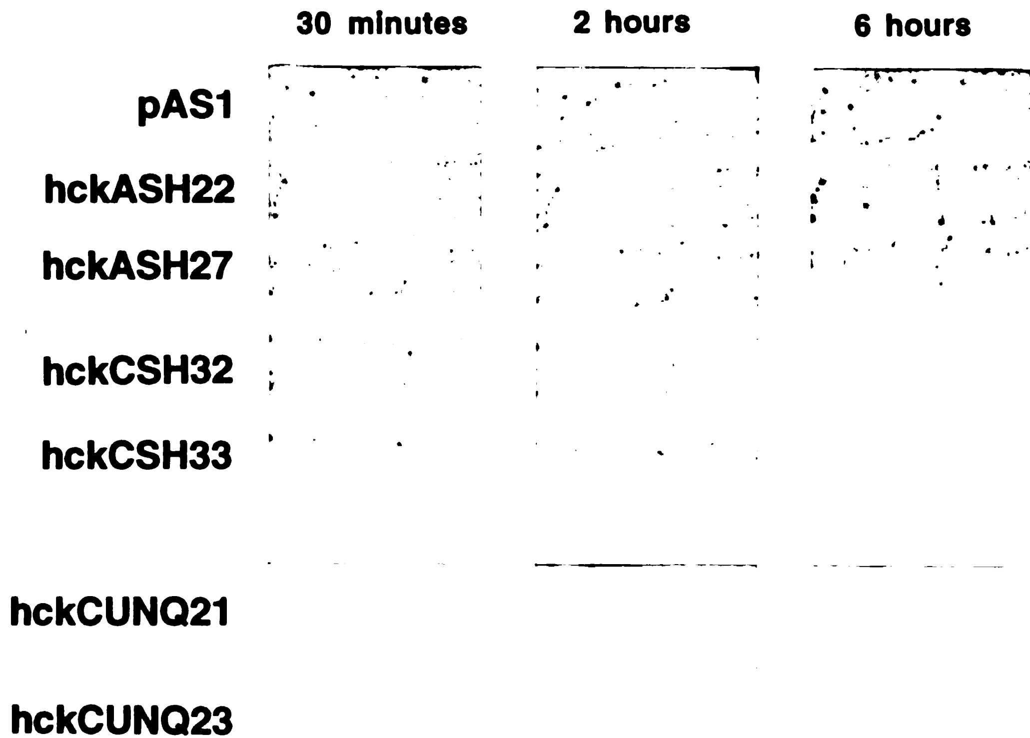
None of the constructs exhibited any detectable β-gal activity in a 30 minute incubation with the standard assay (Figure 3.2). At 2 and 6 hours of incubation, however, both HckCSH and HckASH exhibited considerable β-gal activity (Figure 3.2, top panels). The smaller construct, HckCUNQ did not demonstrate any activity even after an incubation time of 6 hours (Figure 3.2, bottom panels). The high level of β−gal activity seen with the larger constructs during longer incubations meant that a short assay would have to be used for the screen.

The library screened with these constructs was prepared by Steve Elledge and his colleagues in the two-hybrid, transcriptional activation domain vector (See Materials and Methods). RNA for the library was derived from human peripheral B cells (Durfee *et al.*, 1993) Hck is expressed in human B cell lines but had not been previously described in a similar preparation (Willman *et al.*, 1991). A PCR assay was performed on a preparation of DNA to determine if Hck was expressed in this library. When oligos delineating the amino terminal portion of Hck were used in a PCR reaction with the library DNA as a substrate, a band of the appropriate size (~750 nucleotides) was observed when stained with EtBr (data not shown). Reactions run in parallel containing a single oligo or using a DNA preparation from a HeLa Cell library as a substrate did not produce a band of this size (data not shown). The presence of Hck in this library made it an appropriate choice for the identification of Hck interacting proteins.

Figure 3.2: Basal β -gal Activity of Gal4-Hck Constructs.

Y153 cells carrying the constructs indicated were patched, in triplicate, onto Trp- plates and grown overnight at 30°C. The patches were replicated onto filters and grown overnight at 30°C. βgal assays were performed as described in Materials and Methods for the times indicated. The numbers; 22, 27, 32, 33, 21, 23, refer to individual isolates of these constructs.

Basal ß-gal Activity of Gal4-hck Constructs



Identification of clones that interact with Hck constructs in the yeast two-hybrid assay.

To better understand the physiological role of Hck, we wanted to identify proteins with which it interacts. Toward this end, we used the Gal4-Hck constructs described above to screen Elledge's B cell library expressed from the two-hybrid vector pSEll07 (See Materials and Methods). The yeast strain Y153 was transformed separately with each of the Gal4-Hck constructs; HckCUNQ, HckCSH and HckASH and grown under Leucine selection to retain the pAS1 plasmid. Large scale preparations of transformation competent Y153 cells carrying the appropriate Gal4-Hck constructs were then made and transformed with DNA from the B cell library. These double transformants were allowed to recover for a short time in liquid culture under double selection for both plasmids before being plated under Trp-His-Leu- triple selection with 50mM 3AT. The number of colonies present after five days at room temperature under these selection conditions were tabulated for each construct (Table 3-I).

As expected from the mock library platings described in Chapter 2, triple selection of yeast expressing the larger constructs HckCSH and HckASH, gave an enrichment of ~1000X. This resulted in 3600 His+ colonies with HckCSH and 2600 with HckASH after screening over 2.5 million transformants with each construct (Table 3-I). The smaller construct, HckCUNQ, that had not shown any activity in the mock library plating discussed in Chapter 2, proved to allow more stringent growth selection than the larger constructs, but still resulted in ~900 colonies from the 1.6 million transformants screened (Table 3-I). These numbers compare favorably with

Table 3-I: Summary of Two-Hybrid Screens.

The screen carried out in this study was performed as described in Materials and Methods. The number of transformants screened was estimated from a small aliquot of the transformation mix that was plated on non-selective media. His+ colonies represent the number of colonies present on ~30 15 cm^3 Trp-Leu-His- 50mM 3AT plates after 5 days at room temperature. His+/βgal+ colonies represent the number of colonies which both grew under histidine selection and resulted in a positive signal in the βgal filter assays. Construct dependent clones refers to those clones which gave a positive signal in both assays in the presence of the various Hck constructs but not in the presence of unrelated constructs such as lamin, p53, cdk2 and SNF1. The results of other screens are shown for comparison. ND indicates no data was cited in the respective publications.

Table 3-I:
Summary of Two-hybrid Screens

Constructs	Transformants screened	His^+ Colonies	His^+/ßgal$^+$ Colonies	Plasmids rescued	Construct dependent clones	References
HckCUNQ	1.6×10^6	900	13	13	8	this study
HckCSH	3.2×10^6	3600	108	11*	7	this study
HckASH	2.5×10^6	2600	146	7*	5	this study
Rb	2.0×10^6	25,000	139	ND	28	(Durfee, *et al.*, 1993)
SRF	5.0×10^5	ND	42	ND	32	(Dalton, *et al.*, 1992)
Jun	1.0×10^5	ND	12	ND	11	(Chevray, *et al.*, 1992)
SNF1	3.0×10^5	ND	144	ND	16	(Yang, *et al.*, 1992)
Btk	4.5×10^6	3000	70	12	ND	(Cheng, *et al.*, 1994)
Ras	1.4×10^7	229	100	ND	21	(Vojtek, *et al.*, 1993)
Ras	3.0×10^5	ND	3	ND	1	(Hofer, *et al.*, 1994)
Fas	3.0×10^8	395	84	ND	2†	(Sato, *et al.*, 1995)

* This also represents the number of plasmids attempted

† In addition to the standard negative controls, CD40 was used as a specific negative cont

screens run by other labs, being more stringent than that seen with Rb (25,000 His+ colonies) (Durfee *et al.*, 1993) comparable to that observed with the non-receptor tyrosine kinase Btk (3000 His+ colonies) (Cheng *et al.*, 1994) and less stringent than screens run with Ras and Fas (229 and 395 His+ colonies, respectively) (Table 3-I) (Hofer *et al.*, 1995; Sato *et al.*, 1995; Vojtek *et al.*, 1993).

The His+ colonies identified with each of the Gal4-Hck constructs were then screened for β–gal activity. Those colonies that were positive for β–gal activity, were transferred to new selective plates and re-tested both for growth and β–gal activity. This was done to eliminate any clones that did not consistantly activate both reporter genes. After two rounds of selection and screening, the number of His+/β–gal+ colonies remaining for each construct was 13 for HckCUNQ, 108 for HckCSH and 146 for HckASH (Table 3-I). Once again these numbers fell within the range observed in two-hybrid screens run by other labs (Table 3-I), indicating that our bait proteins were no more permissive than other bait proteins successfully employed in other screens.

Once putative interacting proteins had been identified, it was necessary to carry out additional tests in an effort to eliminate false positive clones. False positives can arise in any number of ways. First, a mutation could have arisen in Y153 or in the Gal4-Hck constructs so that transcription from the two reporter genes would no longer depend on the presence of an interacting transcriptional activation domain fusion protein. False positives such as these are easily eliminated by testing the library clones in fresh cells with new copies of the Gal4-Hck constructs. To test for this class of false

positives, library plasmids from all of the double positive clones identified with HckCUNQ and a sample of those identified with the two larger constructs were rescued into *E. coli* and subsequently used to transform the yeast strain Y190. Y190 carrying each of the library clones isolated in the screen were then mated to Y187 carrying one of the Gal4-Hck constructs. All of the library clones tested in this way continued to activate transcription from the reporter genes in the presence of the Gal4-Hck constructs, indicating that none of the clones isolated represented this trivial class of false positives.

A second class of false positives often observed in the two-hybrid system are those that are dependent on the presence of a DNA binding domain construct but which are not specific for the construct used in the library screen (Bartel *et al.*, 1993). This class of false positives may consist of a library protein that recognizes the Gal4 portion of the fusion construct or may allow transcription to occur by some other unknown mechanism. These false positives can be identified by testing the ability of the library clones to activate transcription from the reporter genes in the presence of a heterologous DNA binding domain fusion protein. In this instance, we tested all of the library clones that had been rescued against DNA binding constructs containing the heterologous proteins lamin, cdk2, p53 and SNF1. This was done by mating Y190 cells carrying the library plasmids to Y187 cells containing these heterologous constructs. Five of the library clones isolated with HckCUNQ, four of those rescued from the HckCSH group and two of those rescued from

the HckASH group proved to be in this second class of false positive clones (Tables 3-I and 3-II).

In growth assays against heterologous proteins, some of the clones we report as being Hck dependent, demonstrated absolutely no growth when tested against the heterologous protein, SNF1. For example library clones 6SH3357 and 7CU2389 proved to be entirely dependent on the presence of Hck for growth (Figure 3.3). Other library clones, however, such as 16CU2364 and 16CU2315 did show a minimal ability to promote growth with SNF1 under selective conditions (Figure 3.3). When the basal level of transcriptional activation was tested using the βgal assay, however, none of the library clones categorized as Hck dependent demonstrated any ability to activate transcription in the presence of heterologous proteins, even with overnight incubations in the β-gal assay (data not shown). Therefore, by all of the criteria available for testing clones in the two-hybrid system, the library clones remaining appeared to be Hck dependent for their ability to activate the reporter genes His3 and LacZ (Table 3-I). They thus seemed likely to represent authentic potential binding partners for Hck.

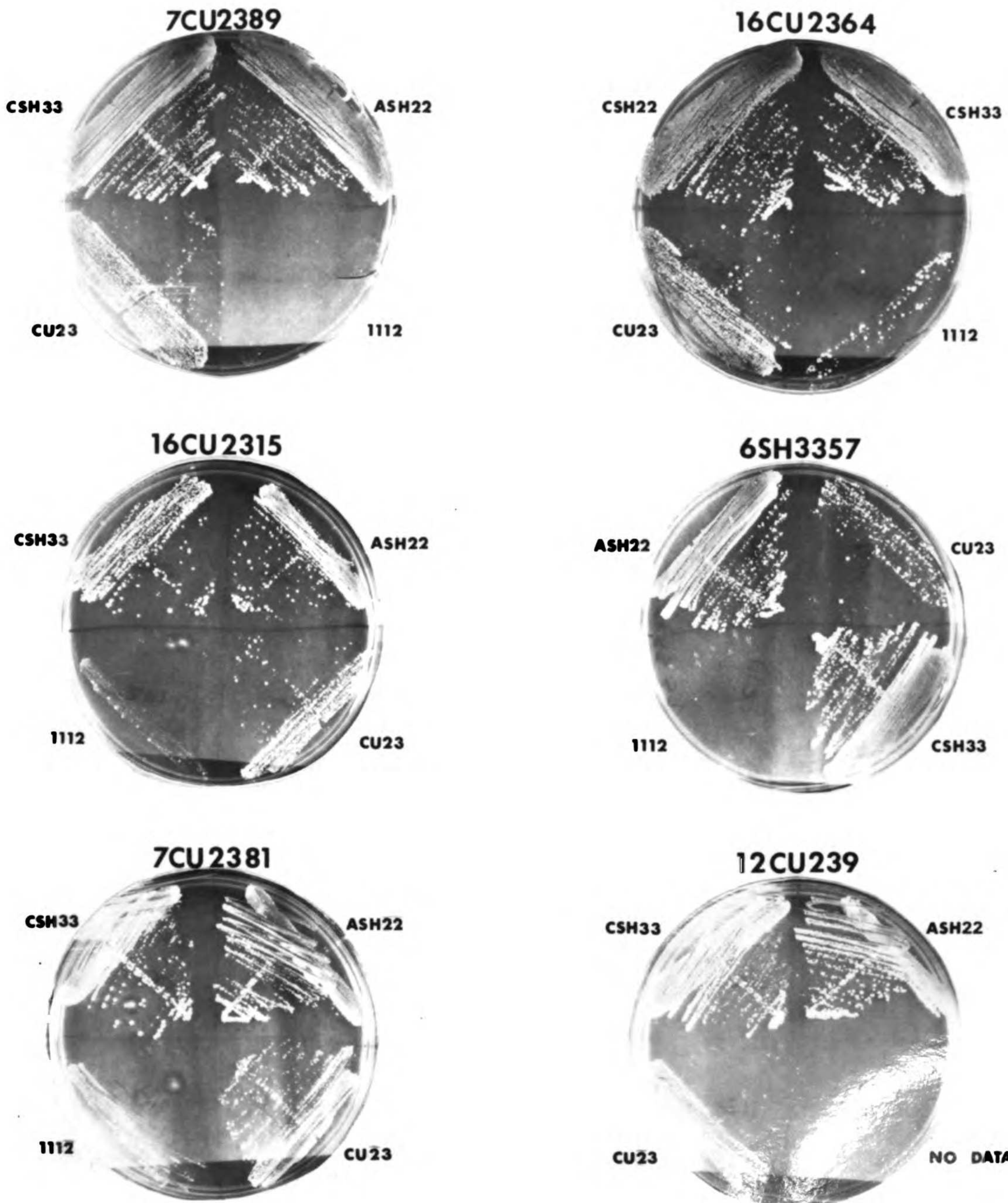
Sequence identity and other characteristics of the library clones

As noted above, the number of positive clones identified with the two larger constructs was not excessive if compared to the number of positive clones identified in other similar screens (Table 3-I). We were still concerned about the validity of these potential interactions though, because of the high basal activity seen with these Hck constructs in both the growth and β-gal assays (Table 2-I

Figure 3.3: Growth of Library Clones on Trp-Leu-His- 3AT Media.

Library clones in Y190 were crossed with Y187 carrying HckCSH (CSH33), HckASH (ASH22), HckCUNQ (CU23) or SNF1 (1112) and tested for the ability to grow on selective media.

Growth of Library Clones on Trp-Leu-His-(3AT) Media



and Figure 3.2). This high background activity could have allowed very low affinity interactions to appear as positive signals. To demonstrate that these interactions were specific for Hck, we wanted to map the binding of the interacting proteins to one of the three domains present in the large constructs; the SH2, SH3 or unique domain. Of the 12 library clones identified as interacting with the large Hck constructs only 4 gave positive results when tested against the unique domain construct. When isolated SH2 and SH2/SH3 domain constructs were tested in the two-hybrid assay, however, both of these constructs displayed a level of basal transcriptional activity that was in excess of that observed with HckASH or HckCSH. Similar results were seen when the SH2 and SH3 domains of Src were tested as autonomous domains in the yeast two-hybrid system (S. Martin, pers. comm.) Due to this high basal activity, these constructs could not be used to further map the potential binding sites of this group of library clones onto the Hck protein.

To evaluate these library clones further, we sequenced across the junction where the transcriptional activation domain is fused to the library cDNA. We then ran these sequences through the non-redundant protein sequence database using the BLAST+ server to evaluate whether these were previously known proteins (Altschul *et al.*, 1990). We were presented with a plethora of protein identities. Many of the clones we had identified in our library screen with the large gal4-Hck constructs were known proteins in the wrong frame. (Since we know the reading frame of the transcriptional activation

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+ non-redundant protein sequence dtaabase (nr-aa) is constructed from Swissprot, pir, prf, genpept and genpept-upd. The sequences were last evaluated in nr-aa May 1996.

domain, we can determine what frame is being utilized in the cDNA. A protein in the wrong frame refers to a sequence that was 90-100% identical to a known protein, but was not being read in its correct frame in the clones we identified. In this instance, +3 is 'in frame' with the transcriptional activation domain.) Examples of known proteins in the wrong frame included; a ribosomal protein in the -3 frame, ferritin in the +1 frame (twice), and IgG λ chain variable region in the +2 frame. Some of the clones were also being read in the correct frame. These included a voltage dependent anion channel, a translated alu sequence found in one isoform of a β_1 integrin, the heat shock protein Hsp60, and the cytoskeletal protein actin. Only one of the clones failed to display any significant homology to sequences in the non-redundant databases in 1993. Of these clones, the four that mapped to the unique domain of Hck were the alu sequence, the unknown protein, HSP60 and actin.

From this information we were unable to determine the significance, if any, of these potential interactions. Due to the high level of basal activity observed with HckCSH and HckASH, and the lack of reasonable clones to pursue, we performed no additional analysis on this group of clones. It is possible that within the group of ~250 clones identified with HckCSH and HckASH some informative clones may have existed. However, since the HckCUNQ construct displayed a much lower level of background activity than the larger constructs, we decided to concentrate our efforts on the library clones identified when the unique domain of Hck was used as bait in the two-hybrid assay.

Table 3-II: Summary of Library Clones.

Library clones were tested for the ability to grow on selective media using lamin, cdk2, p53 or SNF1 as bait. Clones positive in this assay were not processed further. Clones 19CU2342, 18CU235 and 1CU239 were partially sequenced and then pursued no further. Clones 16CU2364 and 7CU2389 encode identical sequences. ND indicates not determined.

TABLE 3-II: Summary of Library Clones

Library Clone	lamin	cdk2	p53	SNF1	Insert (kb)	ORF (aa)	Gal4 fusion (kd)	gst fusion (kd)	mRNA (kb)	Expression	Homology
16CU2364	-	-	-	-	0.7	108	29	ND	2.5	U937, IM-9	7CU2389
7CU2389	-	-	-	-	0.6	108	28	35	2.5	U937, IM-9	16CU2364
16CU2315	-	-	-	-	1.0	117	30	40	1.5	all cells assayed	Actin
6SH3357	-	-	-	-	1.1	120	29.5	40	1.5	all cells assayed	Actin
12CU239	-	-	-	-	1.3	225	25	ND	>9	peripheral blood leukocytes	none
7CU2381	-	-	-	-	1.1	ND	38	48	2.0	all cells assayed (except K562)	Thr-tRNA synthetase
19CU2342	-	-	-	-	0.7	ND	ND	ND	ND	ND	Ferritin (+1 frame)
18CU235	-	-	-	-	0.7	ND	ND	ND	ND	ND	λ chain - variable (+2 frame)
1CU2329	-	-	-	-	0.9	ND	ND	ND	ND	ND	elongation factor 1α
16CU2320	+	+	-	-	ND	ND	ND	ND	ND	ND	Alu sequence
22CU2346	+	+	-	-	ND	ND	ND	ND	ND	ND	ND
15CU2323	+	+	-	-	ND	ND	ND	ND	ND	ND	ND
25CU2393	+	+	-	-	ND	ND	ND	ND	ND	ND	ND
7CU2390	-	+	-	-	ND	ND	ND	ND	ND	ND	ND

The characteristics of the Hck dependent clones identified with HckCUNQ are indicated in Table 3-II. As seen in the group of clones identified with the larger Hck constructs, this group included two clones that were previously known proteins that were being expressed in the wrong reading frame. These were ferritin (+1 frame) and an IgG λ chain variable region (+2 frame). These two examples were the same as those seen with HckCSH and HckASH. The significance of this is unclear. Both sequences contain an open reading frame of ~90 amino acids that are contiguous with the Gal4 transcriptional activation domain. In these reading frames, these two protein sequences do not demonstrate any obvious similarity to one another when tested using BLOCKMAKER (Altschul *et al.*, 1990). Furthermore, the IgG λ chain clone does not contain a contiguous cluster of charged residues like that observed in CD4 that might mediate a weak ionic interaction with Hck. The ferritin clone does, however, contain one cluster of five positively charged amino acids (+ + + X X + +) that could pair with negatively charged residues in HckCUNQ. It is possible though, that these two clones appeared as positives in the two-hybrid screen not because of any real affinity for Hck but by virtue of an interaction with the transcriptional machinery that we do not understand. Jeno Gyuris and colleagues are currently compiling a list of frequently encountered 'junk' clones identified using the two-hybrid screen. It would be interesting to learn if these two sequences have been encountered by other researchers.

We also found two RNA binding proteins among the positive clones encountered when we screened the library with HckCUNQ.

These were elongation factor 1α and a threonyl-tRNA synthetase. Hck has not previously been implicated in the regulation of RNA binding proteins or in the regulation of translation. Src, however, has been seen in association with the RNA binding protein SAM68 (Fumagalli *et al.*, 1994; Taylor *et al.*, 1995). We did not pursue these potential partners for Hck however, because other users of the two-hybrid system have reported that RNA binding proteins were frequently observed 'junk' clones in their screens (S. Goff, S. Martin per. comm.) Once again, it will be interesting to see if this observation is upheld in the compilation being made by Jeno Gyuris and his colleagues.

The remaining clones, 16CU2364, 7CU2389, 16CU2315 and 12CU239 could not be immediately eliminated from further consideration. 12CU239, 16CU2364 and 7CU2389 have not been previously described in any of the databases searched with BLAST (Table 3-II and Figures 3.4 and 3.5) (Altschul *et al.*, 1990)). Analysis of the sequences for 16CU2364 and 7CU2389, however, did show that these two clones encode the same protein. They proved to be independent isolates since 7CU2389 does not extend as far as 16CU2364 at the 3' end of the insert (Figure 3.4). No additional information, however, was available from the nucleotide sequences of these two clones or from that of clone 12CU239. They show no homology with other known proteins or with each other using BLAST or BLOCKMAKER (Altschul *et al.*, 1990; Henikoff *et al.*, 1995).

One of the features that might be expected of a factor that is uniquely involved in the regulation of Hck would be that it is expressed only in the cells in which expression of Hck is also

Figure 3.4: Sequence of 16CU2364 and 7CU2389

The DNA sequence and corresponding amino acids are shown for the clones 16CU2364 and 7CU2389. The right hand facing arrow indicates the start of each clone. The left hand facing arrow indicates where 7CU22389 ends. Charged amino acids are indicated.

```
                                                                     60
CGAGGCCACG AAGGCCTTGA GAAAGTAAAT TTATACTCAG GTTCTAATTT TAATAGGGAA
 R  G  H   E  G  L  E   K  V  N   L  Y  S   G  S  N  F   N  R  E>
                    -   +                                    +  -
                                                                    120
                                             *
GGAAGAAGTT ACAGCTCAGT GCACCATGAA GTTGAGACAG AGATGGAGAC ACCTCAGCCC
 G  R  S   Y  S  S  V   H  H  E   V  E  T   E  M  E  T   P  Q  P>
    +                   +  +  -     -       -     -
                                                                    180
CACCTCTCTG GAACAGGAAA GATGATTGGG GAGGGAGCAC AGGTCAGCGT GGGAAGAGGG
 H  L  S   G  T  G  K   M  I  G   E  G  A   Q  V  S  V   G  R  G>
 +                  +             -                         +
                                                                    240
                    *
TCATGGTGGA CATGGGGGTG GGGTGGTCTC CCCACCTCCT CACATTATGC CTACACGAAC
 S  W  W   T  W  G  W   G  G  L   P  T  S   S  H  Y  A   Y  T  N>
                                               +
                                                                    300
                                                                   *
ACAGACACAT GCAGTTGCCT TGCAGAAACC AAGTCAGGGT TCTTCAAGTC ACAAAGGGAA
 T  D  T   C  S  C  L   A  E  T   K  S  G   F  F  K  S   Q  R  E>
    -                      -      +               +         +  -
                                                                    360
GGGCAGGAAC AACTCTTGCC TCTCAGTCCC ACACAAGGCA GCTGTCTCAC ACTTTACAAG
 G  Q  E   Q  L  L  P   L  S  P   T  Q  G   S  C  L  T   L  Y  K>
       -                                                       +
                                                                    420
                                             *
CTGTGAGAGA CACATCAGAG CCCTGGGCAC TGTCACTGCT TGCAGCCTGA GAGTAGCTCC
 L  *

                                                                    480
CTCCTTTTCT ATCTGAGCTC TTCCTCCTCC ACATCACAGC AGCGACCACA GCTCCAGTGA

                                                                    540
                    *
TCACAGCTCC AAAGAGAACC AGGCCAGCAA TGATGCCCAC GATGGGGATG GTGGGCTGGG

                                                                    600
                                                                   *
AAGACGGCTC CCATCTCAGG GTGAGGGGCT TGGGCAAACC CTCATGCTGC ACATGGCAGG

TGTATCTCTG CTCCTGTCCA GCTTCGTGGC CTCGAGAGAT CTATGAATCG TAGA
```

Figure 3.5: Sequence of 12CU239

The DNA sequence and correspondinig amino acids are shown for the clone 12CU239. Charged amino acids are indicated.

```
                                                                      60
CGAGGCCACG AAGGCCGCCC CTTTCAGCTT CTGGGGTCCC TGGGCCCCAG CCCTGGCCCG
 R  G  H   E  G  R  P   F  Q  L   L  G  S   L  G  P  S   P  G  P>
                 +

                                                                     120
                                                 *
GCCAGGGAGG GGGTGGGGTG CAGCTGGGAG AGTGGCAGAG CCGAGAACGG AGTCACGGGA
 A  R  E   G  V  G  C   S  W  E   S  G  R   A  E  N  G   V  T  G>
    +  -                      -         +      --

                                                                     180
GCCGGTAAGC GGCGCTGGAA CTTCGAGCAG ATCTCCTTCC CCAACATGGC TTCAGACAGC
 A  G  K   R  R  W  N   F  E  Q   I  S  F   P  N  M  A   S  D  S>
       +   +  +            -                                -

                                                                     240
                    *
CGCCACACCC TTCTGCGCGC CCCAGCCCCA GAGCTGCTCC CGGCCAACGT GGCTGGGCGG
 R  H  T   L  L  R  A   P  A  P   E  L  L   P  A  N  V   A  G  R>
 +  +            +                 -                             +

                                                                     300
                                                                   *
GAGACAGACG CTGAGTCCTG GTGCCAGAAG CTGAACCAGC GCAAGGAGAA GCTCTCCAGG
 E  T  D   A  E  S  W   C  Q  K   L  N  Q   R  K  E  K   L  S  R>
 -     -      -                +            +  +  -  +             +

                                                                     360
CGGGAACGGG AGCACCACGC GGAGGCCGCG CAGTTCCAGG AAGATGTCAA CGCCGATCCC
 R  E  R   E  H  H  A   E  A  A   Q  F  Q   E  D  V  N   A  D  P>
 +  -  +   -  +  +      -                   -  -            -

                                                                     420
                                                *
GAGGTGCAGC GCTGCTCCAG CTGGCGGGAG TACAANGAGC TGCTGCAGCG GCGGCCGGTG
 E  V  Q   R  C  S  S   W  R  E   Y  X  E   L  L  Q  R   R  P  V>
 -         +               +  -         -            +   +

                                                                     480
CAGAGGAGCC AGCGCCGGGC CCCTCACCTG TGGGGCCAGC CCGTCACCCC GCTGCTGAGT
 Q  R  S   Q  R  R  A   P  H  L   W  G  Q   P  V  T  P   L  L  S>
    +         +  +         +

                                                                     540
                    *
CCTGGCCAGG CCAGCTCCCC AGCCGTGGTC CTTCAGCATA TCTCTGTGCT GCAGACAACA
 P  G  Q   A  S  S  P   A  V  V   L  Q  H   I  S  V  L   Q  T  T>
                                        +

                                                                     600
                                                                   *
CACCTTCCTG ATGGAGGTGC CCGGCTGTTG GAGAAGTCTG GGGGCTTGGA AATCATCTTT
 H  L  P   D  G  G  A   R  L  L   E  K  S   G  G  L  E   I  I  F>
 +         -            +         -  +                -

                                                                     660
GATGTTTACC AGGCCGACGC TGTGGCCACA TTCCGAAAGA ATAACCCTGG CAAACCCTAT
 D  V  Y   Q  A  D  A   V  A  T   F  R  K   N  N  P  G   K  P  Y>
 -               -                   +  +                +

                                                                     720
                                                *
GCCCGGATGT GCATTAGTGG ATTTGATGAG CCTGTCCCAG ACCTCTGCAG CCTCAAGCGG
 A  R  M   C  I  S  G   F  D  E   P  V  P   D  L  C  S   L  K  R>
    +                      -  -             -                    +

                                                                     780
TTGTCTTACC AGAGTGGGGA TGTCCCTCTG ATCTTTGCCC TGGTGGATCA TGGTGACATC
 L  S  Y   Q  S  G  D   V  P  L   I  F  A   L  V  D  H   G  D  I>
                    .                          -             -
                                                                     840
```

```
TCCTTCTACA GCTTCAGGGA CTTCACGTTG CCCCAGGATG TGGGGCACTG ACCTCACAGC
 S  F  Y   S  F  R  D   F  T  L   P  Q  D   V  G  H  X>
                 +  -                    -            +

                                                             900
                                                               *
TCTGCAGAGG ATGGAGCTTG CTCCGGGGGA CCGGGACTGT CTGTTCTCAG GGTACCATCT

                                                             960
CGGCTGCCTC CTGTACCCAG ACTCTAACCT GTAACTTCAG AGGCCAGTCT GGGCCTTGGC

                                                            1020
                                          *
CCTGTGTGTC TGATACTCAC NGAGTGAAAC TGTGACCCTC TCCCTTCCCT GCTGCCTTGC

                                                            1080
ANTGACCCCT CTGGAACTCA TGACTCNATT TTAATGACCC ANGANGTGGG GCANAAGANA

                    *
NGACNGTGTG CCTTTANCTA NAGGGTGCCT GCTTCGTGCT ATAAAGCCA
```

observed. This would be analogous to the situation seen with CD4 and Lck: Expression of CD4 is only observed in cells that are also seen to express Lck. To test whether this condition held for any of our clones we examined the expression pattern of 12CU2389 and 16CU2364/7CU2389 on Northern blots and found that they displayed a limited pattern of expression that describes a subset of those cells seen to express Hck. In confirmation of previous data from Northern blots (Quintrell *et al.*, 1987; Tsygankov and Bolen, 1993), expression of Hck was observed in the myelomonocytic cell lines Ml-1 (myeloblast), Hl-60 (promyelocyte), and U937 (monocytes) as well as the B cell line IM-9 (Figure 3.6). Expression of 7CU2389/16CU2364 was observed in these same cell lines with the exception that no expression was observed in the Hl-60 cells (Figure 3.7). No expression of 12CU239 was ever observed in the human cell lines used in this study. It wasn't until a Northern blot containing RNA from human tissue was probed with a fragment of 12CU239 that any expression was seen (Figure 3.8). This discrepancy may be due to a true absence of expression in the human cell lines examined; however, another trivial possibility is that transfer of the large message observed for 12CU239 (>9 Kb, Figure 3.8) was not achieved in the 1 hour procedure used to prepare the blots made from the human cell lines (see Materials and Methods). The expression of 12CU239 observed in peripheral blood leukocytes most likely represents expression in mature activated B cells, as no expression was observed in either the thymus or the spleen (Figure 3.8). The observation that 16CU2364/7CU2389 and 12CU239 are expressed in

Figure 3.6: Expression of Clones in Human Cell Lines.

Northern blots were performed as described in Materials and Methods. The same blot was stripped and reprobed successively with the probes indicated at left.

Expression of Clones in Human Cell Lines

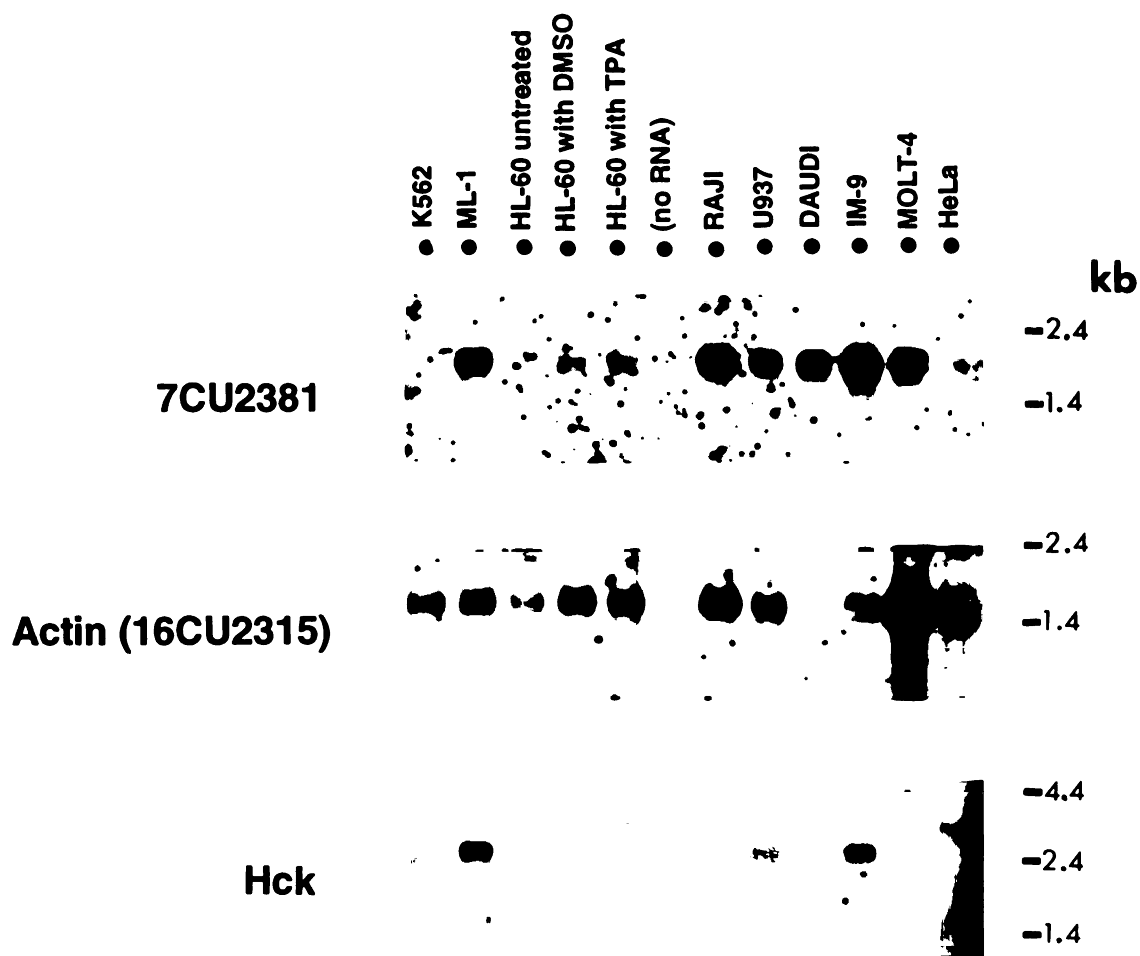


Figure 3.7: Expression of Clones 7CU2389 and 16Cu2364 mRNA in Human Cell Lines. Northern blots were performed as described in Materials and Methods.

Expression of Clones 7CU2389 and 16CU2364 mRNA in Human Cell Lines

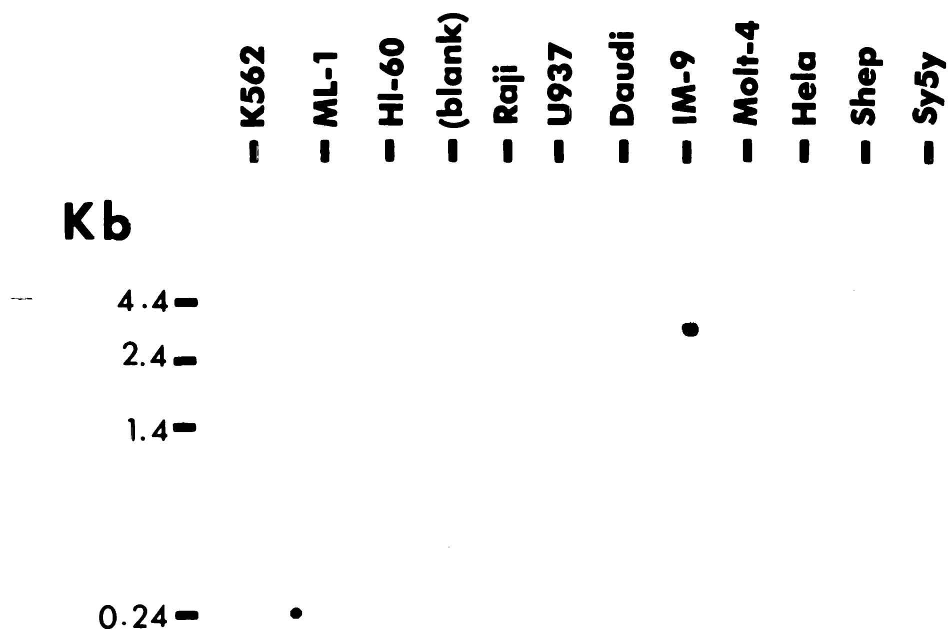
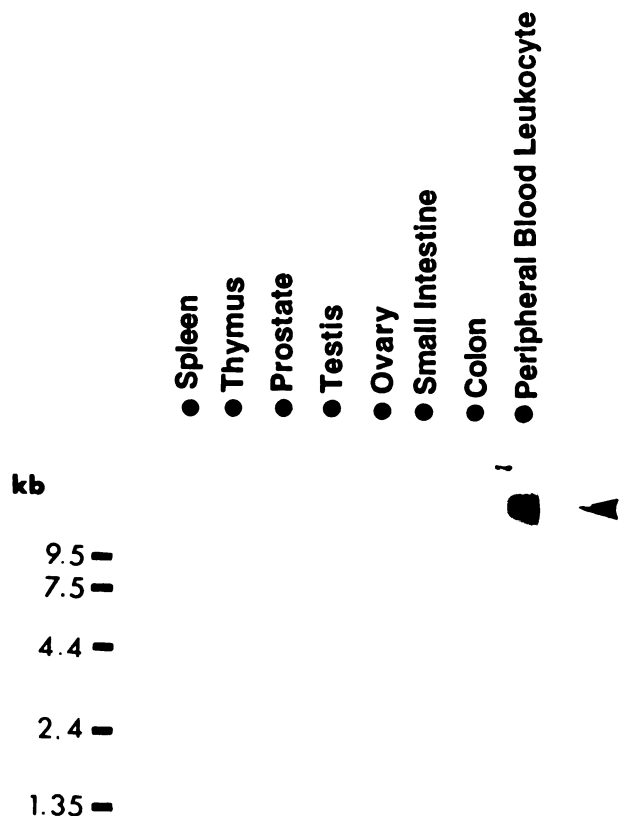


Figure 3.8: Expression of 12Cu239 in Human Tissue.

Northern blots were performed as described in Materials and Methods.

Expression of 12CU239 in Human Tissue



a subset of the cells which express Hck, make these two classes of clones viable candidates for proteins that play a significant role in the regulation of Hck.

Clone 16CU2315 encodes a partial sequence of the cytoskeletal protein actin. Clone 6SH3357, identified in the two-hybrid screen with HckCSH, also contained a partial sequence of actin. The two clones differed from one another by nine nucleotides with 16CU2315 beginning at amino acid 245 and 6SH3357 starting with amino acid 248 (Figure 3.9). This region represents the carboxy terminal one third of actin. We were intrigued by this result since experiments in this lab have shown that an amino terminal peptide of Src is able to bind to actin and behaves as an actin crosslinking protein (Feder, 1993; Feder and Bishop, 1996).

Other kinases have been shown to bind to actin and the sequences responsible for this binding have been defined. Thinking that Hck might bind actin through a similar sequence, we examined Hck to determine if our kinase contained any of the defined actin bining motifs. Using BLOCKMAKER (Henikoff *et al.*, 1995), the unique domain of Hck does not appear to contain any of the actin binding sites observed in c-abl (McWhirter and Wang, 1993), the EGF receptor (den Hartigh *et al.*, 1992) or PKC-ε (Prekeris *et al.*, 1996).

The library clones do not demonstrate interactions with Hck in vitro.

We wanted to confirm the interactions observed in the two-hybrid system between Hck and the various library clones by using an independent assay system. We subcloned the library inserts 7CU23289, 16CU2315 (actin) and 7CU2381 (tRNA synthetase) into

Figure 3.9: Protein Sequence of Actin

The complete amino acid sequence of human γ-actin is shown. The sequence contained in clone 6SH3357 is indicated in bold. The start of Clone 16CU2315 is indicated by the arrow. Charged residues are indicated.

```
                                                               50
MEEEIAALVI DNGSGMCKAG FAGDDAPRAV FPSIVGRPRH QGVMVGMGQK

                                                              100
                                                                *
DSYVGDEAQS KRGILTLKYP IEHGIVTNWD DMEKIWHHTF YNELRVAPEE

                                                              150
HPVLLTEAPL NPKANREKMT QIMFETFNTP AMYVAIQAVL SLYASGRTTG

                                                              200
                                                                *
IVMDSGDGVT HTVPIYEGYA LPHAILRLDL AGRDLTDYLM KILTERGYSF

                                                              250
TTTAERETVR DIKEKLCYVA LDFEQEMATA ASSSSLEKSY ELPDGQVITI

                                                              300
                                                                *
GNERFRCPEA LFQPSFLGME SCGIHETTFN SIMKCDVDIR KDLYANTVLS
  + +   -           -       -      +  -  - +  +-

                                                              350
GGTTMYPGIA DRMQKEITAL APSTMKIKII APPERKYSVW IGGSILASLS
           -+  +-          +  +       -  +

TFQQMWISKQ EYDESGPSIV HRKCF
         +   -  -      +++
```

the pGEX vector system to create GST-clone fusion proteins. We expressed these fusion proteins in *E. coli* and purified the resulting proteins on glutathione agarose columns (Figure 3.10, bottom panel). An extract of NP-40 soluble material from U937 cells that contained both forms of Hck, was incubated on the columns, followed by extensive washing. Free glutathione was added to the column to elute the gst-fusion proteins and any proteins bound to them. Both forms of Hck, p59 and p61, were seen to pass through the column without binding to any of the library clones (Figure 3.10, top panel). These binding assays were performed in the presence of the detergent NP-40 and 150 mM salt. These conditions are necessary to solubilize Hck away from cell membranes and for the solubilized Hck to remain in solution. These same conditions, however, have the potential to interfere with Hck's ability to bind to other proteins.

To test for binding activity in the absence of detergent, we employed the Hck construct p59G2A in which the glycine at position two has been changed to an alanine (Robbins *et al.*, 1995). This protein does not become myristoylated, is not found in association with membranes and is therefore soluble in buffers that do not contain detergents (Robbins *et al.*, 1995). Extracts made from NIH3T3 cells expressing p59G2A were prepared in PBS buffer. These extracts were incubated on glutathione columns to which the library gst-clones, 7CU239, 16CU2315 and 7CU2381, had been bound. After extensive washing with PBS, the columns were eluted with free glutathione. No Hck protein was seen to bind to any of the library clones, even in the absence of detergent (Figure 3.11).

Figure 3.10: Wild Type Hck Does Not Bind to Library Clones *in vitro*.

The western blot in the bottom panel shows the gst constructs loaded onto each column. In the bottom panel, bacterial extract is the crude lysate that was loaded onto the glutathione agarose columns; FT (flow through) shows the gst-fusion protein remaining in the extract after being passed over the glutathione agarose column; eluate represents the gst-clone protein that bound to the glutathione agarose column and was subsequently eluted with 10mM free glutathione. The expected sizes for each GST fusion protein is 35 kd for 7CU2389, 40 kd for 16CU2315, 48 kd for 7CU2381 and 29 kd for GST. Samples were normalized for protein content.

The western blot in the top panel demonstrates that Hck does not bind to any of the clone containing glutathione columns. In the top panel, U937 crude is the total sample dissolved in SDS loading buffer from U937 cells; NP-40 extract is the NP-40 soluble fraction from U937 cells and represents the sample that was loaded onto the columns; FT represents the portion of the NP-40 extract that did not bind to the library clones which were bound to the glutathione column; eluate represents the protein that elutes from the columns in the presence of 10mM free glutathione. Human Hck runs ona polyacrylamide gel as a doublet of 59 and 61 Kd as indicated by the double arrows. Samples were normalized for protein content. Both blots were probed with αHck-gst antibodies which recognizes both Hck and Gst.

WT hck Does Not Bind to Library Clones *in vitro*

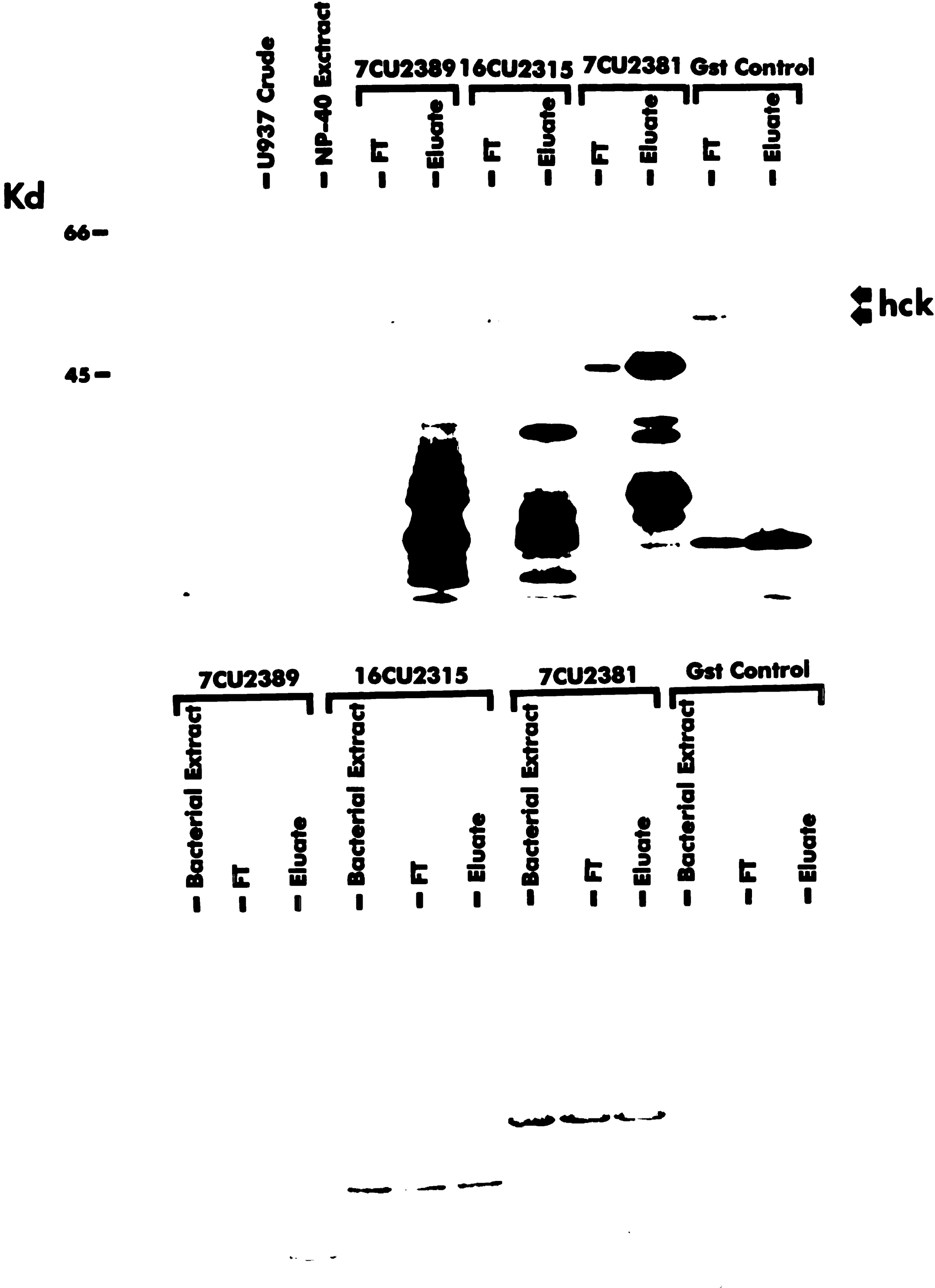
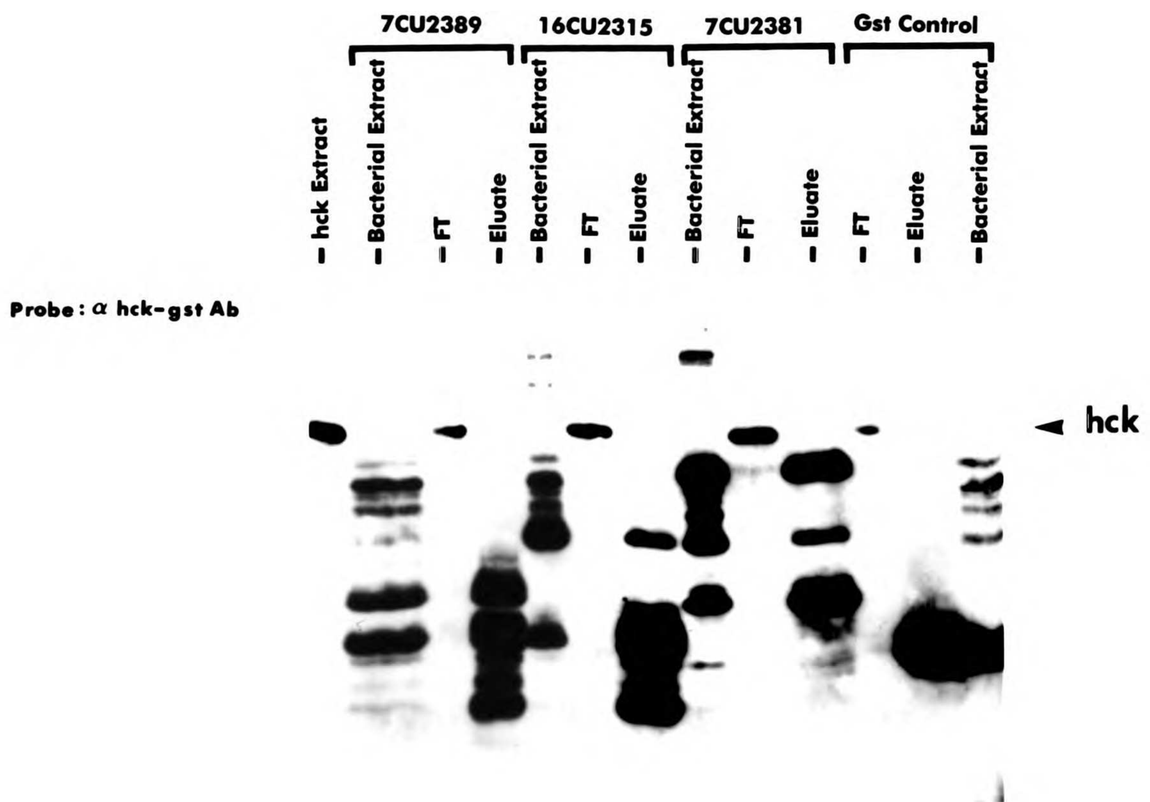


Figure 3.11: Myr (-) Hck Does Not Bind the Library Clones in vitro.

Western blot showing the results of binding experiments with GST-clone fusion protiens. Bacterial extracts (bacterial extract) containing gst-constructs of the library clones indicated were loaded over the glutathione column. After extensive washing, extracts containing myr(-) Hck (Hck extract) were loaded over the column (FT; flow through), washed and eluted with 10mM free glutathione (eluate.)

myr (-) hck Does Not Bind Library Clones *in vitro*



To ensure that Hck was not present in any of the eluates from columns in Figures 3.10 and 3.11, the eluates from each column were subject to precipitation with a Hck specific antibody, followed by incubation in kinase assay buffer with γ- labeled ATP. No Hck kinase activity was present in any of the eluates when compared to the control, gst alone (Figure 3.12).

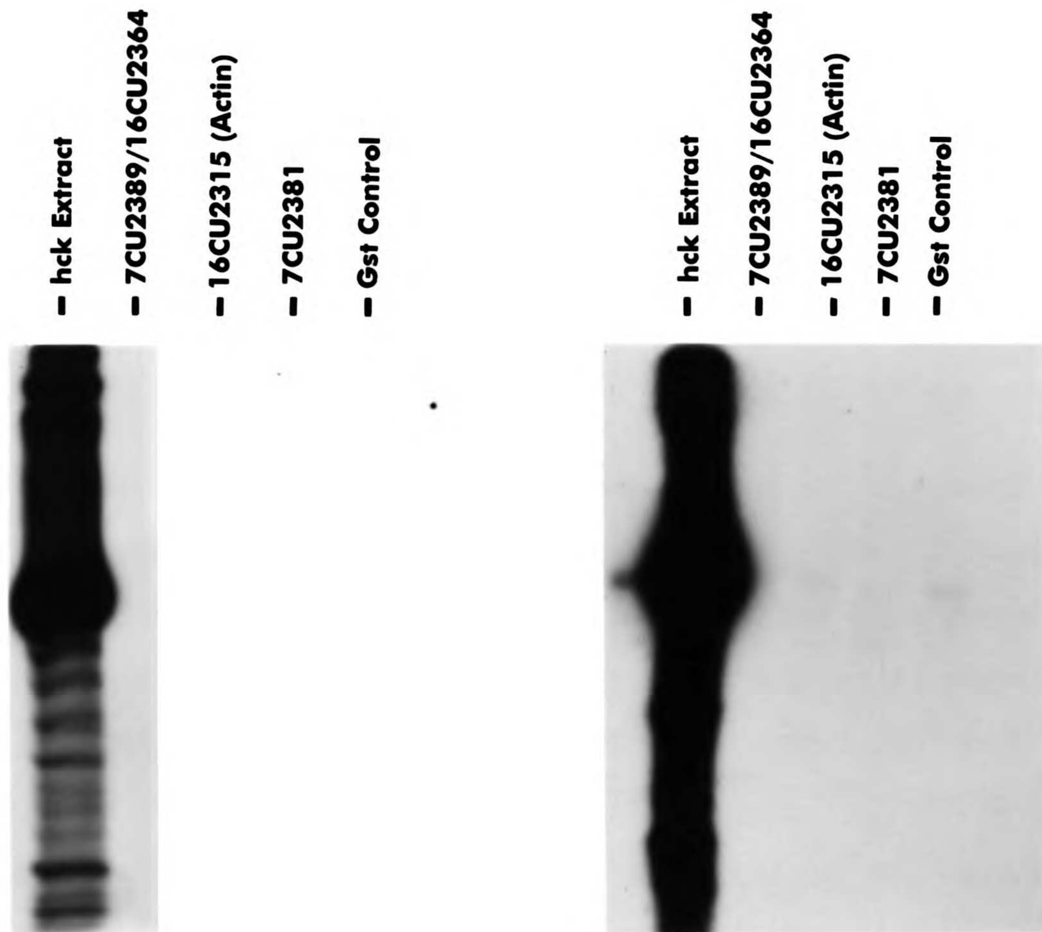
Hck kinase activity was observed in the U937 cell extract and the p59G2A extract that was passed over the glutathione columns (Figure 3.12, hck extract, left and right panels). Eluates from the glutathione columns were also tested in the CDNB assay for the presence of gst activity. Glutathione s-transferase activity was observed for each of the fusion proteins, comparable to that seen with the commercially purchased enzyme (data not shown). The presence of enzymatic activity in both of these assays implies that each sample at the completion of the procedure contained at least some glutathione s-transferase and Hck kinase in their wild type, active conformations. Therefore, the failure to see any binding activity is not likely to be due to denaturation of the proteins during their isolation or when exposed to the glutathione agarose columns. This set of experiments demonstrates that, under the conditions used, Hck does not associate in vitro with any of the library clones tested. Binding of Hck to the library clone 12CU239 was not tested because this clone could not be subcloned into the pGEX vector.

A previous study in the two-hybrid system showed that this assay is sensitive enough to detect the transient interaction of a serine/threonine kinase with its substrate (Haefner *et al.*, 1995). Therefore, we tested to see whether any of the library clones

Figure 3.12: Hck Ip Kinase Assay of Eluates from GST columns.

Hck Kinase assays were performed on eluates from Figure 3.2 (left panel) and Figure 3.13 (right panel) as described in Materials and Methods. No Hck kinase activity was seen to elute with any of the library clones tested.

hck Ip, Kinase Assay of Eluates from Gst Columns



expressed as gst-fusion proteins could act as a substrate for Hck. A Hck construct expressed with a 6-His tag was used to isolate Hck away from most other cellular proteins. This partially purified Hck was added to the gst-fusion constructs bound to glutathione agarose beads in the presence of γ-labeled ATP and Hck kinase buffer. After a short incubation, the supernatant containing Hck was removed, the beads were washed and samples of both the supernatant and the beads were run out on a gel (Figure 3.13). No radioctive bands corresponding to the size of the fusion proteins were apparent. Using this assay, it does not appear that the fusion proteins tested are substrates for p59Hck.

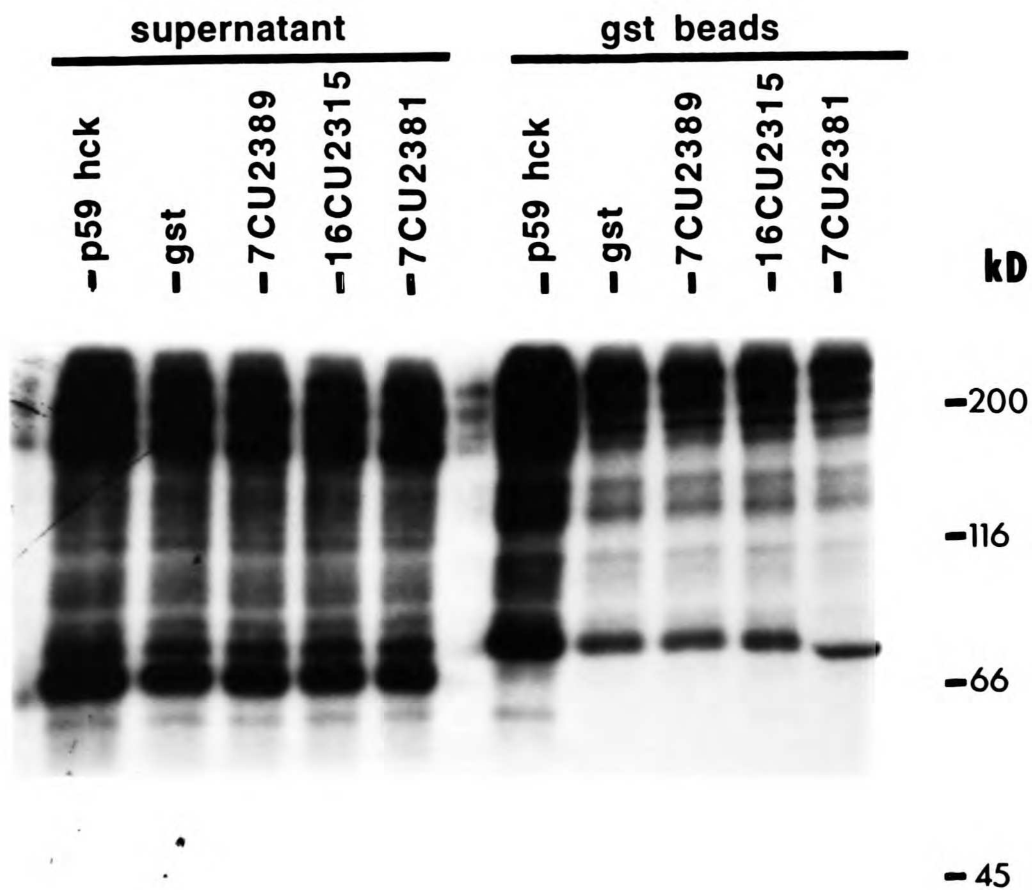
Discussion

We have used the two-hybrid genetic system in an attempt to identify proteins from human B cells that interact with the unique domain of the Src family kinase, Hck. We identified three potential Hck interacting proteins; the previously unknown proteins 7CU2389/16CU2364 and 12CU239 as well as the cytoskeletal protein, actin. The criteria that support these as authentic interactions are that, in screening greater than a million transformants, we identified multiple isolates of two of these proteins (actin and 7CU2389/16CU2364) and two of the clones display a pattern of expression consistent with the limited expression pattern observed for Hck (12CU239 and 7CU2389/16CU64). Unfortunately, we were unable to demonstrate the validity of these interactions using an

Figure 3.13: The Library Clones are not Substrates for the Kinase p59 Hck.

Kinase assays were performed with ^{32}P γATP as described in Materials and Methods. Phosphorylation of the gst-library fusion proteins was not observed and no obvious diminution or enhancement of kinase activity was observed.

The Library Clones are Not Substrates for the Kinase p59 hck



independent assay. At this time the significance of these interactions with the Src family kinase, Hck remain indeterminate.

Actin and the Src Family of Protein Tyrosine Kinases

In our two-hybrid screen, we identified actin as a potential binding partner for the Src family kinase, Hck. Although we were unable to prove by in vitro binding that this Hck/actin interaction is authentic, several lines of evidence suggest that it might be significant.

Feder and Bishop (Feder and Bishop, 1996) have shown that a myristoylated, amino terminal peptide of Src can bind to and be specifically cross-linked to actin in platelet membrane preparations. They went on to show that this peptide co-sediments with purified filamentous actin from rabbit skeletal muscle. Even more surprising than the results that Src bound to actin was their finding that both the full length, purified Src protein and the myristoylated peptide behaved as actin crosslinking proteins in vitro. This means that Src caused filamentous actin to become arranged into a mesh like gel. Actin crosslinking activity requires that two actin binding sites exist on the same actin binding protein. In the case of the Src peptide, the myristoyl moiety seems to constitute at least a portion of one of these two binding sites, as an unmyristoylated peptide is unable to compete with the myristoylated peptide for actin binding (Feder, 1993; Feder and Bishop, 1991; Feder and Bishop, 1996).

A great deal of information exists to suggest that Src is involved in the regulation of the cytoskeleton. One of the early observations made of v-Src transformed cells was that these cells

displayed reduced adherence to the substratum, were more rounded in appearance and contained fewer intact stress fibers (actin filaments) and adhesion plaques than their wild type counterparts (Ballmer-Hofer *et al.*, 1988; Tsen *et al.*, 1980). Furthermore, microinjection of purified v-Src into cells caused immediate (although transient) dissolution of the actin stress fibers (Maness and Levy, 1983). Biochemical fractionation also demonstrated that >70% of v-Src present in an RSV transformed cell was associated with the Triton X-100 resistant fraction of the cell (Hamaguchi and Hanafusa, 1987). This fraction is largely free of lipid (>90%) and is highly enriched in cytoskeletal components (Hamaguchi and Hanafusa, 1987). In addition, immunoelectron microscopy studies showed that v-Src co-localized with both actin cables and adhesion plaques in Triton X-100 extracted cell preparations (Henderson and Rohrschneider, 1987). All of these observations support a role for Src in the regulation of the actin cytoskeleton and even suggest that Src may bind directly to actin. These experiments, however, were carried out with the transforming protein v-Src. Evidence for the direct interaction of c-Src with the cytoskeleton in vivo is a bit more circumstantial.

Under normal, resting conditions, c-Src is not found in association with the Triton insoluble cytoskeleton (Kaplan *et al.*, 1992). However, in circumstances where Src becomes activated or adopts an open conformation (Liu *et al.*, 1993; Roussel *et al.*, 1991). Src is seen to transit to the Triton insoluble fraction and/or to focal adhesions (Clark and Brugge, 1993; Fox *et al.*, 1993; Kaplan *et al.*, 1994; Kaplan *et al.*, 1995). Circumstances under which Src adopts an

open conformation include when Src is expressed in Csk null cells# (Kaplan *et al.*, 1994), when Src is mutated so that it can not become phosphorylated at the negative regulatory site (i.e. Y^{527} to F^{527}) (Kaplan *et al.*, 1994), when a truncated protein including only the unique, SH3 and SH2 domains of Src is expressed (Kaplan *et al.*, 1994) when signaling occurs through the PDGF and EGF receptors (Weernink and Rijksen, 1995) or when integrin binding to fibronectin occurs, leading to receptor clustering (Clark and Brugge, 1993; Fox *et al.*, 1993; Kaplan *et al.*, 1995).

The interpretation of these results is that when c-Src is activated by dephosphorylation of Y^{527}, the SH2, SH3 and unique domains adopt a conformation that favors their binding to focal adhesions or to the actin cytoskeleton. Evidence from Feder would indicate that binding to actin occurs directly through the combined action of the myristoyl moiety and some sequence specific information in the first 15 amino acids of Src (Feder and Bishop, 1991; Feder and Bishop, 1996). This model is consistent with data provided by Kaplan et. al. in studies of Src binding to focal adhesions in vivo (Kaplan *et al.*, 1994). They demonstrated that myristoylation, the first 17 amino acids of Src and the SH3 domain were all necessary for Src to localize to focal adhesions. In the absence of the SH3 domain, however, the construct Src251Δ92-144 still displayed some Triton insolubility. I would interpret this to mean that myristoylation in conjunction with the first 15 to 17 amino acids of

Csk is the c-Src kinase and is responsible for phosphorylating Src on the negative regulatory tyrosine Y^{527}. In the absence of Csk, Y^{527} is not phosphorylated and Src is found in an open conformation (Chow and Veillette, 1995; Imamoto and Soriano, 1993). (Imamoto and Soriano, 1993)

Src provides a weak interaction with actin while the SH3 domain directs concentrated binding to focal adhesions. This SH3 mediated localization of Src to focal adhesions may occur through Src's binding to the actin binding protein paxillin, which is enriched in focal adhesions (Weng *et al.*, 1993). Src is also linked to focal adhesions through SH2 mediated binding to the autophosphorylation site on the Focal Adhesion Kinase (FAK) (Schaller *et al.*, 1994; Schlaepfer *et al.*, 1994). Thus, by having the ability to bind to actin, paxillin and through FAK, the ECM[1], Src is in place to play a key role in regulating adhesion, cell shape, and other cytoskeletal dependant processes.

Recently, Hck was implicated in a similar role to that of Src in connecting the actin cytoskeleton with integrin receptors. Bone marrow derived neutrophils from Hck-/-, Fgr-/- mice were shown to be defective in cell spreading and the ability to form tight adhesions to the extracellular matrix (Lowell *et al.*, 1996). Use of immobilized anti-integrin mAbs as adhesion sites, rather than the ECM, highlighted $\beta3$ and $\beta2$ integrins as the cell adhesion molecules involved in the defective responses (Lowell *et al.*, 1996). These results and those outlined in the previous paragraphs for Src, encourage us to believe that the interaction observed between Hck and actin in the two-hybrid assay may have some functional relevance, though it is unfortunate that we were unable to observe binding of Hck to the gst-actin constructs in vitro. However, comparisons with Src binding to actin readily provides an explanation for this failure. First, in her platelet membrane binding

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[1]FAK binds to the cytoplasmic tail of $\beta1$ integrins which bind to the ECM (Schlaepfer *et al.*, 1994).

studies, Feder observed that the presence of detergent inhibited the binding of the myristoylated Src peptide to actin (Feder, 1993). Since our first set of binding experiments (Figure 3.10) involved the use of wild type, detergent solubilized p59Hck, by analogy to Src we would expect the binding of Hck to actin to have been inhibited by the presence of detergent.

Second, in her binding crosslinking studies, Feder showed that a myristoylated 14 amino acid, amino terminal Hck peptide had some limited ability to displace the myristoylated 15 aa Src peptide from its binding site on actin, while the unmyristoylated Hck counterpart was unable to do so [1] (Feder and Bishop, 1991). These data, in conjunction with the idea that the myristate provides at least a portion of one of the two actin binding sites present on the Src peptide, would explain why we were unable to observe binding of myr (-) Hck to gst-actin (Figure 3.11). I would hypothesize that the binding of Hck to actin observed in the the two-hybrid system, which is in the absence of myristoylation on Hck, is too weak to replicate <u>in vitro</u> in the absence of myristoylation.

To pursue this interaction further, experiments like those of Feder and Kaplan should be performed with Hck (Feder and Bishop, 1991; Feder and Bishop, 1996; Kaplan *et al.*, 1994). To test for <u>in vitro</u> binding between Hck and actin it will be necessary to carry out these studies with myristoylated Hck amino terminal peptides in the absence of detergent. The ability of a myristoylated Hck peptide to

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[1]Kd N-myrSrc(15) to actin=5.2μ M
IC_{50} of N-myrSrc(15) in competition with N-myrSrc(15) = 0.9μ M
IC_{50} of N-myrHck(14) in competition with N-myrSrc(15) = 17μ M
IC_{50} of Hck(14) in competition with N-myrSrc(15) > 200μ M (Feder and Bishop, 1991),

cosediment with F-actin would provide the most clear-cut evidence that Hck, like Src can bind directly to actin. In vivo, wild type Hck under resting conditions, like Src, does not display any co-localization with actin stress fibers or focal adhesions (N. Quintrell and S. Robbins unpublished observations). However, it would be interesting to examine the localization of activated Hck or an amino terminal construct of Hck similar to Src251. The results from Lowell et. al., also suggest that binding to substrates recognized by β3 or β2 integrins should also cause Hck to become localized to focal adhesions.

A Better Mousetrap

Other than the limited pattern of expression observed with 7CU2389/16CU2364 and 12CU239, we have no reason to believe that these two classes of clones are any more significant than the clones we classified as 'junk' by virtue of their sequence identity. Since the sequences of 12CU239 and 7CU2389/16CU2364 have not previously been reported and contain no recognizable similarities with other known proteins, it is possible that these clones are RNA binding proteins or are being read in the 'wrong frame' like the other clones identified in our screen that we dismissed.

One model for how these irrelevant clones appear in the two-hybrid assay is that they recognize some features of the bait protein or are stabilized by its binding at the promoter, but specific binding occurs to other transcriptional components bound at the promoter. After the completion of this study, a yeast strain was derived in an attempt to avoid this problem. This new strain was designed so that,

with the exception of the gal4-binding sites, the promoters for LacZ and His3 reporter genes are completely dissimilar (Feilotter *et al.*, 1994). If a clone is not specific for the bait protein in this strain, you would not expect to see a transcriptional signal from both reporter genes.

As with any genetic screen, the significance of the clones identified with the two-hybrid system needs to be confirmed by a second assay. In classical genetic system, it is usually possible to design a second assay, different than the one used for the screen, that can provide additional information about the clones and their relevance to the system in question. Depending on the kind of screen utilized, additional information may be obtained in a number of ways: Mutations in, or overexpression of, a new gene can be studied to see whether it yields a phenocopy of mutations in the 'bait' gene, hinting that the new gene at least plays a role in similar processes. New genes can also be evaluated for suppression or enhancement of the phenotype of various alleles of the 'bait' gene or of other genes whose products are known to be involved in the same pathways (Haefner *et al.*, 1995) Analogous options are not readily available in the two-hybrid assay. The one option that can be employed in the two-hybrid screen, is to test the candidate interacting proteins against a non-functional mutant of the bait protein (Hofer *et al.*, 1994; Sato *et al.*, 1995; Vojtek *et al.*, 1993). If Src's SH2 domain is used as an example, one could create mutants in the conserved FLVRES sequence that has been shown to be critical for phosphotyrsine binding. Protiens identified by the yeast two-hybrid screen as likely to interact with the SH2 domain could be re-screened

against such a FLVRES mutant. Proteins whose interactions with SH2 were authentic would be expected to fail to interact with such a mutant. Unfortunately, no similar mutations are known for the unique domain of Hck. A detailed deletion analysis or scanning alanine mutagenesis in the context of the two-hybrid system could provide some additional information on precisely how Hck interacts with these clones, but without a functional correlate of these mutants in vivo, the results provided by tests such as these would be of limited usefulness (for example, (Amberg *et al.*, 1995)).

With these limitations, many researchers have turned to in vitro binding assays to confirm the results they observed in vivo with the two-hybrid assay (Boldin *et al.*, 1995; Durfee *et al.*, 1993; Luban *et al.*, 1993). Our attempts to confirm the interaction of clones 12CU239 and 7CU2389/16CU2364 with Hck in vitro were unsuccessful. The reasons for this failure are not clear. It may be that the interactions we have identified are too weak to be observed in vitro, or were not favored by the conditions we employed in our in vitro binding studies.

Another possibility for why we did not see an interaction in vitro is that, in the context of the full length Hck molecule, the unique domain was not in a configuration that favored interactions with the proteins we identified. Precedent certainly exists for this in interactions with the SH2 and SH3 domains of Src family members. For instance, the association of Btk with Fyn was initially identified using the two-hybrid system. This interaction was confirmed in vitro by utilizing gst-fusion proteins of isolated domains of Fyn and was shown to be mediated by the SH3 domain of Fyn. However,

when full length Fyn was employed, no binding was observed between Fyn and Btk (Cheng *et al.*, 1994). The presumption is that in the full length Fyn protein, the SH3 domain is involved in stabilizing the intra-molecular binding between the SH2 domain and pY^{527}. As a consequence, the SH3 domain is unavailable for binding to other protein partners. Indirect evidence also exists to support the involvement of the unique domain this intramolecular stabilization of the 'closed' conformation of Src. One study has shown that phosphorylation of serine and threonine residues in the unique domain of Src by Cdc2 causes the SH2 domain to be more accessible to phosphorylated peptide and pY^{527} to be more amenable to dephosphorylation (Stover *et al.*, 1994). Once again, the model is that the unphosphorylated unique domain is stabilizing the SH2-pY^{527} interaction and this stabilization is relieved by phosphorylation of T^{34}, T^{46} or S^{72} in the unique domain of Src (Kaech *et al.*, 1993; Stover *et al.*, 1994). If the unique domain is involved in stabilization of the closed conformation of Src, then it is reasonable to postulate that some sequences in the unique domain might not be available to participate in interactions with other molecules when Src is in the closed conformation. With this model in mind, we might have had more success observing interactions between the library clones and Hck if we had employed a less than full length Hck construct in our in vitro binding studies.

With the exception of the interaction of CD4 with Lck, interactions between Src family kinases and other molecules have been notoriously difficult to identify. Most of the studies have relied on the use of GST-fusion proteins with the unique domain to

Table 3-III: Unique domain interactins of Src family kinases

A number of studies have identified protein-protein interactions between Src family kinases and other signaling molecules. This table summarizes the interactions which have been mapped to the unique domains of the Src family kinases.

Amino acids indicates the region of each Src family kinase involved in the recognition of the indicated binding partners (GAP, gtpase activating protein; PLCγ1, phospholipase C γ1; MAPK, mitogen activated kinase; Igα immunoglobulin chain α; CD45, transmembrane phosphatase; TCRζ , T cell receptor zeta chain.)

Table 3-III:
Unique Domain Interactions of Src Family Kinases

Src Family Kinase	Amino Acids	Binding Partner	gst	in vivo	comments	Reference
Blk	1-27	GAP, PLCγ1, MapK	yes	no		(Pleiman, *et al.*, 1993)
Lyn	1-27	GAP, PLCγ1, MapK	yes	no		(Pleiman, *et al.*, 1993)
Fyn	1-10	GAP & PLCγ1	yes	yes		(Pleiman, *et al.*, 1993)
Blk	1-51	(Igα)	no	yes	no direct binding demonstrated; substrate recognition only	(Saouaf, *et al.*, 1995)
Lck	34-81	CD45	yes	yes (weak)	Binding seen to full length Lck construct in vitro	(Gervais, *et al.*, 1995; Ng, *et al.*, 1996)
Lck	Cys 21+23	CD4	no	yes		(Shaw, *et al.*, 1990; Turner, *et al.*, 1990)
Fyn	1-10	Igα	yes	no	**DCSM** protein motif on Igα	(Pleiman, *et al.*, 1994)(Clark, *et al.*, 1994)
Lyn	1-27	Igα	yes	no		(Pleiman, *et al.*, 1994)
Fyn	1-10	TCRζ	no	yes	digitonin; high level of expression; 1-5% Fyn associated	(Gauen, *et al.*, 1992)

demonstrate binding of these kinases to other proteins (Table 3-III). In one study, where the researchers attempted to confirm their in vitro binding results in vivo, they were never able to observe an interaction between the Src family members in question and the signaling molecules they believed were bound to them (Pleiman *et al.*, 1993). In those instances where interactions have been observed in vivo, it has been in systems where one or both of the proteins have been overexpressed and gentle lysis conditions have been employed (Gauen *et al.*, 1992; Pleiman *et al.*, 1993). Even then, only a small percentage (~5%) of the Src family kinase was seen to associate with the upstream target molecule (Gauen *et al.*, 1992). With such inefficient binding the norm in this family of kinases, we may have been working at the limits of this assay system.

A Changing Model of Src Regulation

One of the factors influencing the success of this project was the validity of the model on which we based the premise that the unique domain is critical for defining the specific function of each of the Src family members. As outlined in the first chapter, this theory was based on two suppositions: First that since the SH2, SH3 and catalytic domains of the Src family members are largely conserved within the family, specificity in the action of these kinases must arise from sequences in the unique domain of the protein (Bolen *et al.*, 1991); and second, that the unique domain would achieve this specificity through protein-protein interactions with other cellular targets (Shaw *et al.*, 1990; Turner *et al.*, 1990). The picture of the regulation of Src Kinases that has emerged from research over the

past five years, indicates, however, that the model we began this work on was perhaps a bit simplistic.

We have now made much more quantitative measurements of the specificity of the SH2, SH3 and catalytic domains of the Src family kinases and have found that they **do** discriminate between slightly different sequences. For example, although the catalytic domain of Src is able to phosphorylate a variety of substrates in vitro, it demonstrates a preference for an optimal sequence under competitive conditions (Songyang *et al.*, 1995). Under these conditions, the optimal substrate for Src was identified as EEIYGEFF which differs subtly from the optimal substrate sequence for the related family member Lck which is XEXIYGVLF (Songyang *et al.*, 1995). This study indicates that although Src family members under in vitro conditions may be able to phosphorylate a wide range of substrates, preferences do exist and the specificity of the kinase domain likely does contribute to the proper physiological activity of these kinases.

Our view of the specificity of the SH2 and SH3 domains of Src family members has also been subject to scrutiny. Under competitive selection, optimal binding sequences for SH2 and SH3 domains have been determined for some family members (Alexandropoulos *et al.*, 1995; Birge and Hanafusa, 1993; Cheadle *et al.*, 1994; Lee *et al.*, 1995; Songyang *et al.*, 1993; Sparks *et al.*, 1994). What emerges from these studies is that although overlapping specificity does exist in SH2 and SH3 recognition of binding sites, certain peptide sequences do bind to only one or a subset of Src family members. For instance, one set of peptides examined bound

equally well to the SH3 domains of Src, Lyn, Fyn and Hck. However, a proline rich peptide from Btk was seen to bind to Fyn, Lyn and Hck SH3 domains but not to Src's SH3 domain (Alexandropoulos *et al.*, 1995). In another example, the SH3 domain of Hck is able to bind to a proline rich sequence of the HIV protein Nef (Kd=250 nM) while Fyn is unable to do so. However, changing a single amino acid in the SH3 domain of Fyn caused it to bind to Nef with a specificity comparable to that seen with Hck (Kd=380nM) (Lee *et al.*, 1995). This is a dramatic example of how differences in only a very limited number of amino acids can dramatically change the binding specificity of the SH3 domain and presumably, therefore, alter the physiological activity of one of these kinases.

Taken together these studies present a model whereby the unique function of each of the Src family kinases can be conferred through the substrate specificity of the catalytic domain and differences in SH3 sequence recognition. In light of this revised model of Src specificity, some of the demand in our model for the unique domain to recognize distinct protein partners has been dissipated. Does the unique domain thus confer no selectivity to Src family members? Recent research argues that the unique domain does indeed confer some specificity to the function of the Src family kinases by mediating protein/protein interactions, however, a more complex mechanism by which the unique domain controls specificity has also emerged.

A number of studies have demonstrated that specific sequences in the unique domain of Src family kinases are responsible for interactions with upstream signaling molecules as predicted in

our original model (Table 3-III). For instance, AA 1-10 of Fyn directs its binding to TCR ζ chain (Gauen *et al.*, 1992) while AA 1-51 of Blk are responsible for the ability to demonstrate functional reconstitution of signaling through the Igα chain in a heterologous cell system (Saouaf *et al.*, 1995) and amino acids 34-81 of Lck direct its binding to the transmembrane tyrosine phosphates CD45 (Ng *et al.*, 1996). One of the unanticipated features of unique domain interactions with upstream signaling molecules has been that some of these disparate sequences in the unique region direct binding to the same cellular partners! For instance, the first 27 amino acids of Blk, Fyn and Lyn direct their binding to the signaling molecules GAP and PLCγ (Pleiman *et al.*, 1993).

Other work has revealed roles for the unique domain that were unanticipated five years ago. One observation that induced us to pursue protein partners that bound to the unique domain of Hck was that two isoforms of Hck exist that differ in their unique amino terminal domains (Lock *et al.*, 1991; Robbins *et al.*, 1995). In addition, experiments by S. Robbins and N. Quintrell early in the course of this work determined that the two forms of Hck displayed significantly different patterns of subcellular distribution (Robbins, 1995; Robbins *et al.*, 1995). Initially we interpreted this to mean that the two isoforms bound to very different protein receptors in the cell. However, later experiments went on to show that many of the Src family of protein tyrosine kinases are modified on their amino terminus not only by myristic acid but also by palmytic acid. This second fatty acid modification has two functions. First it acts to bind Src family members more tightly to membranes (Kwong and

Lublin, 1995; Resh, 1994) than myristoylation alone is able to achieve. Since palmitoylation is reversible, whereas myristoylation is not, this modification also has the potential to confer regulation to the membrane binding event (Alland *et al.*, 1994; Koegl *et al.*, 1994; Paige *et al.*, 1993; Resh, 1994; Robbins *et al.*, 1995; Shenoy-Scaria *et al.*, 1994). The second function of this additional fatty acid modification is to direct the palmitoylated proteins to specific membrane sub compartments (Robbins *et al.*, 1995). At this time it is unclear if the differences in subcellular localization observed between p59Hck and p61Hck are due solely to these differences in fatty acid modification or if additional protein sequences contribute to their differential subcellular distribution.

In addition to these fatty acid modifications, another factor contributing to the membrane attachment of Src family members are a group of charged lysine residues in the amino terminus of many of these kinases (Silverman and Resh, 1992; Silverman *et al.*, 1993). In Src itself, which does not become palmitoylated, the amino terminus displays a net positive charge of +5. Since the inner leaflet of the plasma membrane is enriched in acidic phospholipids, this cluster of basic residues can contribute significantly to Src's affinity for membranes. In one study, a molar partition coefficient was determined for the Src polybasic sequence binding to acidic phospholipid vesicles. It was seen to be $10^3 M^{-1}$ which compares favorably to the $10^4 M^{-1}$ partition coefficient provided by the myrisitate moiety alone (Buser *et al.*, 1994). Together, the polybasic region in the amino terminus of Src and the myrisitic acid moiety act to bind Src tightly to membranes, just as the combination of myristic

and palmytic acid act synergisticly to bind other Src family members to the inner leaflet of the plasma membrane (Buser *et al.*, 1994; Sigal *et al.*, 1994).

A second unanticipated feature of the unique domain that is only now beginning to be appreciated is the modulatory effects on these kinases of serine and threonine phosphorylations in the unique region. For instance, phosphorylation on Lck at Ser^{59} changes the binding specificity of the SH2 domain of Lck (Carrera *et al.*, 1995; Joung, 1995) and serine and threonine phosphorylations in the unique domain of Src affect accessibility of the SH2 domain and the negative regulatory site at Y^{527} (Stover *et al.*, 1994). It is unclear how these mechanisms work. It is clear, however, that none of these regulatory events were duplicated in the two-hybrid system we used: Due to the way in which our GAL4-Hck fusion proteins were constructed, no fatty acid modifications were expected to occur. When we knew only of the myristoylation event, we accepted this limitation, however, when a second fatty acid modification was also shown to be important in the subcellular distribution of Hck, this limitation was of greater concern. In addition, since we used an isolated unique domain construct in our screen instead of a full length Hck kinase, we also biased ourselves against any interactions that required a concerted binding mechanism involving all of the regulatory domains.

Summary

We have used the two-hybrid genetic system to identify protein from human B cells that interact with the unique domain of

the Src family kinase, Hck. In the end, we identified three potential Hck interacting proteins; the previously unknown proteins 7CU2389/16CU2364 and 12CU239 as well as the cytoskeletal protein, actin.

The clones 7CU2389/16CU2364 and 12CU239 are expressed in a subset of the cells where Hck expression is observed. This pattern of expression is consistent with that of a factor that might be uniquely involved in the regulation of Hck. It might be reasonable therefore, to continue to pursue the meaning of these interactions by performing additional experiments with these two classes of clones. Antibodies against the new proteins or expression of a tagged molecule in human cells could be used to test whether these clones display the same subcellular distribution seen with Hck and whether they could be crosslinked in vivo to this Src family kinase.

Although we were unable to demonstrate it here, the interaction observed between Hck and actin in the two-hybrid assay may be shown, with time, to have some functional significance for physiologic role of Hck in hemopoietic cells. Results with cells from hck defecient mice indicate that, in certain specialized cell types, Hck may play a role similar to Src's in regulating the cytoskeleton.

It is unfortunate that our screen did not yield more clear cut results that would have allowed us to make a more definitive statement about the function of Hck in human cells. In part, we were limited by the two-hybrid screen available to us at the time, as well as by the inherent shortcomings of such a genetic system. We were also bounded by our own incomplete understanding of the nature of

Chapter IV

SUMMARY AND PERSPECTIVES

With the discovery of v-Src and recognition of its role in cellular transformation, researchers initiated a field of study that has already spanned twenty years and now encompasses nine different family members, acting in a variety of different cell types and signaling systems throughout the body. Although the dissection of Src's role and the roles of the other family members in cell growth and differentiation has been more difficult and torturous than anticipated at the outset, considerable progress has been achieved in our understanding of where, when and how these molecules act in the cell. A number of signaling cascades have been identified where members of this family play a crucial role in the successful activation of these systems. In the following paragraphs, I will review some of these signaling systems and the role of src family kinases in each.

Src Family Kinases in the B and T cell Antigen Receptor Complexes.

Many of the functions of the immune system are controlled by molecular recognition events mediated by multi-subunit immune recognition receptors. The T cell antigen receptor complex (TCR) and the B cell antigen receptor complex (BCR) and receptors that recognize the Fc portion of soluble antibodies are all examples of such multi-component recognition systems (Cambier and Jensen, 1994). Src family kinases have been shown to be involved in the intracellular signaling events initiated from these complexes

subsequent to ligand binding. For the T cell and B cell complexes, the roles of the Src family members involved have been well characterized.

Under resting conditions, members of the Src family of protein tyrosine kinases are found associated with the TCR and BCR. Lyn and Fyn are bound to the Ig-α subunit of the BCR by virtue of sequences in their unique amino terminal domains (Clark *et al.*, 1994; Pleiman *et al.*, 1994). Similarly in the T cell complex, Fyn binds to the zeta subunit in an analogous fashion (Gauen *et al.*, 1992). In addition, the co-receptor CD4 binds Lck under resting conditions in T cells and acts to escort this kinase into the TCR complex when co-ligation of CD4 and the receptor occurs (Shaw *et al.*, 1989; Shaw *et al.*, 1990; Simpson *et al.*, 1989; Turner *et al.*, 1990). In an analogous situation, CD19 acts as a co-receptor for the B cell antigen complex and contributes additional Lyn kinase to the BCR complex upon co-legation of CD19 with the antigen receptor (Cambier *et al.*, 1994). By an unknown mechanism, binding of antigen to the receptor subunits on the cell surface transmits a signal to the Src family kinases present in the complexes, leading to the phosphorylation of ITAM sequences imbedded in the intracellular subunits of these receptors (DeFranco, 1995; Howe and Weiss, 1995). ITAM sequences are conserved motifs found in the Ig-α and Ig-β chains of the BCR, the ε, δ, ζ, γ chains of the TCR and in a number of Fc receptor subunits (DeFranco, 1995; Howe and Weiss, 1995). These sequences contain tyrosine residues which are phosphorylated by members of the Src family of protein tyrosine kinases. Phosphorylation of these residues provides binding sites for another family of cytoplasmic tyrosine

kinases, Zap70 and Syk. Binding of these kinases and their subsequent phosphorylation by the Src family kinases are thought to lead to the activation of Zap70 and Syk (DeFranco, 1995; Howe and Weiss, 1995). Phosphorylated ITAM sequences are also recognized by the SH2 domain of Src family kinases (Clark *et al.*, 1994; Pleiman *et al.*, 1994). Binding to these phosphorylated sites alters the conformation of the Src family kinases leading to the exposure of binding sites on the Src family kinases to which other signaling components can bind, such as PI3 kinase, GAP and PLCγ (Clark *et al.*, 1994; Pleiman *et al.*, 1993).

Ligand binding to the TCR and BCR result in a variety of outcomes, ranging from a simple proliferative response to cellular differentiation and activation of effector functions like lymphokine production. At least three main signaling pathways seem to be involved in triggereing these downstream responses; PlCγ1, PI3 Kinase and the Ras pathway (DeFranco, 1995; Zenner *et al.*, 1995). The precise mechanisms by which activation of Src family kinases leads to the activation of these pathways is still not entirely clear. As in other receptor signaling systems, activation is thought to occur through a variety of mechanisms including conformational changes induced by SH2 or SH3 binding as seen with PI3 Kinase, direct activation through tyrosine phosphorylation as observed with PLCγ1 and formation of signaling complexes at the cell membrane that facilitate activation of signaling molecules such as Ras (Zenner *et al.*, 1995). The questions of precisely how activation of Src family members is achieved following ligand binding and how the subsequent induction of kinase activity leads to the triggering of

downstream signaling pathways are still not understood. These questions need further experimental consideration before a complete picture of the role of Src family kinases in BCR and TCR signaling can be constructed.

Src's Role in Mitogenesis, Mitosis and Adhesion

Proliferating cells undergo an orderly progression through the cell cycle from G1 to S (DNA synthesis) to G2 and on to mitosis which is then followed by cell division and re-entry into G1. The products of proto-oncogenes have been shown to impinge upon this cycle, often providing signals that allow cells to pass from 1 phase into the next. Src and two other family members Fyn and Yes, have recently been shown to act in two places in the cell cycle in fibroblasts: during mitosis and in the induction of DNA synthesis after growth factor stimulation (Taylor and Shalloway, 1996).

In quiescent fibroblasts, the addition of PDGF (platelet derived growth factor) or EGF (epidermal growth factor) causes these cells to re-enter the cell cycle and undergo DNA synthesis. The effects of both PDGF and EGF are mediated by transmembrane receptor tyrosine kinases. Ligand binding to these receptors causes dimerization of the receptors and subsequent transphosphorylation of tyrosines in the cytoplasmic portion of each receptor (Cantley *et al.*, 1991). These phosphorylated tyrosines provide binding sites for SH2 containing signaling molecules including Src, Fyn and Yes (Mori *et al.*, 1993). Micro-injection experiments with a neutralizing Ab that recognizes these three Src family members demonstrated that the

activity of these Src family kinases is necessary for the cells to enter S phase after EGF or PDGF stimulation (Roche *et al.*, 1995b; Twamley *et al.*, 1993). This requirement for Src family kinases in the stimulation of DNA synthesis, however, is not an integral step in the cell cycle. Rather, it seems that these kinases initiate a pathway that impinges upon the basic cell cycle machinery since this neutralizing antibody does not block the stimulation of DNA synthesis in response to other growth factors like bombesin and lysophosphatidic acid (Roche *et al.*, 1995b). The downstream effects of Src activation and the cell cycle component through which the stimulation of DNA synthesis is achieved are unknown at this time.

The same approach, micro-injection of neutralizing antibodies against Fyn, Yes and Src was used to show that these kinases are essential for progression into mitosis. Both neutralizing antibody and the SH2 domain of Fyn, which presumably acts in a dominant negative fashion blocked the micro injected fibroblasts at the G2/M transition, prior to nuclear envelope breakdown.

Src, Fyn and Yes all become activated during mitosis (Roche *et al.*, 1995a). For Src, at least, its thought that this is achieved through phosphorylation of serine and threonine residues in its unique amino terminal domain which are carried out by cdc2 (Morgan *et al.*, 1989; Shenoy *et al.*, 1992; Shenoy *et al.*, 1989). These phosphorylations destabilization the closed conformation of the enzyme and promote full activation of the kinase through dephosphorylation of tyrosine 527 (Bagrodia *et al.*, 1991; Shenoy *et al.*, 1992). The critical mitotic targets of Src, Fyn and Yes are entirely unknown. Clearly, to complete the picture of Src's role in mitosis and in growth factor

stimulated mitogenesis, greater knowledge of the targets of these enzymes needs to be achieved.

As discussed in Chapter 3, Src also plays a role in cellular adhesion. The details of Src's function in focal adhesions are still being refined. It is clear, however, that Src is involved in regulating the dynamic process of assembly and dis-assembly of focal adhesions. This idea is supported by the observations that the absence of Src in fibroblasts leads to less stable focal contacts while the presence of Src251 gives rise to increased cellular adhesion (Kaplan *et al.*, 1994; Kaplan *et al.*, 1995). At least some of this regulation is achieved through control of FAK kinase activity. In seemingly contradictory sets of observations, Src is able to both promote FAK kinase activity as wells as inhibit it (Bibbins, 1995; Calalb *et al.*, 1995; Schaller *et al.*, 1994). Src binding through its SH2 domain to the autophosphorylation site on FAK inhibits FAK function while Phosphorylation of FAK by Src stimulates FAK's kinase activity (Bibbins, 1995; Calalb *et al.*, 1995; Schaller *et al.*, 1994). Results from FAK deficient mice indicate that FAK kinase activity promotes the disassembly of focal adhesions (Ilic *et al.*, 1995; Ilic *et al.*, 1996). Therefore Src's inhibition of FAK would inhibit disassembly while phosphorylation of FAK would stimulate dissolution of the structures. Src may also play a structural role in the assembly of focal adhesions through recruitment and cross linking of a variety of focal adhesion components through SH2 SH3 and unique domain mediated interactions.

Other questions that remain concerning Src's role in focal adhesions include how Src's activity in these structures relate to its

role in more classical signaling cascades. For instance, evidence exists to indicate that a signal may be generated from focal adhesions. In particular, binding of integrins to the ECM causes the activation of FAK and Src and the recruitment of components of the Ras pathway to focal adhesions (Schlaepfer *et al.*, 1994). Is this signal on its own sufficient to promote mitogenesis? This is probably not the case. One model to explain the activation of the Ras pathway upon integrin binding is that adhesion plays the role of a co-receptor for other signaling systems, such as PDGF stimulation. Just as in B cells and T cells where CD4 and CD19 contribute additional Src family activity to the primary signaling occurring through the antigen receptors, so might signaling initiated at focal adhesions enhance the impact of growth factor stimulation. This is a particularly pleasing hypothesis when you consider that normal fibroblasts require adhesion to the substratum to undergo DNA synthesis in response to growth factors. A co-receptor function for focal adhesion signaling would provide a mechanism by which these events could be coordinated.

Hck and Signal Transduction Cascades

In the previous paragraphs we have seen examples of how other src family members are involved in regulating a number of different signaling systems. What evidence exists implicating Hck in similar signaling cascades?

As discussed in Chapter 3, recent results from Lowell et al, point to a role for Hck in cellular adhesion in neutrophils (Lowell *et*

al., 1996). The molecular mechanisms of Hck's role in adhesion is yet to be established. Initial studies should investigate whether Hck plays a role in neutrophil analogous to Src's role in adhesion in fibroblasts. Experiments conducted thus far, rule out the involvement of FAK in Hck's regulation of adhesion: FAK is not expressed in neutrphils (Lowell *et al*., 1996). The possibility exists, however, that a FAK like kinase does exist in neutrophils which is sufficiently different from FAK that it can not be regulated by Src itself and would therefore require the activity of another Src family member such as Hck. The downstream effects of Hck function in neutrophil adhesion may also differ considerably from the proposed role of adhesie signaling in fibroblasts. Neutophils are post mitotic cells and therefore are no longer undergoing DNA synthesis. Instead of coordinating cell adhesion with mitogenesis, Hck signaling in adhesive neutrophils may help to coordinate activity such as the release of oxidative radicals. Since these compounds can be quite damaging to surrounding tissue, it is important that release only occurs when the cells are tightly adhered to their target and then releasae should only occur into the pocket formed by this tight adhesion. The identification of Hck's binding partners during neutrophil adhesion and following the activity of Hck during adhesion dependent processes in neutrophils are reasonable experimental approaches to further pursuing Hck's roles in adhesion.

Limited evidence exists implicating Hck in signaling through multi-subunit]unit immune recognition complexes similar to the role of other Src family kinases in signaling through the BCR and TCR. While the initiation of signaling through BCR and TCR has been well

defined, less work has been directed at signaling through the Fc receptor subunit. Since the Fc receptors contain ITAM sequences like those of the BCR and TCR, the involvement of a Src family kinase inn the initiation of signaling is suspected. Both Hck and Lyn have been implicated in the regulation of these receptors (Ghazizadeh *et al.*, 1994; Wang *et al.*, 1994; Zhou *et al.*, 1995). In two studies, Hck and Lyn kinase activity was seen to be stimulated about two fold in response to Fc cross linking (Ghazizadeh *et al.*, 1994; Wang *et al.*, 1994). Both kinases were also seen to associate with a larger receptor complex, although no direct binding has been seen to any specific subunits (Ghazizadeh *et al.*, 1994; Wang *et al.*, 1994; Zhou *et al.*, 1995). Our laboratory was unable to replicate these results and as such we are unsure how to interpret these studies. It may be that the activation is only evident in certain cell types or under particular in vitro conditions that we were unable to duplicate.

Hck deficient mice do not show any defects in Fc signaling systems while Lyn mice do display impaired functioning through their FcRI receptors (Dunn, 1995; Lowell *et al.*, 1994). Since the two kinases may be able to functionally substitute for one another, definitive evidence for the role of these kinases in Fc signaling awaits production of Lyn/Hck doubly deficient mice.

While no evidence exists supporting a role for Hck in signaling systems analogous to Src's role in PDGF signaling, Hck has been implicated in signal transduction through the LPS receptor (Beaty *et al.*, 1994; English *et al.*, 1993; Stefanova *et al.*, 1993). In a number of studies, stimulation of macrophages with LPS or cross linking of the LPS receptors led to a modest increase in Hck kinase activity.

Unlike the PDGF or the TCR or BCR, the LPS receptor is attached to the surface of the cell through a GPI linkage. This means that it exists wholly on the cell surface with no apparent means of contacting the intracellular kinase, Hck. A transmembrane co-receptor that would link the two molecules through protein/protein interactions has been proposed, but non has yet been identified. The GPI anchor of LPS receptor does direct this molecule to a specific lipid compartment on the cell surface known as cavveoli (Lisanti *et al.*, 1995; Mayor *et al.*, 1994; Sargiacomo *et al.*, 1993). As discussed in Chapter 3, palmitoylation of Hck also directs this kinase to the caveolea fraction of the cell membrane. So, even without the existence of a direct link between the LPS receptor and Hck, the two proteins co-localize in the cell membrane as if poised to act together. Much more work needs to be done to elucidate Hck's role in LPS signaling and its role in caveolea. In situ crosslinking studies in isolated caveolea is one method that could be pursued to identify the protein partners of the Hck kinase in this subcellular fraction. Efforts t characterize the activity f Hck in caveolea and examine its phosphorylation state after various treatments may also yield clues to Hck's function.

Appendix A

MATERIALS AND METHODS

Yeast and Bacteria-Methods and Strains

Bacteria and yeast were manipulated using standard protocols (Guthrie and Fink, 1991; Sambrook *et al.*, 1989).

Yeast transformations were performed using a modified protocol. Cells were harvested at a density of 2 x 10^7 cells/ml after an overnight incubation at 30°C. The cells were washed and resuspended in LiAc/TE [100 mM LiAc in 100 mM Tris(hydroxymethyl)aminomethane (Tris) pH 7.5, 10 mM disodium ethylenediamine tetra acetate (EDTA)] 0.01-1 ug of plasmid DNA and 200 ug denatured Salmon Sperm DNA (SS DNA) were added to the LiAc treated cells. PEG mix [40% polyethylene glycol (PEG) 6000 in 100 mM LiAc/TE] was added at a volume of 10:1 to the cell suspension. This mixture was incubated at room temperature for 15 minutes. The cells were heat shocked for 15 minutes at 42°C, spun down and resuspended in TE before plating on appropriate media (Gietz *et al.*, 1992).

The following yeast strains were used: **EGY.048** (*Mata, ura 3, trp1, his3,* LEU2::*pLexAop6*-Leu2) (Gyuris *et al.*, 1993); **Y153** (*Mata, leu2-3, -112, ura3-52, trp1-901, his3-Δ200, ade2-101, gal4Δgal80Δ,* URA3::GAL-lacZ, LYS2::GAL-His3) (Durfee *et al.*, 1993); **Y190** (*Mata, leu2-3, -112, ura3-52, trp1-901, his3Δ200, ade2-101, gal4Δgal80Δ,* URA3::GAL-lacZ, LYS2::GAL-His3, cyh^r) (Harper *et al.*, 1993); **Y187** (*Matα, gal4Δgal80Δ, his3-Δ200, trp1-901, ade2-101, ura3-52, leu2-3,-112,* URA3::GAL-lacZ met$^-$, LYS2::GAL-His3) (Harper *et al.*, 1993).

Electrocompetent *E. coli* were prepared by washing a mid-log phase culture several times in cold water and once in cold 20% glycerol. The washed cells were aliquoted in 20% glycerol and either used immediately or frozen at $^{-}80^{o}C$ for future use. Electroporations were carried out using a Biorad Gene-Pulser and micro-cuvettes according to the standard protocol of Biorad. In brief, 1 pg-100 ng DNA, suspended in a small volume of water (o.5- 2.0μl), was added to 20 μl of electrocompetant *E. coli* and pulsed in the Gene-Pulser at 2,500 Volts, 25 μfaraday and 200 ohm. The cells were allowed to recover in Luria Broth [LB; 1% Bacto-Tryptone (Difco), 0.5% Bacto Yeast Extract (Difco), 1% NaCl] at 37°C for 45 minutes before plating on Leu^- minimal media bacterial plates.

The following bacterial strains were used: **DH5α** (F^-, φ80d*lac*ZDM15 Δ(*lac*ZYA-*arg*F)U169*deo*R *rec*A1 *end*A1 *hsd*R17(r_K^- ,m_X^+)*sup*E44λ^- *thi*-1 *gyr*A96 *rel*A1) (GIBCO BRL 8265-017); **BL21(DE3)plysS** [F^-*ompT* $hsdS_B$ ($r_B^-m_B$) *gal* *dcm* Δ (*srl-recA*)*306*::Tn*10* (DE3)pLysS (Novagen)]; **BNN132** is JM07 [*end*A1, *gyr*96, *thi*, *hsd*R17, *supE*44, *rel*A1, Δ(*lac-pro*AB), (F', *tra*D36, *pro*AB^+, *lacI*^{q} *lac*ZΔM15)] with λKC, a Kanomycin resistant lambda lysogen containing the *cre* gene (Faulkner *et al.*, 1992) and **MH6** (D(*lac*Z) x74, *galU*, *galK*, *str*A, hsr^-, hsm^+ *leu*3600, pyrF::TN5.)

DNA, plasmids and constructs

DNA was manipulated by standard methods (Sambrook *et al.*, 1989). DNA sequencing was performed on double stranded DNA (dsDNA) templates using the Sequenase system (United States Biochemical) with the addition of Terminal Deoxy Transferase (GIBCO

BRL) for 30 minutes prior to the addition of Stop buffer. DNA fragments were purified from low melt agarose (Pharmacia) using GENECLEAN (Bio101), GeneCAPSULE (Geno Technology, Inc.) or Wizard preps (Promega). Polymerase Chain reactions (PCR) were carried out with PFU DNA Polymerase (Stratagene), 50 μM deoxyribonucleoside triphosphates (dNTPs; Promega), 1 ng template DNA, 1 μM of each primer and 5% dimethyl sulfoxide (DMSO.) The plasmids and constructs used are described in Tables A-1 and A-2 respectively.

Cell Culture

The hemopoietic cell lines used (K562, ML-1, Hl-60, Raji, U937, Daudi, Im-9, and Molt-4) were obtained from the UCSF cell culture facility. These cells were propagated in RPMI 1640 with N-2-hydroxyethylpiperaine-N'-2-ethanesulfonic acid (Hepes) containing penicillin (100U/ml) and streptomycin (100ug/ml.) The media was supplemented with 10% Fetal Bovine Serum (FBS), 12 mM sodium pyruvate and 50 μM Beta-mercaptoethanol (BME). The Hela cells used were obtained from L. Deiss and the 3T3 fibroblasts containing 6his-hck were derived by S. Robbins (unpublished.) These cell lines were propagated in Dulbecco's Modified Eagle's minimal medium (DME) H21, containing penicillin (100U/ml), streptomycin (100ug/ml) and supplemented with 10% FBS. The Shep and Sy5y cells used were obtained from A. Foster-Barber. The cells were propagated in RPMI-1640 with Hepes containing penicillin (100U/ml) and streptomycin (100ug/ml.) The media was supplemented with 10% FBS and 12mM sodium pyruvate.

TABLE A-1

Parent Vectors for the Two-Hybrid System

Vector	Selectable Marker	Promoter	Origin of Replication	Fusion	Reference
pAS1	Amp, Trp1	ADH	2μ	Gal4(1-147)HA	(Durfee, *et al.*, 1993)
pSE1107	Amp, Leu2	ADH	2μ	Gal4(768-881)	(Durfee, *et al.*, 1993)
Lex202pl	Amp, His3	ADH	2μ	LexA(1-202)	(Ruden, *et al.*, 1991)
pJG4-5	Amp, Trp1	Gal1	2μ	SV40NLS; B42TA; HA	(Zervos, *et al.*, 1993)
JK103	Amp, Ura3	LexA, Gal1	2μ	LacZ	(Kamens, *et al.*, 1990)
pMA41	Amp,Trp1	Gal1	2μ	LexA(1-87)Gal4(74-881)	(Brent, *et al.*, 1985)

Table A-1: Parent Vectors for the Two-Hybrid System.

pAS1 and pSE1107 were obtained from Steve Elledge. pAS1 contains the Gal4 DNA binding domain and pSE1107 contains the GAL4 transcriptional activation domain. Lex202pl, PJG4-5 and JK103 were obtained from Roger Brent. Lex202pl contains the LexA DNA binding domain, pJG4-5 contains an acidic domain from E. coli (B42) that acts as a transcriptional activator in eukaryotic cells and JK103 contains lacZ driven from a LexA inducable promoter. pMA41 was obtained from Yokum Li and contains a LexA DNA Binding Domain- Gal4 transcriptional activation domain fusion.

Table A-II: Plasmid Constructs for Expression in Yeast and Bacteria.

The CD4Small construct contains the complete cytoplasmic domain of murine CD4 (amino acids 394- amino acids 431.) CD4Small was constructed using PCR (as described) with pSMCD4 (D. Littman) as a template and the oligonucleotides indicated. The CD4Large construct contains the complete cytoplasmic and transmembrane domains and one half of the extracellular domain of murine CD4 (amino acids 250-amino acids 431.) CD4Large was constructed using PCR with pSMCD4 as a template and the oligonucleotides indicated. The CD4Cys- construct is the same as CD4Small except that alanine was substituted for cys^{418} and cys^{420}. CD4Cys- was constructed by PCR using CD4-MCA1/2 (D. Littman) as a template and the oligonucleotides indicated. The $CD4T_2$ construct is a truncation of the cytoplasmic domain of murine CD4 at amino acid 408. $CD4T_2$ was constructed by PCR using pSMCD4 as a template and the oligonucleotides indicated. The Lck construct contains the first one half of the unique domain (31 amino acids) of murine Lck. Lck was constructed by PCR using pSMlck (D. Littman) as a template and the oligonucleotides indicated. The LckCys- construct is the same as the Lck construct except that alanine was substituted for Cys^{20} and Cys^{23} lckCys- was made by PCR using LC3/4 (D. Littman) as a template and the oligonucleotides indicated. The HckAUNQ construct contains the entire unique domain of human Hck beginning at the ATG codon. The HckCUNQ constructs also contain the entire unique domain of human Hck but begin at the upstream CTG. The HckASH and HckCSH constructs contain the entire unique, SH3 and SH2 domains of human

Hck and begin at the ATG or upstream CTG respectively. All of the Hck containing constructs were made using PCR with pSP72-hck (S. Robbins) as a template and the oligonucleotides indicated. The library clones pACT 7CU22389, pACT 16CU2315, and pACT 7CU2381, were subcloned into pGEX-5X-2 using PCR with the oligonucleotides indicated.

Oligo #1 (CD45'S) GGGCTCGAGAGGTGCCGGCACCAACA; Oligo#2 (CD43') GGGCTCGAGGATGAGATTATGGCTCT; Oligo#3 (CD45'L) GGG CTCGAGCAGCTGAAGGAAACGCT; Oligo#4 (CD43'T_2) GGGCTCGAGCATCT GAGACATCGTG; Oligo#5 (Lck5') CCGTTCGGCTGTGTCTGCAGCTCA; Oligo#6 (Lck3') CCGAATTCCAGTGGGACTATGGGATA; Oligo#7 (HckA5') CCGAATTCGGGTGCATGAGTCCAA; Oligo#8 (HckUNQ3') GGGATTCCA GGGCACCCGATGA; Oligo#9 (HckC5') CCGTTCGGGGGGCGCTCAAGCTG; Oligo#10 (HckSH3') CCGATTCGGCTTCTGGGGCTTGG; Oligo#11 (pAS5') TTCGGATCCTCGAGCCACGAAGGGCC; Oligo#12 (pAS3') GGGGAATCTTC ATAGATCTCTGAG.

TABLE A-2

Plasmid Constructs for Expression in Yeast and Bacteria

Construct	Vector Backbone	Cloning Site	Template DNA	5' Oligo	3' Oligo
CD4small	pJG4-5	XhoI	pSMCD4 (Turner, *et al.*, 1990)	#1 (CD45'S)	#2 (CD43')
CD4large	pJG4-5	XhoI	pSMCD4	#3 (CD45'L)	#2
CD4cys-	pJG4-5	XhoI	CD4-MCA-1/2 (Turner, *et al.*, 1990)	#1	#2
$CD4T_2$	pJG4-5	XhoI	pSMCD4	#1	#4
Lck	Lex202pl	EcoRI	pSMlck (Turner, *et al.*, 1990)	#5 (Lck5')	#6 (Lck3')
Lckcys-	Lex202pl	EcoRI	LC3/4 (Turner, *et al.*, 1990)	#5	#6
HckAUNQ	Lex202pl	EcoRI	pSP72-hck (Robbins, *et al.*, 1995)	#7 (HckA5')	#8(HckUNQ3')
HckCUNQ	Lex202pl	EcoRI	pSP72-hck	#9 (HckC5')	#8
HckCSH	Lex202pl	EcoRI	pSP72-hck	#9	#10(HckSH3')
HckASH	Lex202pl	EcoRI	pSP72-hck	#7	#10
HckCUNQ	pAS1	EcoRI	pSP72-hck	#9	#8
HckCSH	pAS1	EcoRI	pSP72-hck	#9	#10
HckASH	pAS1	EcoRI	pSP72-hck	#7	#10
pGEX789	pGEX-5X-2†	BamHI EcoRI	pACT-7CU2389	#11(pAS15')	#12 (pAS13')
pGEX1615	pGEX-5X-2	BamHI EcoRI	pACT-16CU2315	#11	#12
pGEX781	pGEX-5X-2	BamHI EcoRI	pACT-7CU2381	#11	#12

† Pharmacia

Antibodies

The rabbit polyclonal antiserum to Hck that was used for Western blots and immunoprecipitations was made against a HCK-GST fusion protein by U. Lichtenberg as described (Robbins *et al.*, 1995). The crude sera recognized both Hck and Glutathione S-Transferase fusion proteins on Western Blots. The rabbit polyclonal antiserum used to recognize the transcriptional activation domain of Gal4 was kindly provided by I. Sedowski. The rabbit polyclonal antiserum used to recognize the LexA DNA binding domain was kindly provided by R. Brent.

Yeast Growth Assays

A 2 ml liquid culture of EGY.048 transformed with the appropriate construct was grown in raffinose minimal media supplemented with the appropriate amino acids at 30°C for 2-4 hours. An OD_{600} (Optical Density, λ=600) reading was taken and all of the cultures within a single experiment were normalized to a standard OD (OD_{600}=0.7 to 1) by the addition of media. Ten fold dilutions were made with PBS into a replica plating tray (DanKar Scientific) and replicas were made onto $Trp^-His^-Leu^-$ galactose plates. Growth was assessed after 3 days at 30°C.

Two-Hybrid Library

The phage library was obtained from S. Elledge. "The mRNA was prepared from EBV transformed human peripherial lymphocytes" (Durfee *et al.*, 1993). The sized cDNA [>600 base pairs (bp)] was inserted into a phagemid vector (pACT) and packaged into

bacteriophage (Durfee *et al.*, 1993). Plasmid DNA was prepared from the phagemid vector by *cre* mediated recombination in bacterial strain BNN132. BNN132 was grown overnight at 37°C in LB, 50 mM kanamycin, 0.2% maltose and 10 mM $MgSO_4$. In the morning, the culture was diluted ten fold and grown an additional 1.5 hours. The cells were harvested, washed and resuspended in 0.01 mM $MgSO_4$ to an OD_{600}=2.0. 1 x 10^6 plaque forming units of phage were added to100 µl of the cell suspension and allowed to absorb for 30 minutes at 30°C. The cells were allowed to recover at 37°C for 1 hour, with aeration, and then plated on LB plates with 50 mM Carbanacillin and 0.2% glucose. The colonies obtained after 24 hours were scraped into TE and DNA was prepared by the standard alkaline lysis method (Sambrook *et al.*, 1989).

Library Screen and β–gal Assay

Yeast strain Y153 was transformed with the bait constructs HckCUNQ, HckASH or HckCSH (Table-A2). These cells were then transformed with the plasmid library as follows: Trp^- media was inoculated with Y153 containing one of the bait plasmids and grown overnight at 30°C. After 12 hours, the culture was diluted 1:10 in the same media and grown an additional 4-5 hours until the culture reached mid-log phase (OD_{600}=0.5-0.8). The cells were harvested and washed one time in TE (1/100 volume of the starting culture) and one time in LiSorb (100 mM LiAOc, 10 mM Tris-HCl pH 8.0, 1mM EDTA, 1 M sorbitol; 1/100 volume of the starting culture). These twice washed cells were resuspended in LiSorb (1/500 volume of the

starting culture.) To these cells was added 25 ug library DNA prepared as described above, 5 ug of sonicated SS DNA, and 3.5 ml PEG mix (40% PEG 6000 in LiSorb) . This suspension was incubated at 42°C for 15 minutes. 5 ul of this sample was plated on $Trp^{-}Leu^{-}$ selective media to test the transformation efficiency. The remainder of the sample was inoculated into 100 ml of $Trp^{-}His^{-}Leu^{-}$ media and allowed to recover, with aeration, at 30°C for 1-3 hours. This culture was spun down and resuspended in 3 ml $Trp^{-}His^{-}Leu^{-}$ media and equally divided among 10 15cm^3 plates of $Trp^{-}His^{-}Leu^{-}$ 50 mM 3AminoTriazole (3AT; Sigma). Each of the bait constructs underwent transformation with the plasmid library on two separate occasions.

After 5 days at room temperature, the plates were scored for initial His^{+} clones. The positive clones were either lifted directly from the plates onto nylon membranes and assayed for β-galactosidase activity (β-gal) or first patched onto selective media then transferred and assayed for β-gal activity. β-galactosidase filter assays were performed as follows: Colonies were either replica plated onto filter paper (Whatmann 50) or lifted directly from plates onto nylon filters (Hybond-N, Amersham). The cells were permeabilized by submerging the filters in liquid nitrogen for 10 seconds. The filters containing the permeablized cells were placed on a second filter (Whatmann 3) which had been saturated with Z buffer (40 mM $NaHPO_4$, 60 mM Na_2HPO_4, 1 mM $MgSO_4$, 10 mM KCl and 200 μM BME, added fresh) containing 0.03% 5-Bromo-4-Chloro-3-indolyl-β-D-galactoside (X-gal) in Dimethylformamide (DMF) and allowed to develop at 30°C for 30 minutes. Colonies that gave a blue color

reaction were scored positive and re-tested for both growth on His$^-$ selective media and β -gal activity.

Plasmids from clones that re-tested positive were rescued using a standard protocol (Guthrie and Fink, 1991). Cells were sheared using glass beads in a solution of 100 mM NaCl, 10 mM Tris pH 8.0, 1mM EDTA and 0.1%SDS. The resulting supernatent was phenol/chloroform extracted. The DNA was recovered and washed on glassmilk (Bio101), followed by precipitation with ethanol.

The recovered DNA was electroporated into MH6 bacterial cells. These cells were plated on LEU$^-$ minimal media to select for the library plasmids. The plasmids recovered in this way were subsequently transformed into the yeast strain Y190 containing the original bait construct (Table A2,) or into WT Y190. Library clones that still displayed activity with the original bait plasmid were mated to Y187 carrying a number of irrelevant transcriptional activation domain fusion proteins (lamin, p53, cdk2, SNF1) as controls for Hck dependent activity (Harper *et al.*, 1993).

RNA Isolation and Northern Blot

RNA was isolated from 2 x 10^8 cells in culture using the Fastrack mRNA Isolation Kit (Invitrogen, Version 3.5). 1 ug of RNA was precipitated and taken up in MOPS buffer [20 mM 3-[N-morpholino]-propanesulfonic acid (MOPS), 5 mM NaOAc, 0.5 mM EDTA] with 5% formaldehyde, 40% formamide, 0.25% bromophenol blue and 0.25% xylene cyanol. The samples were loaded onto a 1% agarose gel submerged in a MOPS running buffer with 0.22M formaldehyde and run at 80 volts for several hours (Tsang *et al.*,

1993). After the dye front had advanced 12-14 cm, the gel was removed and rinsed in diethyl pyrocarbonate (DEPC) treated water. The samples were transferred to a nylon membrane using a descending, alkaline transfer method (Chomczynski, 1992). In brief, a stack of paper towels were put down followed by a stack of dry 3MM filter paper, followed by a sheet of Hybond-N that had been soaked in transfer buffer (3 M NaCl, 8 mM NaOH, 2 mM N-laurel Sarcosine, pH=11.4-11.45.) This was followed by the rinsed gel, followed by 3MM filter paper that was soaked in transfer buffer, followed by a light plastic cover all of which was then completely covered in Saran Wrap. The transfer was allowed to proceed for 1 hour at room temperature. Following transfer, the membrane was neutralized in 200 mM Sodium Phosphate buffer (pH=6.8) and the RNA was cross-linked to the membrane by UV irradiation for 90 seconds in a StrataLinker (Stratagene).

The blot was pre-hybridized in Rapid-Hybe buffer (Amersham) for 15 minutes at 65°C. The blot was hybridized, using 2 ug of a heat denatured, radiolabelled DNA probe, for 1 hour at 65°C in Rapid-Hybe buffer. The DNA probes were prepared using the Random Primed DNA labeling Kit (Boehringer Mannheim Biochemica) followed by ethanol precipitation. The hybridized blots were washed once at room temperature for 20 minutes in 2X SSC buffer (1X SSC buffer; 150 mM NaCl, 15 mM in Sodium Citrate) and 0.1% SDS. This was followed with two 15 minute washes in 0.5X SSC buffer.

The Northern blot containing RNA from human tissue was purchased from Clonetech [Human Multiple Tissue Northern (MTN) Blot II]. DNA probes were prepared as above and the hybridization

was carried out as specified by Clonetech. The blot was prehybridized in hybridization buffer (5X SSPE (20X SSPE; 3M NaCl, 0.2M $NaH_2PO_4 \cdot H_2O$, 0.02M Na_2EDTA, pH=7,) 10X Denhardt's (50X Denhardt's; 5% Ficoll, 5% Polyvinylpyrrolidone, 5% BSA,) 100 µg SS DNA, 2% SDS) for 1 hour at 65°C. The probe was boiled for 5 minutes and added to fresh hybridyzation buffer at 65°C. The prehybridized blot was added to this mix and incubated overnight at 65°C with gentle agitation. The blots were washed three times for 15 minutes at room temperature in 2X SSC, 0.05% SDS and two times 20 minutes in 0.1X SSC, 0.1% SDS at 50°C.

Western Blots

Samples were separated using sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) (Suzuki *et al.*, 1992). The protein was transferred to nitrocellulose (Schleicher & Schuell), using a semi-dry transfer apparatus (E&K scientific) at $3mA/cm^2$ for 30 minutes. The filters were blocked for 30 minutes in Tris Buffered Saline with Tween (TBST; 10 mM Tris-HCl pH 8.0, 200 mM NaCl, 5 mM KCl, 0.5% Tween-20) with 5% heat inactivated calf serum (CS) and 2% non-fat dry milk. The primary antibody was incubated with the blot in TBST, 5% CS and 2% milk for 1.5 hours and then washed three times for fifteen minutes each in TBST with 5% CS, 2% milk and 0.5% Nonidet-40 (NP-40). This was followed by the addition of the secondary Ab (donkey anti-rabbit HRP, Amersham) in TBST with 5% CS and 2% milk for 30 minutes. Finally, the blot was washed three more times in the NP-40 containing buffer and developed with enhanced chemiluminescence substrate (Amersham).

Preparation of Yeast Cell Lysates

Yeast cell lysates were prepared by the method of Dr. Matthias Peters (Herskowitz lab) as follows: A saturated culture of yeast was harvested by centrifugation. The cells were resuspended in cold TCA buffer [20 mM Tris pH 8.0, 50 mM ammonium acetate, 2 mM EDTA, 1 mM Pefabloc (Boehringer Mannheim,) 10 ng/ml aprotinin, 10 ng/ml leupeptin.] Cold TCA (trichloroacetic acid) was added to a final concentration of 10%. TCA washed glass beads were added to just below the meniscus of the solution and the entire mix was vortexed twice on high for 30 seconds each. The resulting supernatant was collected and the protein pelleted by centrifugation in a microfuge for 10 minutes at 4°C. The pellets were resuspended in laemmli loading buffer (3.5% SDS, 15% glycerol, 40 mM Tris pH 8.0, 8 mM EDTA, 120 mM Dithiothreitol (DTT), 0.1% bromophenol blue) and loaded onto a polyacrylamide gel.

Preparation of Hck Containing Extracts

Cell extracts were made from U937 cells that had been differentiated with TPA as described (Robbins *et al.*, 1995). 2 x 10^7 differentiated cells were washed twice in cold PBS and then harvested with a rubber policeman into cold PBS. The cells were incubated for 20 minutes on ice in swelling buffer (10 mM KCl, 10 mM Hepes pH 7.5, 1 mM DTT, 1 mM EDTA 1 mM Pefabloc, 10 ng/ml aprotinin, 10 ng/ml leupeptin) and lysed by 50 strokes in a dounce homogonizer. Nuclei and unlysed cells were removed from the lysate by a low speed centrifugation (10 minutes at 1000 X g). The

supernatant was made 150 mM in NaCl and 1% in NP-40. The remaining insoluble material was cleared from the lysate by a 50,000 X g centrifugation for 1 hour at 4°C. The cleared lysate containing soluble p59Hck and p61Hck was loaded onto the glutathione-agarose fusion protein columns as described below.

Cell extracts were made from NIH 3T3 cells expressing the Hck mutant p59G2A (S. Robbins, unpublished). 1 x 10^8 cells were washed twice in cold PBS and then harvested with a rubber policeman into cold PBS. The cells were incubated for 20 minutes on ice in swelling buffer (10 mM KCl, 10 mM HEPES pH 7.5, 1mM DTT, 1mM EDTA 1mM Pefabloc, 10 ng/ml aprotinin, 10 ng/ml leupeptin) and lysed by 20 strokes in a dounce homogenizer. Nuclei and unlysed cells were removed from the lysate by a low speed centrifugation (10 minutes at 1000 X g). The remaining insoluble material was cleared from the lysate by a 50,000 X g centrifugation for 1 hour at 4°C. The resulting supernatant was dialyzed overnight in PBS. This sample was loaded onto the glutathione agarose columns that had been pre-loaded with the GST-Library fusion proteins as described below.

Preparation of Glutathione-Agarose Columns Containing GST Fusion Proteins.

BL21(DE3)plysS were transformed with the constructs pGEX789, pGEX1615 and pGEX781 (Table A-2). The resulting transformants were screened for expression of GST-fusion proteins as described by Pharmacia (Pharmacia Biotech GST Gene Fusion System). In brief, transformants containing the appropriate insert

were grown for several hours at 37°C to mid-log phase. The cultures were induced with 1mM Isopropylthio-β-D-galactoside (IPTG) and grown for an additional 2 hours at 25°C. The cells were harvested by centrifugation, resuspended in PBS and lysed by sonication (Branson Sonifier Cell Disruptor 200). The resulting lysates were cleared by centrifugation. The GST-fusion proteins were precipitated from the extracts with glutathione agarose and released by the addition of SDS loading buffer. Samples were loaded onto 10% polyacrylamide gels. Following electrophoresis, the gels were stained with bromophenol blue to detect the presence of protein.

Transformants demonstrating expression of the appropriate fusion proteins were used to produce fusion proteins for use on columns. Overnight cultures of pGEX transformed Bl21(DE3)plysS were diluted 1:10 in the morning and allowed to grow for another 1.5 hours at 37°C. The cultures were induced with 1 mM IPTG and grown for an additional 4 hours at 25°C. The cultures were harvested by centrifugation and lysed in PBS by sonication (2 x 30 seconds at 90 cycles/second using a micro tip adapter.) The lysates were cleared by centrifugation for 10 minutes at 15,000 x g. The resulting supernatants were passed through a 19 gauge needle several times to shear any DNA and put through a 2 μ filter to remove any remaining particulate matter. The cleared lysates were loaded onto 1.5 ml glutathione agarose columns (Sigma) prepared in econo columns (Biorad) using gravity flow in the cold. The loaded columns were washed twice with 10 ml of PBS, twice with 10 ml of 500mM KCl in PBS and again three times with 10 ml PBS. Hck containing extracts were then loaded onto the columns and incubated

on the columns for 30 minutes at 4°C to allow for binding. For extracts containing wild type Hck protein, the columns were washed three times 10 ml in Hck Binding Buffer (150 mM NaCl, 10 mM Hepes pH 7.5, 0.5% NP-40, 1 mM DTT, 1 mM EDTA and 1 mM Pefabloc, 10 ng/ml aprotinin, 10 ng/ml leupeptin). For lysates made from p59G2A Hck containing cells, PBS was substituted for Hck Binding Buffer in the last wash steps. The columns were eluted with 2X 1 ml 10 mM glutathione in Hck Binding Buffer or PBS, as appropriate.

Kinase reactions

Hck autophosphorylation reactions were performed following immunoprecipitation with the anti-hck antibody described above. The eluates from the GST columns above were subjected to immunoprecipitation by the following procedure: 2 µl of Hck antisera was added to 500 µl of elute. To this was added protein A and the samples were allowed to incubate for 1 hour at 4°C with rocking. The protein A and bound protein were washed three times in Kinase Assay Buffer (20 mM Tris-HCl pH 7.2, 10 mM $MgCl_2$, 10 mM $MnCl_2$ and 0.1% NP-40) with 500 mM NaCl and one time in Kinase Assay Buffer alone. Kinase Assay Buffer containing 16.7 µCi γATP was added to each of the washed immunoprecipitations and incubated at room temperature for 10 minutes. Following incubation, the reactions were washed three times in Wash Buffer (100 mM NaCl, 10 mM Tris pH 7.2, 1 mM EDTA, 1% NP-40, 0.3% SDS) and solubilized away from the protein A with SDS loading buffer. The samples were run out on a polyacrlamide gel. The gel was then dried under vacuum and exposed to film.

Hck Kinase reactions were also performed with GST-fusion proteins as potential substrates. The GST-fusion proteins were purified as described above except that the final wash steps were performed with Kinase Assay Buffer instead of PBS. Partially purified p59Hck was added to these glutathione bound fusion proteins and a standard kinase assay was performed, as outlined above. The first wash step following the kinase reaction was analyzed for Hck activity.

Partial Purification of p59Hck

1.5 x 10^8 NIH 3T3 cells expressing 6his-p59Hck (Robbins, 1995; Winzerling *et al.*, 1992) were washed twice in PBS and lysed in NP-40 Lysis Buffer (150 mM NaCl, 1.0% NP-40, 50 mM Tris pH 8.0.) Cell debris was removed by centrifugation at 12,000 Xg for 10 minutes. Ni+ agarose (Qiagen) was added to the lysates and incubated for 10 minutes at 4^oC. The lysates were washed twice in NP-40 Lysis Buffer, followed with a single wash step in NP-40 Lysis Buffer containing 500 mM NaCl. p59Hck was eluted from the Ni+ agarose resin with 20 mM Tris pH 7.2, 0.1% NP-40 and 100 mM EDTA. The resulting supernatants were dialyzed for 12 hours against Kinase Assay Buffer, with three changes of buffer.

Appendix B

REFERENCES

Alexandropoulos, K., Cheng, G., and Baltimore, D. (1995): Proline-rich sequences that bind to Src homology 3 domains with individual specificities. *Proc Natl Acad Sci U S A* **92**, 3110-4.

Alland, L., Peseckis, S. M., Atherton, R. E., Berthiaume, L., and Resh, M. D. (1994): Dual myristylation and palmitylation of Src family member p59fyn affects subcellular localization. *J Biol Chem* **269**, 16701-5.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990): Basic local alignment search tool. *J Mol Biol* **215**, 403-10.

Amberg, D. C., Basart, E., and Botstein, D. (1995): Defining protein interactions with yeast actin in vivo. *Nat Struct Biol* **2**, 28-35.

Bagrodia, S., Chackalaparampil, I., Kmiecik, T., and Shalloway, D. (1991): Altered tyrosine 527 phosphorylation and mitotic activation of p60c-src. *Nature* **349**, 172.

Ballmer-Hofer, K., Ziegler, A., and Burger, M. M. (1988): Association of Polyoma Virus Middle T antigen and pp60src with cytoskeletal elements. *Oncogene* **3**, 365-71.

Bartel, P., Chien, C. T., Sternglanz, R., and Fields, S. (1993): Elimination of false positives that arise in using the two-hybrid system. *Biotechniques* **14**, 920-4.

Beaty, C. D., Franklin, T. L., Uehara, Y., and Wilson, C. B. (1994): Lipopolysaccharide-induced cytokine production in human monocytes: role of tyrosine phosphorylation in transmembrane signal transduction. *Eur J Immunol* **24**, 1278-84.

Bibbins, K. (1995): Regulation of pp60c-src and pp125 FAK by Src SH2 domain, University of California, San Francisco.

Bibbins, K., Boeuf, H., and Varmus, H. E. (1993): The binding of the Src SH2 domain to phosphopeptides is determined by residues in both the SH2 domain and the phosphopeptide. *Proc. Natl. Acad. Sci. USA* **88**, 10696-10700.

Birge, R. B., and Hanafusa, H. (1993): Closing in on SH2 specificity. *Science* **262**, 1522-24.

Bishop, J. M. (1983): Cellular Oncogenes and Retroviruses. *Ann. Rev. Biochem.* **52**, 301-354.

Bishop, J. M. (1985): Viral oncogenes. *Cell* **42**, 23-38.

Bishop, J. M. (1987): The molecular genetics of cancer. *Science* **235**, 305-311.

Bishop, J. M. (1996): The discovery of proto-oncogenes. *The FASEB Journal* **10**, 362-364.

Boldin, M. P., Varfolomeev, E. E., Pancer, Z., Mett, I. L., Camonis, J. H., and Wallach, D. (1995): A novel protein that interacts with the death domain of FAS/Apo1 contains a sequence motif related to the death domain. *The Journal of Biological Chemistry* **270**, 7795-7798.

Bolen, J. P., Thompson, P. A., Eiseman, E., and Horak, I. D. (1991): Expression and ineractions of the *Src* family of tyrosine protein kinases in T lymphocytes. *Adv. Canc. Res.* **57**, 103-149.

Boschelli, F., Uptain, S. M., and Lightbody, J. J. (1993): The lethality of p60v-src in *Saccharomyces cerevisiae* and the activatin of p34CDC28 kinase are dependent on the integrity of the SH2 domain. *J. Cell Sci.* **105**, 519-528.

Brent, R., and Ptashne, M. (1985): A Eukaryotic Transcriptional Activator Bearing the DNA Specificity of a Prokaryotic Repressor. *Cell* **43**, 729-736.

Brugge, J. S., and Erikson, R. L. (1977): Identification of a transformation specific antigen induced by an Avian Sarcoma Virus. *Nature* **269**, 346-347.

Brugge, J. S., Jarosik, G., Andersen, J., Queral-Lustig, A., Fedor-Chaiken, M., and Broach, J. R. (1987): Expression of Rous Sarcoma Virus transforming protein $pp60^{vsrc}$ in *Saccharomyces cerevisiae* cells. *Mol. Cell. Biol* **7**, 2180-2187.

Buser, C., Sigal, C., Resh, M., and McLaughlin, S. (1994): Membrane binding myristylated peptides corresponding to the NH2 terminus of Src. *Biochemistry* **33**, 13093.

Buser, C. A., Kim, J., McLaughlin, S., and Peitzsch, R. M. (1995): Does the binding of clusters of basic residues to acidic lipids induce domain formation in membranes? *Molecular Membrane Biology* **12**, 69-75.

Buss, J. E., Kamps, M. P., and Sefton, B. M. (1984): Myristic acid is attached to the transforming protein of Rous Sarcoma Virus during or immediately after synthesis and is present in both soluble and membrane-bound forms of the protein. *Mol. Cell. Biol.* **4**, 2697-2704.

Calalb, M. B., Polte, T. R., and Hanks, S. K. (1995): Tyrosine phosphorylation of focal adhesion kinase at sites in the catalytic domain regulates kinase activity: a role for Src family kinases. *Mol Cell Biol* **15**, 954-63.

Cambier, J., Pleiman, C., and Clark, M. (1994): Signal transduction by the B cell antigen receptor and its coreceptors. *Ann. Rev. Immunol.* **12**, 457.

Cambier, J. C., and Jensen, W. A. (1994): The hetero-oligomeric antigen receptor complex and its coupling to cytoplasmic effectors. *Curr Opin Genet Dev* **4**, 55-63.

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

Carrera, A. C., Paradis, H., Borlado, L. R., Roberts, T. M., and Martinez, C. (1995): Lck unique domain influences Lck specificity and biological function. *J Biol Chem* **270**, 3385-91.

Celenza, J. L., and Carlson, M. (1989): Mutational analysis of the Sacchaaromyces cereviia SNF1 protein kinase and evidence for functional interaction with the SNF4 protein. *Molecular and Cellular Biology* **9**, 5034.

Chan, A. C., Desai, D. M., and Weiss, A. (1994): The role of protein tyrosine kinases and protein tyrosine phosphatases in T cell antigen receptor signal transduction. *Annu Rev Immunol* **12**, 555-92.

Cheadle, C., Ivashchenko, Y., South, V., Searfoss, G. H., French, S., Howk, R., Ricca, G. A., and Jaye, M. (1994): Identification of a Src SH3 domain binding motif by screening a random phage display library. *J Biol Chem* **269**, 24034-9.

Cheng, G., Ye, Z. S., and Baltimore, D. (1994): Binding of Bruton's tyrosine kinase to Fyn, Lyn, or Hck through a Src homology 3 domain-mediated interaction. *Proc Natl Acad Sci U S A* **91**, 8152-5.

Cheng, H., Nishio, H., Hatase, O., Ralph, S., and Wang, J. H. (1992): A synthetic peptide derived from p34cdc2 Is a specific and efficient substrate of *src*-family tyrosine kinases. *J. Biol. Chem.* **267**, 9248-9256.

Chevray, P. M., and Nathans, D. (1992): Protein interaction cloning in yeast: identification of mammalian proteins that react with the leucine zipper of Jun. *Proc Natl Acad Sci U S A* **89**, 5789-93.

Chien, C. T., Bartel, P. L., Sternglanz, R., and Fields, S. (1991): The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. *Proc Natl Acad Sci U S A* **88**, 9578-82.

Chomczynski, P. (1992): One-Hour Downward Alkaline Capillary Transfer for Blotting of DNA and RNA. *Analytical Biochemistry* **201**, 134-139.

Chow, L. M., and Veillette, A. (1995): The Src and Csk families of tyrosine protein kinases in Hemopoietic cells. *Seminars in Immunology* **7**, 207-226.

Clark, E. A., and Brugge, J. S. (1993): Redistribution of activated pp60c-src to integrin-dependent cytoskeletal complexes in thrombin stimulated platelets. *Mol. Cell. Biol.* **13**, 1863-1871.

Clark, M. R., Johnson, S. A., and Cambier, J. C. (1994): Analysis of Ig-alpha-tyrosine kinase interaction reveals two levels of binding specificity and tyrosine phosphorylated Ig-alpha stimulation of Fyn activity. *Embo J* **13**, 1911-9.

Collett, M. S., Erikson, E., and Erikson, R. L. (1979): Structural analysis of the Avian Sarcoma Virus transforming protein: sites of phosphorylation. *J. Virol.* **29**, 770-781.

Collett, M. S., and Erikson, R. L. (1978): Protein kinase activity associated with the Avian Sarcoma Virus *src* gene product. *The Proceedings of the National Acadamy of Science, USA* **75**, 2021-2024.

Cooper, J. A. (1989): The src-family of protein-tyrosine kinases. In B. Kemp, and P. F. Alewood (Eds): *Peptides and Protein Phosphorylation*, CRC Press Inc.

Cooper, J. A., and Howell, B. (1993): The when and how of Src regulation. *Cell* **73**, 1051-1054.

Courtneidge, S. A., Levinson, A. D., and Bishop, J. M. (1980): The protein encoded by the transforming gene of avian sarcoma virus (pp60src) and a homologous protein in normal cells (pp60proto-src) are associated with the plasma membrane. *Proc Natl Acad Sci U S A* **77**, 3783-7.

Cross, F. R., Garber, E. A., Pellman, D., and Hanafusa, H. (1984): A short sequence in the p60src N terminus is required for p60src myristylation and membrane association and for cell transformation. *Mol. Cell. Biol.* **4**, 1834-1842.

da, S. A., Janssen, O., and Rudd, C. E. (1993): T cell receptor zeta/CD3-p59fyn(T)-associated p120/130 binds to the SH2 domain of p59fyn(T). *J Exp Med* **178**, 2107-13.

Dalton, S., and Treisman, R. (1992): Characterization of SAP-1, a protein recruited by serum response factor to the c-fos serum response element. *Cell* **68**, 597-612.

DeFranco, A. (1995): Transmembrane signaling by antigen receptors of B and T lymphocytes. *Current Biology* **7**, 163.

den Hartigh, J. C., van Bergen en Henegouwek, P. M. P., Verkleij, A. J., and Boonstra, J. (1992): The EGF Receptor is an actin-binding protein. *Journal of Cell Biology* **119**, 349-355.

Hibbs, M.L., Tarlinton D.M, Armes, J. Grail, D., Hodgson, G., Maglitto, R., Stacker, S.A., Dunn, A. (1995): Multiple defects in the immune system of Lyn-deficient mice, culminating in autoimmune disease. *Cell* **83**(2), 301.

Durfee, T., Becherer, K., Chen, P. L., Yeh, S. H., Yang, Y., Kilburn, A. E., Lee, W. H., and Elledge, S. J. (1993): The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. *Genes Dev* **7**, 555-69.

Durfee, T., Gorovits, B., Hensey, C., and Lee, W.-H. submitted..

Durfee, T., Mancini, M. A., Jones, D., Elledge, S. J., and Lee, W. H. (1994): The amino-terminal region of the retinoblastoma gene product binds a novel nuclear matrix protein that co-localizes to centers for RNA processing. *J Cell Biol* **127**, 609-22.

Eiseman, E., and Bolen, J. B. (1990): src-related tyrosine protein kinases as signaling components in hematopoietic cells [published erratum appears in Cancer Cells 1990 Dec;2(12):405]. *Cancer Cells* **2**, 303-10.

Emmelot, P., and Bentvelzen, P. (1972): RNA Viruses and Host Genomes in Oncogenesis, American Elsevier Publishing Co., New York.

English, B., Ihle, J., Myracle, A., and Taolin, Y. (1993): Hck tyrosine kinase activity modulates tumor necrosis factor production by murine macrophages. *J. Exp. Med.* **178**, 1017.

Faulkner, L., Patel, M., Brickell, P. M., and Katz, D. R. (1992): Regulation of c-fgr messenger RNA levels in U937 cells treated with different modulating agents. *Immunology* **76**, 65-71.

Fearon, E. R., Finkel, T., Gillison, M. L., Kennedy, S. P., Casella, J. F., Tomaselli, G. F., Morrow, J. S., and Van Dang, C. (1992): Karyoplasmic interaction selection strategy: a general strategy to detect protein-protein interactions in mammalian cells. *Proc Natl Acad Sci U S A* **89**, 7958-62.

Feder, D. (1993): Unpublished Observations.

Feder, D., and Bishop, J. M. (1991): Identification of platelet membrane proteins that interact with amino-terminal peptides of pp60c-src. *J Biol Chem* **266**, 19040-6.

Feder, D., and Bishop, J. M. (1996): Amino terminal peptides of pp60cSrc interact with actin and integrins. *Manuscript in preparation.*

Feilotter, H. E., Hannon, G. J., Rudell, C. J., and Beach, D. (1994): Constructin of an improved host strain for two-hybrid screening. *Nucleic Acids Res.* **22**, 1502-1503.

Fields, S., and Song, O. (1989): A novel genetic system to detect protein-protein interactions. *Nature* **340**, 245-246.

Fields, S., and Sternglanz, R. (1994): The two-hybrid system: an assay for protein-protein interactions. *Trends Genet* **10**, 286-92.

Florio, M., Wilson, L. K., Trager, J. B., Thorner, J., and Martin, G. S. (1994): Aberant protein phosphorylation at Tyrosine is responsible for the growth-inhibitory action of pp60v-*src* expressed in the yeast *Saccharomyces cerevisiae. Mol. Biol. Cell* **5**, 283-296.

Formosa, T., Barry, B., Alberts, B., and Greenblatt, J. (1991): Using Protein Affinity Chromatography to Probe the Structure of Protein Machines. *Methods Enzymol.* **208**, 24-45.

Fox, J. E., Lipfert, L., Clark, E. A., Reynolds, C. C., Austin, C. D., and Brugge, J. S. (1993): On the role of the platelet membrane skeleton in mediating signal transduction. Association of GP IIb-IIIa, pp60c-src, pp62c-yes, and the p21ras GTPase-activating protein with the membrane skeleton. *J Biol Chem* **268**, 25973-84.

Fumagalli, S., Totty, N. F., Hsuan, J. J., and Courtneidge, S. A. (1994): A target for Src in mitosis. *Nature* **368**, 871-874.

Garber, E. A., and Hanafusa, H. (1987): NH2-terminal sequences of two Src proteins that cause aberrant transformation. *Proc. Natl. Acad. Sci. USA* **84**, 80-85.

Gauen, L. K. T., Kong, A. T., Samelson, L. E., and Shaw, A. (1992): p59Fyn tyrosine kinase associates with multiple T-Cell receptor subunits through Its unique amino terminal domain. *Molecular and Cellular Biology* **12**, 5438-5446.

Gentry, L. E., Chaffin, K. E., Shoyab, M., and F., P. A. (1986): Novel serine phosphorylation of pp60c-src in intact cells after tumor promoter treatment. *Mol. Cell. Biol.* **6**.

Gervais FG and Veillette A (1995). The unique amino-terminal domain of p56lck regulates interactions with tyrosine protein phosphatases in T lymphocytes. *Mol Cell Biol* **15**(5): 2393.

Ghazizadeh, S., Bolen, J. B., and Fleit, H. B. (1994): Physical and functional association of Src-related protein tyrosine kinases with Fc gamma RII in monocytic THP-1 cells. *J Biol Chem* **269**, 8878-84.

Gietz, D., St. Jean, A., Woods, R. A., and Schiestl, R. H. (1992): Improved Method for High Efficiency Transformation of Intact Yeast Cells. *Nucleic Acids Research* **20**, 1425.

Goddard, C., Arnold, S., and Felsted, R. L. (1989): High affinity binding of an N-terminal myristoylated p60*src* peptide. *J. Biol. Chem.* **264**, 15173-15176.

Gordon, J. I., Duronio, R. J., Rudnick, D. A., Adams, S. P., and Glaxer, L. (1991): Protein N-myristoylation. *J. Biol. Chem* **266**, 8647-8650.

Gould, K. L., Woodgett, J. R., Cooper, J. A., Buss, J. E., D., S., and Hunter, T. (1985): Protein kinase C phosphorylates pp60c-src at a novel site. *Cell* **42**, 849-857.

Guarente, L. (1993): Strategies for the Identification of Interacting Proteins. *Proc. Natl. Acad. Sci. USA* **90**, 1639-1641.

Guthrie, C., and Fink, G. R. (1991): Guide to Yeast Genetics and Molecular Biology. In J. N. Abelson, and M. I. Simon (Eds): *Methods in Enzymology*, Harcourt Brace Jovanovich, New York.

Gyuris, J., Golemis, E., Chertkov, H., and Brent, R. (1993): Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2. *Cell* **75**, 791-803.

Haefner, B., Baxter, R., Fincham, V. J., Downes, C. P., and Frame, M. C. (1995): Cooperation of Src homology domains in the regulated binding of phosphatidylinositol 3-kinase. A role for the Src homology 2 domain. *J Biol Chem* **270**, 7937-43.

Hamaguchi, M., and Hanafusa, H. (1987): Association of p60Src with Triton X-100 resistant cellular structure correlates with morphological transformation. *Proc. Natl. Acad. Sci. USA* **84**, 2312-2316.

Hanks, S. K., Quinn, A. M., and Hunter, T. (1988): The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science* **241**, 42-51.

Hardy, C. F., Sussel, L., and Shore, D. (1992): A RAP1-interacting protein involved in transcriptional silencing and telomere length regulation. *Genes Dev* **6**, 801-14.

Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K., and Elledge, S. J. (1993): The p21 Cdk-interacting Protein Cip1 is a Potent Inhibitor of G1 Cyclin-Dependent Kinases. *Cell* **75**, 805-816.

Hatakeyama, M., Kono, T., Kobayashi, N., Kawahara, A., Levin, S. D., Perlmutter, R. M., and Taniguichi, T. (1991): Interaction of the IL-2 receptor with the src-family kinase p56lck: identifiction of novel intermolecular association. *Science* **252**, 1523-1528.

Henderson, D., and Rohrschneider, L. (1987): Cytoskeletal association of pp60Src. *Experimental Cell Research* **168**, 411-421.

Henikoff, S., Henikoff, J. G., Alford, W. J., and Pietrokovski, S. (1995): Automated construction and graphical presentation of protein blocks from unalighned sequences. *Gene* **163**, GC 17-26.

Hirai, H., and Varmus, H. (1990): Site-directed mutagenesis of the SH2- and SH3-coding domains of c-*src* produces varied phenotypes, including oncogenic activation of p60c-*src*. *Mol. Cell. Biol.* **10**, 1307-1318.

Hofer, F., Fields, S., Schneider, C., and Martin, G. S. (1994): Activated Ras interacts with the Ral guanine nucleotide dissociation stimulator. *Proc Natl Acad Sci U S A* **91**, 11089-93.

Hofer, U., Wehrle, B., and Ballmer, H. K. (1995): Membrane association of polyomavirus middle-T antigen in an in vitro system. *Virus Res* **35**, 169-80.

Holtzman, D. A., Cook, W. D., and Dunn, A. R. (1987): Isolation and sequence of a cDNA corresponding to a *src*-related gene expressed in murine Hemopoietic cells. *The Proceedings of the National Academy of Sciences, USA* **84**, 8325-8329.

Hope, I. A., and Struhl, K. (1986): Functional Dissection of a Eukaryotic Transcriptional Activator Protein, GCN4 of Yeast. *Cell* **46**, 885-894.

Howe, L. R., and Weiss, A. (1995): Multiple kinases mediate T-cell-receptor signaling. *Trends Biochem Sci* **20**, 59-64.

Ilic, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobure, K., Nakatsuji, N., Nmura, S., Fujimaooto, J., Okada, M., and Yamamoto, T. (1995): Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK deficient mice. *Nature* **377**, 539.

Ilic, D., Kanazaawa, S., Furuta, Y., Yamamoto, T., and Aizawa, S. (1996): Impairment of mobility in endodermal cells by FAK deficiency. *Experimental Cell Research* **222**, 298.

Imamoto, A., and Soriano, P. (1993): Disruption of the csk gene, encoding a negative regulator of Src family tyrosine kinases, leads to neural tube defects and embryonic lethality in mice. *Cell* **73**, 1117-24.

Jahner, D., and Hunter, T. (1991): The stimulation of quiescent rat fibroblasts by v-src and v-fps oncogenic protein-tyrosine kinses leads to the induction of a subset of immediate early genes. *Oncogene* **6**, 1259-1268.

Joung, I. (1995): Modification of Ser59 in the unique N-terminal region of tyroine kinase p59Lck regulates specificity of its Src homology 2 domain. *Proceedings of the National Academy of Sciences USA* **92**, 5778-82.

Kaech, S., Schnierle, B., Wyss, A., and Ballmer-Hofer, K. (1993): Myristylation and amino-terminal phosphorylation are required for activation of pp60c-src during mitosis. *Oncogene* **8**, 575-581.

Kamens J, Richardson P, Mosialos G and Brent R. (1990). Oncogenic Transformation by V-rel Requires an Amino-terminal Activation Domain. *Mol Cell Biol* **10**: 2840.

Kaplan, J. M. (1990): Genetic and Biochemical Analysis of Transformation by pp60Src , University of California, San Francisco.

Kaplan, J. M., Varmus, H. E., and Bishop, J. M. (1990): The src protein contains multiple domains for specific attachment to membranes. *Mol Cell Biol* **10**, 1000-9.

Kaplan, K., Swedlow, J. R., Varmus, H. E., and Morgan, D. O. (1992): Association of p60c-src with endosomal membranes in mammalian fibroblasts. *J. Cell Biol.* **118**, 321-333.

Kaplan, K. B., Bibbins, K. B., Swedlow, J. R., Arnoud, M., Morgan, D. O., and Varmus, H. E. (1994): Association of the amino terminal half of c-Src with focal adhesions alters their properties and is regulated by phosphorylation of tyrosine 527. *EMBO J.* **13**, 4745-4756.

Kaplan, K. B., Swedlow, J. R., Morgan, D. O., and Varmus, H. E. (1995): c-Src enhances the spreading of src-/- fibroblasts on fibronectin by a kinase independent mechanism. *Genes and Development* **9**, 1505-1517.

Karin, M., and Smeal, T. (1992): Control of transcription factors by signal transduction pathways: The beginning of the end. *TIBS* **17**, 418-422.

Keegan, L., Gill, G., and Ptashne, M. (1986): Separation of DNA Binding from the Transcription-Activating Function of a Eukaryotic Regulatory Protein. *Science* **231**, 699-703.

Kefalas, P., Brown, T. R. P., and Brickell, P. M. (1995): Signalling by the p60c-src family of protein-tyrosine kinases. *Int. J. Biochem. Cell Biol.* **27**, 551-563.

Kemp, B. E., and Pearson, R. B. (1990): Protein kinase recognition sequence motifs. *TIBS* **15**, 342-6.

Kishone, and Shah (1988): Amino acid biosynthesis inhibitors as herbicides. *Ann. Rev. Biochem.* **57**, 627.

Kmiecik, T. E., and Shalloway, D. (1987): Activation and suppression of pp60c-src transforming ability by mutation of its primary sites of tyrosine phosphorylation. *Cell* **49**, 65-73.

Koch, C. A., Anderson, D., Moran, M. F., Ellis, C., and Pawson, T. (1991): SH2 and SH3 domains: Elements that control interactions of cytoplasmic signaling proteins. *Science* **252**, 668-674.

Koegl, M., Zlatkine, P., Ley, S. C., Courtneidge, S. A., and Magee, A. I. (1994): Palmitoylation of multiple Src-family kinases at a homologous N-terminal motif. *Biochem J*, 749-53.

Kornbluth, S., Jove, R., and Hanafusa, H. (1987): Characterization of avian and viral p60^{src} proteins expressed in yeast. *Proceedings of the National Academy of Sciences, USA* **84**, 4455-4459.

Kriegler, M. (1990): *Gene Tansfer and Expression: A Laboratory Manual*. Stockton Press. New York.

Krueger, J. G., Garber, E. A., Goldberg, A. R., and Hanafusa, H. (1982): Changes in amino-terminal sequences of pp60src lead to decreased membrane association and decreased *in vivo* tumorigenicity. *Cell* **28**, 889-896.

Krueger, J. G., Wang, E., and Goldberg, A. R. (1980): Evidence that the src gene product of Rous sarcoma virus is membrane associated. *Virology* **101**, 25-40.

Kwong, J., and Lublin, D. M. (1995): Amino-terminal palmitate or polybasic domain can provide required second signal to myristate for membrane binding of p56lck. *Biochem Biophys Res Commun* **207**, 868-76.

Kypta, R. M., Goldberg, Y., Ulug, E. T., and Courtneidge, S. A. (1990): Association between the PDGF receptor and members of the src family of tyrosine kinases. *Cell* **62**, 481-92.

Lee, C. H., Leung, B., Zheng, J., Cowburn, D., Kuriyan, J., and Saksela, K. (1995): A single amino acid in the SH3 domain of Hck determines its high affinity and specificity in binding to Hiv-1 Nef protein. *Embo J.* **14**, 5006-5015.

Levin, S. D., Abraham, K. M., Anderson, S. J., Forbush, K. A., and Perlmutter, R. M. (1993): The protein tyrosine kinase p56lck regulates thymocyte development independently of its interaction with CD4 and CD8 coreceptors [corrected] [published erratum appears in J Exp Med 1993 Sep 1;178(3):1135]. *J Exp Med* **178**, 245-55.

Levinson, A. D., Courtneidge, S. A., and Bishop, J. M. (1981): Structural and functional domains of the Rous sarcoma virus transforming protein (pp60src). *Proc Natl Acad Sci U S A* **78**, 1624-8.

Levinson, A. D., Oppermann, H., Levintow, L., Varmus, H. E., and Bishop, J. M. (1978): Evidence that the transforming gene of Avian Sarcoma Virus encodes a protein kinase associated with a phosphoprotein. *Cell* **15**, 561-572.

Li, B., and Fields, S. (1993): Identification of mutations in p53 that affect its binding to SV40 large T antigen by using the yeast two-hybrid system. *Faseb J* **7**, 957-63.

Liebl, E. C., England, L., DeClue, J. E., and Martin, G. S. (1992): Host range mutants of *v-src*: alterations in kinase activity and substrate interactions. *J. Virol.* **66**, 4315-4324.

Lindberg, R. A., Quinn, A. M., and Hunter, T. (1992): Dual specificity protein kinases: Will any hydroxyl do? *Trends In Biochemistry* **3**, 114-119.

Lisanti, M. P., Tang, Z., Scherer, P. E., Kubler, E., Koleske, A. J., and Sargiacomo, M. (1995): Caveolae, transmembrane signalling and cellular transformation. *Mol Membr Biol* **12**, 121-4.

Liu, X., Brodeur, S. R., Gish, G., Zhou, S., Cantley, L. C., Laudano, A. P., and Pawson, T. (1993): Regulation of the c-Src tyrosine kinase activity by the Src SH2 domain. *Oncogene* **8**, 1119-1126.

Lock, P., Ralph, S., Stanley, E., Boulet, I., Ramsay, R., and Dunn, A. R. (1991): Two isoforms of murine *Hck* generated by utilization of alternative translational initiation codons, exhibit different patterns of subcellular localization. *Molecular and Cellular Biology* **11**, 4363-4370.

Lowell, C. A., Fumagalli, L., and Berton, G. (1996): Deficiency of Src-family Kinases p59/61Hck and p58c-Fgr Results in Defective Adhesion-Dependent Neutrophil Functions. *submitted.*

Lowell, C. A., Soriano, P., and Varmus, H. E. (1994): Functional overlap in the src gene family: inactivation of hck and fgr impairs natural immunity. *Genes Dev* **8**, 387-98.

Luban, J., Alin, K. B., Bossolt, K. L., Humaran, T., and Goff, S. P. (1992): Genetic assay for multimerization of retroviral gag polyproteins. *J Virol* **66**, 5157-60.

Luban, J., Bossolt, K. L., Franke, E. K., Kalpana, G. V., and Goff, S. P. (1993): Human Immunodeficiency Virus Type 1 Gag protein binds to Cyclophilins A and B. *Cell* **73**, 1067-1078.

Luo, K. X., and Sefton, B. M. (1990): Cross-linking of T-cell surface molecules CD4 and CD8 stimulates phosphorylation of the lck tyrosine protein kinase at the autophosphorylation site. *Mol Cell Biol* **10**, 5305-13.

Ma, J., and Ptashne, M. (1987): A new class of yeast transcriptional activators. *Cell* **51**, 113-119.

Malek, S. N., and Desiderio, S. (1993): SH2 domains of the protein-tyrosine kinases Blk, Lyn, and Fyn(T) bind distinct sets of phosphoproteins from B lymphocytes. *J Biol Chem* **268**, 22557-65.

Maness, P. F., and Levy, B. (1983): Highly purified pp60src induces the actin transformation in microinjected cells and phosphorylates selected cytoskeletal proteins *in vitro*. *Mol. Cell. Biol.* **3**, 102-112.

Margolis, B. (1992): Proteins with SH2 domains: Transducers in the tyrosine kinase signaling pathway. *Cell Growth and Differentiation* **3**, 73-80.

Mayor, S., Rothberg, K. G., and Maxfield, F. R. (1994): Sequestration of GPI-anchored proteins in caveolae triggered by cross-linking. *Science* **264**, 1948-51.

McWhirter, J. R., and Wang, J. Y. (1993): An actin-binding function contributes to transformation by the Bcr-Abl oncoprotein of Philadelphia chromosome-positive human leukemias. *Embo J* **12**, 1533-46.

Miyajima, A., Kitaamura, N., Harada, T., Yokota, K., and Arai, K. (1992): Cytokine Receptors and Signal Transduction. *Annu. Rev. Immunol.* **10**, 295-331.

Molina, T. J., Kishihara, K., Siderovski, D. P., van Ewijk, W., Narendran, A., Timms, E., Wakeham, A., Paige, C. J., Hartmann, K. U., Veillette, A., Davidson, D., and Mak, T. W. (1992): Profound block in thymocyte devolopment in mice lacking $p56^{lck}$. *Nature* **357**, 161-164.

Morgan, D., Kaplan, J., Bishop, J., and Varmus, H. (1989): Mitosis specific phosphorylation of pp60c-ssrc by p34cdc2 assoociated protein kinase. *Cell* **57**, 775.

Mori, S., Ronnstrand, L., Yokote, K., Engstrom, A., Courtneidge, S., Claesson-Welsh, L., and Heldin, C. (1993): *EMBO J* **12**, 2257.

Muller, A. J., Pendergast, A. M., Havlik, M. H., Puil, L., Pawson, T., and Witte, O. (1992): A limited set of SH2 domains binds BCR thhrough a high-affinity phosphotyrosine-independent interaction. *Mol. Cell. Biol.* **12**, 5087-5093.

Murphy, S., Bergman, S. M., and Morgan, D. O. (1993): Suppression of c-Src activity by C-terminal Src kinase involves the c-Src SH2 and SH3 domains: analysis wih *Sacharomyces cerivisiae. Mol. Cell. Biol.* **13**, 5290-5300.

Mustelin, T., and Burn, P. (1993): Regulation of Src family tyrosine kinases in lymphocytes. *TIBS* **18**, 215-220.

Narayanaswami, V., Kim, J., and McNamie, M. (1993): Protein Lipid interactions and Torpedo Californica nicotinic acetylcholine receptor function. *Biochemistry* **32**, 12413-12419.

Ng, D. H., Watts, J. D., Aebersold, R., and Johnson, P. (1996): Demonstration of a direct interaction between p56Lck and the cytoplasmic domain of CD45 *in vitro*. *Journal of Biological Chemistry* **271**, 1295-1300.

O'Brien, M. C., Fukui, Y., and Hanafusa, H. (1990): Activation of the proto-oncogene p60c-*src* by point mutations in the SH2 domain. *Mol. Cell. Biol.* **10**, 2855-2862.

Okada, M., Howell, B., Broome, M. A., and Cooper, J. A. (1993): Deletion of the SH3 domain of Src interferes with regultion by the phosphorylated carboxy terminal tyrosine. *J. Biol. Chem.* **268**, 18070-18075.

Paige, L. A., Nadler, M. J. S., Harrison, M. L., Caassady, J. M., and Geahlen, R. L. (1993): Reversible palmitoylationof the protein tyrosine kinase p56Lck. *J. Biol. Chem.* **268**.

Panchamoorthy, G., Fukazawa, T., Stolz, L., Payne, G., Reedquist, K., Shoelson, S., Zhou, S., Cantley, L., Walsh, C., and Band, H. (1994): Physical and functional interactions between SH2 and SH3 domains of the Src family protein tyrosine kinase p59fyn. *Mol Cell Biol* **14**, 6372-85.

Parsons, S. J., and Creutz, C. E. (1986): p60c-src activity detected in the chromaffin granule membrane. *Biochem Biophys Res Commun* **134**, 736-42.

Pawson, T. (1991): Signal transduction in the control of cell growth and development. *TIGS* **7**, 343-345.

Phizicky, E. M., and Fields, S. (1995): Protein-Protein Interaction: Methods for Detection and Analysis. *Microbiological Reviews* **59**, 94-123.

Pleiman, C. M., Abrams, C., Gauen, L. T., Bedzyk, W., Jongstra, J., Shaw, A., and Cambier, J. C. (1994): Distinct p53/p56Lyn and p59Fyn Domains Associate with Nonphosphorylated and Phosphorylated Ig-α. *Proc. Natl. Acad. Sci. USA* **91**, 4268-4272.

Pleiman, C. M., Clark, M. R., Gauen, L. K., Winitz, S., Coggeshall, K. M., Johnson, G. L., Shaw, A. S., and Cambier, J. C. (1993): Mapping of sites on the Src family protein tyrosine kinases p55blk, p59fyn, and p56lyn which interact with the effector molecules phospholipase C-gamma 2, microtubule-associated protein kinase, GTPase-activating protein, and phosphatidylinositol 3-kinase. *Mol Cell Biol* **13**, 5877-87.

Potts, W. M., Reynolds, A. B., Lansing, T. J., and Parsons, J. T. (1988): Activation of pp60c-src transforming potential by mutations altering the structure of an amino terminal domain containing residues 90-95. *Oncogene Research* **3**, 343-355.

Prekeris, R., Mayhew, M. W., Cooper, J. B., and Terrian, D. M. (1996): Identification and localization of an actin-binding motif that is unique to the epsilon isoform of protein kinase C and participates in the regulation of synaptic function. *The Journal of Cell Biology* **132**, 77-90.

Purchio, A. F., Erikson, F., Brugge, J., and Erikson, R. L. (1978): Identification of a polypeptide encoded by the Avian Sarcoma Virus Gene. *Proc. Natl. Acad. Sci., USA* **75**, 1567-1571.

Purchio, A. F., Shoyab, M., and Gentry, L. E. (1985): Site specific increased phosphorylation of pp60c-src after treatment of RSV-transformed cells with a tumor promoter. *Science* **229**, 1393-1395.

Quintrell, N., Lebo, R., Varmus, H., Bishop, J., Pettenati, M., LeBeau, M., Diaz, M., and Rowley, J. (1987): Identification of a human gene (HCK) that encodes a protein-tyrosine kinase and is expressed in hemopoietic cells. *Molec. Cell. Biol.* **7**, 2267-2275.

Ramos-Morales, F., Doute, M., and Fischer, S. (1994): P56lck: a transducing protein that binds to SH2 containing proteins and to phosphotyrosine containing proteins. *Cell Mol Biol (Noisy-le-grand)* **40**, 695-700.

Ren, R., Mayer, B., Cicchetti, P., and Baltimore, D. (1993): Identification of a ten-amino acid proline-rich SH3 binding site. *Science* **259**, 1157-1161.

Rendu, F., Lebret, M., Danielian, S., Fagard, R., Levy-Toledano, S., and Fischer, S. (1989): High pp60c-src level in human platelet dense bodies. *Blood* **73**, 1545-51.

Resh, M. (1994): Myristylation and palmitylation of src family members: The fats of the matter. *Cell* **76**, 411-413.

Resh, M. D. (1988): Reconstitution of the Rous Sarcoma Virus transforming protein pp60v-src into phospholipid vesicles. *Mol. Cell. Biol.*, 1896-1905.

Resh, M. D. (1989): Specific and saturable binding of pp60v-*src* to plasma membranes: evidence for a myristyl-*src* receptor. *Cell* **58**, 281-286.

Resh, M. D., and Erikson, R. L. (1985): Highly specific antibody to Rous sarcoma virus src gene product recognizes a novel population of pp60v-src and pp60c-src molecules. *J Cell Biol* **100**, 409-17.

Resh, M. D., and Ling, H. (1990): Identification of 32K plasma membrane protein that binds to the myristylted amino-terminal sequence of p60v-*src*. *Nature* **346**, 84-86.

Robbins, S. (1995): Unpublished observations.

Robbins, S., Quintrell, N., and Bishop, J. M. (1995): Myristoylation and differential palmitoylation of the HCK protein-tyrosine kinases govern their attachment to membranes and association wih caveolae. *Molecular and Cellular Biology* **15**, 3507-3515.

Roche, S., Fumagalli, S., and Curtneidge, S. (1995a): Requirement for Src family tyrosine kinases in G2 ffor fibroblast cell division. *Science* **269**, 1567.

Roche, S., Koegl, M., Barone, M. V., Roussel, M. F., and Courtneidge, S. A. (1995b): DNA synthesis induced by some but not all growth factors requires Src family protein tyrosine kinases. *Mol Cell Biol* **15**, 1102-9.

Rodgers, W., and Glaser, M. (1991): Characterization of lipid domains in Erythrocyte membranes. *Proc. Natl. Acad. Sci. USA* **88**, 1364-1368.

Rodgers, W., and Glaser, M. (1993): Distributions of proteins and lipids in the Erythrocyte membrane. *Biochemistry* **32**, 112591-12598.

Roth, C. W., Richert, N. D., Pastan, I., and Gottesman, M. M. (1983): Cyclic AMP treatment of Rous Sarcoma Virus-transformed Chinese Hamster Ovary cells increases phosphorylation of pp60c-src and increases pp60c-src kinase. *J. Biol. Chem* **258**, 10768-10773.

Roussel, R. R., Brodeur, S. R., Shalloway, D., and Laudano, A. P. (1991): Selective binding of activated pp60c-src by an immobilized synthetic phosphopeptide modeled on the carboxyl terminus of pp60c-src. *Proc Natl Acad Sci U S A* **88**, 10696-700.

Rudd, C. E., Janssen, O., Prasad, K. V. S., Raab, M., da Silva, A., Telfer, J. C., and Yamamoto, M. (1993): src-related protein tyrosine kinases and their surface receptors. *Biochimica et Biophysica Acta* **1155**, 239-266.

Rudd, C. E., Trevellyan, J. M., Dasgupta, J. D., Wong, L. L., and Schlossman, S. F. (1988): The CD4 receptor is complexed in detergent lysates to a protein-tyrosine kinase pp58 from hman T lymphocytes. *Proc. Natl. Acad. Sci. USA* **85**, 5190-5194.

Ruden, D. M., Ma, J., Li, Y., Wood, K., and Ptashne, M. (1991): Generating Yeast Transcriptional Activators Containing No Yeast Protein Sequences. *Nature* **350**, 250-252.

Sadowski, I., Stone, J. C., and Pawson, T. (1986): A noncatalytic domain conserved among cytoplasmic protein-tyrosine kinases modifies the kinase function and transforming activity of Fujinami Sarcoma Virus p130*gag-fps*. *Mol. Cell. Biol.* **6**, 4396-4408.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989): *Molecular Cloning: A Laboratory Manual.* CSH Press. New York.

Samelson, L. E., Phillips, A. F., Luong, E. T., and Klausner, R. D. (1990): Association of the Fyn protein-tyrosine kinase with the T-cell antigen receptor. *Proc Natl Acad Sci U S A* **87**, 4358-62.

Saouaf, S. J., Kut, S. A., Fargnoli, J., Rowley, R. B., Bolen, J. B., and Mahajan, S. (1995): Reconstitution of the B cell antigen receptor signaling components in COS cells. *The Journal of Biological Chemistry* **270**, 27072-27078.

Sargiacomo, M., Sudol, M., Tang, Z., and Lisanti, M. P. (1993): Signal transducing molecules and glycosyl-phosphatidylinositol-linked proteins form a caveolin-rich insoluble complex in MDCK cells. *J Cell Biol* **122**, 789-807.

Sato, T., Irie, S., Kitada, S., and Reed, J. C. (1995): FAP-1: a protein tyrosine phosphatase that associates with Fas. *Science* **268**, 411-5.

Schaller, M. D., Hildebrand, J. D., Shannon, J. D., Fox, J. W., Vines, R. R., and Parsons, J. T. (1994): Autophosphorylation of the focal Adhesion Kinase, pp125FAK Directs SH2 Dependent Binding of pp60Src. *Mol. Cell. Biol.* **14**, 1680-1688.

Schlaepfer, D. D., Hanks, S. K., Hunter, T., and van der Geer, P. (1994): Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. *Nature* **372**, 786-791.

Seidel-Dugan, C., Meyer, B. E., Thomas, S. M., and Brugge, J. S. (1992): Effects of SH2 and SH3 deletions on the functional activities of wild-type and transforming variants of c-*src*. *Mol. Cell. Biol.* **12**, 1835-1845.

Shaw, A. S., Amrein, K. E., Hammond, C., Stern, D. F., Sefton, B. M., and Rose, J. K. (1989): The *lck* tyrosine protein kinase interacts with the cytoplasmic tail of the CD4 glycoprotein through Its unique amino-terminal domain. *Cell* **59**, 627-636.

Shaw, A. S., Chalupny, J., Whitney, A., Hammond, C., Amrein, K. E., Kavathas, P., Sefton, B. M., and Rose, J. K. (1990): Short related sequences in the cytoplasmic domains of CD4 and CD8 mediate binding to the amino-terminal domain of the $p56^{lck}$ tyrosine protein kinase. *Molecular and Cellular Biology* **10**, 1853-1862.

Shenoy, S., Chackalaparampil, I., Bagrodia, S., Lin, P.-H., and Shalloway, D. (1992): Role of p34cdc2 mediated phosphorylations in two-step activation of pp60c-src during mitosis..

Shenoy, S., Choi, J., Bagroia, S., Copeland, T., Maller, J., and Shalloway, D. (1989): Purified maturatin promoting factor phosphorylates pp60c-src at the sites phosphorylated during fibroblast mitosis. *Cell* **57**, 763.

Shenoy-Scaria, A. M., Dietzen, D. J., Kwong, J., Link, D. C., and Lublin, D. M. (1994): Cysteine3 of Src family protein tyrosine kinase determines palmitoylation and localization in caveolae. *J Cell Biol* **126**, 353-63.

Sigal, C., Zhou, W., Buser, C., McLaughlin, S., and Resh, M. (1994): Amino-terminal basic residues of Src mediate membrane binding through electrostatic interaction with acidic phospholipids. *Proc. Natl. Acad. Sci. USA* **91**, 12253.

Sigal, C. T., and Resh, M. D. (1993): The ADP/ATP carrier Is the 32-kilodalton receptor for the NH2-terminally myristylated Src peptide but not for pp60src polypeptide. *Mol. Cell. Biol.* **13**, 3084-3092.

Silverman, L., and Resh, M. (1992): Lysine residues form an integral component of a novel NH2-terminal membrane targeting motif for myristylated pp60v-src. *The Journal of Cell Biology* **119**, 415

.

Silverman, L., Sudol, M., and Resh, M. D. (1993): Members of the src family of nonreceptor tyrosine kinases share a common mechanism for membrane binding. *Cell Growth Differ* **4**, 475-82.

Simpson, S. C., Bolen, J. B., and Veillette, A. (1989): CD4 and p56lck can stably associate when co-expressed in NIH3T3 cells. *Oncogene* **4**, 1141-43.

Songyang, Z., Carraway, K. L., Eck, M. J., Harrison, S. C., Feldman, R. A., Mohommadl, M., Schlessinger, J., Hubbard, S. R., Smith, D. P., Eng, C., Lorenzo, M. J., Ponder, B. A. J., Mayer, B. J., and Cantley, L. C. (1995): Catalytic Specificity of protein tyrosine kinases is critical for selective signalling. *Nature* **373**, 536-538.

Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J., and et, a. l. (1993): SH2 domains recognize specific phosphopeptide sequences. *Cell* **72**, 767-78.

Soriano, P., Montgomery, C., Geske, R., and Bradley, A. (1991): Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. *Cell* **64**, 693-702.

Sparks, A. B., Quilliam, L. A., Thorn, J. M., Der, C. J., and Kay, B. K. (1994): Identification and characterization of Src SH3 ligands from phage-displayed random peptide libraries. *The Journal of Biological Chemistry* **269**, 23853-23856.

Spector, D., Varmus, H. E., and Bishop, J. M. (1978): *Proc. Nat. Acad. Sci. USA* **75**, 4102-4106.

Stanley, E., Ralph, S., McEwen, S., Boulet, I., Holtzman, D. A., Lock, P., and Dunn, A. R. (1991): Alternatively spliced murine lyn mRNAs encode distinct proteins. *Mol Cell Biol* **11**, 3399-406.

Stefanova, I., Corcoran, M. L., Horak, E. M., Wahl, L. M., Bolen, J. B., and Horak, I. D. (1993): Lipopolysaccharide induces activation of CD14-associated protein tyrosine kinase p53/56lyn. *J Biol Chem* **268**, 20725-8.

Stehelin, D., Varmus, H. E., and Bishop, J. M. (1976): DNA related to the transformng gene(s) of Avian Sarcoma Viruses is present in normal avian DNA. *Nature* **260**, 170-173.

Stephenson, J. R. (1980): Molecular Biology of RNA Tumor Viruses, Academic Press, New York.

Stover, D. R., Liebetanz, J., and Lydon, N. B. (1994): Cdc2 mediated modulation of pp60c-src activity. *Journal of Biological Chemistry* **269**, 26885-26889.

Straus, D. B., and Weiss, A. (1992): Genetic evidence for the involvement of the lck tyrosine kinase in signal transduction through the T cell antigen receptor. *Cell* **70**, 585-593.

Sudol, M., Greulich, H., Newman, L., Sarkar, A., Sukegawa, J., and Yamamoto, T. (1993): A novel Yes-related kinase, Yrk, is expressed at elevated levels in neural and hematopoietic tissues. *Oncogene* **8**, 823-31.

Superti, F. G., and Courtneidge, S. A. (1995): Structure-function relationships in Src family and related protein tyrosine kinases. *Bioessays* **17**, 321-30.

Superti-Furga, G., Fumagalli, S., Koegl, M., Courtneidge, S. A., and Draetta, G. (1993): Csk inibition of Src activity requires both the SH2 and SH3 domains of Src. *EMBO J.* **12**, 2625-2634.

Suzuki, K., Watanabe, M., and Miyoshi, J. (1992): Differences in effects of oncogenes on resistance of gamma rays, ultraviolet light, and heat shock. *Radiat Res* **129**, 157-62.

Swierczynski, S. L., and Blackshear, P. J. (1995): Membrane associtionof the myristoylated alanine-rich C kinase substrate (MARCKS) protein. *J. Biol. Chem.* **270**, 13436-13445.

Swope, S. L., and Huganir, R. L. (1994): Binding of the nicotinic acetylcholine receptor to SH2 domains of Fyn and Fyk protein tyrosine kinases. *J Biol Chem* **269**, 29817-24.

Tamura, T., Friis, R. R., and Bauer, H. (1984): pp60c-src is a substrate for phosphorylation when cells are stimulated to enter cell cycle. *FEBS Lett.* **177**, 152-156.

Taniguchi, T. (1995): Cytokine signaling through nonreceptor protein tyrosine kinases. *Science* **268**, 251-255.

Taylor, S. J., Anafi, M., Pawson, T., and Shalloway, D. (1995): Functional interaction between c-Src and its mitotic target, Sam 68. *J Biol Chem* **270**, 10120-4.

Taylor, S. J., and Shalloway, D. (1996): Src and the control of cell division. *BioEssays* **18**, 9-11.

Treisman, R. (1992): The serum response element. *TIBS* **17**, 423-426.

Tsang, S. S., Yin, X., Guzzo-Arkuran, C., and Davison, A. J. (1993): Loss of Resolution in Gel Electrophoresis of RNA: A Problem Associated with the Presence of Formaldehyde Gradients. *BioTechniques* **14**, 380-381.

Tsen, S. D., Lee, R., Damiani, B., Hsieh, P., and Chen, L. B. (1980): F-Actin inhibits protein kinase activity associated with the src-gene product. *Cold Sring Harbor Symp. on Quant. Biol.* **XLIV**, 967-974.

Tsygankov, A., and Bolen, J. (1993): The Src family of tyrosine protein kinases in Hemopoietic signal transduction. *Stem Cells* **11**, 371-380.

Turner, J., Brodsky, M., Irving, B., Leven, S., Perlmutter, R., and Littman, D. (1990): Interaction of the unique N-terminal region of tyrosine kinase p56lck with cytoplasmic domains of CD4 and CD8 is mediated by cysteine motifs. *Cell* **60**, 755-765.

Twamley, G. M., Kypta, R. M., Hall, B., and Courtneidge, S. A. (1992): Association of Fyn with the activated platelet-derived growth factor receptor: requirements for binding and phosphorylation. *Oncogene* **7**, 1893-901.

Twamley, S. G., Pepperkok, R., Ansorge, W., and Courtneidge, S. A. (1993): The Src family tyrosine kinases are required for platelet-derived growth factor-mediated signal transduction in NIH 3T3 cells. *Proc Natl Acad Sci U S A* **90**, 7696-700.

Van Aelst, L., Barr, M., Marcus, S., Polverino, A., and Wigler, M. (1993): Complex Formation Between RAS and RAF and other Protein Kinases. *Proc. Natl. Acad. Sci. USA* **90**, 6213-6217.

Vasavada, H. A., Ganguly, S., Germino, F. J., Wang, Z. X., and Weissman, S. M. (1991): A contingent replication assay for the detection of protein-protein interactions in animal cells. *Proc. Natl. Acad. Sci. USA* **88**, 10686-10690.

Veillette, A., Bolen, J., and Bookman, M. A. (1989a): Alterations in tyrosine protein phosphorylation induced by antibody-mediated cross-linking of the CD4 receptor of T lymphocytes. *Molecular and Cellular Biology* **9**, 4441-4446.

Veillette, A., Bookman, M. A., Horak, E. M., and Bolen, J. B. (1988): The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrsoine protein kinase p56lck. *Cell* **55**, 301-308.

Veillette, A., Bookman, M. A., Hraak, K. M., Samelson, L. E., and Bolen, J. B. (1989b): Signal transduction through the CD4 receptor involves the activation of the internal membrane tyrosine-protein kinase p56lck. *Nature* **338**, 257-259.

Veillette, A., Caron, L., Fournel, M., and Pawson, T. (1992): Regulation of the enzymatic function of the lymphocyte-specific tyrosine protein kinase p56lck by the non-catalytic SH2 and SH3 domains. *Oncogene* **7**, 971-80.

Veillette, A., Zuniga-Pflucker, J. C., Bolen, J. B., and Kruisbeek, A. M. (1989c): Engagement of CD4 and CD8 expresed on immature

thymocytes induces activation of intracellular tyrosine phosphorylation pathways. *J. Exp. Med.* **170**, 1671-1680.

Vojtek, A. B., Hollenberg, S. M., and Cooper, J. A. (1993): Mammalian Ras interacts directly with the serine/threonine kinase Raf. *Cell* **74**, 205-14.

Wang, A. V., Scholl, P. R., and Geha, R. S. (1994): Physical and functional association of the high affinity immunoglobulin G receptor (Fc gamma RI) with the kinases Hck and Lyn. *J Exp Med* **180**, 1165-70.

Weernink, P. A., and Rijksen, G. (1995): Activation and translocation of c-Src to the cytoskeleton by both platelet-derived growth factor and epidermal growth factor. *J Biol Chem* **270**, 2264-7.

Weiss, A. (1993): T cell antigen receptor signal transduction: A tale of tails and cytoplasmic protein-tyrosine kinases. *Cell* **73**, 209-212.

Weiss, R., Teich, N., and Varmus, H. E. (1982): RNA Tumor Viruses, Cold Spring Harbor Press, Cold Spring Harbor, New York.

Weng, Z., Taylor, J. A., Turner, C. E., Brugge, J. S., and Seidel-Dugan, C. (1993): Detection of Src Homology 3-binding proteins, including paxillin, in normal nd v-Src- transformed Balb/c 3T3 Cells. *Journal of Biological Chemistry* **268**, 14956-14963.

Wildin, R. S., Garvin, A. M., Pawar, S., Lewis, D. B., Abraham, K. M., Forbush, K. A., Ziegler, S. F., Allen, J. M., and Perlmutter, R. M. (1991): Developmental regulation of lck gene expression in T lymphocytes. *J Exp Med* **173**, 383-93.

Willman, C. L., Stewart, C. C., Longacre, T. L., Head, D. R., Habbersett, R., Ziegler, S. F., and Perlmutter, R. M. (1991): Expression of the c-fgr and hck protein-tyrosine kinases in acute myeloid leukemic blasts is associated with early commitment and differentiation events in the monocytic and granulocytic lineages. *Blood* **77**, 726-34.

Winzerling, J. J., Berna, P., and Porath, J. (1992): How to Use Immoblized Metal Ion Affinity Chromatography, pp. 4-13: *Methods: A Companion to Methods in Enzymology*, Academic Press.

Yamanashi, Y., Miyasaka, M., Takeuchi, M., Ilic, D., Mizuguchi, J., and Yamamoto, T. (1991): Differential responses of p56lyn and p53lyn, products of alternatively spliced lyn mRNA, on stimulation of B-cell antigen receptor. *Cell Regul* **2**, 979-87.

Yang, L., and Glaser, M. (1995): Membrane domains containing phosphatidylserine and substrate can be important for the activation of protein kinase C. *Biochemistry* **34**, 1500-1506.

Yang, X., Hubbard, E. J., and Carlson, M. (1992): A protein kinase substrate identified by the two-hybrid system. *Science* **257**, 680-2.

Yi, T. L., Bolen, J. B., and Ihle, J. N. (1991): Hematopoietic cells express two forms of lyn kinase differing by 21 amino acids in the amino terminus. *Mol Cell Biol* **11**, 2391-8.

Yu, H., Rosen, M. K., Shin, T. B., Seidel-Dugan, C., Brugge, J. S., and Schreiber, S. L. (1992): Solution structure of the SH3 domain of Src and identification of its ligand binding site. *Science* **258**, 1665-1668.

Zenner, G., Dirk zur Hausen, J., Burn, P., and Mustelin, T. (1995): Towards unraveling the complexity of T cell signal transduction. *BioEssays* **17**, 967.

Zervos, A. S., Gyuris, J., and Brent, R. (1993): Mxi1, a Protein That Specifically Interacts with Max to Bind Myc-Max Recognition Sites. *Cell* **72**, 223-232.

Zhang, Q. X., Walker, F., Burgess, A. W., and Baldwin, G. S. (1995): Reduction in platelet-derived growth factor receptor mRNA in v-src-transformed fibroblasts. *Biochim Biophys Acta* **1266**, 9-15.

Zhou, M. J., Lublin, D. M., Link, D. C., and Brown, E. J. (1995): Distinct tyrosine kinase activation and Triton X-100 insolubility upon Fc gamma RII or Fc gamma RIIIB ligation in human polymorphonuclear leukocytes. Implications for immune complex activation of the respiratory burst. *J Biol Chem* **270**, 13553-60.

Ziegler, S., Marth, J., Lewis, D., and Perlmutter, R. (1987): Novel protein-tyrosine kinase gene (hck) preferentially expressed in cells of hematopoietic origin. *Molec. Cell. Biol.* **7**, 2276-2285.

Ziegler, S. F., Wilson, C. B., and Perlmutter, R. M. (1988): Augmented expression of a myeloid-specific protein tyrosine kinase gene (*hck*) after macrophage activation. *Journal of Experimental Medicine* **168**, 1801-1810.

APPENDIX C

ENHANCING GRADUATE TRAINING AT UCSF

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ENHANCING GRADUATE TRAINING

Introduction

Scientists in the biomedical arena are witnessing dramatic changes in their work environment. A tighter national economy[1,2], the restructuring of the health care industry[3,4] and a continuing expansion in the number of scientists[5,6,7,8,9] has led to an increase in competition for funds and positions. Young scientists in search of their first job or initial grant will attest to the scarcity of viable academic posts and the intensity of competition for funding.[8,9]

Researchers in the physical sciences have been experiencing this squeeze for some time[5,10,11], but it is a fairly recent phenomenon for biomedical scientists.[8] This pressure will become even more acute as federal deficit reduction schemes shave money from the NIH 4budget[12,13,14] and academic health centers continue to lose patients and revenue to managed care organizations.[3,4]

Despite these constraining market forces, the number of Ph.D. students being trained for academic positions continues to rise. The most current data available from the National Science Foundation indicate that there has been a 23.8% increase in the number of doctorates awarded in the biological sciences from 1988 to 1993.[15] According to the 1995 report by Massy and Goldman, *The Production and Utilization of Doctorates in the US*, this increase translates to an

oversupply of Ph.D.s in the biosciences. Only a small fraction of those Ph.D.s currently being trained will be needed to fill academic positons and taking into account the demand for Ph.D.s in academe, industry, government and non-research areas, the report concludes that there is still a 28% excess in the supply of Ph.D.s in the biosciences.[9] Thus, almost a third of today's doctoral students will end up under-employed in jobs that do not require a Ph.D.

The available statistics on bioscience Ph.D.s indicate that recent graduates may be responding to the shrinking academic job market by remaining longer in graduate school and shifting their career goals away from academe. Data collected by the NSF clearly show that the time to degree for life science Ph.D.s has been steadily increasing. In 1972 the median time to degree was 5.5 years which by 1982 had lengenthed to 6 years and by 1993 had increased to 6.8 years.[15] While it may not be possible to attribute this increase wholly to the poor job market, over this same time period, the percentage of students in all fields who have undefined career plans after graduation has increased from 23.4% in 1970 to 40% in 1993.[15] Although only anecdotal evidence is available to indicate whether postdocs are taking longer to find permanent employment[15], the poor job market may also help explain the burgeoning number of postdoctoral fellows. From 1982 to 1992 the number of postdocs in the biological sciences nearly doubled from 7700 to 13,000, a 71% increase.[15] Finally, employment of Ph.D.s is shifting from academe to industry. According to data from the NRC Survey of Doctorate Recipients, more than 50% of recent Ph.D.s in the biological sciences have sought employment outside the tenure track of academic

institutions.[15] The trend from 1977 to 1991 shows a decrease in academic and government employment with a concomitant increase in business, industry or other employment.[15] This shift in career goals is evident even among current UCSF graduate students. In a survey that included 44 PIBS and BMS students in their third year or beyond, 38 (86%) entered graduate school intending to become academic principle investigators, of which 23 (52%) have now decided to pursue other career paths. These numbers suggest that nearly 70% of doctoral candidates at UCSF are considering careers other than academic principle investigators.[16]

Clearly, the role of today's biomedical scientist is expanding and diversifying beyond the traditional arena of research-based academic science. It follows that graduate training should be adapted to respond to these changes. The graduate programs at UCSF are top-ranked in the country and prepare their students to be excellent original researchers, but how well are these students prepared to use their knowledge and apply their skills to the changing demands of society and the marketplace?

In discussing this question with UCSF students and postdocs, we identified four shortcomings in our current graduate program. They parallel ones outlined in the National Academy report on restructuring graduate education.

Acceptance of and Access to Non-Academic and Non-Traditional Careers

In 1991, 45.7% of recent graduates nationwide in the biological sciences were employed in academe, 5.7% in government and 25.4%

in business and industry.[15] Unfortunately comparative numbers for UCSF graduates are not available. Despite the employment of more than 50% of graduates outside of academe, there is very little information available to students concerning these other career options. Many faculty mentors are unfamiliar with non-academic job opportunities and tend to consider these positions of lesser value. Since faculty are not likely to have extensive knowledge in these other areas, students typically receive little preparation or guidance when moving into these new careers.

However, there is a pressing need for this kind of information. In our recent survey of 130 UCSF graduate students and postdocs, 78% supported the idea of a career office; one person comments, "it's time to complete our graduate education through career education." Another writes, "...we should work to make (non-academic) career goals equally desirable, so that scientists can exit from the academic track without being made to feel like failures in any respect."[16]

Time to Degree

In agreement with the National Academy report on graduate education, we recognize that graduate training lasts too long.[15] Through our discussions with graduate students and postdocs, we have identified two components that contribute to a student's extended length of stay in graduate school. First, there are no clear departmental guidelines on what comprises an acceptable dissertation. At this point, time to degree is subjective and inconsistent, and largely determined by the student's faculty advisor. Second, once a student joins a lab, the education component of

graduate education is de-emphasized.[15] Since the majority of graduate education is financially supported through faculty research grants, the emphasis tends to shift to producing results for the faculty research project. As a result, a student may spend several years learning valuable lessons from failed experiments but making little tangible progress toward an actual degree.

Interpersonal Skills and Management Training

Interpersonal and management skills are not taught and are rarely discussed in graduate programs. However, both of these skills are highly valued by employers in industry and business, and the lack of such skills in science graduates is an impediment to making the transition from academe to industry. The National Academy report includes business employer perspectives, describing that Ph.D.s are valued for their analytical and problem-solving skills, but "their effectiveness could be enhanced through practical experiences/traineeships, functioning as a member of a team, strengthened interpersonal skills, (and the) ability to communicate clearly the purpose of the activity in question."[15] Furthermore, the weak management skills demonstrated by some faculty can contribute to ineffective graduate student mentoring and serve as poor role models for those students leaving academe.

Support Systems for Graduate Students

There are no formal support systems to help students cope with the failure and disappointment that are inherent to graduate

school and bench research. This leads to unnecessarily high levels of frustration and despondency. Younger graduate students need to be better prepared for this possibility. As students progress further in their training, they encounter different types of challenges and anxieties, from choosing a thesis lab, to dealing with orals anxiety, forging a working relationship with one's thesis advisor, and deciding on a suitable career path. Support should be available to alleviate the stresses at each of these stages, and an improved mentorship program should be provided to all graduate students.

UCSF faculty, students, and postdocs discussed these issues in a problem-solving workshop at the symposium, "Uncertainty and Opportunity: Careers in Science," held earlier this summer. They formulated possible solutions to the problems, and identified the ones of highest priority. The purpose of this report is to summarize these solutions, and present detailed proposals of the ones considered most important. We hope this will encourage the faculty to consider modifications to our current graduate program. Following the recommendations put forth in the National Academy report, our goal is to retain the existing strengths of the UCSF graduate programs while substantially increasing the information available to, the versatility of, and the career options afforded to students by their doctoral education.

REFERENCES

1. Finn, Robert (1995). "Layoffs in Biotechnology Signify Growing Pains for an Industry in Transition, Analysts Contend," **The Scientist** 9, no.9, p1.

2. Kilborn, Peter (1993). "The Ph.D.s are Here, but the Lab Isn't Hiring," **The New York Times**, July 18, 1993.

3. Editorial (1995). "The Assault on Teaching Hospitals," **The New York Times,** May 1995.

4. Marshal, Eliot (1995). "Heavy Weather Ahead for Clinical Research," **Science**

5. Goodstein, David (1995). "Implications for Student Support," paper presented at the AAAS Annual Colloquim on Science and Technology Policy (Washington, DC: April 12, 1995).

6. Goodstein, David (1995). "The Big Crunch," paper available on the Internet, http://www.caltech.edu/~goodstein/

7. Fleet, James (1995). "Young Researchers' Disillusionment Bodes Ill for Future of Science," **The Scientist** 9, no. 11, p11.

8. McIntosh, Richard (1994). "Funding Constraints and Population Growth: The Cell Biologists Nightmare," **The ASCB Newsletter** 17, no. 11, p1.

9. Massy, William and Goldman, Charles (1995). The Production and Utilization of Science and Engineering Doctorates in the

United States. The Stanford Institute for Higher Education Research, Stanford CA.

10. CPST (1995). "Supply and Demand: Physicists in Academe," **Comments CPST** 32, no. 1, p7.

11. Finn, Robert (1995). "Discouraged Job-Seekers Cite Crisis in Science Career Advice," **The Scientist** 9, no. 11, p1.

12. (1995). "Scientists Mobilize to Fight Cuts," **Science** 268, p1120.

13. Teich, Albert and Gramp, Kathleen (1995). "Overview of Federal Budget Proposals for R&D in FY 1996," paper presented at the AAAS Annual Colloquim on Science and Technology Policy, (Washington, DC: April 12, 1995).

14. AAAS (1995). "R&D Budget and Policy Reports," available on the Internet, http://www.aaas.org/.

15. COSEPUP, "Reshaping the Graduate Education of Scientists and Engineers," Committee on Science, Engineering, and Public Policy, National Academy of Sciences, National Academy of Engineering, and National Institute of Medicine (Wahington, D.C.: National Academy Press, 1995).

16. Hans, Sherrie and Awabdy, Tanya (1995). Unpublished information from "1995 Career Development Survey" (UCSF).

17. Kerr, Elizabeth (1995). "Too Many Scientists, Too Few Academic Jobs," **Nature Medicine** 1, no. 1, p14.

Proposal for a Career Office for Scientists at UCSF

Introduction/Overview

The proposal for a Career Office for Scientists at UCSF draws primarily from job placement needs that have resulted from an increasingly constrained job market for Ph.D.s in science. The April 1995 report of the National Academy entitled "Reshaping the Graduate Education of Scientists and Engineers" (1) describes the current climate of graduate education in science and engineering as a time of change. The end of the Cold War, the rapid growth of international competition in technology-based industries, and a variety of constraints on federal research spending have altered the job market for scientists and engineers. Although the demand for scientists and engineers has remained strong (as evidenced by low and stable unemployment rates), the three areas of primary employment for Ph.D. scientists and engineers -- universities and colleges, industry, and government -- are currently undergoing simultaneous change.

Many recent graduates are frustrated by their inability to find basic-research positions (2-7). Today, there are far more seekers of jobs as professors in academe and as basic researchers than there are available positions (5). Fortunately, expansion in applied research and non-research employment has absorbed many of the still growing cohort of Ph.D. graduates. However, there are worrisome indicators of weakness in the market, such as long delays in initial

placement of new graduates, the fact that some graduates are employed in positions that do not require a PhD, and the increasing length of postdoctoral assignments.

A UCSF Career Office for Scientists is proposed as a means of responding to the needs of current students and postdoctoral fellows at UCSF. In discussions on reforming graduate education at UCSF the suggestion to create a career office arose from three of the four working groups formed at the symposium "Uncertainty and Opportunity: Careers in Science." The activities proposed for the office come from suggestions made at the symposium and input received from a survey of career needs conducted in February of 1995 at UCSF.

Proposal

Users

The primary users of the Career Development Office would be graduate academic students and postdoctoral fellows. Ideally the office should serve graduate students and postdoctoral fellows campus wide. It is possible, however, that one or more graduate programs could sponsor such an office. Under these circumstances, access might be reserved for graduates and postdocs within that program.

The secondary users of the Career Development Office would be faculty members and the UCSF administration. Faculty members could rely on the office for accurate and up-to-date job market information when advising students and postdocs in their career choices. Both faculty members and the administration could also use

the resources and services proposed for the office in obtaining additional grant support, particularly for grants like the NIH biotechnology training grants, and in garnering additional financial support from the biotech and pharmaceutical industries. Lastly, a career development office would be yet another inducement to draw graduate students and postdocs to the UCSF campus.

Activities

1. To establish contacts with industry and other outside organizations for the purposes of maintaining a database of current job openings, establishing internship programs, organizing yearly job fairs, recruiting industry scientists and managers to speak regularly on campus and encourage students and postdocs to give talks at local biotech and pharmaceutical companies.

2. To establish education or internship programs with other local universities to provide alternative training vehicles for Ph.D.s in areas such as science writing, editing, business administration, law, education and science and health policy.

3. To collect resources such as job hunting guides, resume writing instructions and overviews of the current job market relevant to biomedical scientists (See Appendix A-1).

4. To sponsor frequent career panels and skill building workshops directed at both traditional academic endeavors as well as

alternative careers. For example, a career panel might consist of 2-3 scientists who are currently working in fields such as science writing or investment banking who would comment on their experiences. A skill building workship might focus on topics such as how to write a grant or honing interview skills.

5. To collect fellowship information for both traditional postdoctoral posts as well as alternative careers. Work to establish programs where none currently exist.

6. To establish a database to track graduate students and postdoctoral fellows after they leave UCSF (See Appendix A-2 for an example of a career information record). This information and the contacts it would provide would be particularly valuable for those seeking jobs in alternative careers.

7. Use the career-tracking database as a watch-dog to identify trends and potential problems regarding time to degree, attrition, types of careers sought, length of post-doctoral appointments, etc. for graduate students and post-doctoral fellows in various departments.

8. To establish and maintain a World Wide Web page including the proposed alumni directory, job openings, a schedule of office activities and links to other career sites and employment data important to biomedical scientists (See Appendix A-3 for current examples).

Oversight The services offered by the Career Development Office should be reviewed quarterly by a user oversight committee. This committee could consist of the Director of the Career Development Office, graduate students, postdocs and representatives from the faculty and administration. This committee will serve to insure that the office continues to serve the needs of graduate students and postdocs. In addition, the participation of faculty and the administration on this committee will help to link the activities of the Career Development Office to other activities on campus.

Resources

Space: The Career Development Office requires a space allocation large enough to house at least two employees, a small resource library with several filing cabinets, and a small reading/work space for students and postdocs. The optimal location for such an office would be on the main Parnassus campus. Graduate students and postdocs are both transiently employed at UCSF and tend to be more closely affiliated with their programs or mentors than to UCSF as a whole. As such, they are often unaware of programs and services available to them at UCSF except those that can be easily accessed and are highly visible. One possibility to explore is whether the Career Development Office would fit with the mission of the UCSF library and could be housed in this facility.

Personnel: As a minimum, one full-time Ph.D. level director with full-time administrative assistance and part-time computer support. As the program is outlined here, the director is expected to be a

dynamic, pro-active individual who can build bridges between the UCSF research community and outside entitities. The director would be charged with raising funds, developing programs both on site and at other institutions and interfacing with students, faculty and the administration. The success of this office would rest heavily on the skills and committment of this individual.

Office: Two to three Macintosh computers with a laser writer and ethernet connections, Xerox machine, filing cabinets, desks, chairs, work tables, and shelving are required. Reference books (job searches, resume preparation, interview skills...) and periodicals (tracking financial and business trends in the biotech industry) should be available in the office. Easy access to a public photocopier will also be required by those visiting the office.

Finances: To establish the office, money for space renovations and the purchase of the equipment outlined above would be necessary. An annual budget would have to be established to support the personnel, overhead costs, periodical subscriptions, limited travel funds for the director, upkeep and expansion of the computers, stipend support for internships and speaker fees. Not all of these activities need to be supported by an annual operating budget. Some items, like stipend support for internships, could be administered by the office, but in a separate pool from the operating budget.

The administrative placement of the Career Development Office will naturally influence the financial resources available to the office. This office, however, need not be wholly dependent on campus

resources. Since one of the goals of the office is to facilitate information and personnel transfer to biotech and pharmaceutical companies, there is great potential for leaning on industry sources for the establishment of this office and ongoing support.

Electronic Information: The office should communicate with users via the Internet. This will include email distribution of newsletters and workshop announcements, and a World Wide Web home page containing information for those new to the office and its services, as well as updates to current users. Links on the home page could be made to databases kept by the office such as the alumni tracking informations and biotech job listings as well as links to relevent information and institutions outside UCSF. We expect that electronic communications will result in greater accessability and increased efficiency while helping to limit overhead costs.

Addendum

Please note that while students and postdocs are eager to see an office established that would accomodate all of the programs and activities outlined in this proposal, this does not preclude the establishment of an office or program(s) that fullfill only portions of what has been outlined here. For instance, a department or program could independently establish an internship program, hold monthly skill building workshops, and maintain an alumni database accessible to students and postdocs. We encourage programs to implement some of the suggestions made here, as such pilots will help to

convince students, faculty and administrators that such programs are feasible, useful and sorely needed.

References

1. "Reshaping the Graduate Education of Scientists and Engineers," Committee on Science, Engineering, and Public Policy, National Academy of Sciences, National Academy of Engineering, Institute of Medicine, April, 1995, NAS Press, Washington, D.C.

2. "Career-building sites on the Internet: hunting for jobs electronically," The Scientist, Jan. 23, 1995, p. 22.

3. "Discouraged job-seekers cite crisis in science career advice," The Scientist, May 29, 1995 , p. 1.

4. "Young researchers' disillusionment bodes ill for future of science," The Scientist, May 29, 1995, p. 11.

5. "Transferable skills: a scientist's asset," The Scientist, May 29, 1995, pp. 12.

6. "Finding good scientific career advice when job prospects are tight," The Scientist, May 29, 1995, p. 15.

7. "Science careers: playing to win," (1995) Science. **265:** 1905.

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Proposal to Decrease the Time to Degree

Acquiring the intellectual and technical skills necessary to conduct successful scientific research is an intrinsically difficult and often lengthy process. In consequence, graduate training can seldom be brief. Nonetheless, we must ask whether students really need to invest six, seven or more years in graduate school to be qualified to receive a doctorate. Addressing this broad issue really involves answering two very basic questions: first, what exactly is a doctorate and second, what do students actually do with all the time they spend in graduate school?

Unlike students in professional schools, graduate students follow no clearly defined course of study which, when completed, leads to the receipt of the desired degree. Certainly graduate students must complete specified classes, but by far the bulk of a student's time in graduate school is spent conducting laboratory research. By its nature, the outcome and rate of progress of this research cannot be predicted in advance. To evaluate and -- ideally -- to help guide this research, a student convenes a thesis committee. This body also has the ultimate authority to determine whether the student's research meets the criteria for conferral of a doctorate. What, then, are these criteria?

In practice, they are whatever a thesis committee decides they are. As a result, the requirements for receiving a doctorate vary as widely as do educational philosophies of individual faculty members. Some professors would consider adequate a thesis that contains a well-controlled series of experiments that answer a clearly defined scientific question regardless of whether that answer was 'positive' or publishable. Others would grant a doctorate only for research that yielded several first-author publications in major journals. These disparities in educational outlook produce corresponding disparities in what is required of students to obtain a degree.

Certainly no policy can or should abolish these differences in educational philosophy, but a policy to rationalize the standards required of a graduate thesis can mitigate the inequities these differences produce. ***We thus propose that a committee of faculty and students be formed in each department to establish guidelines for thesis committees to use in determining whether a student's work meets the requirements for the award of a Ph.D.***. In addition to bringing greater cohesion to thesis committee decisions, such guidelines should also be helpful to students in measuring their progress toward graduation.

Though a thesis committee can have a significant effect on a student's experience in graduate school, by far the most profound influence on that time is usually made by the student's principal advisor. When a student selects and is accepted into a lab to conduct

thesis research, he or she enters into a set of mutual obligations with the faculty advisor. On one side, the student implicitly agrees to work for many years on a project whose results will be published under the names of both the student and the advisor. In return for this investment of time and effort, the student should receive some regular guidance and assistance from his/her faculty advisor.

The failure to receive adequate guidance often answers the question of what students do with at least a large portion of their time in graduate school. They drift. Years of hard work are devoted by many students to projects that are either conceptually flawed or slowed by avoidable technical difficulties. While each successful student ultimately manages to correct these flaws and generate productive research, this process of self-correction is a slow, frustrating and inefficient procedure. While no-one would suggest that students be led by the hand through their thesis, it is reasonable to expect continuous educational input by faculty advisors. Many highly placed members of the scientific community[1] feel that the emphasis on education must be re-introduced to most graduate programs. Once a student enters a lab, the obligation of all of the faculty to teach a student should continue regardless of how much experience a student has or how reticent they are to receive advice. Students continue to need help both technically and conceptually in order to become productive members of the scientific community. If they do not receive or accept such advice, not only will they remain in graduate school far longer than is beneficial, but the future

contribution of this individual to science may be lost through attrition.

Specific policies can be implemented that will help to ensure that students receive more effective guidance during their thesis research. To reinforce the idea that having a student in the lab generates a set of obligations for both the student and the advisor, ***we propose that each student and advisor adopt a compact specifying what is expected from each by the other***. This compact would be prepared and mutually agreed upon prior to each thesis committee meeting. It would cover both general expectations, such as weekly meetings between the student and faculty member or the preparation of a quarterly research summary by the student, as well as the specific research goals that the student and advisor expect will be reached within a specified time frame.

The first of these compacts would be presented to the thesis committee prior to the student's initial committee meeting and would be subject to further modification at that time. The modified compact should be signed by the student and members of the thesis committee and be filed in the students permanent educational record. In subsequent years, the student should always submit a written summary to the committee several days prior to a meeting and the committee members should read and consider the contents of that summary before they meet with the student in order to be well prepared to offer advice and criticism. The committee should also review whether the goals from the previous compact had been

met and if not, then why. Unmet goals might result from many causes: unexpected results from the students work or another lab, technical difficulties or muddled ideas by the student. In such cases, the thesis committee can recommend specific remedies such as attending a Cold Spring Harbor course, visiting another lab to learn a technique or re-thinking the problem. Crucially, the thesis committee can also assess whether the failure to reach specified goals resulted from either the student or the advisor having deviated significantly from the obligations agreed to in the compact. Appropriate questions in this latter regard might include asking whether the student had worked with the expected level of diligence or whether the advisor had provided adequate guidance and technical assistance.

In addition to its retrospective function in reviewing the past student/advisor compact, the thesis committee would also have a prospective function in evaluating the compact for the upcoming year or years. By the student's third or fourth year, the compact should include specific goals which, when reached, would be considered sufficient for graduation. By having the thesis committee affirm these goals, the student and committee would have a clear mutual agreement in advance as to what was expected for graduation.

Even with the improvements to the current system outlined above, some students will still spend longer in graduate school than the desired norm and we propose that special attention should be

focused on these cases. Specifically, ***we propose that a standing committee of faculty be formed to review the progress of those students whose time in graduate school has exceeded some specified length.*** (We suggest 5.5 years.) This committee would meet regularly with the student's advisor and the student to inquire as to the reasons for the student's protracted stay and to insure that all reasonable measures were being taken to speed that student's departure. This committee could also serve as a forum to which a student could appeal if he or she felt that the thesis committee had deviated significantly from the standards for evaluating thesis research or if the faculty advisor had failed markedly to fulfill the obligations specified in the student/advisor compact. Conversely, if a student felt the need to remain in graduate school after the agreed requirements for graduation have been met, this committee would insure that his or her reasons were valid and that the extra time would be kept to a minimum.

In summary, our proposals have several objectives, all designed to reduce the amount of time students spend in graduate school as well as to improve the quality and productivity of that time. First, by rationalizing standards for graduation, the proposals seek to provide students with a clearer understanding of what is required to receive a doctorate. Second, through the mechanism of student/advisor compacts, they seek to insure that both students and advisor clearly understand what each expects from the other. Furthermore, these compacts should provide a clear plan of research with agreed goals leading to graduation. Finally, by establishing an

oversight committee of faculty our proposals seek to insure that greater attention is focused on those students (and their advisors) who remain at UCSF the longest.

As described elsewhere in this report, the frustrations of working for many years at the bench with little guidance and often even less success can take a huge psychological toll on students. The length and poor quality of the time many students spend in graduate school affects not only their own mental health but will ultimately affect the academic health of the PIBS programs. If this program continues to develop a reputation for protracted graduation times and, as a consequence, for low student morale, then its ability to attract the most talented incoming students may be impaired. While no set of policies can guarantee every student a smooth and swift ride through graduate school, the proposals we have outlined above should reduce the amount of time students spend hopelessly lost or stuck in traffic along the way.

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1. "Reshaping the Graduate Education of Scientists and Engineers," Committee on Science, Engineering, and Public Policy, National Academy of Sciences, National Academy of Engineering, Institute of Medicine, April, 1995, NAS Press, Washington, D.C.

Proposal for a Management Workshop

Introduction

Learning to manage people is often a far more daunting task for a scientist than learning to operate new high-tech equipment. Many would attribute this to the fact that while instruments and experiments can be carefully controlled and contained, individual laboratory workers cannot. However, what many of us do not consciously recognize is that just as we can receive specific training in the use of a confocal microscope or an electron spray mass spectrometer, we can also be trained to be better managers. While management training cannot eliminate the variables encountered with each student, it will help the head of a lab to be more effective in guiding a student to apply those differences in ways that benefit them both. In this proposal we present suggestions for initiating a management training program for junior faculty.

Overview

In a discussion held at the recent symposium, "Uncertainty and Opportunity: Careers in Science," students, postdocs and faculty addressed the issue of restructuring graduate training at UCSF. The intent of the organizers in initiating this dialogue was to determine the best ways to improve the laboratory work environment and to better educate, train and prepare students for jobs in the future. One of the issues discussed was the insufficient training academic scientists have in management and interpersonal skills. Improving these skills would allow students to interact more effectively with

their advisor and other laboratory members, making students more productive and enhancing their value to future employers in industry or business. Improving these skills among faculty would assist them in becoming more effective lab mangers and stronger mentors for their students.

A number of ideas on how to improve the management skills of scientists were introduced during this discussion. First, a management workshop should be established for new and continuing faculty. If this workshop is beneficial, then a similar program should be considered for graduate students and postdocs. Second, a formal mentoring program should exist to pair beginning faculty with those who are more advanced in their careers so that junior faculty may receive more advice and guidance. Third, a policy on mentorship should be written that specifies the mentoring obligations a faculty member has toward his/her students. Finally, to allow students and postdocs to practice management skills, they should be encouraged to serve on departmental committees. This proposal addresses only the management workshop for faculty. It is our hope that the other issues will be considered in the future.

Proposal

The proposal we would like to see implemented is a 1-2 day management workshop for all faculty members, but focusing on junior faculty. After speaking with many people qualified to run such a workshop, we feel the strongest candidate is Dr. Jennifer Chatman, an assistant professor in the Haas School of Business at the University of California at Berkeley. Dr. Chatman's skills come highly

recommended by several business professors as well as students. She has experience in management training, not only in business and in business schools, but she has also worked with scientists in a research environment (see Appendix B-1). Our decision to recommend Dr. Chatman, however, was also strongly influenced by her interest and enthusiasm. In her role as a professor of business management, she is very interested in developing new areas of management training such as the one we have proposed for academic scientists. She has expressed her willingness to meet with groups of faculty, students and postdocs to define the specific needs of these groups and then to tailor a workshop to address those needs. Even without that assessment, however, Dr. Chatman provided us with an outline of the kinds of issues which could be included in a management training course (Appendix B-10).

In brief, Dr. Chatman recommends five distinct sessions on related topics. These sessions blend discussions intended to increase conceptual understanding and problem diagnosis with skill-building and problem solving techniques. For example, the workshops would open with a session on how to make better decisions by first discussing research findings that show the kinds of cognitive biases people are most vulnerable to. Participants then try to avoid these biases by applying this knowledge to a real organizational decision. Similarly, in a session on *Managing and Motivating Others,* participants examine the array of motives people have at work and compare a variety of possible ways of managing subordinates, peers and even their bosses. This understanding of business contexts and repertoire of relevant skills and behaviors should allow participants

to function more effectively in all environments, including our target, the academic research lab.

While we strongly favor inviting Dr. Chatman to run a management seminar, other resources available on campus could be used as an alternative or in addition to Dr. Chatman's proposed program. The Faculty, Staff and Student Assistance Program (FSSAP) is available on campus to provide ongoing guidance to reinforce topics discussed in a workshop. The Director of FSSAP, Dr. Teddy Adelstein, and his staff are available to design and put on a workshop or to work in conjunction with Dr. Chatman to do so. In particular, Dr. Yvette Guerrero will be joining the FSSAP staff in mid-September 1995 and could be a contact/resource person for development of the proposed management workshop. She is a Ph.D. Psychologist as well as an RN and has experience training and assisting staff in the medical profession (see Appendix B-11).

In order to facilitate implementation of the workshop and any on-going guidance program, a dedicated liaison should be selected. Krista Timlin, a UCSF employee with a degree in organizational management has expressed interest in working as a consultant on this project. Departmental MSOs would also be well-suited for this role, since they have significant management experience and work directly with faculty, students, and postdocs on a daily basis. A dedicated liaison will ensure that the project moves forward and will serve as a focal point to assess specific needs to be addressed in the workshop as well as to develop ongoing support and training programs. The incorporation of a management workshop and

ongoing training should develop the management skills of faculty and improve the mentoring of students in the programs involved.

Finally, it is important to record feedback from the management workshop or any other activities undertaken by FSSAP. A suggestion, therefore is that a yearly survey of students, postdocs, research associates and faculty be conducted to asses the development of management skills. Viewing survey results will define areas for future improvement by individual faculty members.

Scope and Cost

At this time we recommend that all new faculty in the basic sciences be required to attend a management training seminar and all established faculty be strongly encouraged to do so. The funding source will largely determine the participants as well as the scope of any program. A designated faculty sponsor and his/her administrative assistant are necessary to ensure continuity. We would be able to organize a management training session as early as January 1996.

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Proposal for Graduate Student Retreat

Introduction

The idea of a graduate student retreat grew in large part from the career development symposium held on June 10 at UCSF. One of the issues discussed at the symposium focused on fostering and maintaining a sense of graduate student well being, a task which is both complicated and difficult. Discussion at the symposium, as well as the results of a survey taken in February, show there exists a great deal of discontent among graduate students with respect to a number of different issues. These issues include:

- The need for faculty acceptance toward the pursuit of alternate career paths as well as more information about such opportunities.
- More active guidance and structure in the graduate program that would encourage students to set timely goals for the completion of their degree.
- Better support systems for coping with failure (perceived or real) in the laboratory.
- The current under-funded and overcrowded job market (primarily, but not limited to, the academic principle investigator track).

To address these issues, the faculty, student and postdoc participants in the discussion on student well being made two major recommendations. The first is the formation of a career office, which would coordinate a number of different services for graduate students and postdocs (please see the Career Office proposal). The second major recommendation was to organize an annual graduate student retreat to actively address many of the concerns presented above.

General Description

While Asilomar continues to be the premiere gathering for PIBS, its size and focus on research precludes it from being a forum in which graduate student-specific concerns can be addressed. Asilomar is an excellent forum for exposing students to current research at UCSF, but it does not offer graduate students an opportunity to discuss the difficulties they might encounter while performing such research.

The graduate student retreat would be a combination of presentations and activities promoting both good science and student well being. Students and interested faculty would come together in a forum that focuses on issues specifically affecting graduate students. Students will have the opportunity to present and discuss their projects. Younger students will be able to ask for feedback on experimental plans, and older students can share experiences such as choosing a working thesis project or overcoming technical difficulties. The goals of the retreat are to give support and guidance to students

who feel adrift, to build self esteem through mentoring of other students, and to initiate dialogue that helps to build a stronger safety net for students when they do encounter problems. The retreat is designed to achieve these goals through a series of activities including; two sessions for scientific troubleshooting, the presentation of campus resources available to students, an open forum for the discussion of any new/unaddressed issues important to graduate students, and the nucleation of small, structured support groups that would meet on an ongoing basis once students return to campus.

Specifics

Who would attend?

All graduate students in PIBS would be invited and encouraged to attend. Ideally, in future years, students from other programs (e.g. BMS, biophysics, pharm chem) would also be invited, but their attendance would be dependent on the willingness of the other departments to share costs.

A small number of faculty members (~10) would also be invited to attend. A letter would be sent to all faculty asking only those *very* interested in participating in a retreat of this type to contact the organizers.

Where will the retreat be held?

One possibility is the Marconi Conference Center at Point Reyes.

When will the retreat be held?

We recommend an overnight retreat, starting at 1 PM on a Thursday until around 1 PM the next day. However, if the cost prohibits approval of the retreat, it could be condensed to a one-day event.

What will the schedule be (approximate)?

Day 1

1:00 - 1:30: *Introduction and welcome.* The organizers will introduce themselves as well as the other student and faculty participants. It will be important for all of the graduate students to introduce themselves as many of the older students do not know the younger members of the program and vice versa. The format, goals, and motivation of the retreat will be described. In particular, we would like to emphasize that this is an opportunity to mentor and be mentored by their peers. Everyone should make an effort, during meals and other social breaks, to get to know students in years above and below them.

1:30 - 5:30: *Scientific Problem Solving.* Upper level students will present their projects, explain current research dilemmas, or

describe the process they have gone through in solving past dilemmas. The tone here should be positive rather than negative, with an emphasis on problem solving and perseverance. The goal is to give students who have encountered a scientific roadblock a chance to receive input, as well as to show other students that they are not alone in dealing with a difficult project that refuses to work. Student presenters would be chosen ahead of time to speak for about 10 minutes. Following each presentation would be a 10-20 minute discussion to get feedback and suggestions from other students and faculty. There would be time for about 10-12 presentations. There will be break at about 3:30.

5:30 - 7:00: Dinner

7:00 - ?: *Poster session* (leading to a party.) All students would be strongly encouraged to present a poster. Second year students (or other students with few or no results) would be asked to present a poster describing the project they intend to accomplish with the goal of soliciting advice from older students on different approaches that could be attempted.

Day 2

9:30 - 1:00: *Student well being session.*

- Short presentations by a number of faculty members discussing the history of their lab research, including past experiences they have had where a project or another aspect of their career did not go as smoothly as they might have wished and how they worked through/around/over the difficulty. Many graduate students in their first years experience what they view as the first academic failure of their career. This can result in a state of mind that can be unhealthy and unproductive. The purpose of the faculty presentation is to *strongly* make the point that failure in lab, while seemingly hopeless, is in fact often not as insurmountable as it seems and is also a normal component of performing laboratory research. Failure of a project does not equate to the failure of the student. (30 minutes - 3 faculty members)

- Presentation of the student well being packet (please see Appendix C). This packet includes a list of faculty who have volunteered to act as confidential advisors to students as well as other campus resources available to students such as counseling services, student organizations and support offices. In this presentation we would highlight the value of utilizing these services and emphasize that there is certainly no stigma attached to those who do use them. (10 minutes - student)

- Large group discussion of problems students see with graduate school. This is a time for the attending faculty members to listen, ask questions, present the faculty viewpoint, and make suggestions on how to deal with these concerns *without becoming didactic*. One of

the observations made at the career development symposium was that a large difference of opinion exists between students and faculty concerning the problems that exist in graduate school and the best ways to deal with these problems. This discussion would initiate a dialogue that should help to enlighten both students and faculty about the others point of view. (45 minutes - facilitated by student)

- Break (15 minutes)

- Introduce the idea of forming student support groups to deal with conflicts and problems arising in graduate school. This method involves the formation of small, structured, regularly meeting, confidential support groups which focus on solving specific problems brought up for discussion by a member of the group. (15 minutes, presented by a current participant in such a group.)

- Break into small (10 person) groups for a discussion/problem solving session. This is an active demonstration of how these ongoing support groups would operate, as well as an opportunity for students to obtain advice on problems they are currently wrestling with. Students willing to nucleate support groups would be asked to help start such groups back at UCSF and we will distribute a list of interested nucleators to all students. (90 minutes - facilitated by a student with a faculty member in each group and emphasis on mixing years within each group to foster mentoring from older students to younger students.)

Cost

Administrative: Organization would require a staff member to help coordinate mailings to students and faculty, as well as conference center reservations/payment, ordering food, easels for the poster session, party materials, etc. This person would also have to interact with the appropriate people in other participating departments (if any). There would also be some follow up mailings needing staff assistance.

Marconi Conference Center: At ~$80/person, including food, the estimated cost with an attendance of 80% of the PIBS students is $10,000.

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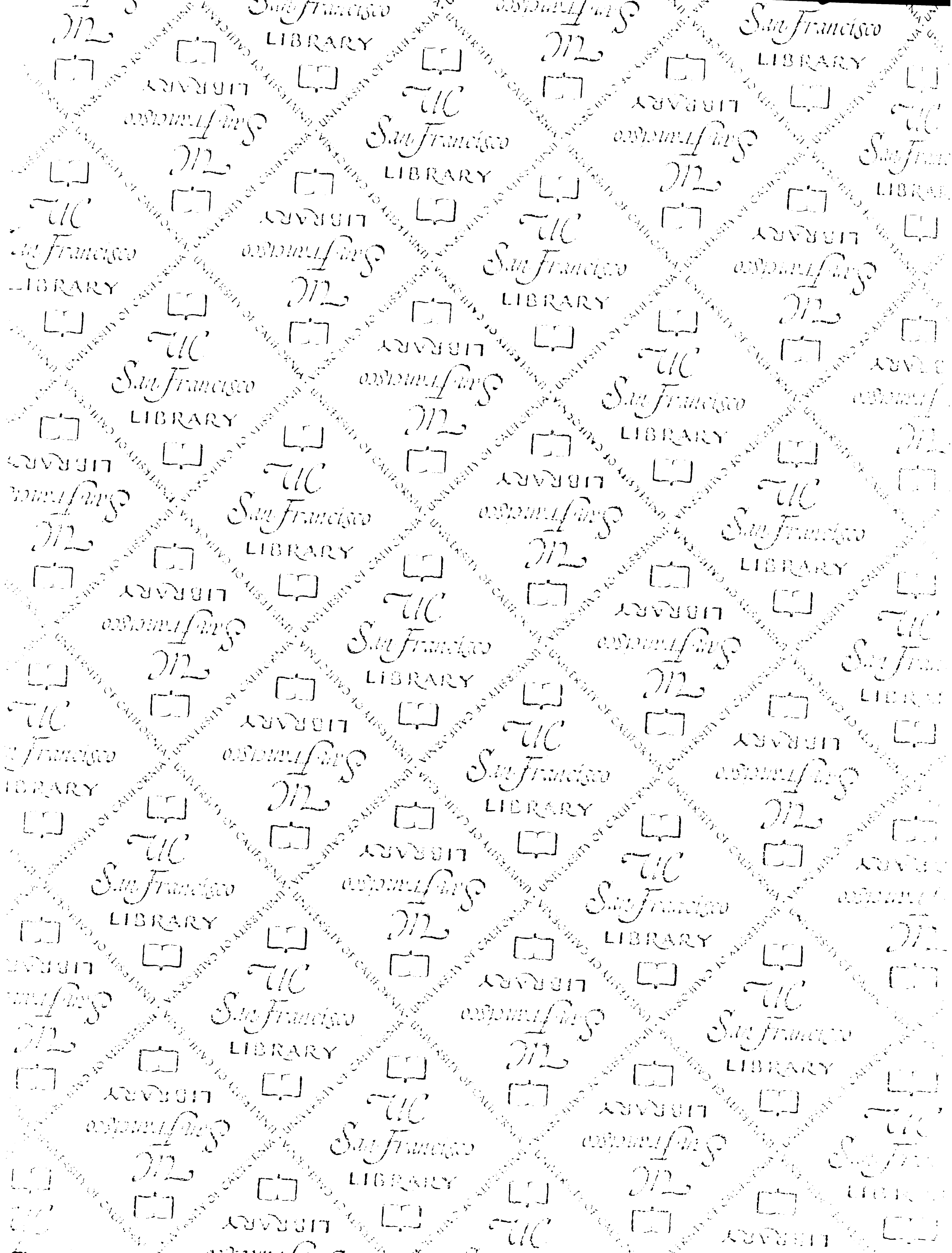
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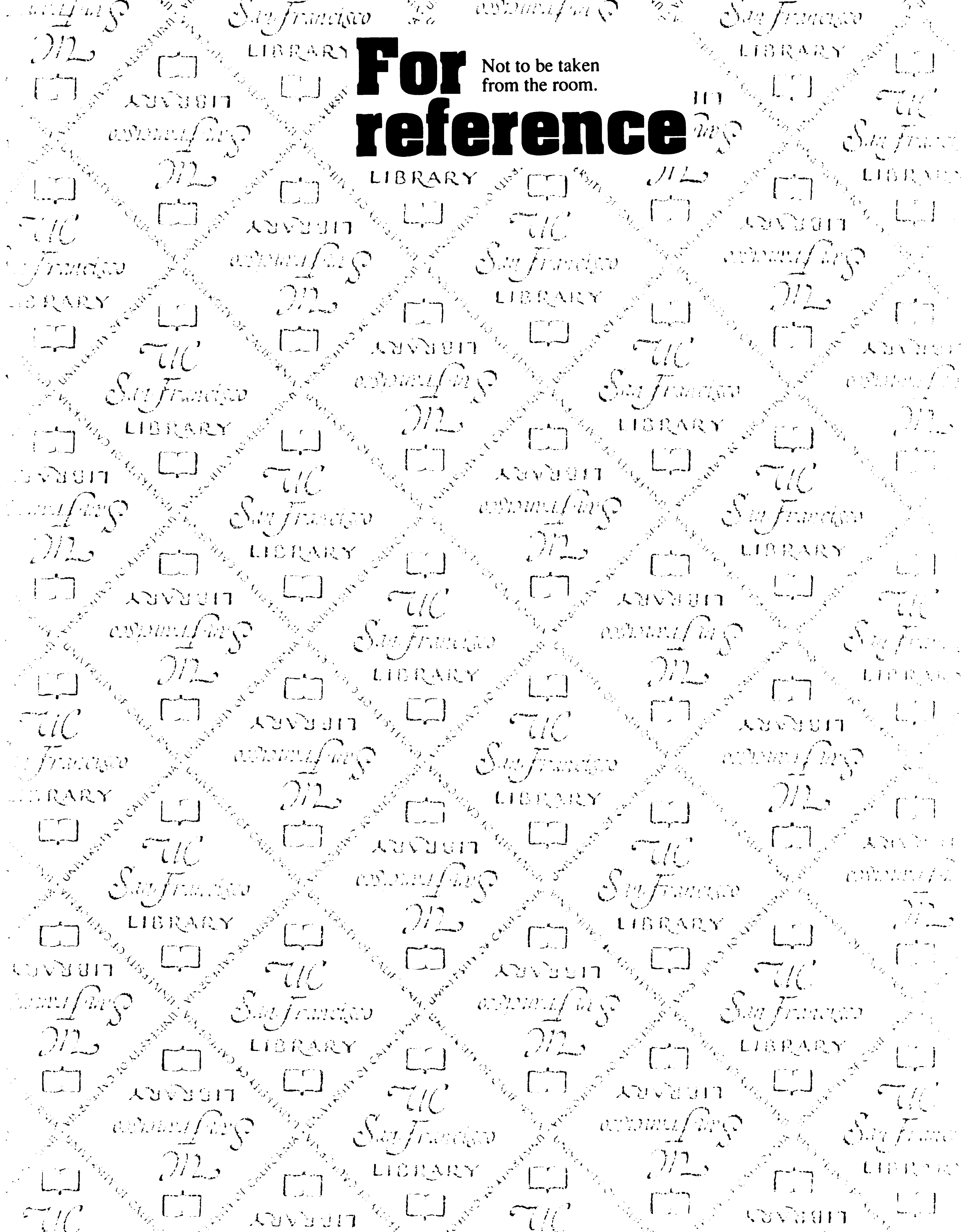

For reference
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