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Biochemical analysis of essential proteins involved in T cell antigen receptor mediated-signal transduction

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BIOCHEMICAL ANALYSIS OF ESSENTIAL PROTEINS INVOLVED IN T CELL ANTIGEN RECEPTOR MEDIATED-SIGNAL TRANSDUCTION

by

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DISSERTATION

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Dedication

To my parents, John and Jane Sieh, for teaching me the meaning of love.
To my beloved husband, Mark Andrew Moore, whose unwavering faith in my abilities has sustained me throughout the years.

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The text of Chapters II and III is a reprint of the material as it appears in the journals listed below. The coauthor listed in this publication directed and supervised the research which forms the basis for the thesis. Dr. Art Weiss was the principal investigator supporting this research. Dr. Joseph Bolen, Dr. Andreas Batzer and Dr. Joseph Schlessinger provided reagents and antibodies that made this thesis possible.

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The following figures have been modified from the following published works:

Figures 1 and 3: Weiss, A. *T lymphocyte activation*, in Fundamental Immunolgy, W. E. Paul, Editor. 1993. Raven Press: New York. p. 467-504.

Figure 2: van Oers, N. S. C. and Weiss, A. 1995. The Syk/ZAP-70 protein tyrosine kinase connection to antigen receptor signalling processes. Sem. Immunol. **7**: 227-236.

ABSTRACT

Biochemical Analysis of Essential Proteins Involved in T cell Antigen Receptor-Mediated Signal Transduction

Monica Sieh Moore

Mounting an effective immune response requires the concerted interaction of T and B lymphocytes activated by foreign antigens. Recognition of an antigen by the T cell receptor (TCR) initiates a biochemical cascade that culminates in proliferation, lymphokine production or cytotoxic activity. One of the earliest biochemical events leading to these cellular responses is the activation of Lck, a lymphocyte specific protein tyrosine kinase (PTK). In this thesis, the "intramolecular model" for regulation of the Src family PTK as it applies to Lck, was addressed. Studies with leukemic T cell lines and their CD45-deficient mutants, as well as phosphopeptides encompassing the negative regulatory site (Y505) demonstrate that Lck can be modulated by CD45 *in vivo*. Moreover, this was a specific effect since Fyn, another member of the Src family PTKs, was not equally affected by the presence of CD45. These findings not only support the"intramolecular model" proposed for regulation of Src-like kinases, but also provide insight into the molecular mechanism by which CD45 functions as a positive regulator in TCR signaling.

Although activation of the cytoplasmic PTKs Lck/Fyn and ZAP-70 results in Ras-GTP accumulation, the biochemical pathway linking these two events is poorly understood. In this thesis, a novel inducible tyrosine phosphorylated 36-38 kDa protein (pp36-38) is described. The importance of pp36-38 is underscored by experiments demonstrating that pp36-38 can associate with Grb2-SOS complexes as well as with PLC γ1, providing a

possible link between cytoplasmic PTKs and the Ras pathway. A Grb2-GST fusion protein was used to affinity purify pp36-38 from forty liters of Jurkat T cells. Five proteins were isolated including dynamin, Sam68, Cbl and two unknown proteins of 80 kDa and 36 kDa. Notably, dynamin, Sam68 and Cbl were shown to interact with Grb2, indicating that this affinity purification is a reasonable approach for purifying pp36-38. Therefore, the gene encoding the purified 36 kDa was cloned and antisera against p36 were generated. However, studies with these anti-p36 antisera revealed that the purified p36 protein was an *in vitro* artifact and did not correspond to the pp36-38 seen associated with Grb2 *in vivo*. A new protocol for the purification of pp36-38 is presented and discussed herein.

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CHAPTER 1

AN OVERVIEW OF T CELL RECEPTOR SIGNAL TRANSDUCTION

Preface

T and B lymphocytes play a key role in mounting an effective cellular and humoral immune response. The ability of T lymphocytes to distinguish between self and foreign antigens is mediated by the T cell antigen receptor (TCR), an oligomeric complex consisting of at least six transmembrane polypeptides. The signaling cascade initiated in response to TCR activation is similar to that evoked by many mitogens and growth factors: an increase in tyrosine kinase activity, a rapid induction of tyrosine phosphoproteins followed by activation of the γ1 subunit of phospholipase C (PLC γ1). Activation of PLC γ1 leads to increases in cytoplasmic free Ca^{2+}, Ras-GTP accumulation and activation of several serine/threonine kinases including members of the MAP kinases (Mitogen Activated Protein), ultimately leading to gene transcription (1). This introduction will review our current understanding of some of the proximal signal transduction events mediated by engagement of the TCR complex. Additionally, it will highlight the importance of the experiments described herein that attempt to understand how tyrosine kinases are regulated and how the TCR couples to Ras activation.

Proximal signal transduction pathways mediated by the TCR

T Cell Antigen Receptor Structure

The TCR is a multichain complex composed of a disulfide-linked Ti αβ or γδ heterodimer non-covalently associated with the invariant CD3 complex. Association between the Ti αβ chains and the CD3 complex is obligatory for the efficient receptor assembly and expression on the cell surface. The CD3 complex is composed of the γ, δ and ε chains, along with the disulfide-linked ζ chain homodimer or ζη heterodimer. Within each TCR complex, there appears to be two copies of CD3ε that can associate with either the δ or γ chains to form dimers. While the Ti chains recognize both the major histocompatibility complex (MHC) as well as the antigenic peptide displayed in the cleft of an MHC molecule, the function of the associated CD3 and ζ chains is to couple the Tiαβ or Tiγδ to the intracellular signal transduction machinery.

Initial clues to this function came from studies using monoclonal antibodies reactive with extracellular domains of the CD3 complex that were able to recapitulate TCR signal transduction. Furthermore, stimulation/aggregation of chimeric molecules, consisting of the cytoplasmic domains of the CD3ε or ζ subunits fused to a heterologous extracellular domain, was able to mimic the events initiated by TCR engagement including tyrosine phosphorylation, PLC γ1 activation, increases in Ca^{+2}, and cytotoxic responses (reviewed in Ref.1). This important finding greatly simplified the multimeric TCR complex into a single transmembrane protein that was more amenable to genetic manipulation. In this manner, the sequence in the cytoplasmic domains of the CD3 and ζ chains that is required for proper signaling was mapped to a motif consisting of two YXXI/L sequences

separated by six to eight amino acids. This motif, now known as ITAM (Immunoreceptor Tyrosine-based Activation Motif) was first identified by Reth as being present in the γ, δ, ε and ζ chains of the TCR receptor, as well as in the Ig-α and Ig-β chains of the B cell receptor and the γ-chain of the FcγRIII receptor (2). It is still not known whether the CD3γ and δ chains can also mimic the TCR-mediated signals. The ITAMs in the TCR complex, present in a single copy in the CD3 ε, γ and δ chains, or in six copies in the ζ-chain homodimer, is depicted in Figure 1. Since TCR stimulation induces cellular protein tyrosine phosphorylation and chimeric molecules containing ITAMs have been coimmunoprecipitated with protein tyrosine kinase (PTK) activity (3, 4), the functionality of the ITAM can be partially explained by its ability to associate with cytoplasmic PTKs. The three candidate cytoplasmic PTKs that have been implicated in mediating the TCR signaling events will be discussed below.

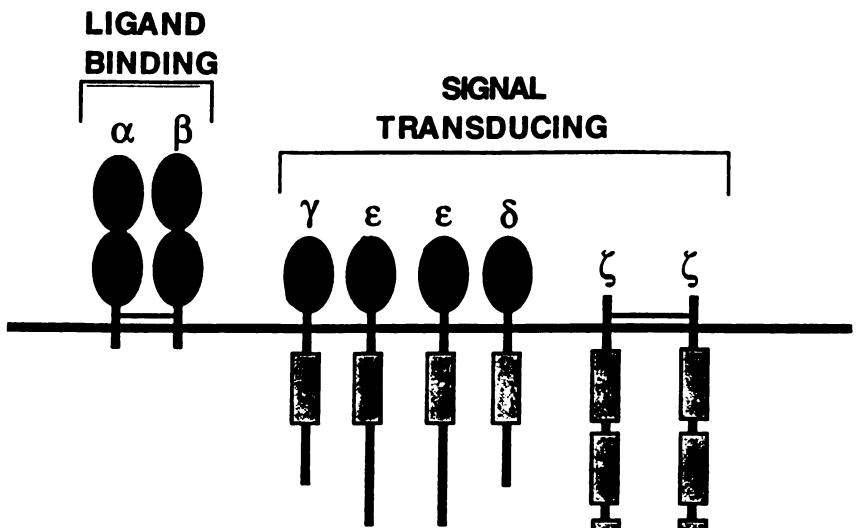
Evidence for Fyn involvement in TCR-mediated signaling

One candidate PTK, Fyn, a member of the Src family of tyrosine kinases, is present either exclusively in hematopoeitic cells (FynT) or primarily in the brain (FynB). The molecular basis for generating these two isoforms of Fyn is the tissue-specific alternative splicing of the mutually exclusive forms of exon 7 (5). FynT was first implicated in TCR signaling in studies demonstrating that small amounts of FynT coimmunoprecipitated with the TCR complex under mild non-ionic detergent conditions (6). Notably, the stoichiometry of this association seems to be quite low since sensitive *in vitro* kinase assays were required to detect this interaction. Though further structure-function analyses mapped this association to the first 10 amino acids of FynT, and to the ITAMs in the CD3 and ζ chains, this association was mapped using an

Figure 1. The T cell antigen receptor complex

Schematic illustration of the clonotypic Tiα/β heterodimer associated with the signal transducing CD3 and ζ chains. The immunoreceptor tyrosine-based activation motif (ITAM), depicted as rectangular boxes, is present singly in each CD3 chain or triply in the ζ chain.

T CELL ANTIGEN RECEPTOR COMPLEX



= ITAM (Immune-receptor tyrosine-based activation motif)

. . Y . . L . . . . Y . . L

overexpression system (7), suggesting that the interaction of Fyn with the TCR may be indirect or very weak.

More compelling evidence supporting the notion that FynT indeed contributes to TCR-triggered signals comes from additional genetic and functional studies. First, overexpression of FynT in thymocytes of transgenic mice enhances TCR-mediated tyrosine protein phosphorylation, calcium flux and cellular proliferation, whereas overexpression of a kinase inactive form of FynT inhibits TCR-induced thymocyte proliferation (8). Second, expression of a constitutively activated version of FynT (Y528F) augments the responsiveness of an antigen-specific T-cell line (BI-141) (8). Third, stimulation of the TCR induces a transient and reproducible, albeit modest, increase in Fyn PTK activity towards an exogenous substrate (9). These data, in conjuction with the physical association described between the TCR and FynT, suggest that FynT participates in the signal transduction induced by the engagement of the TCR.

However, targeted disruption of the *fyn* gene by homologous recombination did not affect the total number of T cells in either the periphery or in the various thymocyte populations. Similarly, TCR signaling in peripheral T cells and immature double positive (CD4+CD8+) thymocytes was normal in the fyn-deficient mice. However, Fyn does seem to be required for proper TCR signaling in CD4 or CD8 single positive thymocytes, since they displayed a striking defect in the ability to respond to TCR stimuli including anti-CD3 antibody or concanavalin A, a mitogenic lectin (10, 11). Taken together, the evidence suggests that Fyn plays a role in signaling mediated by the TCR, though its role may be restricted to a subset of mature thymocytes.

Evidence for the involvement of Lck in TCR-mediated signaling

There is substantial genetic and biochemical evidence supporting the involvement of Lck, another member of the Src family of tyrosine kinases, in TCR-mediated signal transduction. Unlike the other ubiquitously expressed members of the Src family of tyrosine kinases, such as Src, Yes and Yrk (reviewed in Ref.12), the expression of Lck is restricted to T lymphocytes, NK cells and some B cells. In fact, Lck was originally identified in a murine T cell lymphoma (LSTRA) in which insertion of a Moloney Murine Leukemia Virus (MoMuLV) long terminal repeat (LTR) within the Lck gene caused a several-fold increase in Lck expression relative to normal lymphocytes (Marth et al., 1985). The first suggestive evidence that Lck was involved in T cell signaling came from the discovery that Lck could directly associate with CD4 and CD8 (13, 14). Quantitative evaluations showed that 50-90% of Lck is associated with CD4 in CD4-positive T lymphocytes, and 10-25% is complexed to CD8α in CD8-positive T cells. These non-covalent interactions are dependent on pairs of cysteine residues located in the unique domain of Lck, and in the cytoplasmic domains of CD4 and CD8α (15, 16). Thus, it was postulated that binding of CD4 or CD8 to MHC molecules allows Lck to be in close juxtaposition to the TCR complex during antigen recognition, making Lck an attractive candidate for a regulated PTK involved in TCR signal transduction. Indeed, coaggregation of CD4 with the TCR enhances both proximal and distal signaling events associated with the engagement of the TCR alone (17, 18). More importantly, CD4 and CD8 mutants unable to associate with Lck have a dramatically diminished ability to enhance antigen responsiveness (19-21), whereas, reconstitution with CD4 molecules capable of associating with Lck restores TCR function (21).

The strongest genetic evidence supporting an essential function for Lck in TCR-mediated signal transduction comes from isolation of mice or T cell lines deficient in Lck. An Lck-deficient variant of the Jurkat leukemic cells (JCaM1.6) and of a cytotoxic T cell clone (CTLL-2), exhibited markedly reduced TCR-induced tyrosine protein phosphorylation, calcium flux and effector functions (22), which were corrected by re-introduction of wild-type Lck (22). Unlike Fyn, disruption of Lck by homologous recombination in the mouse had a profound effect on thymic development, demonstrated by a remarkable decrease in numbers of double and single positive thymocytes and peripheral T cells, while the numbers of progenitor double negative thymocytes appeared to be normal (23). Taken together, these data demonstrate that Lck plays a distinct role in TCR signaling that cannot be compensated by the endogenous expression of Fyn and Yes kinases.

Although the catalytic activity of Lck is clearly involved in TCR-mediated signaling, it is curious that under certain conditions, CD4-associated Lck can enhance TCR-mediated signals by a mechanism independent of its enzymatic activity. This process apparently involves the SH2 domain of Lck, underscoring the importance of this domain in recruiting other tyrosine phosphorylated molecules or protecting certain tyrosine phosphoproteins from dephosphorylation (24). Consistent with the notion that an increase in the catalytic activity of Lck may not be sufficient to initiate a T cell response, there is only a minimal increase in its kinase activity (2-fold) following TCR stimulation in Jurkat cells (25) but none detected in peripheral blood T lymphocytes or in two leukemic T cell lines (9). Additionally, a comparison of the phosphorylation status of Lck in wild type versus CD45-deficient T cell leukemic lines (26, 27) suggests that a large proportion of Lck molecules are basally active. Furthermore, transfection of a constitutively active Lck (F505)

into a CD4 negative T cell hybridoma (BI-141) is not sufficient to initiate the TCR signaling cascade (28).

Thus, the initiating step of the T cell signaling cascade is likely to involve localization of Lck to the TCR complex by co-aggregation of CD4 or CD8, rather than an increase in the enzymatic activity of Lck. Although crosslinking CD4 and CD8 with the TCR can clearly enhance TCR signaling as mentioned above, the isolation of T cell lines that do not express CD4 or CD8 and that are still able to induce a normal TCR-mediated response (29) seems to argue against a simple substrate proximity model. Furthermore, there is indirect evidence suggesting that Lck, independently of CD4 or CD8, may be in close proximity to the TCR complex, albeit in low stoichiometry. First, overexpression of CD4 in a T cell clone inhibited the ability of this cell to be stimulated by anti-TCR mAbs, presumably by "sequestering Lck" away from the TCR complex (30). Second, reconstitution of JCaM1.6 cells with a myristylation mutant of Lck failed to restore TCR signaling (D. Straus, personal communication). Since this Jurkat variant lacks CD8 and expresses only low levels of CD4, this suggests that plasma membrane localization is essential for Lck function in TCR signal transduction. Consistent with this notion, Lck is both myristylated and palmitylated (31, 32) and may be targetted to the plasma membrane in close proximity to the TCR complex, independently of its association with CD4 or CD8. Third, the TCR ζ chain can be co-immunoprecipitated with Lck in a heterologous overexpression system (33, 34) and *in vivo*, albeit in low stoichiometry (35). Thus, it seems that a small amount of Lck could be constitutively associated with the TCR complex, and that another mechanism must exist for regulating the ability of TCR- associated Lck to phosphorylate the ITAMs.

One possible mechanism, based on studies with growth hormones (36), is that engagement of the TCR complex induces a conformational change in the ITAMs, allowing its subsequent phosphorylation by the Lck or Fyn molecules that are weakly associated with the TCR complex. Phosphorylation of the ITAMs, in turn, would lead to the initiation of the signaling cascade as will be discussed later in this chapter. Alternatively, it is possible that engagement of the TCR complex leads to a co-aggregation and activation of the weakly associated cytoplasmic tyrosine kinases. In either case, it is clear that delineating the factors that regulate the activity of Lck, is required before achieving a complete understanding of its role in TCR signal transduction.

Evidence for ZAP-70 involvement in TCR-mediated signaling

A third PTK implicated in proximal TCR signaling is ZAP-70, designated for zeta associated protein. ZAP-70 is a member of the Syk-family of PTKs and structurally distinct from members of the Src family PTKs. It contains two tandemly arranged SH2 (Src-homology 2) domains and a C-terminal kinase domain. However, unlike Lck and Fyn, the Syk-family PTKs are not myristylated and do not contain SH3 (Src-homology 3) domains, or known sites of negative regulation at their C-termini. Although structurally similar to Syk, ZAP-70 is expressed exclusively in thymocytes, peripheral T cells and natural killer cells (NK cells). In contrast, Syk is predominantly expressed in B cells, myeloid cells and thymocytes, but drastically reduced in peripheral T cells (37).

There is strong correlative biochemical evidence for the involvement of ZAP-70 in TCR function. First, ZAP-70 was originally identified and cloned as a 70 kDa tyrosine phosphoprotein that associated with the TCR ζ chain following TCR stimulation (3). Further studies have shown that the CD3ϵ

subunit can also associate with ZAP-70 following TCR stimulation (38, 39). Additionaly, *in vitro* studies utilizing phosphopeptides encompassing the individual ITAMs of CD3ε, δ and γ chains bound to ZAP-70, suggest that ZAP-70 interacts with subunits of the TCR. Second, the ability of the stimulated TCR to initiate a signaling cascade strongly correlates with its ability to associate with ZAP-70 (40, 41). Phosphopeptides that block the interaction of ZAP-70 with the ζ chain can also inhibit T cell signaling events (42). Furthermore, overexpression of the SH2 domains of ZAP-70 can block TCR-mediated signaling (43, 44) by inhibiting the tyrosine phosphorylation of downstream substrates (43). Taken together, the data strongly suggest that binding of ZAP-70 to an ITAM is required for proper TCR-mediated signaling.

Genetic evidence also supports a possible role for ZAP-70 in thymic development and in TCR signal transduction. A group of SCID (severe combined immunodeficiency) patients have been described as having a reduced number of CD8 T cells in the periphery. Although these patients had peripheral CD4 T cells, these cells were unable to proliferate in response to TCR-dependent stimuli. At least three distinct genetic mutations, including a three amino acid insertion and a 13 base pair deletion within the kinase domain of ZAP-70, have been identified. These mutations result in drastically reduced amounts of ZAP-70 protein expression and/or function, demonstrating that ZAP-70 activity is required for proper TCR signaling in peripheral CD4+ T cells and for thymic development (45-47). Consistent with these results, targeted disruption of the *zap70* gene in mice caused a profound block in T cell ontogeny, supporting the notion that ZAP-70 is required for proper TCR-mediated signaling in thymocytes (48). Interestingly, thymocyte cell lines derived from a ZAP-70 deficient patient expressed elevated levels of Syk PTK, suggesting that Syk may be able to compensate for the absence of

ZAP-70 in mediating receptor signaling (49). The targeted disruption of both *zap-70* and of *syk* gene in mice may help elucidate the functional role of Syk in the thymus.

A sequential model for activation of PTK involved in TCR signaling

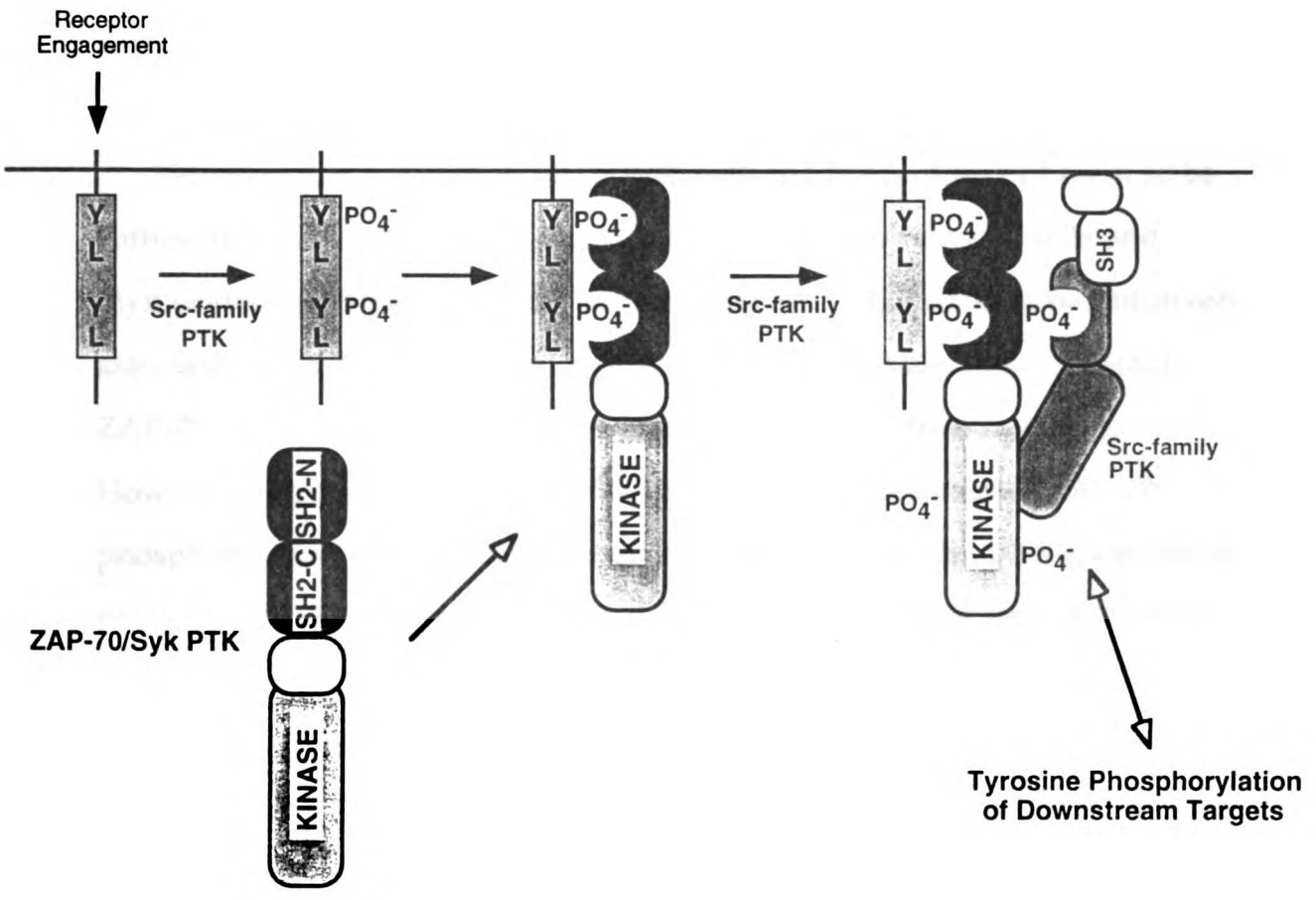
While the active TCR complex is likely to consist of ZAP-70 bound to a phosphorylated ITAM [reviewed above), there is compelling evidence that a member of the Src family of PTKs is required for phosphorylating the ITAM. First, in the Lck deficient mutant JCaM1.6, there is no inducible tyrosine phosphorylation of ITAM and subsequent association with ZAP-70 (38). Second, in a heterologous system, the association of ZAP-70 with the CD8/ζ chimera requires the co-expression of Lck or Fyn (3). Additionally, co-transfection of Lck, but not of ZAP-70, along with the CD8/ζ chimera induces the phosphorylation of the ζ chain (3, 34). Third, Lck but not ZAP-70 can efficiently phosphorylate the TCR ζ chain *in vitro* (A. Chan, unpublished observation). Together, these data indicate that the Src-family PTKs, most notably Lck, plays a role upstream of ZAP-70 in TCR-mediated signal transduction by inducing the phosphorylation of the ITAM (Figure 2).

Binding of ZAP-70 to the phosphorylated ITAM either in the CD3 or ζ chains (38, 50) involves the two SH2 domains of ZAP-70 (34, 40, 51) Although ZAP-70 can bind to peptides in which a single tyrosine in the ITAM is phosphorylated, maximal ZAP-70 binding is achieved when both tyrosines in the ITAM are phosphorylated (52, 53; and reviewed in 54). This notion is further supported by the crystal structure of the two tandem SH2 domains of ZAP-70, which demonstrates that the region between the two ZAP-70 SH2 domains forms a coiled-coil structure (55). This allows the two ZAP-70 SH2

Figure 2. A sequential model for the activation of PTKs associated with the TCR complex.

Interaction of antigen with receptor leads to phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) within the T-cell receptor (TCR) chains by a Src family kinase. ZAP-70 is recruited to the phosphorylated ITAMs by interaction with its SH2 domains. ZAP-70 is phosphorylated by Src family kinases, possibly leading to modulation of its activity.

Antigen Receptor Signal Transduction



domains to simultaneously bind the closely spaced phosphotyrosines (only 6 to 8 residues apart) of the ITAM. Furthermore, spacing between the tandem SH2 domains of ZAP-70 as well as between the two tyrosines of the ITAM, are critical for functional interactions *in vivo* (44). Thus, phosphorylation of the ITAMs on both tyrosines forms the basis for the simultaneous association with the two SH2 domains of ZAP-70 and likely influences the ensuing downstream TCR-mediated effector functions.

However, the association of ZAP-70 with the ITAM does not seem to be sufficient to activate ZAP-70. In freshly isolated lymph node T cells and thymocytes, the basally tyrosine phosphorylated TCR ζ chain is constitutively associated with ZAP-70 but the cells are not constitutively active. Notably, ZAP-70 is only minimally tyrosine phosphorylated in these cells (56). However, engagement of the TCR leads to a marked increase in ZAP-70 phosphorylation and the concomitant induction of cellular phosphotyrosine proteins (57). Furthermore, stimulation with an antagonist peptide induces the phosphorylation of the low molecular weight isoform of TCR ζ chain and its association with ZAP-70 but fails to induce ZAP-70 phosphorylation and T cell activation (58). Thus, there seems to be a causal association between ZAP-70 phosphorylation and its catalytic activity.

There is indeed evidence that ZAP-70 kinase activity can be regulated by its phosphorylation status. Recently, both electrospray ionization mass spectrometry and biochemical approaches have identified several tyrosine residues in ZAP-70 that are phosphorylated *in vivo* following stimulation of the TCR (59, 60). At least three tyrosines (Y492, Y493 and Y292) have been identified as *in vivo* phosphorylation sites upon TCR stimulation. Individually mutating the autophosphorylation sites Y492 and Y493, located within the kinase domain, have beeen shown to increase or inhibit the

kinase activity of ZAP-70, respectively (42, 60). Thus, it seems that tyrosine phosphorylation of ZAP-70 may regulate its kinase activity or serve as a docking site for binding to other proteins.

Consequences of TCR-mediated PTK activation: Activation of the phosphatidylinositol (PI) second messenger pathway

In the case of growth factor receptors, ligand binding induces dimerization, activation of tyrosine kinase activity and autophosphorylation. The tyrosine-phosphorylated regions of the receptor, in turn, function as high affinity binding sites for a series of SH2 domain and PTB (phosphotyrosine binding) domain-containing proteins (61, 62). Both the SH2 and PTB domains are capable of binding to phosphorylated tyrosine residues. However, whereas the PTB domain recognizes phosphotyrosine in the context of amino-terminal residues, the carboxy-terminal residues seem to determine the binding specificity to the SH2 domain (reviewed in Ref. 62).

Unlike the growth factor receptors, it is still unclear how engagement of the TCR and activation of the relevant cytoplasmic PTKs lead to the subsequent phosphorylation of cellular proteins, partly because it has been difficult to isolate the *in vivo* substrates for these kinases. Even so, there is an increasing amount of evidence demonstrating that tyrosine phosphorylation of cellular proteins such PLC γ1 (63-65), Vav (66), SLP-76 (67), pp36-38 (68-70), ZAP-70 (3), Cbl (71), Shc (72), MAPK (73), Grb2 associated p116 (69) and ezrin (74) may regulate ensuing cellular interactions and/or increases in catalytic activity.

In the case of PLC γ1, phosphorylation on two critical tyrosine residues has been shown to be necessary for *in vivo* function and for enhancing its catalytic activity *in vitro* (75, 76). Tyrosine phosphorylation of PLC γ1

increases its affinity towards the profilin bound phosphatidylinositol-4,5-biphosphate (PIP_2)(77) and leads to PIP_2 hydrolysis into the two second messengers diacylglycerol (DAG) and inositol-1,4,5-triphosphate ($InsP_3$) (78, 79). InsP3 binds to the specialized homotetrameric InsP3 receptor that spans the endoplasmic reticular (ER) membrane and triggers release of $[Ca^{2+}]i$ from the ER (80).

In T cells, the increase in intracellular calcium occurs in two phases, an initial peak and a plateau phase persisting for several hours. The sustained increase is thought to be required for many cellular responses, including a commitment to interleukin-2 production (IL-2) (81, 82). The release of calcium from intracellular stores and the ER accounts for the initial peak, whereas the plateau phase is thought to be mediated by the influx of extracellular calcium across the plasma membrane via capacitative entry mechanisms (reviewed in Ref. 79). The capacitative entry mechanism is thought to play a key role in replenishing the intracellular calcium stores and generating calcium waves (reviewed in Ref. 80). Although several second messengers have been proposed as a mechanism by which depleted stores signal the $[Ca^{2+}]i$ entry channel (reviewed in Ref. 80), an interesting candidate is the $[Ca^{2+}]i$ influx factor (CIF), a diffusable phosphorylated intermediate initially isolated from Jurkat T cells stimulated with phytohemagglutinin (PHA) (83). More recently, several mutant Jurkat T cell defective in the capacitance entry mechanism have been isolated (84, 85). Characterization of this entry mechanism should greatly enhance our understanding of how calcium concentrations are regulated in T cells.

The other second messenger produced as a result of PLC $\gamma 1$ activation is DAG, a lipid moiety that activates the PKC family of serine/threonine kinases by increasing the affinity of this kinase for $[Ca^{2+}]i$ and phospholipid

(reviewed in Ref. 86). Although phosphatidylinositol hydrolysis generates an initial peak of DAG, hydrolysis of other phospholipids, such as phosphatidylcholine, produces diacylglycerol at a relatively later phase in cellular responses. Generation of diacylglycerol is thought to be critical for sustaining PKC activation (reviewed in Ref. 87). There are multiple isozymes of PKC expressed in T cells (88, 89). Interestingly, transfection of activated forms of PKC-epsilon and, to a lesser extent PKC-alpha but not PKC-zeta, can regulate the transcription factors AP-1 and nuclear factor of activated T cells (NF-AT), suggesting that these isoforms may have distinct functions.

The importance of increasing the concentration of intracellular calcium and activating PKC in T cells is underscored by the effects of calcium ionophores and phorbol esters. More specifically, calcium inonophores and phorbol esters can mimic the distal events mediated by TCR engagement (89; reviewed in Ref. 90). Consistent with this, inhibition of calcium increases with chelators of extracellular calcium or inhibition of PKC activation by PKC inhibitors block T cell activation (91). Furthermore, constitutively active forms of calcineurin or PKC also induce IL-2 production (92, 93). Thus, understanding how the mobilization of $[Ca^{2+}]i$ and activation of PKC contribute to the subsequent cellular events is of ultimate importance.

It is likely that the increase in $[Ca^{2+}]i$ influences calmodulin-dependent events (94) Studies have revealed that calcineurin (PP2B), a calcium- and calmodulin- activated serine/threonine phosphatase, is a target of cyclophilin-cyclosporin A and FK506-FKBP complexes (reviewed in Ref. 95, 96). Since then, several studies have linked these binding events with functional properties of T lymphocytes and, in so doing, have confirmed that calcineurin is a necessary and late-acting cytoplasmic mediator of the signal arising from the TCR (97, 93). Thus, the increase in intracellular calcium is

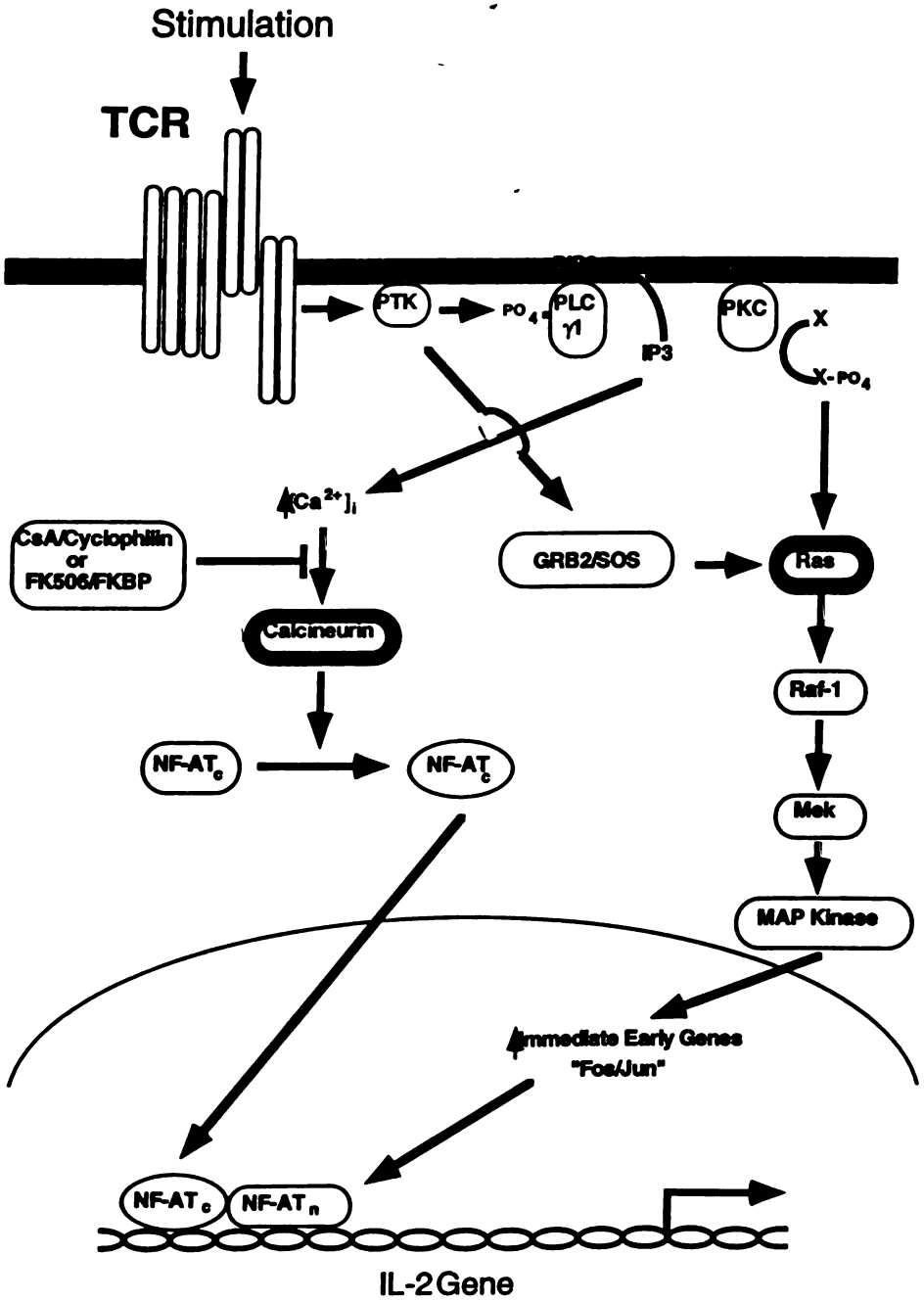
thought to activate the phosphatase activity of calcineurin, which then dephosphorylates NFATp/c (nuclear factor of activated T cells) allowing it to translocate from the cytoplasm to the nucleus (reviewed in Ref. 98). Although one of the candidate NFATp/c proteins is a substrate for calcineurin *in vitro* (99), it remains to be demonstrated that the dephosphorylation of this NFATp/c indeed regulates its translocation to the nucleus. NFATp/c, in conjunction with AP1 family members, c-fos and c-jun, form the nuclear NFAT component that regulates the promoters of cytokine genes such as IL-2. The full activation of AP1 is not only dependent on new protein synthesis but recent studies suggest that it may require both calcium- and PKC-dependent pathways (100, 101).

The identification of PKC substrates is of considerable interest since a number of cellular proteins are phosphorylated on serine and threonine residues following treatment with phorbol esters. Among these cellular substrates are the CD3γ and δ chains, CD4, the IL-2 receptor, MAP kinase and the Raf-1 kinase (reviewed in Ref. 90). Whether these proteins are direct substrates for PKC, or are substrates for PKC regulated kinases remains to be determined. Notably, treatment of T cells with phorbol ester is sufficient to activate Ras, a small molecular weight GTP-binding protein (102). Ras is in an active conformation when it is bound with GTP, and inactive when bound to GDP. The conversion between the GDP and GTP bound states is catalyzed by nucleotide exchange factors, while the hydrolysis of GTP to GDP is facilitated by GAP (GTPase activating protein) (reviewed in Ref. 103). Thus, the finding that phorbol esters, by activating PKC, could inhibit the activity of GAP and activate Ras, provided the first clue that Ras participates in T cell signaling.

The importance of Ras in T cell signaling is underscored by studies demonstrating that constitutively active Ras is capable of activating the transcriptional factors such as AP1 and of synergizing with ionomycin to activate the IL-2 gene (104, 105). Although an increasing number of Ras effectors have been identified (106), including PI3K (107), Rac (108, 109) and RasGAP (110), the transmission of signal from Ras to the nucleus is proposed to involve the serine/threonine protein kinase Raf. Activated Ras binds to the N-terminal domain of Raf, recruiting it from the cytosol to the plasma membrane where it is tyrosine phosphorylated and activated (111). Although there is suggestive evidence that Raf is additionally modified in order to be fully activated, the nature of this modification is not yet clear (112). Once Raf is activated, it phosphorylates and activates the dual specificity serine/threonine and tyrosine kinase MEK1/MEK2 or MAPKK (MAP Kinase Kinase), which in turn, phosphorylates and activates MAPK (ERK1 and 2). A fraction of the activated ERK1/ERK2 population translocates into the nucleus and phosphorylates transcription factors, such as TCF/Elk1 (reviewed in Ref. 111). Phosphorylation of Elk1 by ERK1/2 seem to be obligatory for the transcription of c-fos gene, a component of the AP1 transcription factor required for activation of NFAT (111).

The other component of AP1 is c-jun. It is now known that phosphorylation of c-jun both positively and negatively regulates its transcriptional activity (113). Recently, cloning of the kinase responsible for phosphorylating c-jun, JNK1, has demonstrated that it is a member of the family of MAPK. However, JNK seems to be differentially regulated from MAP kinases, in that its activation in T cells requires co-stimulation of both calcium- and PKC-dependent pathway (101, 114). This working model for TCR-mediated signaling is summarized in Figure 3. Interestingly, JNK can

Figure 3. A working model of TCR-mediated signal transduction pathways. Engagement of the TCR leads to the tyrosine phosphorylation of a number of cellular substrates, including PLC γ1. Tyrosine phosphorylation of PLC γ1 leads to the ativation of the phosphatidylinositol pathway, which in concert with other signaling pathways culminate in lymphokine gene transcription. Arrows do not necessarily indicate direct interactions.


Stimulation
TCR
PTK
PO4
PLC γ1
IP3
PKC
X
X-PO4
[Ca2+]i
CsA/Cyclophilin or FK506/FKBP
Calcineurin
GRB2/SOS
Ras
Raf-1
Mek
MAP Kinase
NF-ATc
NF-ATc
Immediate Early Genes "Fos/Jun"
NF-ATc
NF-ATn
IL-2 Gene

also be activated synergistically by the TCR and the costimulatory receptor CD28 (101).

How the TCR activates Ras is not clear. As previously mentioned, one mechanism involves the activation of PKC and the concomitant decrease in GAP activity. However, inhibition of PKC function does not completely inhibit Ras activation by TCR stimulation (115), suggesting that there is a PKC independent pathway connecting the TCR to Ras. Furthermore, this PKC independent pathway linking the TCR complex to Ras involves a tyrosine kinase since this pathway could be inhibited with the use of tyrosine kinase inhibitors (115). However, the proteins involved in this pathway have not been identified yet.

Considerable progress has been made in understanding the activation of Ras by receptor PTKs. Genetic studies with the epidermal growth factor receptor (EGFR) have shown that Grb2 (Sem5 in *Caenorhabditis elegans* and Drk in *Drosophila melanogaster*), a highly conserved adaptor protein that has a single SH2 domain flanked by two SH3 domains, is involved in coupling receptor PTKs to Ras activation (reviewed in Ref. 116). Biochemical dissection of the pathway revealed that Grb2 is responsible for linking the receptor PTKs to a guanine nucleotide exchange factor SOS, enabling the tyrosine kinase to modulate Ras activity (116). In addition to coupling receptor PTKs to the Ras pathway, there is suggestive evidence that Grb2 may also link cytoplasmic PTKs to the Ras pathway via its association with the proto-oncogene Shc (117). Given that the TCR complex can associate with cytoplasmic PTK (discussed above), it is likely that these proteins may play a role in linking the TCR complex to Ras. The experiments described in this thesis attempt to address this issue.

CHAPTER 2

REGULATION OF LCK BY CD45

Summary

Understanding the function and regulation of Lck, a lymphocyte specific PTK, is of great importance in TCR-mediated signal transduction. An "intramolecular model" has been proposed to explain how the Src family PTKs are negatively regulated. According to this model, phosphorylation of the carboxy-terminal tyrosine (Y505 in Lck), enables it to associate with its own SH2 domain, rendering the kinase inactive and/or unable to phosphorylate key substrates. The implication of this model as it pertains to Lck, is that dephosphorylation of Y505 by a phosphatase, such as CD45, would free the SH2 domain of Lck and allow it to bind to other phosphoproteins.

To address whether this intramolecular model of negative regulation applied to Lck *in vivo*, two sets of leukemic T cell lines and their CD45-deficient mutants, as well as phosphopeptides encompassing the Y505 of Lck were used. The prediction of this model is that in CD45 deficient cells, the Y505 of Lck will be hyperphosphorylated and bind to its own SH2 domain, and the SH2 domain of Lck would be unavailable to bind to an exogenous Y505-containing phosphopeptide. However, in cells containing CD45, the Y505 of Lck will not be hyperphosphorylated and the SH2 domain would be available to bind to the exogenous Y505-containing phosphopeptide. The results described herein are consistent with the intramolecular model. Interestingly, the binding of the SH2 domain of Fyn, another member of the Src family PTKs, did not seem to be regulated by CD45 to the same extent. These findings not only increase our understanding of how Lck is regulated *in vivo*, but also provide insight into the molecular basis of how CD45 functions as a positive-regulator in TCR signaling.

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CD45 specifically modulates binding of Lck to a phosphopeptide encompassing the negative regulatory tyrosine of Lck

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CD45 is a tyrosine phosphatase expressed in all hematopoietic cells which is important for signal transduction through the T cell antigen receptor (TCR). Studies using CD45-deficient cells have revealed that Lck, a tyrosine kinase thought to be essential for TCR signaling, is hyperphosphorylated on Y505 in the absence of CD45. This site of tyrosine phosphorylation negatively regulates the function of the Src family of kinases. Here we provide evidence that CD45 can modulate the binding of the Lck to an 11 amino acid tyrosine phosphorylated peptide containing the carboxy-terminus of Lck (lckP). Significantly, CD45 did not influence the binding of Fyn, PLCγ1, GAP and Vav to the same phosphopeptide. Lck protein which bound the peptide was dephosphorylated on Y505 and consisted of only 5–10% of the total cellular Lck. Interestingly, there was a marked increase in binding 15–30 min after CD4 or TCR cross-linking. Taken together, our data suggest that CD45 specifically modulates the conformation of Lck in a manner consistent with the intramolecular model of regulation of Src-like kinases.

Key words: binding/CD45/Lck/negative regulation/tyrosine phosphorylation

Introduction

CD45 is a plasma membrane tyrosine phosphatase expressed on all hematopoietic cells, except mature erythrocytes. Alternative splicing of exons 4, 5 or 6 can generate eight isoforms of CD45 with different extracellular domains that can be differentially expressed in tissues or upon activation (for a review, see Trowbridge, 1991). CD45 is thought to play a critical role in antigen receptor-induced responses of T and B cells (Pingel and Thomas, 1989; Koretzky *et al.*, 1990, 1991, 1992; Justement *et al.*, 1991; Weaver *et al.*, 1991). Helper and cytotoxic mouse T cell lines which are deficient in CD45 expression have impaired ability to respond to antigen (Pingel and Thomas, 1989; Weaver *et al.*, 1991). Studies using CD45-deficient human T cell leukemic lines have shown that CD45 is required for antibody-mediated coupling of the T cell antigen receptor (TCR) to the phosphatidylinositol second messenger pathway (Koretzky *et al.*, 1990), as well as for the increase in tyrosine phosphorylated proteins induced following TCR stimulation (Koretzky *et al.*, 1991). Although it is clear that CD45 plays a role in TCR signaling, the mechanism by which it does so has not been elucidated.

Lck, a lymphocyte specific member of the Src family of tyrosine kinases (Src, Fyn, Blk, Yes, Lyn, Fgr and Hck) (Veillette and Bolen, 1989; Dymecki *et al.*, 1990), is expressed in all T cells and in some B cells (Marth *et al.*, 1985). The amino-terminal region of Lck non-covalently associates with the cytoplasmic tail of the co-receptor molecules CD4 and CD8 (Veillette *et al.*, 1988b; Turner *et al.*, 1990). Cross-linking of CD4 induces an increase in Lck tyrosine kinase activity, as determined by an increase in Lck autophosphorylation at Y394, induction of several tyrosine phosphorylated proteins as well as phosphorylation of exogenous substrates (Marth *et al.*, 1985; Veillette *et al.*, 1989a,b; Veillette and Ratcliffe, 1991). Simultaneous engagement of CD4 molecules with the TCR complex has been shown to enhance signaling through the TCR (Ledbetter *et al.*, 1988), an effect thought to be mediated by Lck (Veillette *et al.*, 1989a; Abraham *et al.*, 1991; Glaichenhaus *et al.*, 1991).

Although the signaling defect in CD45-deficient cells has not been determined, increasing evidence implicates Lck as a key substrate for CD45. In studies using CD45-deficient T lymphoma cells, the carboxy-terminal tyrosine of Lck (Y505) was hyperphosphorylated relative to wild-type cells (Ostergaard *et al.*, 1989), suggesting that CD45 can dephosphorylate this residue *in vivo*. Furthermore, Lck can be co-precipitated with CD45 (Schraven *et al.*, 1991) and its kinase activity is increased when incubated with CD45 immunoprecipitates (Mustelin *et al.*, 1989; Ostergaard and Trowbridge, 1990). These findings are significant since phosphorylation of Y505 may suppress the oncogenic potential and kinase activity of Lck. Specifically, mutation of Y505 of Lck and expression of this mutant protein increases the amount of cellular tyrosine phosphoproteins and transforms 3T3 cells (Amrein and Sefton, 1988; Marth *et al.*, 1988).

Because of the documented transforming potential of the Src family of kinases, considerable effort has been made to understand the mechanism by which these kinases are negatively regulated (Veillette and Bolen, 1989; Nada *et al.*, 1991; and references therein). Transformation assays in fibroblasts have indicated that phosphorylation of the carboxy-terminal tyrosine decreases the kinase activity of Src (Carwright *et al.*, 1987; Kmiecik and Shalloway, 1987; Hirai and Varmus, 1990a,b). Recent studies have also demonstrated that point mutations within the SH2 (Src-homology region 2) domain affect the tyrosine kinase activity of Src (Hirai and Varmus, 1990a; O'Brien *et al.*, 1990). Because SH2 domains are known to bind tyrosine phosphorylated proteins (reviewed in Koch *et al.*, 1991), an 'intramolecular model' has been proposed to explain how point mutations within the SH2 domain could affect the tyrosine kinase activity of Src. According to this 'intramolecular model', phosphorylation of the carboxy-terminal

tyrosine enables it to associate with its own SH2 domain, rendering the kinase inactive and/or unable to phosphorylate key substrates (Cantley *et al.*, 1991; Koch *et al.*, 1991). Dephosphorylation of this carboxy-terminal tyrosine increases the protein tyrosine kinase activity and the oncogenic potential of the kinase. Consistent with this 'intramolecular model', structural differences between c-src and F527c-src have been reported (MacAuley and Cooper, 1989).

We have studied the intramolecular model of negative regulation as it applies to Lck using two sets of leukemic T cell lines and their CD45-deficient mutants (Koretzky *et al.*, 1991, 1992). Since Lck isolated from CD45-deficient cells is expected to be hyperphosphorylated on Y505 (Ostergaard *et al.*, 1989), the intramolecular model predicts that the phosphorylated Y505 would bind to its own SH2 domain, preventing a phosphotyrosine peptide containing Y505 (lckP peptide) from binding to the SH2 domain. On the other hand, Lck from CD45+ cells should not be hyperphosphorylated on Y505 and, thus, its SH2 domain should be available to bind such a phosphopeptide. The data in this paper are consistent with this model and demonstrate that CD45 expression can specifically modulate binding of Lck, but not of Fyn, PLCγ1, GAP and Vav to the phosphopeptide.

Results

Lck from Jurkat but not from J45.01 lysate preferentially binds the lckP peptide

We synthesized an 11 amino acid (aa) tyrosine phosphorylated (lckP) and the corresponding non-phosphorylated (lck) peptides encompassing the carboxy-terminal tyrosine of Lck. These peptides were covalently coupled to Sepharose beads and incubated with equivalent amount of lysate from Jurkat (CD45+) or J45.01 (CD45−) cells. Lck from the Jurkat lysate bound to the lckP peptide whereas Lck from J45.01 cells did not bind efficiently (Figure 1A, lanes 5 and 6), even though the cells had equivalent amounts of Lck (Figure 1A, lanes 1 and 2). Binding to the peptide was dependent on its phosphorylation as the non-phosphorylated peptide did not bind Lck from either cell (Figure 1A, lanes 7 and 8). The two Lck bands observed with Jurkat cells were not consistently observed and may correspond to differentially phosphorylated forms of Lck, the upper band being hyperphosphorylated on ser/thr residues (Veillette *et al.*, 1988a; Ramer *et al.*, 1991). Similar phosphopeptides containing the tyrosine near the carboxy-terminus of Src (srcP) and Fyn (Figure 1A, lanes 3 and 4, and data not shown, respectively) were also able to efficiently bind Lck from the CD45+ Jurkat cells, but not from the CD45-deficient cells (J45.01). In contrast, a very different phosphopeptide spanning the autophosphorylation site of Lck (AutoP) did not bind Lck from Jurkat cells (Figure 1B). This indicates that Lck binding to the lckP peptide is not only dependent on the presence of a phosphorylated tyrosine residue, but also on the sequences immediately surrounding that tyrosine. It is probable that the autophosphorylation site (Y394) and the negative regulatory tyrosine (Y505) have distinct functions and will bind to distinct sets of proteins *in vivo*.

To confirm that the binding of Lck is dependent on the presence of CD45, we tested whether reconstitution of CD45 on J45.01 cells could restore Lck binding to the lckP peptide. J45.LB3 and J45.LA2 are derived from J45.01 cells which have been stably transfected with the 180 kDa isoform of CD45, but still only express 20–30% of the level of CD45 on Jurkat (Koretzky *et al.*, 1992). Equal amounts of lysate from Jurkat, J45.01 and the CD45+ J45.01 transfectants were incubated with the lckP peptide and the binding of Lck to the peptide was assessed. There was an increase in Lck bound to the lckP in both of the independently CD45 reconstituted J45.01 cells (Figure 2A, compare lanes 2, 3 and 4). The lower amount of Lck bound from the CD45 reconstituted J45.01 cell compared to Jurkat (Figure 2A, lanes 1, 3 and 4) is likely to be due to lower surface expression of CD45 in the reconstituted cells (data not shown). The dependency on the presence of CD45 for Lck binding to the lckP peptide was also observed in HPB1.1 and HPB.053, independent CD45+ and CD45-deficient T cell leukemic lines, respectively (Figure 2B). When lysates of CD45+ and CD45− cells were mixed, Lck still bound to the LckP peptide (data not shown), suggesting that CD45− cells do not contain an inhibitory protein that prevents Lck from binding to the lckP peptide.

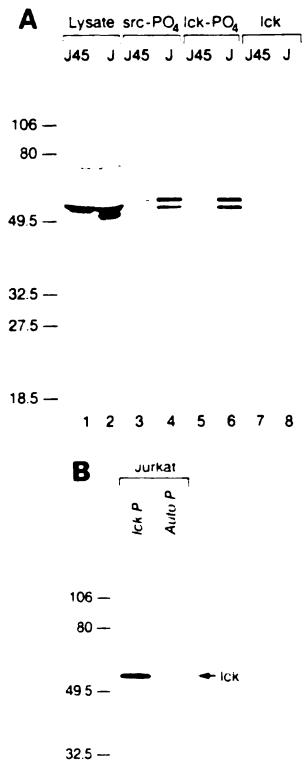

Fig. 1. (**A**) Precipitation of Lck from Jurkat and J45.01 cells with Lck or Src phosphopeptides. Jurkat (J; lanes 2, 4, 6 and 8) or J45.01 (J45; 1, 3, 5 and 7) lysates were precipitated with the lckP peptide (lanes 5 and 6), srcP peptide (lanes 3 and 4) or lckP peptide (lanes 7 and 8) coupled to Sepharose beads. Bound proteins were eluted, separated by 8.5% SDS–PAGE under reducing conditions and transferred to nitrocellulose for immunoblotting with an anti-Lck mAb. (**B**) Precipitation of Lck with lckP or AutoP peptides. Jurkat lysates were incubated with lckP or AutoP peptides. Bound Lck was eluted with reduced sample buffer and visualized by immunoblotting with the Lck-specific mAb. Mobilities of mol. wt standards are indicated in kilodaltons.

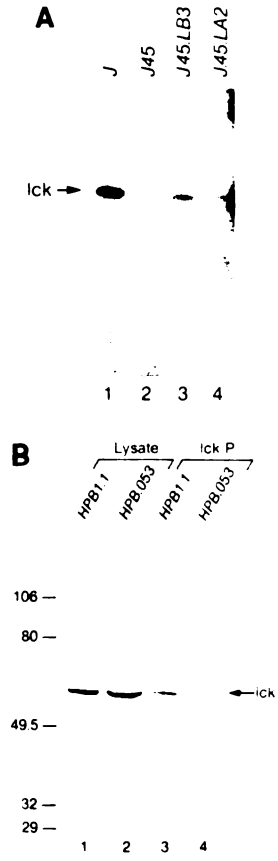

Fig. 2. (A) Precipitation of Lck from J45.01 stable transfectants. Lysates from Jurkat (J), J45.01 (J45) and two stably CD45 transfected J45 cells (J.45.LB3 and J.45.LA2) were precipitated with the lckP peptide. Eluted proteins were detected by immunoblotting with an anti-Lck mAb. (B) Precipitation of Lck from HPB.1.1 (CD45+) and HPB.053 (CD45−) with lckP peptide. HPB.1.1 (lanes 1 and 3) and HPB.053 (lanes 2 and 4) lysates were prepared and separated by 8.5% SDS−PAGE (lanes 1 and 2) or precipitated with lckP peptide coupled to Sepharose beads (lanes 3 and 4). The bound proteins were eluted and detected by immunoblotting with an anti-Lck mAb. Mol. wt standards are indicated in kilodaltons.

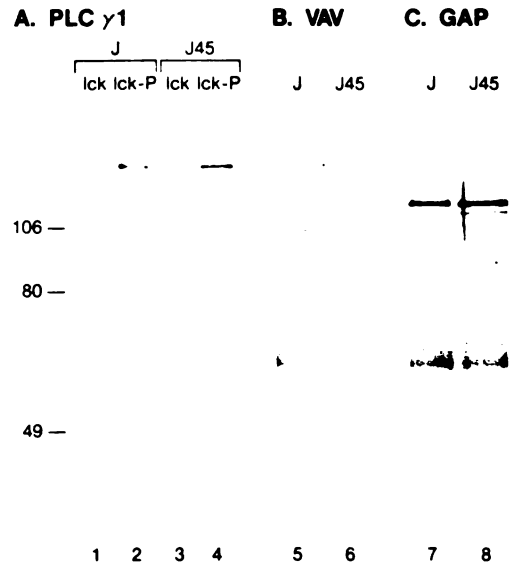

Fig. 3. Precipitation of PLCγ1, Vav and GAP with lckP peptide. Lysates from Jurkat (J) or J45.01 (J45) cells were incubated with lckP or Lck peptides coupled to Sepharose beads. Bound proteins were eluted and separated by SDS−PAGE and blotted with (A) anti-PLCγ1 mAb, (B) anti-Vav antiserum, (C) anti-GAP antiserum. Mobilities of standards (in kilodaltons) are shown on the left. In panel A, PLCγ1 corresponds to the 150 kDa band; in panel B, Vav corresponds to the 95 kDa band; in panel C, GAP corresponds to the 110 kDa band.

Proteins which have SH2 domain can also bind to the lckP peptide

Numerous studies have demonstrated that SH2 domains are able to bind to tyrosine phosphorylated proteins (reviewed in Cantley *et al.*, 1991; Koch *et al.*, 1991). Therefore, we determined whether proteins which contain at least one SH2 domain, namely GAP, Vav and PLCγ1, expressed in the wild-type and CD45-deficient cells, could also bind the lckP peptide. Equivalent amounts of lysate from Jurkat and J45.01 were incubated with the lckP peptide, the bound proteins were eluted, resolved in an SDS−polyacrylamide gel and blotted for PLCγ1 (Figure 3A, lanes 1−4), Vav (Figure 3B, lanes 5 and 6) and GAP (Figure 3C, lanes 7 and 8). All three proteins that contain one or more SH2 domains bound the lckP peptide and binding was dependent on the tyrosine phosphorylation of the peptide (Figure 3A, lanes 1 and 3, and data not shown). However, unlike Lck, these proteins bound equally well whether from Jurkat or J45.01 lysates. Since Jurkat and J45.01 contain equivalent amounts of cytoplasmic PLCγ1, GAP and Vav (data not shown), these results indicate that although PLCγ1, GAP and Vav contain an SH2 domain, they differ from Lck in that they do not depend on the presence of CD45 to bind the lckP peptide. Although we have not directly shown that binding to the lckP peptide is mediated by the SH2 domain, we found that binding to F527 c-src to the lckP peptide is dependent on the SH2, but not SH3 domain, using lysates from 3T3 cells expressing various Src mutants (data not shown). This is also consistent with a previous study using the phosphopeptide containing the carboxy-terminus of c-src which bound F527c-src (Roussel *et al.*, 1991).

Binding of Fyn to the lckP peptide does not depend on CD45

We have also analyzed whether Fyn, another member of the Src family of tyrosine kinases expressed in T cells, could bind the lckP peptide. Fyn has been co-immunoprecipitated with the TCR complex (Samelson *et al.*, 1990), making it a possible candidate for a TCR-activated tyrosine kinase (Cooke *et al.*, 1991). Jurkat and J45.01 lysates were prepared and incubated with the lckP peptide. Bound proteins were eluted and resolved in an 8.5% SDS−PAGE and blotted for the presence of Fyn. Fyn bound equally well to the lckP peptide whether from Jurkat or J45.01 lysates (Figure 4A). This result was also confirmed in the independent CD45+ and CD45− cells, HPB.1.1 and HPB.053 cells (Figure 4B). Thus, Fyn differs from Lck in its ability to bind to lckP peptide independently of CD45.

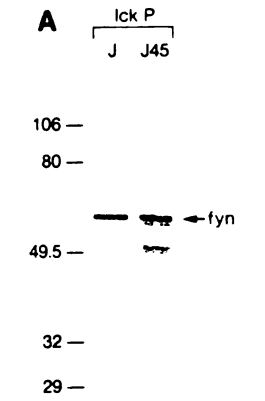

Fig. 4. (**A**) Precipitation of Fyn with lckP peptide from Jurkat and J45.01 cells. Jurkat (J) and J45.01 (J45) lysates were prepared and incubated with the lckP peptide coupled to Sepharose beads. The bound proteins were separated by SDS–PAGE and transferred to nitrocellulose for immunoblotting with an anti-Fyn antiserum. (**B**) HPB.1.1 (CD45+) or HPB.053 (CD45–) lysates were prepared (lanes 1 and 2) or precipitated with lckP peptide coupled to Sepharose beads (lanes 3 and 4). The eluted proteins were separated by SDS–PAGE and processed as in (A). Mobilities of mol. wt standards are indicated in kilodaltons.

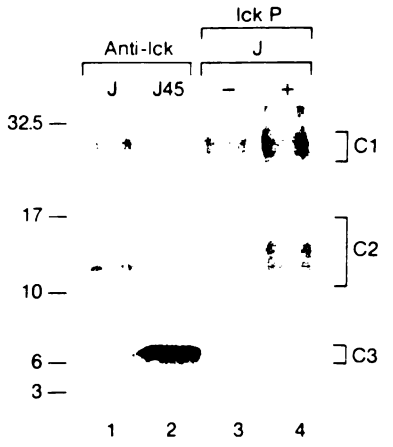

Fig. 5. CNBr cleavage of ^{32}P-labeled Lck proteins. Jurkat (J) and J45.01 (J45) were cultured in [^{32}P]orthophosphorous and the phospholabeled Lck polypeptides were immunoprecipitated with either anti-Lck heteroserum or the LckP peptide and digested with CNBr. The products of these reactions were resolved on a tricine–glycine gel. **Lanes 1 and 2**: Lck precipitated from Jurkat or J45 cells with anti-Lck heteroserum, respectively. **Lanes 3 and 4**: Lck precipitated with the lckP peptide from unstimulated Jurkat (–) or after 30 min of stimulation with anti-TCR mAb (+), respectively. C1 corresponds to fragments containing unidentified phosphoserines/phosphothreonines in the amino-terminal portion of the molecule. C2 contains the autophosphorylation site (Y394). C3 contains the negative regulatory site (Y505).

Only Lck which is dephosphorylated at Y505 binds to the lckP peptide

As described previously, the intramolecular model predicts that only Lck dephosphorylated on Y505 would be able to bind to lckP peptide. In order to examine the phosphorylation status of the Lck that binds to the lckP peptide, Jurkat and J45.01 cells were cultured in the presence of [^{32}P]orthophosphorous to label endogenous phosphoproteins. Lysates were prepared and precipitated with either anti-Lck antibody or the lckP peptide. The bound Lck was eluted, run on SDS–polyacrylamide gel and transferred to nylon membrane, followed by CNBr cleavage. The products of the digestion were run in a tricine–glycine gel used for resolving small peptide fragments. CNBr cleavage of Lck generated the three characteristic groups of fragments containing different phosphorylation sites: C1 contains unidentified ser/thr phosphorylation sites; C2 contains Y394, the autophosphorylation site; C3 contains Y505, the negative regulatory site (Abraham and Veillette, 1990; Luo *et al.*, 1990; Veillette and Fournel, 1990). As previous studies have shown with murine CD45-negative lymphomas (Ostergaard *et al.*, 1989), Lck isolated from J45.01 with anti-Lck antiserum was found to be hyperphosphorylated on Y505 relative to Jurkat cells (Figure 5, lanes 1 and 2), suggesting that the majority of the lck from Jurkat is dephosphorylated at this residue. Moreover, consistent with the predictions, the Lck from Jurkat cells which bound the lckP peptide was completely dephosphorylated at Y505 (lane 3). Although we have obtained both partial and complete CNBr cleavage of the Lck that bound to the lckP peptide, no band at C3 was observed in three independent experiments. Stimulation with anti-TCR did not change the phosphorylation status at this residue of the Lck bound to the peptide (Lane 4). Interestingly, a small fraction of the total Lck from Jurkat was phosphorylated on Y505 (lane 1), demonstrating that there are two pools of Lck in Jurkat cells: one which is dephosphorylated on Y505 and binds the lckP peptide, and another which is phosphorylated on this residue and is unable to bind to the peptide.

Only a small fraction of Lck is dephosphorylated at Y505 and able to bind to the lckP peptide in CD45+ cells

From the preceding experiment, it appears that unstimulated Jurkat cells contain a pool of Lck that is both constitutively dephosphorylated on Y505 and able to bind the lckP peptide. Based on the transfection studies with F505 mutants of Lck in fibroblasts (Carwright *et al.*, 1987; Hirai and Varmus, 1990a), this should represent an active or activatable pool of Lck kinase. Therefore, it was of interest to quantitate the relative amount of Lck that is able to bind the lckP peptide.

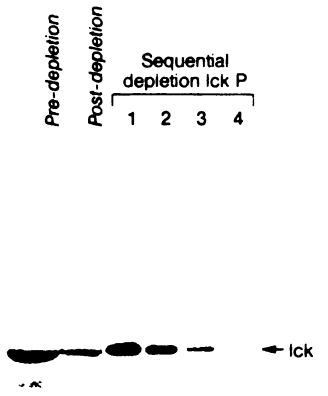

Fig. 6. Sequential depletion study with lckP peptide. Jurkat cells (50×10^6) were lysed and incubated sequentially with the lckP peptide coupled to beads. One-twentieth of the pre- and post-depletion lysates, as well as the total bead eluates, were detected quantitatively by Western blotting for Lck with anti-Lck mAb followed by ^{125}I-labeled goat anti-mouse IgG.

For such experiments, we performed several rounds of sequential depletion with the lckP peptide and quantitated the total amount of bound and unbound Lck. As shown in Figure 6 (lanes 1–4), decreasing amounts of Lck bound the lckP peptide following sequential rounds of adsorption with the lckP peptide coupled to Sepharose beads, indicating that this Lck pool could be depleted. When the pre-depletion and post-depletion lysates (lanes 1 and 2) were normalized for protein concentration, we estimated that only 5–9% of the total Lck in CD45+ Jurkat cells was able to bind to the peptide. Thus, although the majority of the lck in Jurkat cells seems to be dephosphorylated on Y505 (Figure 5), only a relatively small proportion is able to bind the lckP peptide.

Cross-linking CD4 or TCR induces an increase in the amount of Lck bound to the lckP peptide

It has been previously shown that cross-linking CD4 with anti-CD4 monoclonal antibodies (mAbs) can lead to an increase in kinase activity of Lck (Veillette *et al.*, 1989a,b). However, no substantial increase in kinase activity has been observed when the TCR or CD3 is cross-linked (Veillette *et al.*, 1989b; our unpublished data). Since an increase in kinase activity has been correlated with the dephosphorylation of Y505 in Lck, we expected to see an increase in the amount of Lck bound to the lckP with CD4, but not with TCR cross-linking. To our surprise, both anti-CD4 and anti-TCR cross-linking in Jurkat cells induced a significant increase in the amount of Lck bound to the lckP peptide (Figure 7). Interestingly, this increase occurred fairly late (15–30 min). Most tyrosine phosphorylated proteins peak 1–2 min after stimulation in Jurkat cells (Koretzky *et al.*, 1991). The decreased mobility of a portion of Lck that binds to the lckP following TCR or CD4 stimulation is likely to reflect the ser/thr phosphorylation of Lck (Veillette *et al.*, 1988a; Ramer *et al.*, 1991). This peptide binding experiment

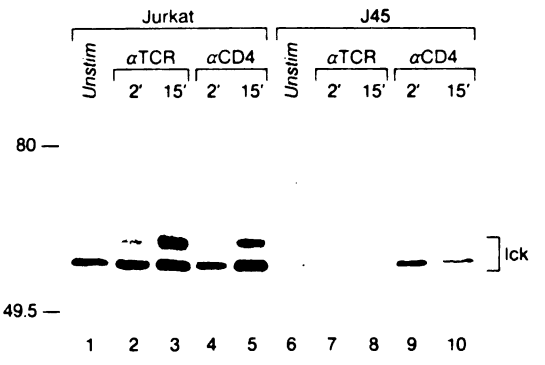

Fig. 7. Cross-linking TCR or CD4 from Jurkat and J45.01 cells. Jurkat (J) and J45.01 (J45) cells were stimulated with either the anti-TCR mAb C305 (lanes 2, 3, 7 and 8) or with anti-CD4 mAb plus rabbit anti-mouse Ig (lanes 4, 5, 8 and 10). Lysates were prepared and incubated with the lckP bound to Sepharose beads. Bound proteins were eluted, analyzed by SDS–PAGE and blotted with anti-Lck mAb. **Lanes 1 and 6**: unstimulated cells; **lanes 2, 4, 7 and 9**: 2 min stimulation; **lanes 3, 5, 8 and 10**: 15 min stimulation..

was confirmed in more detailed kinetic studies (data not shown). In contrast to the results observed with Jurkat cells, no increase in Lck binding to the lckP peptide was seen when the TCR complex on J45.01 was stimulated. A small increase was observed when CD4 was cross-linked. These results indicate that the two pools of Lck can be modulated with regard to peptide binding by TCR or CD4 stimulation, but modulation appears to occur late and is attenuated in CD45-deficient cells.

Discussion

The studies reported here show that CD45 can modulate the binding of Lck to a phosphotyrosine peptide encompassing the Lck negative regulatory tyrosine (Y505). Using this phosphopeptide containing Y505 (lckP), we were able to precipitate Lck more efficiently from CD45+ cells than from CD45-deficient cells derived from two independent T cell leukemic lines. Reconstitution of CD45 expression, albeit at lower levels, in J45.01 cells partially restored Lck binding. This suggests that dephosphorylation of Y505 by CD45 may change the conformation of the kinase and render it able to bind to the lckP peptide.

There is ample evidence in the literature that SH2 domains bind tyrosine phosphorylated proteins (reviewed in Cantley *et al.*, 1991; Koch *et al.*, 1991). Consistent with our results, studies of c-src show that a peptide based on the phosphorylated carboxy-terminus of c-src binds to the SH2 domain of the c-src protein isolated from fibroblasts (Roussel *et al.*, 1991). We obtained similar results using the lckP peptide and Src deletion mutants (data not shown). Moreover, in our studies, proteins which have at least one SH2 domain (PLCγ1, Vav, GAP) all bound the lckP peptide from either CD45+ or CD45-deficient cells (Figure 3A). Thus, it is likely that binding of Lck involves accessibility of the Lck SH2 domain to the lckP peptide. CD45 may modulate the accessibility of the SH2 domain of Lck, but not other SH2 domain-containing proteins, to the lckP peptide. This is presumably mediated by the effect of CD45 on the phosphorylation status of Y505 of Lck.

It is interest that Fyn, another tyrosine kinase expressed in lymphoid cells whose structure is similar to Lck, bound the lckP peptide in the absence of CD45 in two separate CD45-deficient cell lines (Figure 4A and B). This could argue either that the phosphorylation status of Fyn is unaffected by CD45 or that Fyn is not an efficient substrate for CD45 compared to Lck. This implies that other transmembrane tyrosine phosphatases could regulate the phosphorylation status of Fyn. Alternatively, in the absence of CD45 there may be an unidentified protein which specifically associates with Lck, but not with Fyn, preventing Lck from associating with the lckP peptide (Figure 8). T cells express at least one other tyrosine phosphatase, PTPase α, also termed LRP. Our laboratory has detected low levels of PTPase α in both wild-type Jurkat cells and the CD45-negative cell J45.01 (D.Desai and A.Weiss, unpublished data). Thus, Fyn could be a substrate for PTPase α, while Lck is not. The binding of Fyn to the lckP peptide would then be accounted for by the presence of another transmembrane phosphatase that acts on the phosphorylated carboxy-terminal tyrosine of Fyn, but not Lck. Indeed, data from heterologous transfection systems support this notion (J.Sap and J.Schlessinger, personal communication). Preliminary CNBr cleavage and peptide mapping studies of Fyn isolated from CD45+ and CD45− cells suggests that Fyn is not hyperphosphorylated to the same degree as Lck is at the carboxy-terminal negative regulatory site (data not shown), but it can serve as a substrate for CD45 in *in vitro* studies (Mustelin *et al.*, 1992). Further studies are needed to understand the regulation of phosphorylation of Fyn by protein tyrosine phosphatases.

Resting CD45+ Jurkat cells appear to have a pool of Lck that is constitutively dephosphorylated at Y505. If Lck is analogous to Src, this should represent an active or activatable pool of Lck (Carwright *et al.*, 1987; Hirai and Varmus, 1990a). The sequential depletion studies showed that only 5−10% of the total Lck in CD45+ Jurkat cells bound the lckP peptide. However, our CNBr cleavage experiment suggests that the majority of the Lck in Jurkat is dephosphorylated on Y505 when compared to Lck in J45.01 cells (Figure 5, compare lanes 1 and 2). Therefore it is likely that the lckP peptide cannot bind all the Lck dephosphorylated on Y505, perhaps because much of the Lck dephosphorylated at Y505 is already associated with other proteins.

The inaccessibility of a large pool of Lck dephosphorylated at Y505 for the peptide appears to be modulated by either CD4 or TCR cross-linking (Figure 7). It is possible that in resting cells and immediately following T cell stimulation, the majority of the Lck dephosphorylated on Y505 is associated with other protein complexes (Schraven *et al.*, 1990), thus accounting for its inability to bind to the lckP peptide. However, 15−30 min after stimulation these complexes may dissociate due to TCR or CD4-mediated events, allowing Lck to bind the lckP peptide. Further studies are required to identify proteins that may interact with Lck and the mechanism by which this interaction is regulated.

Although the studies presented in this paper are consistent with the intramolecular model of negative regulation of Lck, they are also consistent with models in which there could be intermolecular associations between Lck molecules or between Lck and other cellular components (protein X, Figure 8). In any of these models, the interaction could occur between the phosphorylated Y505 and an SH2 domain.

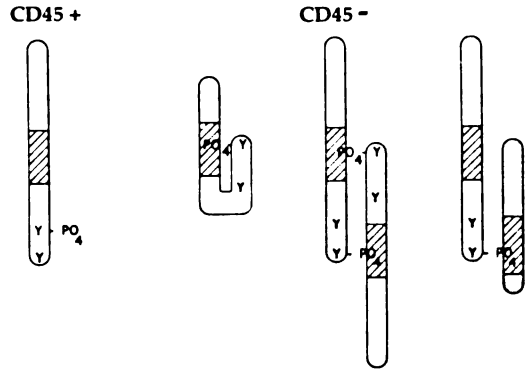

Fig. 8. Regulation of Lck kinase function. Schematic representation of the different models for regulation of Lck. According to the 'intramolecular model', the hyperphosphorylated Y505 interacts with its own SH2 domain (hatched region) in the CD45-deficient cell. Alternatively, intermolecular associations between two Lck molecules or between a Lck molecule and a cellular protein X (shaded protein) are also depicted. However, in any of these models, the Lck in the CD45-deficient cell is thought to be 'inactive' or unable to associate with key substrates.

Furthermore, none of these models is mutually exclusive, and it is possible that a dynamic interplay between these different interactions coexists within the T cell. Finally, even though the peptide containing the autophosphorylation site of Lck (Y394) did not bind to Lck (Figure 1B), it is likely that tyrosine phosphorylation at this residue plays an important role in Lck regulation, perhaps by binding to distinct set of cellular proteins. The availability of CD45-deficient cells, together with the phosphopeptide binding assay, should provide insight into the mechanism by which Lck is regulated.

Materials and methods

Cell lines

The CD45+ leukemic T cells Jurkat and HPB 1.1, as well as the corresponding CD45− deficient mutants J45.01 (Koretzky *et al.*, 1991) and HPB 053 (Koretzky *et al.*, 1990), were grown in RPMI 1640 supplemented with 10% fetal calf serum, glutamine, penicillin and streptomycin (Irvine Scientific). J45.LB3 and J45.LA2 CD45 transfectants (Koretzky *et al.*, 1992) were maintained in the above medium containing Geneticin (GIBCO, Grand Island, NY) at 2 mg/ml.

Antibodies

Polyclonal antibody against unique NH_2-terminal domain of Lck used for immunoprecipitations was previously described. Antibodies or mAbs used in Western blots were kindly provided by the following investigators: anti-Lck hybridoma, from Dr Rafik Sekaly (Ansotegui *et al.*, 1991); anti-PLCγ1 mAb, from Dr Soo Goo Rhee; rabbit anti-Vav antiserum, from Dr Shulamit Katzav; anti-Fyn antibody raised against the unique domain of Fyn, from Dr Andre Veillette; RH6-2A anti-rGAP polyclonal antibody, from Dr Frank McCormack. Stimulating antibodies were as follows: C305 mAb, IgM reactive against the Tα3 was used at a final dilution of 1:1000 of ascitic fluid; anti-CD4 mAb (1:200 dilution) was purchased from Becton-Dickinson Monoclonal Center (Mtipitas, CA) and rabbit anti-mouse IgG (1 mg/ml) was purchased from Zymed (South San Francisco, CA).

Peptides

All the peptides were synthesized by standard Merrifield solid-phase procedures (Merrifield, 1963). For synthesis of the phosphopeptides, the phosphotyrosine was incorporated as the tert-butyloxycarbonyl-O-dibenzyl-phosphono)-L-tyrosine derivative (Peninsula Laboratories). LckP phosphopeptide contains the carboxyl-terminal 11 aa of Lck: Thr-Ala-Thr-Glu-Gly-Gln-TyrPO$_3$H$_2$-Gln-Pro-Gln-Pro. The corresponding non-phosphorylated carboxyl-terminal peptide was synthesized in the same

manner. AutoP peptide contained the autophosphorylation site for Lck: Ile-Glu-Asp-Asn-Glu-Tyr(PO$_3$H$_2$)-Thr-Ala-Arg-Glu-Gly. The scrP contained the carboxyl-terminus tyrosine: Thr-Ser-Thr-Glu-Pro-Gln-Tyr (PO$_3$H$_2$)-Gln-Pro-Gly-Glu. The peptides were covalently coupled to CNBr-activated Sepharose beads (Pharmacia). Briefly, 3 mg of each peptide were incubated for 2 h at room temperature with 1 ml of CNBr-activated Sepharose 4B beads in coupling buffer (0.1 M NaHCO$_3$ pH 8.3, 0.5 M NaCl). Reactive unbound groups were blocked with 0.2 M glycine (pH 8.0) overnight at 4°C. The excess adsorbed protein was extensively washed with coupling buffer followed by acetate buffer (0.1 M NaOAc pH 4.0, 0.5 M NaCl). Covalently coupled beads were stored in 50% (v/v) of coupling buffer.

Protein – phosphopeptide precipitations

Cell lysates were prepared as previously described (Fraser *et al.*, 1989). The equivalent of 4 mg of protein in total lysates was incubated with 50 μl of Sepharose beads covalently coupled to peptide for 2 h at 4°C. The beads were washed five times with the lysis buffer (Fraser *et al.*, 1989) and the bound proteins were eluted by boiling in SDS sample buffer containing 5% β-mercaptoethanol for 3 min prior to SDS–PAGE in 8.5% gels. Gels were pre-equilibrated with transfer buffer (20 mM Tris-base, 150 mM glycine, 20% methanol) for 20 min, electrophoretically transferred into nitrocellulose membranes and blocked with 3% bovine serum albumin (BSA) in TBST (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20) for 1 h. Membranes were incubated with the respective primary antibody for 2 h, washed with TBST, and further incubated for 1 h with a 1:7000 dilution of conjugated alkaline phosphatase goat anti-rabbit or goat anti-mouse (Promega). The immunoblots were washed with TBST and developed by adding nitroblue tetrazolium and 5-bromo-4 chloro-indoyl phosphate substrate as recommended by the manufacturer (Promega). For the quantitative experiments, ^{125}I-labeled goat anti-mouse (Amersham) was used.

In vivo labeling and CNBr cleavage

Jurkat or J45.01 cells (50×10^6) were labeled by incubation for 4 h with 5 mCi of [^{32}P]orthophosphorous in 5 ml of phosphate-free RPMI 1640 supplemented with 3% of phosphate-free fetal calf serum. Jurkat cells were stimulated by addition of 1:1000 dilution of C305 followed by a 30 min incubation at 37°C. Lysates were precleared with normal rabbit serum coupled to protein A beads, followed by precipitation with anti-Lck polyclonal antiserum or the lckP peptide. Bound proteins were eluted in SDS sample buffer, resolved in an 8.5% SDS–PAGE and transferred to Immobilon membranes (Millipore) as described above. The bands corresponding to Lck were located upon short exposure to film, excised from membranes and treated with 60 mg/ml of CNBr according to previously published methods (Veillette *et al.*, 1989b; Luo *et al.*, 1990). Peptides were resolved in a tricine–glycine discontinuous gel of Schagger and Von Jagow (modified by Sigma).

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CHAPTER 3

GRB2 AND PLC γ1 ASSOCIATE WITH A 36-38 KDA PHOSPHOPROTEIN FOLLOWING TCR STIMULATION

Summary

The pathways linking the TCR complex to Ras activation are poorly understood. Although both PKC-dependent and independent mechanisms have been reported, very little is known about the proteins involved in these pathways. However, several proteins, including the adaptor protein Grb2, the proto-oncogene Shc and the guanine-nucleotide exchange factor SOS, have been implicated in linking growth factor receptors to Ras.

In this chapter, the involvement of these proteins in linking the TCR to Ras activation was examined. Although only a very low amount of Shc was inducibly tyrosine phosphorylated in the Jurkat leukemic T cell line, a prominent 36-38 kDa tyrosine phosphoprotein was found to be associated with Grb2 and with PLC γ1 following TCR stimulation. Interestingly, this pp36-38 was exclusively found in the particulate fraction, suggesting that it may serve as a docking protein to recruit different signaling complexes from the cytosol to the plasma membrane. The implication of these findings are further disscussed in this chapter.

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GRB2 and Phospholipase C-γ1 Associate with a 36- to 38-Kilodalton Phosphotyrosine Protein after T-Cell Receptor Stimulation

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GRB2, a 25-kDa protein comprising a single SH2 domain flanked by two SH3 domains, has been implicated in linking receptor protein tyrosine kinases (PTKs) to the Ras pathway by interacting with the guanine nucleotide exchange protein SOS. Previous studies have demonstrated that GRB2 directly interacts with Shc, a proto-oncogene product that is tyrosine phosphorylated upon receptor and nonreceptor PTK activation. In this report, we detected low levels of tyrosine phosphorylation of Shc and induced association with GRB2 upon T-cell receptor (TCR) stimulation. Instead, a prominent 36- to 38-kDa tyrosine phosphoprotein (pp36-38) associated with the SH2 domain of GRB2 and formed a stable complex with GRB2/SOS upon TCR stimulation. Cellular fractionation studies showed that whereas both GRB2 and SOS partitioned to the soluble and particulate fractions, pp36-38 was present exclusively in the particulate fraction. This phosphoprotein had the same apparent mobility in sodium dodecyl sulfate-polyacrylamide gel electrophoresis as the phosphoprotein that associates with phospholipase C-γ1 (PLC-γ1). Furthermore, following partial immunodepletion of GRB2 and of the associated pp36-38, there was a significant reduction in the amount of the 36-kDa phosphoprotein associated with PLC-γ1, suggesting that a trimeric PLC-γ1/pp36-38/GRB2 complex could form. In support of this notion, we have also been able to detect low levels of PLC-γ1 in GRB2 immunoprecipitates. We suggest that pp36-38 may be a bridging protein, coupling different signalling molecules to cytoplasmic PTKs regulated by the TCR.

Activation of resting T cells via the T-cell antigen receptor (TCR) triggers a cascade of intracellular biochemical events that ultimately lead to cellular proliferation and induction of effector functions. The earliest in this series of biochemical events is the induction of protein tyrosine phosphorylation (26, 28). Tyrosine phosphorylation of cellular substrates is essential, since protein tyrosine kinase (PTK) inhibitors can block most if not all of the later events associated with TCR stimulation (21, 29, 38). These results, together with the observation that the TCR does not have any intrinsic enzymatic activity, strongly suggest that cytoplasmic PTKs are regulated by the TCR. At least three candidate PTKs have been implicated in transducing signals from the stimulated TCR: Lck (1, 20, 55), Fyn (13, 48, 57), and ZAP-70 (8, 9).

One of the many cellular proteins that is tyrosine phosphorylated soon after TCR stimulation is the γ1 isoform of phospholipase C (PLC-γ1) (42, 51, 60). Although the biochemical pathway which leads to PLC-γ1 phosphorylation is not completely understood, it is thought to involve Lck (55, 59) or Fyn (23). Both are members of the Src family of kinases. Interestingly, a 35- to 38-kDa tyrosine phosphoprotein in PLC-γ1 immunoprecipitates has been observed following the stimulation of the TCR (19), B-cell antigen receptor (19), or nerve growth factor receptor (40), suggesting that this protein may play a role in PLC-γ1 function. Phosphorylation of PLC-γ1 is responsible for an increase in its catalytic activity (30, 39), resulting in the conversion of phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate and diacylglycerol. Inositol 1,4,5-trisphosphate and diacylglycerol mediate the increase in intracellular calcium levels and the activation of protein kinase C (PKC), respectively. The activation of PKC has been correlated with an increase in Ras activity (14, 27), although there appears to be a PKC-independent mechanism of Ras activation as well (27). The activations of both calcineurin and Ras have been associated with lymphokine gene expression (45), suggesting that these are important events in TCR signal transduction.

The PKC-dependent and -independent mechanisms of Ras activation in T cells are poorly understood. Some investigators have reported that Ras activation occurs primarily by inhibiting GTPase-activating protein function (14); others have suggested the possible involvement of the Vav protein in regulating guanine nucleotide exchange (22). The relative importance of regulating GTPase-activating protein or guanine nucleotide exchange function in T cells is not clear. However, considerable progress has been made in understanding the activation of Ras by receptor PTKs (reviewed in reference 50). Several studies have shown that growth factors can induce the activation of Ras (5, 17, 18, 31, 36, 37, 49) and that mutational inactivation of the *ras* gene blocks the effects of a large number of receptor PTKs (24, 52). Furthermore, expression of constitutively active Ras can bypass the need for receptor PTK activity during vulval differentiation (3) and development of photoreceptor cells (16).

Genetic studies have shown that GRB2 (33) (Sem-5 in *Caenorhabditis elegans* [12] and Drk in *Drosophila melanogaster* [41, 53]), a highly conserved adaptor protein that has a single Src homology 2 (SH2) domain flanked by two SH3 domains, is involved in coupling receptor PTKs to Ras activation (12, 41, 53). Biochemical dissection of the pathway revealed that GRB2 is responsible for linking the receptor PTKs to a

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guanine nucleotide exchange factor SOS, enabling the tyrosine kinase to modulate Ras activity (4, 10, 15, 17, 32, 46, 54).

However, in addition to coupling receptor PTKs to the Ras pathway, there is suggestive evidence that GRB2 may also link cytoplasmic PTKs to the Ras pathway via its association with the proto-oncogene *shc* (43). First, the neurite outgrowth caused by Shc overexpression in PC12 pheochromocytoma cells is Ras dependent (47). Second, upon insulin stimulation (44, 54), upon epidermal growth factor (EGF) stimulation (43), or in v-Src-transformed cells (35, 46), Shc is tyrosine phosphorylated and induced to bind to the SH2 domain of GRB2 (44, 47, 54). Third, Shc associates with murine SOS in v-Src- and v-Fps-transformed cells (15). Since signalling through the TCR is mediated by cytoplasmic kinases, we were interested in determining if the Shc/GRB2/SOS complex played a role in the TCR-mediated activation of Ras by examining the phosphotyrosine proteins associated with GRB2 following TCR stimulation.

In this study, we detected only low levels of tyrosine phosphorylation of Shc and induced association with GRB2 upon TCR stimulation. Instead, a prominent 36- to 38-kDa tyrosine phosphoprotein (also referred to as pp36-38) associated with the SH2 domain of GRB2 and formed a stable complex with GRB2 and SOS upon TCR stimulation. Cellular fractionation studies showed that whereas both GRB2 and SOS partitioned to the soluble and particulate fraction, this novel phosphoprotein was present exclusively in the particulate fraction. This 36- to 38-kDa phosphoprotein had the same apparent mobility in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as the phosphoprotein that associates with PLC-γ1. Following partial immunodepletion of GRB2 and of the associated pp36-38, there was a significant reduction in the amount of pp36-38 associated with PLC-γ1, suggesting that a trimeric PLC-γ1/pp36-38/GRB2 complex could form. In support of this notion, we have also been able to detect low levels of PLC-γ1 in GRB2 immunoprecipitates. We suggest that this pp36-38 may be a bridging protein, coupling different signalling molecules to a cytoplasmic PTKs regulated by the TCR.

MATERIALS AND METHODS

Constructs. The glutathione *S*-transferase (GST)–GRB2 fusion proteins were previously described (2, 33). Briefly, GRB2 cDNA (33) and the appropriate oligonucleotides flanking the domains of interest were used in a PCR. The amplified DNA was cloned into the pGEX-2X bacterial expression plasmid and used to transform *Escherichia coli* competent cells. Expression of the GST fusion proteins was induced with 0.25 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and the proteins were purified by affinity chromatography using glutathione-Sepharose beads. The GRB2 mutant R86K and the mutants corresponding to the *sem-5* allele n1619 (12) were generated as previously described (33). All mutations were confirmed by sequencing.

Antibodies. The polyclonal antiserum against GRB2 used in immunoprecipitations and Western blotting (immunoblotting) was derived from the N-terminal SH3 domain (residues 36 to 50) (33). A polyclonal anti-Shc antibody (purchased from Transduction Laboratories, Lexington, Ky.) was covalently coupled to Sepharose as described elsewhere (25). Immunoblotting with antiphosphotyrosine antibodies was performed with either monoclonal antibody (MAb) 4G10 or antibody RC20 as specified by the manufacturer (Transduction Laboratories). Anti-SOS polyclonal antiserum, which recognizes SOS1, was a generous gift from David Bowtell; anti-mitogen-activated protein kinase (anti-MAPK), anti-CD3E and anti-PLC-γ1 antibodies were purchased from Zymed (South San Francisco, Calif.), Dako (Santa Barbara, Calif.), and Upstate Biotechnology, Inc. (Lake Placid, N.Y.), respectively. For stimulation of the TCR, an anti-Vβ MAb directed against the TCR (C305) was used at a dilution of 1:400 to 1:700 (61).

Cell line, immunoprecipitation, and Western blotting. The human leukemic T-cell line Jurkat was maintained in RPMI 1640 supplemented with 5% fetal calf serum (Intergen), penicillin, streptomycin, and glutamine (Irvine Scientific). Prior to stimulation, cells were incubated in phosphate-buffered saline for 20 min and stimulated with C305 for 2 min at 37°C. Cells were lysed in lysis buffer (1% Nonidet P-40, 150 mM NaCl, 10 mM Tris [pH 7.6]) containing protease and phosphatase inhibitors (55) at 4°C for 30 min. Lysates were centrifuged at 13,000 × *g* for 15 min, and the supernatant was recentrifuged at 100,000 × *g* for 20 min. The lysates were immunoprecipitated with antibody coupled to 25 μl of protein A-Sepharose beads. The immune complexes were resolved by SDS-PAGE and transferred to nitrocellulose. Blots were blocked with 3% bovine serum albumin in TBST (10 mM Tris [pH 7.6], 150 mM NaCl, 0.05% Tween 20) for 1 h prior to immunoblotting as described previously (55).

Partial immunodepletion of GRB2. Jurkat lysates from 100 × 10^6 stimulated cells were prepared as described above and split into two samples. One sample was incubated with an anti-PLC-γ1 or anti-SOS antibody (predepletion sample), while the other sample was immunodepleted of GRB2 and then incubated with an anti-PLC-γ1 or anti-SOS antibody (postdepletion sample). Partial immunodepletion of GRB2 was obtained by subjecting the lysate to five sequential incubations with anti-GRB2 antibody (15 μl) coupled to 25 μl of protein A-Sepharose.

Subcellular fractionation. After stimulation, the cells were incubated in ice-cold hypotonic solution (10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol) for 5 min before sonication. The homogenate was centrifuged at 100,000 × *g* in a Beckman TL-100 centrifuge at 4°C for 30 min. The supernatant cytosol was brought to a final concentration of 1% Nonidet P-40 and 150 mM NaCl and used directly for immunoprecipitation. The pellet was washed with the hypotonic solution and recentrifuged to eliminate any residual cytosolic protein. The washed pellet was resuspended in the hypotonic solution containing 1% Nonidet P-40 and 150 mM NaCl and recentrifuged at 100,000 × *g* for 10 min, and the supernatant was used for immunoprecipitation.

RESULTS

Shc is weakly tyrosine phosphorylated upon TCR stimulation. Shc is tyrosine phosphorylated following EGF (43) or insulin (44, 54) treatment or in cells transformed by the nonreceptor tyrosine kinases v-Src and v-Fps (35). Therefore, we were interested in determining whether TCR stimulation induces Shc tyrosine phosphorylation. Lysates from T cells that had been stimulated with an anti-TCR MAb were immunoprecipitated with an anti-Shc antibody, separated by SDS-PAGE, transferred to nitrocellulose, and probed with an antiphosphotyrosine or anti-Shc antibody. Though Jurkat cells express both the 46- and 52-kDa forms of Shc (Fig. 1B), we detected a slight increase in tyrosine phosphorylation of only the 52-kDa species following TCR stimulation (Fig. 1A). No other phosphotyrosine proteins coprecipitating with Shc were observed. Because we detected only very low levels of Shc tyrosine phosphorylation, we sought to confirm our finding by

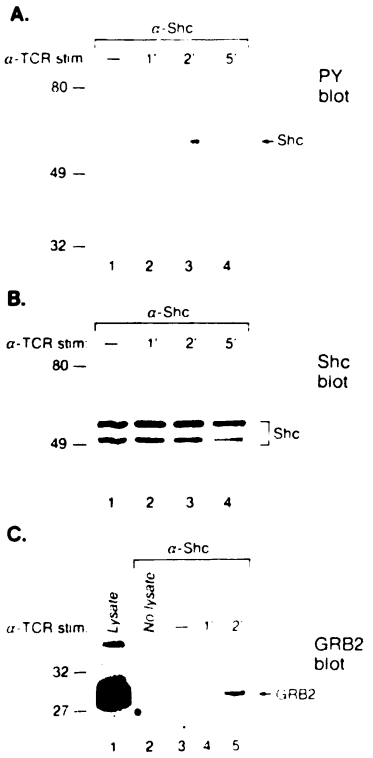

FIG. 1. Shc is weakly tyrosine phosphorylated following TCR stimulation. Jurkat cells were untreated (−) or treated for 1, 2, or 5 min with an anti-TCR antibody (α-TCR), lysed, and immunoprecipitated with an antibody to Shc (α-SHC). The immunoprecipitates were then subjected to Western blot analysis with an antiphosphotyrosine (PY) antibody (A). The blot from panel A was stripped and reprobed with an anti-Shc antibody (B). In panel C, cells were left unstimulated (lane 3) or stimulated for 1 or 2 min as in panel A (lanes 4 and 5). The Shc immunoprecipitates and unstimulated lysate (lane 1) were subjected to immunoblot analysis with an anti-GRB2 antibody. Lane 2 is an Shc immunoprecipitation in the absence of lysate. Sizes are indicated in kilodaltons.

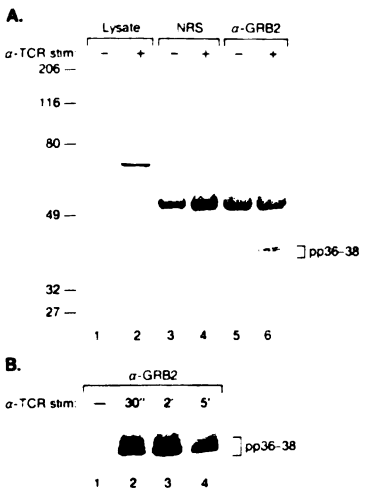

FIG. 2. GRB2 associates with a 36- to 38-kD tyrosine-phosphorylated protein. (A) The equivalent of 50×10^6 Jurkat cells were left unstimulated (−) or stimulated (+) for 2 min as in Fig. 1A, lysed, and immunoprecipitated with either normal rabbit serum (NRS) (lanes 3 and 4) or an anti-GRB2 antibody (α-GRB2; lanes 5 and 6). The immunoprecipitated proteins were then separated by SDS-PAGE and immunoblotted with an antiphosphotyrosine antibody. Lanes 1 and 2 contain lysates from 2×10^6 unstimulated (−) and stimulated (+) cells, respectively. (B) Same as in panel A except that Jurkat cells were left unstimulated (lane 1) or stimulated for 30 sec, 2 min, or 5 min (lanes 2, 3, or 4, respectively). Sizes are indicated in kilodaltons.

determining if there was an induced association between Shc and GRB2. Previously, it has been shown that only the tyrosine-phosphorylated Shc could associate with the SH2 domain of GRB2 (47). Thus, stimulated Jurkat cell lysates were immunoprecipitated with anti-Shc antibodies, and coprecipitating GRB2 protein was assessed by probing the nitrocellulose membrane. As shown in Fig. 1C, there was an increase, albeit low, in the amount of GRB2 associating with Shc following TCR stimulation, supporting the notion that the 52-kDa form of Shc is tyrosine phosphorylated and associates with GRB2 after TCR stimulation.

GRB2 associates with a 36- to 38-kDa tyrosine-phosphorylated protein. Since there was very little GRB2 associating with Shc, we determined whether there were other tyrosine-phosphorylated proteins that might associate with GRB2 following TCR stimulation. Jurkat cells were stimulated, and the lysates were subjected to immunoprecipitation with an anti-GRB2 antibody followed by immunoblotting with an antiphosphotyrosine antibody (Fig. 2A). As previously reported and shown here, stimulation of the TCR leads to phosphorylation of several proteins within the first minute (Fig. 2A, lanes 1 and 2) (26, 28). A prominent phosphotyrosine protein of 36 to 38 kDa (pp36-38) coprecipitated with GRB2 after TCR stimulation (lane 6) but was not present in the unstimulated cell (lane 5) or in the control normal rabbit serum immunoprecipitations (lanes 3 and 4). The diffuse 80- to 90-kDa band seen in unstimulated GRB2 immunoprecipitation was seen only in this particular experiment. Increases in tyrosine phosphorylation of pp36-38 and association with GRB2 occurred very rapidly following TCR activation, peaking 2 min and decreasing 5 min after stimulation stimulation (Fig. 2B). As it has been difficult to detect pp36-38 in GRB2 immunoprecipitates by metabolic labeling, we do not know whether the association is induced.

GRB2 associates with pp36-38 via its SH2 domain. To determine which region of GRB2 was responsible for interacting with pp36-38, wild-type GRB2, GRB2 mutants, as well the SH2 domain of GRB2 were expressed as GST fusion proteins. Lysates from stimulated Jurkat cells were incubated with these fusion proteins, and the bound phosphotyrosine proteins were analyzed. As shown in Fig. 3, the full-length wild-type GRB2 fusion protein bound to the pp36-38, as seen in GRB2 immunoprecipitations (compare lanes 2 and 3). There were several additional tyrosine-phosphorylated proteins that associated with the wild-type GRB2 fusion protein that were not detected in the GRB2 immunoprecipitations (compare lanes 2 and 3). As the use of GST fusion proteins can sometimes lead to artifactual in vitro associations, the relevance of these associations still remain to be assessed.

As expected for a phosphotyrosine-SH2 domain interaction, pp36-38 bound to the SH2 domain of GRB2 alone (Fig. 3, lane

FIG. 3. GRB2 associates with pp36-38 via its SH2 domain. Jurkat lysate that had been stimulated for 2 min with an anti-TCR antibody was incubated with GST alone (lane 1), an anti-GRB2 antibody (α-GRB2; lane 2), full-length GST-GRB2 (lane 3), full-length GST-GRB2 with a substitution of lysine for arginine in the FLVRES motif of the SH2 domain (lane 4), the GST-SH2 domain of GRB2 (lane 5), or the full-length GRB2 with a substitution in the amino-terminal SH3 domain corresponding to the *sem-5* loss of function (lane 6). The coprecipitating proteins were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was divided into two parts. The lower part was probed with an antiphosphotyrosine antibody for visualization of the bound pp36-38 (A); and the upper part was blotted with anti-SOS antibody (B).

5). A point mutation (R86K) within the highly conserved FLVRES motif abrogated binding to pp36-38 (lane 4). To confirm that the R86K mutant was not grossly misfolded, we tested the ability of the fusion proteins to bind to other proteins via its SH3 domain. As shown in Fig. 3B, SOS still bound to the R86K mutant as well as the wild-type GRB2 fusion protein (compare lanes 3 and 4), indicating that the R86K mutant still retained its ability to bind other proteins via its SH3 domains. Furthermore, a point mutation in the SH3 domain of GRB2 corresponding to loss of function in *sem-5* did not affect the binding of GRB2 to pp36-38 (Fig. 3A, lane 6) but markedly compromised SOS binding (Fig. 3B, lane 6). Taken together, the results demonstrate that the association between pp36-38 and GRB2 is likely to occur via the interaction of a tyrosine-phosphorylated residue of pp36-38 with the SH2 domain of GRB2.

GRB2 and SOS form a complex with pp36-38. Several recent studies have demonstrated that SOS and GRB2 form a stable complex mediated by the proline-rich region in the carboxyl terminus of SOS and the SH3 domains of GRB2 (10, 32). Therefore, we examined whether GRB2 formed a complex with SOS in T cells. Anti-SOS immune complexes from unstimulated and TCR-stimulated Jurkat cells were precipitated and Western blotted for the presence of coprecipitating GRB2 protein. Consistent with studies of the EGF receptors (EGFR) (32), GRB2 and SOS form a complex even in unstimulated cells (Fig. 4A). There was not a significant increase in the association between SOS and GRB2 following TCR stimulation (compare lanes 2 and 3). The origin of the upper band seen above GRB2 is not known, but it may be due to the use of an upstream initiation site (33).

Since the SH3 domain of GRB2 has been shown to mediate the binding to SOS while the SH2 domain is responsible for its interaction with pp36-38, it seemed possible that a trimeric complex among pp36-38, GRB2, and SOS could form following TCR stimulation. To address this question, we immunoprecipitated SOS or GRB2 from stimulated cells and probed the membranes with antiphosphotyrosine antibodies. As previously noted, there was a marked increase in the association of pp36-38 with GRB2 following TCR stimulation, even though there was some pp36-38 detected in the basal state (Fig. 4B, lanes 3 and 4). Interestingly, pp36-38 was also present in the SOS immunoprecipitates from unstimulated cells (lane 5). Following TCR stimulation, there was an increase in the amount of pp36-38 associated with SOS (lane 6).

To demonstrate that the association between pp36-38 and SOS was mediated through GRB2, we attempted to immunodeplete GRB2 and determine whether, under those conditions, pp36-38 could associate with SOS. Despite multiple

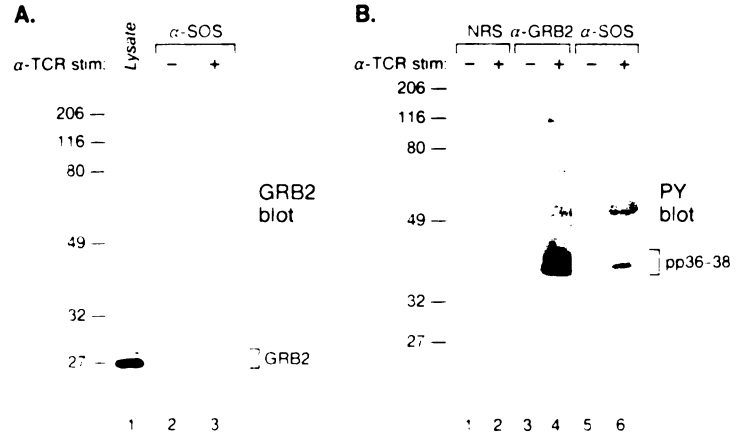

FIG. 4. GRB2 and SOS forms a complex with pp36-38. (A) Jurkat cells were left unstimulated (lane 2) or stimulated for 2 min (lane 3) with an anti-TCR antibody (α-TCR). Lysates were immunoprecipitated with an anti-SOS antibody (α-SOS), washed, and resolved by SDS-PAGE. The Western blots were probed with an anti-GRB2 antibody and visualized by using [^{125}I]protein A. Lane 1 contains lysate from unstimulated cells. (B) Lysates were prepared as in panel A, immunoprecipitated with either normal rabbit serum (NRS; lanes 1 and 2), an anti-GRB2 antibody (α-GRB2; lanes 3 and 4), or an anti-SOS antibody (α-SOS; lanes 5 and 6), and separated by SDS-PAGE. The proteins were transferred to nitrocellulose membranes and blotted with an antiphosphotyrosine antibody. Sizes are indicated in kilodaltons.

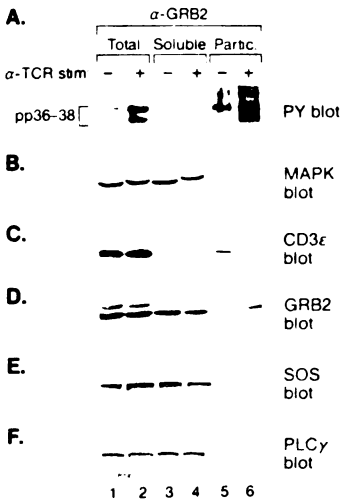

FIG. 5. pp36-38 is detected only in the particulate fraction. Jurkat cells were left untreated (−) or stimulated with an anti-TCR antibody (α-TCR) for 2 min (+) as indicated. Unfractionated lysates (lanes 1 and 2) as well as the soluble (lanes 3 and 4) and particulate (lanes 5 and 6) fractions were prepared and immunoprecipitated with an anti-GRB2 antibody (α-GRB2) (A) or directly resolved by SDS-PAGE (B to F). The proteins were transferred to a nitrocellulose membrane and immunoblotted with an antiphosphotyrosine (PY) (A), anti-MAPK (B), anti-CD3ε (C), anti-GRB2 (D), anti-SOS (E), or anti-PLC-γ1 (F) antibody.

attempts, we have succeeded in obtaining only a partial depletion of GRB2 and of its associated pp36-38 (see Fig. 7B, lanes 1 through 7). Interestingly, even with a partial GRB2 depletion, there was a decrease in the amount of pp36-38 associated with SOS (see Fig. 7B, lanes 8 and 9). Though we cannot rule out the possibility that pp36-38 can also associate with SOS directly, the data suggest that the trimeric pp36-38 GRB2 SOS complex does indeed occur.

pp36-38 is detected only in the particulate fraction. GRB2 is thought to be an adaptor protein, responsible for linking SOS to the autophosphorylated EGFR (4, 15, 17, 32, 46). Whereas the association of EGFR with SOS has not been shown to alter its intrinsic exchange activity, it has been postulated that this association relocalizes SOS to the plasma membrane, modulating its access to its substrate, Ras (4, 32, 46, 54). Therefore, we determined if pp36-38 could, in an analogous manner to the EGFR, be responsible for modulating the location of the GRB2 SOS complex to the plasma membrane. Particulate and soluble fractions were prepared from untreated or anti-TCR-stimulated Jurkat cells. GRB2 from both cellular compartments was immunoprecipitated and blotted with an antiphosphotyrosine antibody. Though we cannot determine the location of the unphosphorylated 36- to 38-kDa protein, fractionation of total lysates from Jurkat cells demonstrated that pp36-38 was detected solely in the particulate fraction (Fig. 5A, lanes 5 and 6), with no detectable levels in the soluble fraction (lanes 3 and 4), despite the fact that some GRB2 partitioned to the soluble fraction (Fig. 5D, lanes 3 and 4). Furthermore, there were approximately equivalent amounts of pp36-38 in the particulate and unfractionated samples, supporting the notion that the GRB2-associated pp36-38 is exclusively found in the particulate fraction.

To ascertain that the fractionation technique was correctly separating cytosolic from membrane-bound proteins, we probed both fractions with antibodies against proteins known to be either located in the cytosol or associated with the membrane. As shown in Fig. 5B, at least 90% of the MAPK partitioned to the soluble fraction, whereas the transmembrane CD3ε chain was found exclusively in the particulate fraction (Fig. 5C). This indicated that a good separation of cytosolic from particulate-associated proteins was obtained.

We then examined the distribution of GRB2 before and after TCR stimulation. GRB2 was found in both the cytosolic and particulate fractions in unstimulated cells (Fig. 5D, lanes 3 and 5). Following TCR stimulation, there was only a small increase in the amount of GRB2 in the particulate fraction (lanes 5 and 6). Similar findings were observed for SOS (Fig. 5E). As a control, we also examined the distribution of PLC-γ1, a protein known to translocate to the plasma membrane following EGF stimulation (56). As shown in Fig. 5F, there was an increase in the amount of PLC-γ1 translocated from the cytosolic to the particulate fraction upon TCR stimulation. These results indicate that although GRB2 and SOS are in the soluble and particulate fractions, the GRB2-associated pp36-38 exclusively partitioned to the particulate fraction.

pp36-38 has the same mobility as the 36-kDa phosphoprotein that associates with PLC-γ1. In previous studies (19, 40), a 35- to 38-kDa protein, heavily tyrosine phosphorylated in T cells following antigen receptor ligation, was observed in PLC-γ1 immunoprecipitates and was shown to interact with the SH2 domain of PLC-γ1 (19). Thus, we were interested in determining whether the pp36-38 that associates with GRB2 might be the same as the one that associates with PLC-γ1 after TCR stimulation. T-cell lysates from unstimulated and stimulated cells were prepared and subjected to immunoprecipitation with an anti-GRB2 or anti-PLC-γ1 antibody. The coprecipitating proteins were resolved in SDS-PAGE and Western blotted with an antiphosphotyrosine antibody. Consistent with previous reports, a 36- to 38-kDa phosphoprotein associated with PLC-γ1 following TCR stimulation (Fig. 6, lanes 5 and 6). The pp36-38 associated with GRB2 was comparable in mobility in SDS-PAGE to the phosphotyrosine protein associated with PLC-γ1 (lanes 4 and 6), suggesting that they may be the same protein.

PLC-γ1, pp36-38, and GRB2 may form a trimeric complex. If the 36-kDa phosphoprotein reported to associate with PLC-γ1 (19) is the same as the pp36-38 seen associated with GRB2, the trimeric PLC-γ1 pp36-38 GRB2 complex may form. To determine whether this was the case, GRB2 immunoprecipitates were resolved by SDS-PAGE and the Western blots were probed with an anti-PLC-γ1 antibody. As shown in Fig. 7A, PLC-γ1 does coprecipitate with GRB2 after TCR stimulation (lanes 3 and 4). It is important to note that only a small fraction of PLC-γ1 seem to be directly or indirectly associated with GRB2 immunoprecipitates from stimulated cells (compare lanes 4 and 5). Moreover, this was seen with only one antiserum which was raised against the SH3 domain of GRB2, although we could detect PLC-γ1 in GRB2-GST fusion precipitates from stimulated cells (data not shown). To demonstrate the complex formation between PLC-γ1, pp36-38, and GRB2 more conclusively, we attempted to deplete the pp36-38 associated with GRB2 and determine whether it affected the ability of the reported 36-kDa phosphoprotein to bind to PLC-γ1. Though we have obtained only a partial depletion of GRB2 and of its associated pp36-38 (Fig. 7B, lanes 1 through 7), there was no detectable 36-kDa phosphotyrosine protein associated with PLC-γ1 after depletion (lanes

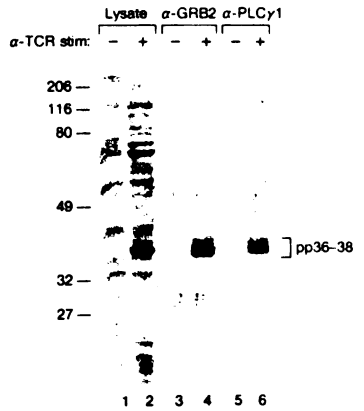

FIG. 6. pp36-38 has the same apparent mobility in SDS-PAGE as the phosphoprotein that associates with PLC-γ1. Jurkat cells were left unstimulated (−; lanes 1, 3, and 5) or stimulated (+; lanes 2, 4, and 6) as in Fig. 5. The lysates were loaded directly into the gel (lanes 1 and 2) or immunoprecipitated with an anti-GRB2 antibody (α-GRB2; lanes 3 and 4) or with an anti-PLC-γ1 MAb (α-PLCγ1; lanes 5 and 6). The proteins were resolved by SDS-PAGE, and the nitrocellulose membrane was immunoblotted with antiphosphotyrosine antibody RC20. Sizes are indicated in kilodaltons.

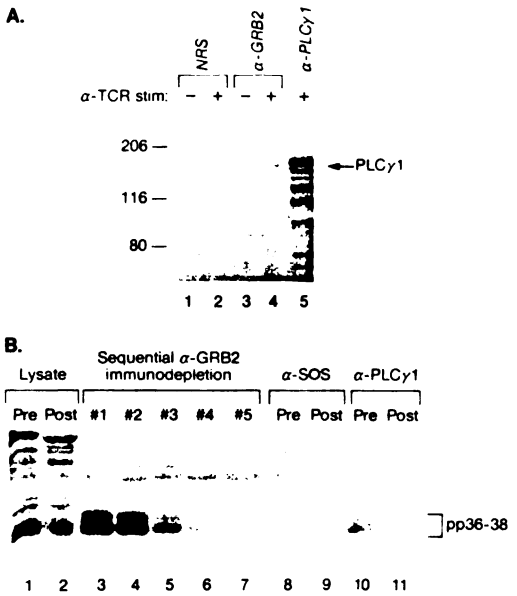

FIG. 7. pp36-38 forms a trimeric complex with PLC-γ1 and GRB2. (A) Jurkat cells were left unstimulated (−; lanes 1 and 3) or stimulated (+; lanes 2, 4, and 5) as in Fig. 5. The lysates were immunoprecipitated with normal rabbit serum (NRS; lanes 1 and 2), an anti-GRB2 antibody (α-GRB2; lanes 3 and 4), or an anti-PLC-γ1 MAb (α-PLCγ1; lane 5). The proteins were resolved by SDS-PAGE, and the nitrocellulose membrane was immunoblotted with an anti-PLC-γ1 MAb. Sizes are indicated in kilodaltons. (B) Lysate from stimulated Jurkat cells was prepared as in Fig. 5 and incubated with an anti-PLC-γ1 or anti-SOS antibodies (α-SOS) (Pre; lane 8 or 10, respectively) or sequentially immunodepleted of GRB2 and then incubated with an anti-PLC-γ1 or anti-SOS antibody (Post; lane 9 or 11, respectively). Partial immunodepletion of GRB2 was obtained by subjecting the lysate to five sequential incubations with the anti-GRB2 antibody (lanes 3 through 7). Jurkat lysate before (Pre) and after (Post) GRB2 depletion were directly loaded for SDS-PAGE (lanes 1 and 2). The proteins were transferred to a nitrocellulose membrane and immunoblotted with antiphosphotyrosine antibody RC20.

6 and 7). Taken together, these data suggest that the PLC-γ1-associated pp36-38 is the same as the pp36-38 that associates with GRB2 and that the trimeric PLC-γ1/pp36-38/GRB2 complex can form following TCR stimulation.

DISCUSSION

There are several small adaptor proteins, such as GRB2, Crk, and Nck, that possess SH2 and SH3 domains but have no covalently linked enzymatic domain. Cellular overexpression of several of these adaptor proteins leads to transformation (11, 34), underscoring the importance of these molecules in signal transduction. A better understanding of how these proteins function in PTK receptor signalling came with the demonstration that GRB2 is involved in Ras activation by receptor PTKs in mammalian cells (33) and in *C. elegans* (12). It is now known that the SH3 domain of GRB2 binds to a proline-rich motif in SOS (10, 32) and that upon receptor activation, this complex is recruited to the plasma membrane via its association with the autophosphorylated EGFR (5, 15). In this study, we were interested in determining whether GRB2 might play a role in the TCR-mediated activation of Ras by identifying the phosphotyrosine proteins that associate with GRB2 upon TCR stimulation. Contrary to what was observed in EGF- and insulin-stimulated cells (43, 44, 54), we detected only low levels of Shc tyrosine phosphorylation following TCR stimulation (Fig. 1A). This is intriguing, since signalling through the TCR is mediated by cytoplasmic kinases and phosphorylation of Shc has been proposed as a mechanism by which nonreceptor tyrosine kinases are coupled to the Ras pathway (15, 46). A recent study demonstrated that Shc is phosphorylated following interleukin-2 receptor stimulation (7). Thus, it is possible that in T cells, Shc interacts with cytoplasmic PTK pathways that are distinct from those involved in TCR signal transduction. Alternatively, it is possible that the low level of tyrosine-phosphorylated Shc associated with GRB2 (Fig. 1C) is physiologically relevant and sufficient for coupling the TCR complex to the Ras pathway.

Though little Shc was associated with GRB2 upon TCR stimulation, we detected a major 36- to 38-kDa tyrosine-phosphorylated protein interacting with GRB2 upon TCR stimulation (Fig. 2A). The interaction between GRB2 and pp36-38 was detected very rapidly following TCR stimulation (Fig. 2B). Similar findings were reported by Buday et al. (6) following the submission of this report. Since the interaction was dependent on phosphotyrosine binding function of the SH2 domain of GRB2 (Fig. 3), we presume that the interaction is induced by pp36-38 phosphorylation. Taken together, the data suggest that upon TCR stimulation, the 36- to 38-kDa protein is phosphorylated by a cytoplasmic kinase proximal to the TCR. This phosphorylation may activate its function or may allow it to bind to GRB2 and/or to the GRB2 SOS complex (Fig. 4). We frequently detected two bands in the pp36-38 region and are currently investigating whether pp36-38 comprises two distinct phosphoproteins or whether it is differentially phosphorylated.

It is intriguing that this phosphoprotein was found only in the particulate fraction and that there was a slight but reproducible increase of GRB2 and SOS to this fraction upon stimulation (Fig. 5). It is possible that this phosphoprotein mediates the redistribution of a fraction of GRB2 and SOS from the soluble to the particulate fraction upon TCR stimulation. Though we cannot determine the component of the particulate fraction that the pp36-38/GRB2 SOS complex associates with, it is tempting to think that it could be the plasma membrane. If so, binding to pp36-38 might be a mechanism by which different SH2-containing effector molecules are recruited to the membrane. The ability of phosphotyrosine-SH2 interactions to direct signalling complexes to translocate from the cytosol to the membrane is not without precedence. In EGF-stimulated cells, the GRB2/SOS complex redistributes to the plasma membrane by binding to the phosphorylated EGFR (5, 15). Once immunologic reagents to pp36-38 are available, a more detailed fractionation study will be required to determine whether p36-38 is indeed located in the plasma membrane fraction and if it is responsible for the translocation of different signalling molecules such as GRB2/SOS and PLC-γ1, to this fraction.

Consistent with previous reports (19, 40), we have observed a 36- to 38-kDa phosphoprotein associating with PLC-γ1 following TCR stimulation (Fig. 6). Furthermore, we have been able to detect low levels of PLC-γ1 coprecipitating with GRB2 following TCR stimulation (Fig. 7A). There are two possibilities that explain the somewhat surprising association between PLC-γ1 and GRB2. The first possibility is that the anti-GRB2 antibody used for the immunoprecipitation has a small degree of cross-reactivity with PLC-γ1. Though unlikely, we cannot rule out this possibility since the anti-GRB2 antibody was generated against the SH3 domain of GRB2 and PLC-γ1 does have an SH3 domain. If this is the case, the epitope in PLC-γ1 for the anti-GRB2 antibody must be accessible only after TCR stimulation, since this antibody does not cross-react with PLC-γ1 from unstimulated cells (Fig. 7A, lanes 3 and 4). The second possibility is that PLC-γ1 can associate with GRB2 either directly or indirectly. Though our immunodepletion data (Fig. 7B) suggest that this interaction maybe mediated by pp36-38, it is also possible that the tyrosine-phosphorylated PLC-γ1 can bind to the SH2 domain of GRB2. Immunologic reagents against pp36-38 will be necessary to determine which of these possibilities is correct.

Since GRB2 is not tyrosine phosphorylated upon TCR stimulation (data not shown), binding to pp36-38 might be a mechanism by which different signalling molecules such as GRB2 or PLC-γ1 are coupled to a cytoplasmic PTK regulated by the TCR, such as ZAP-70, Lck, or Fyn. Interestingly, a similar 36- to 38-kDa phosphotyrosine protein has been reported to associate with the GST-SH2 fusion proteins of Lyn, Fyn, Lck, Blk, PLC-γ1, GTPase-activating protein, and p85 upon TCR stimulation (58). The functional consequences of the interaction between GRB2 and or the GRB2 SOS complex with pp36-38 in TCR-stimulated cells awaits further characterization of this protein. However, it is tempting to speculate that these interactions may be crucial for the induction of the broad repertoire of pleiotropic responses mediated by TCR stimulation.

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CHAPTER 4

PURIFICATION SCHEME FOR THE 36-38 kDA PHOSPHOPROTEIN ASSOCIATED WITH GRB2 AND PLCγ1

Summary

In previous experiments, pp36-38 was shown to bind to Grb2 (68-70, 118, 119), p85 (120) and PLC γ1 (68) *in vivo*, raising the possibility that it is a coupling molecule linking a cytoplasmic tyrosine kinase to various downstream signaling complexes. The elucidation of its exact role in T cell signal transduction awaits its molecular characterization. A biochemical purification scheme for isolating proteins that associate only with the SH2 domain of Grb2 was developed. Grb2GST containing a point mutation in the SH3 domain (P49LGrb2-GST) was covalently crosslinked to glutathione beads and used for affinity purification of pp36-38 from Jurkat lysates. Peptide sequences for five proteins were obtained, including a novel 36 kDa protein, dynamin, Cbl, Sam68 and an 80 kDa serine/threonine phosphoprotein. Degenerate oligonucleotides, based on the peptide sequences of p36, were used to screen a human leukemia cDNA library. One cDNA clone encoding ten of the eleven peptides found in the 36 kDa protein was isolated. Rabbit polyclonal antisera were raised against the amino- or the carboxy-terminal sequences of p36, and were shown to immunoprecipitate and western blot a single 36 kDa protein. However, p36 did not co-immunoprecipitate with Grb2 *in vivo* nor was it found to be tyrosine phosphorylated following TCR engagement. Further studies revealed that p36 bound to the P49LGrb2-GST only when the latter was covalently crosslinked to beads. A new protocol for the purification of pp36 is presented and discussed below.

Preface

Stimulation of the TCR complex induces the tyrosine phosphorylation of a prominent 36-38 kDa protein. Although pp36-38 has not been molecularly characterized, several key signaling complexes such as the p85 subunit of PI3 kinase (120), Grb2-SOS (68-70) and PLC γ1 (68), and SLP-76 (J. Wu and G. Koretsky, personal communication) are able to directly or indirectly associate with pp36-38. There is suggestive data indicating that pp36-38 is critical for increases in the levels of intracellular calcium as well as phosphatidylinositol turnover following engagement of the TCR complex (Motto et al., in press). Furthermore, engagement of the TCR complex in a Jurkat mutant called JCaM2.5 does not induce the tyrosine phosphorylation of p36-38, the concomitant increase in intracellular calcium (121) or Ras activation (J. Wu, personal communication), suggesting that pp36-38 is required for these signaling events.

Interestingly, a 36-38 kDa phosphoprotein have been reported in several different cells including peripheral T cells (70, 122), leukemic T cell lines (120, 68, 69, 118), NK cells (123), PC12 cells (124) and Ramos B cells (125) but has not been detected in fibroblasts stimulated with EGF (125). However, the 36-38 kDa phosphoprotein described in B cells and PC12 cells seems to have different binding properties than the pp36-38 identified in T and NK cells. More specifically, the 36-38 kDa phosphoprotein in B cells is not capable of binding to the SH2 domain of PLC γ1 (125), whereas the pp36-38 isolated in T cells does (125 and our unpublished observation). In PC12 cells, stimulation with NGF induces the phosphorylation of a 38 kDa protein. However, unlike pp36-38 detected in T cells (120), this 38 kDa phosphoprotein does not bind to the SH2 domain of p85 (124). Furthermore, binding of the PC12 38 kDa

protein to PLC γ1 requires both the SH2 domains of PLC γ1 whereas T cell pp36-38 can independently bind to either amino or carboxy SH2 domain of PLC γ1 (our unpublished observation). Although pp36-38 could be differentially tyrosine phosphorylated in different cell types, it is tempting to speculate that pp36-38 is exclusively expressed in T lymphocytes and NK cells, or that the kinase responsible for pp36-38 phosphorylation has a very restricted expression pattern.

There is suggestive evidence that ZAP-70 may be responsible for phosphorylating p36-38. pp36-38 has been reproducibly found in immunoprecipitates of ZAP-70 following TCR stimulation (D. Qian, personal communication). This association is unlikely to be an artifact due to the cross reactivity of the anti-ZAP-70 antibody with pp36-38 because similar results were obtained when anti-Myc antibody was used to immunoprecipitate an epitope-tagged ZAP-70 construct (Q. Zhao, personal communication). Although it remains to be determined whether ZAP-70 directly associates with pp36-38, it is intriguing that the two SH2 domains of ZAP-70 do not seem to mediate the binding to pp36-38 (39), raising the possibility that the kinase domain and/or linker region of ZAP-70 is involved in binding and phosphorylating p36-38. Taken together, there is strong correlative data suggesting that pp36-38 may be an essential coupling protein linking cytoplasmic tyrosine kinases to the downstream signaling complexes. An attempt to purify and clone pp36-38 from stimulated Jurkat cells is described below.

Results

pp36-38 is drastically reduced in JCaM2.5 cells

There is suggestive evidence that pp36-38 is critical for increases in the levels of intracellular calcium as well as phophatidylinositol turnover following engagement of the TCR complex (Motto et al., in press). In order to determine if pp36-38 was required for increases in intracellular calcium, biochemical analysis of a Jurkat mutant, JCam2.5 was undertaken. JCaM2.5 cells were originally isolated based on their failure to increase cytoplasmic free calcium following TCR stimulation (121). However, the TCR on JCaM2.5 cells does partially activate the TCR-associated PTK since several proteins such as the TCR ζ chain (D. Straus, personal communication), Cbl (T. Sosinowski, personal communication) and Vav (J. Wu, personal communication) are still inducibly phosphorylated. Further analysis of JCaM2.5 cells has shown that the inducible tyrosine phosphorylation of PLC γ1 (63) as well as the activation of Ras (J. Wu, personal communication) is compromised. Because pp36-38 can associate with Grb2-SOS (68-70) as well as with PLC γ1 (68), it was of interest to determine if this interaction remained intact in JCaM2.5 cells.

As shown in Figure 1, pp36-38 bound to Grb2 following TCR stimulation in Jurkat cells (lane 4) but not in JCaM2.5 cells (lane 7). Interestingly, although pervanadate stimulation of Jurkat and JCaM2.5 cells induced many tyrosine phosphorylated proteins that bound to Grb2-GST (lane 5 and 8, respectively), very little bound pp36-38 was detected in JCaM2.5 cells (lane 8). These results suggest that JCaM2.5 cells are deficient in, or have drastically reduced amounts of pp36-38. In order to confim this, PLC γ1 was immunoprecipitated from unstimulated and stimulated Jurkat and JCaM2.5

Figure 1. **pp36-38 associated with Grb2 is drastically reduced in JCaM2.5 cells.** The equivalent of 5 x 10^7 Jurkat and JCaM2.5 cells were left unstimulated (lanes 1, 3 and 6), stimulated with anti-TCR antibody (lanes 2, 4 and 7) or treated with pervanadate (lanes 5 and 8). The lysates were incubated with either NRS (lanes 1 and 2) or anti-Grb2 antibody (lanes 3 - 8), and the bound proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes and western blotted with anti-phosphotyrosine mAb 4G10.

Association of pp36-38 with Grb2 in Jcam2 cells

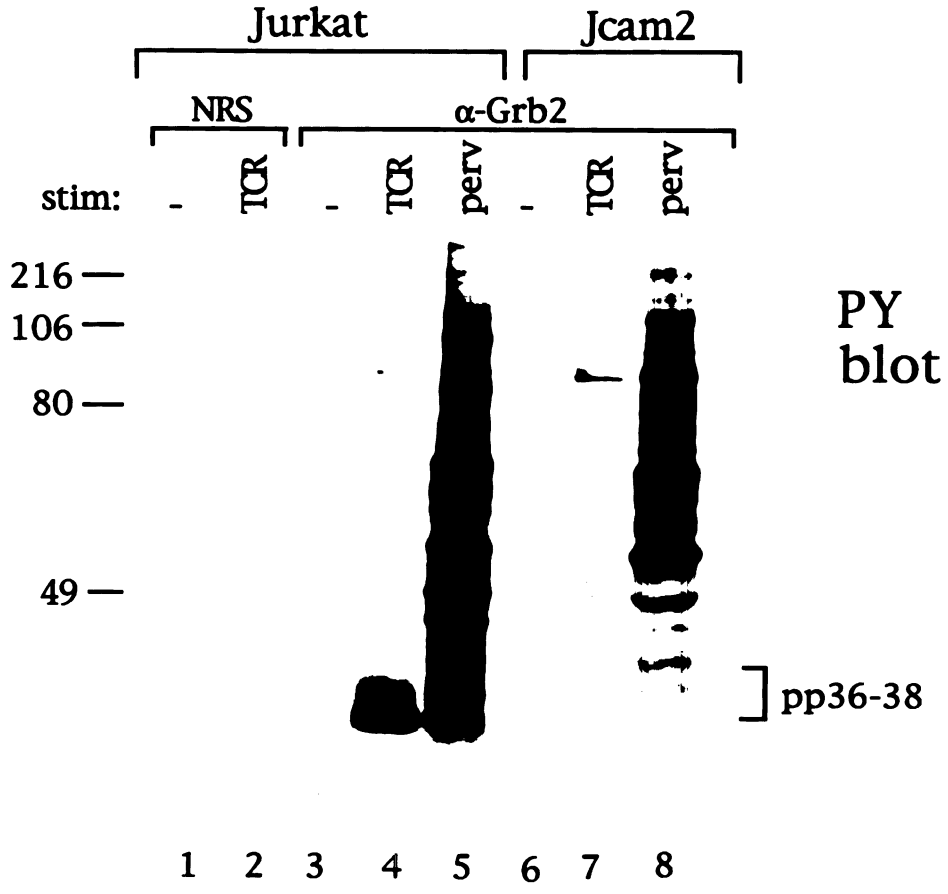
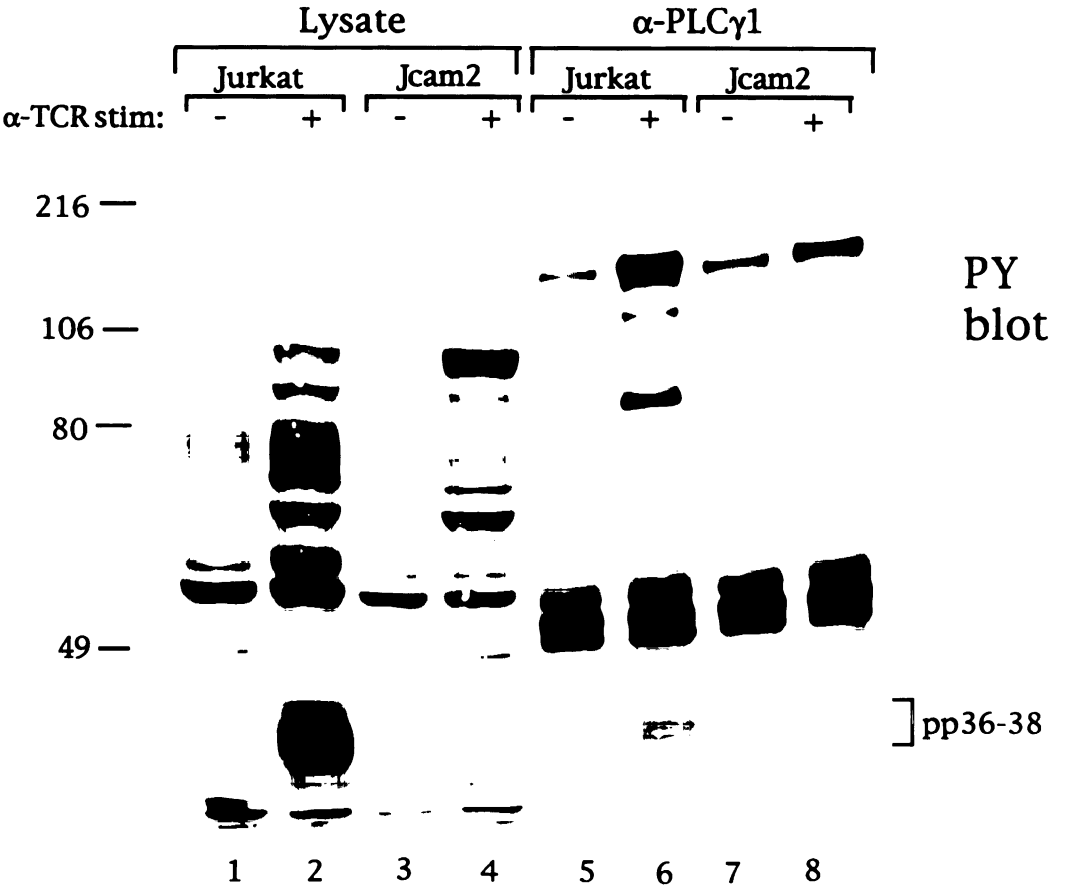


Figure 2. **pp36-38 associated with PLC γ1 is drastically reduced in JCaM2.5 cells.**

The equivalent of 5 x 10^7 Jurkat and JCaM2.5 cells were either left unstimulated (odd numbered lanes) or stimulated with anti-TCR antibody (even numbered lanes). The lysates were either directly loaded onto the SDS-PAGE (lanes 1 - 4) or first immuprecipitated with anti-PLC γ1 antibody (lanes 5 - 8). The proteins were western blotted with anti-phosphotyrosine mAb 4G10.

Association of pp36-38 with PLCγ1 in Jcam2 cells



cells. As shown in Figure 2, stimulation of Jurkat cells induced the phosphorylation of several proteins, one of the most prominent being pp36-38 (lane 2). Consistent with what had been previously reported, stimulation of JCaM2.5 cells did induce the phosphorylation of some proteins (63), but there was no detectable pp36-38 (lane 4). Furthermore, pp36-38 associated with PLC γ1 from stimulated Jurkat cells (lane 6) but not from stimulated JCaM2.5 cells, even though PLC γ1 from JCaM2.5 was inducibly phosphorylated, albeit to a lower extent than in Jurkat cells (Compare lanes 6 and 8). Taken together, these results demonstrate that pp36-38 is drastically reduced in JCaM2.5 cells.

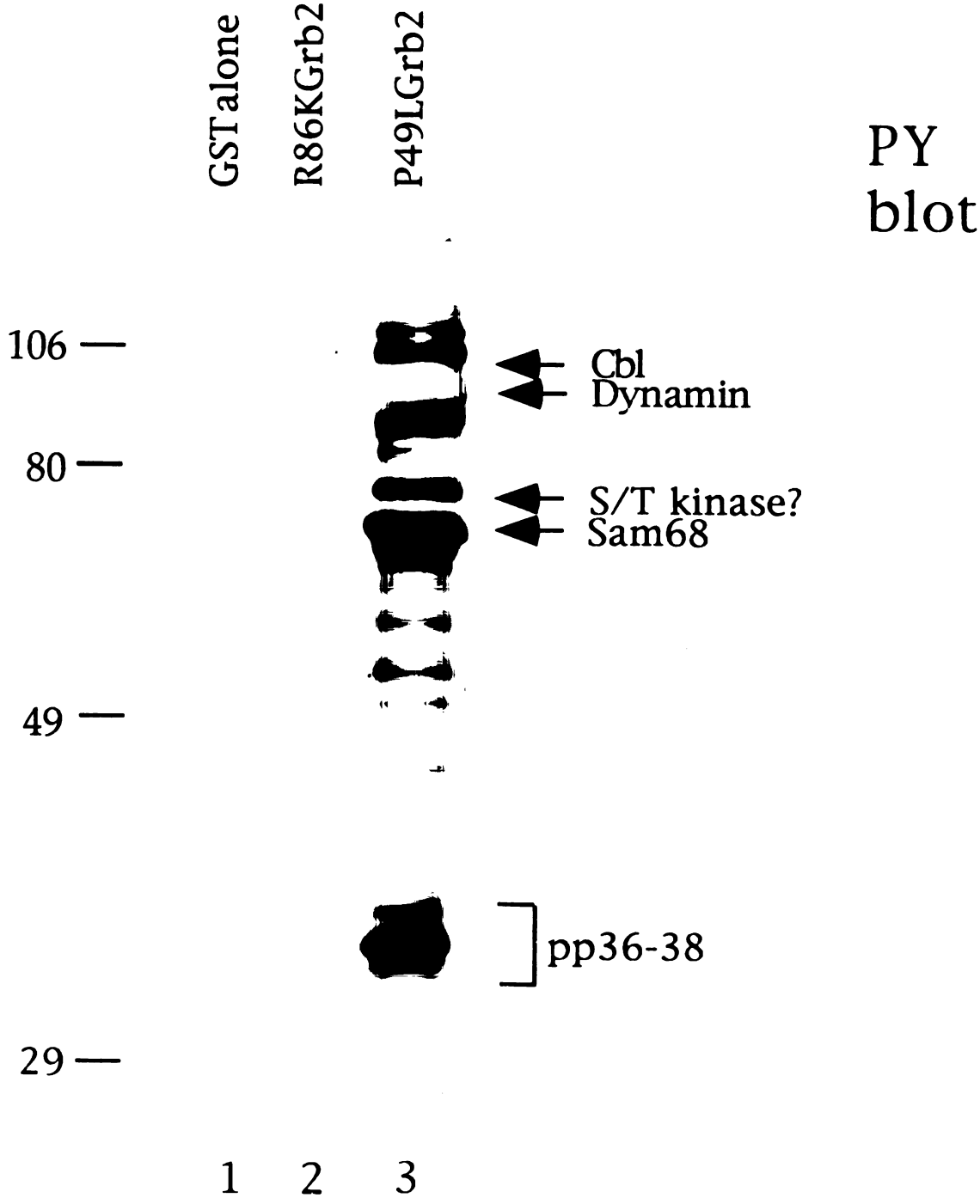
Purification and cloning of p36

In order to purify pp36-38, the equivalent of 8×10^9 Jurkat cells were stimulated with anti-TCR antibody. The lysates were precleared with GST alone, with PTP1C-GST, a fusion protein encompassing the two SH2 domains of PTP1C, followed by another pre-clearing step with R86KGrb2-GST, a fusion protein containing a point mutation in the conserved FLVRES sequence (68) in the SH2 domain. The precleared lysate still containing pp36-38 was incubated with P49LGrb2-GST, a Grb2-GST fusion protein containing a point mutation in the amino-terminal SH3 domain of Grb2. The proteins bound to the P49LGrb2-GST fusion protein were resolved by SDS-PAGE, transferred to Immobilon membranes and western blotted with anti-phosphotyrosine antibody (Figure 3) or directly stained with Ponceau S (data not shown). The rationale for using the P49LGrb2-GST as opposed to a SH2Grb2-GST fusion protein containing only the SH2 domain of Grb2, was that the former migrated as a 49kDa protein and therefore did not overlap with pp36-38.

Figure 3. **Several tyrosine phosphorylated proteins bound to the P49LGrb2-GST fusion protein.**

The equivalent of 8 x 109 Jurkat cells were stimulated with anti-TCR antibody and the lysates were prepared according to the purification protocol described in Experimental Procedures. The proteins that bound to GST (lane 1), R86KGrb2-GST (lane 2) or P49LGrb2-GST (lane 3) were loaded on SDS-PAGE, transferred to Immobilon membrane, and analyzed by western blotting with anti-phosphotyrosine 4G10 mAb (shown in Figure) or stained with Ponceau S (data not shown). The arrows indicate the proteins that were microsequenced from the corresponding Ponceau S stained blot. The identity of the five identified proteins are as shown.

Purification of pp36-38



Furthermore, although the carboxyl-terminal SH3 domain of the P49LGrb2-GST fusion protein was still functional, proteins that required an intact amino- and carboxyl-terminal SH3 domain of Grb2 would be eliminated.

Ponceau S staining showed that five major proteins of molecular weights 120 kDa, 100 kDa, 80 kDa, 70 kDa and 36 kDa copurified with the P49LGrb2 but not with the R86KGrb2 nor with GST (data not shown). Trypsin digestion followed by microsequencing of these five proteins revealed the identity of three out of the five proteins: dynamin (100 kDa), Cbl (120 kDa) and Sam68 (70 kDa) (Figure 3), each of which had previously been shown to interact with Grb2 *in vivo* and/or *in vitro* (71, 150, 174). The peptide sequences for the other two proteins of 36 kDa and 80 kDa (Figure 4) did not match any sequence in the Genebank database.

Degenerate oligonucleotides based on peptide QTYYQV and TFHLAND of p36 were used in polymerase chain reaction (PCR) amplification of whole cell RNA. A specific 870bp fragment was amplified and was then used to screen a leukemic T cell cDNA library. One cDNA encoding ten of the eleven peptides found in p36 was isolated (Figure 5). Search of the EST (Expressed Sequence Tagged) database revealed that the p36 cDNA had 70% identity to the C. elegans cosmid ZK652 (data not shown). Because the partial cDNA isolated was 1171 bp and most likely encoded for a protein larger than 36 kDa, northern blots were probed to determine the exact size and tissue distribution of this gene. As shown in Figure 6, 1.6Kb and 2.4Kb transcripts were widely expressed, with the highest levels present in testes, skeletal muscle and heart. Consistent with the notion that the partial cDNA encoded a protein larger than 36 kDa, sequencing of the amino-terminus showed that this protein was proteolytically cleaved, releasing a 320 amino acid fragment as diagrammed

Figure 4. Peptide sequences for the proteins purified with P49LGrb2-GST.

Proteins were purified as described in Experimental Procedures. Bands corresponding to p36, p70, p80, p100, p120 were excised from Immobilon membranes stained with Ponceau S, digested with trypsin and microsequenced. The unique peptide sequences obtained for p36 and p80 are numbered. The peptides with the consensus VLETVG, VIPFYMXM, PPFSLE and PXNVK were obtained more than once. Peptides used for cloning p36 are underlined. The only peptide not found in the gene encoding p36 is marked by an asterisk (*) whereas the peptide in p80 containing homology to a serine kinase involved in meiosis is marked by two asterisks (**).

Peptide sequences for p36 and p80

p36

1) VLETVGXF
2) TVIPFYMGMR
3) FERPDGSHFDVVR
4) VAQIPIYMEM *
5) SQTEAVTFLANHD
6) LENLDSDVXQL
7) GVVGREPVLSK
8) APPFVARES
9) NPPFSLES
10) QTYYQV
11) FSLSGVLETV

p80

1) PREFXAY
2) PLHVVXK
3) LENLNK
4) ITAVAETFK
5) PINVKL
6) FLIPTLY**
7) LYNLIIK
8) LVLIPAEK
9) VNLTDFR

Figure 5. **cDNA encoding for p36.**

One cDNA clone isolated from a human T cell cDNA library encodes for ten peptides present in the purified p36. Peptides microsequenced are shown in thick lines. A potential site for amino-terminus cleavage is shown by the arrow. Amino- and carboxyl-terminal regions of p36 used to generate antibody are shown in dashed lines.

Partial cDNA sequence of p36

atg tcg ggt ggg act ggg gcg gaa cgc cgc ccg gcc gcg ggc tgg gct ctg tgt cag cag ccg ggc
M S G G T G A E R R P A A G W A L C Q Q P G

ggc gct cgg gcg gga cat ggc agc ctg tac agc ccg gcg ccc tgg cgt ggc gag cgg ctg gtg gtc
G A R A G H G S L Y S P A P W R G E R L V V

ccg gtc gct aac tgg gcc cgg agc caa cgc tct gtg cgg cgg ccg gag ctg gag cct tct cgc cag
P V A N W A R S Q R S V R R P E L E P S R Q

ctc gac cac gac gac gcg gag gca ctc tcg tcc cga aac cga cca gag ggc aaa gtg ttg gag aca
L D H D D A E A L S S R N R P E G K V L E T

gtt ggt gtg ttt gag gtg cca aaa cag aat gga aaa tat gag acc ggg cag ctt ttc ctt cat agc
V G V F E V P K Q N G K Y E T G Q L F L H S

att ttt ggc tac cga ggt gtc gtc ctg ttt ccc tgg cag gcc aga ctg tat gat tgg gat gtg gct tct
I F G Y R G V V L F P W Q A R L Y D W D V A S

gca gct cca gaa aaa gca gag aac cct gct ggc cat ggc cat ggc tcc aag gag gtg aaa ggc aaa
A A P E K A E N P A G H G H G S K E V K G K

act cac act tac tat cag gtg ctg att gat gct gtg act gcc cga cat ata tct cag agt ctc aca gaa
T H T Y Y Q V L I D A V T A R H I S Q S L T E

gct gtt acc ttc ttg gct aac cat gat gac agt cgg gcc ctc tat gcc atc cca ggc ttg gac tat gtc
A V T F L A N H D D S R A L Y A I P G L D Y V

agc cat gaa gac atg ctc ccc tac acc tcc act gat cag gtt ccc atc caa cat gaa ctc ttt gaa aga
S H E D M L P Y T S T D Q V P I Q H E L F E R

ttt ctt ctg tat gac cag aca aaa gca cct cct ttt gtg gct cgg gag agt aag ggc cta aca aga
F L L Y D Q T K A P P F V A R E S K G L T R

gaa gaa tca ccc ctg gct gga gct ctc gat gtt cat cgg gaa aca act gag aac ata cgt gtc act
E E S P L A G A L D V H R E T T E N I R V T

gtc atc ccc ttc tac atg ggc atg agg gaa gtc gga att cca cat gtg tac tgg tgg cgc tac tgt
V I P F Y M G M R E V G I P H V Y W W R Y C

atc cgt ttg gag aac ctt gac agt gat gtg gta cag ctc cgg gag cgg cac tgg agg ata ttc agt
I R L E N L D S D V V Q L R E R H W R I F S

ctc tct ggc acc ttg gag aca gtg cga ggc cga ggg gta gtg ggc agg gaa cca gtg tta tcc aag
L S G T L E T V R G R G V V G R E P V L S K

gag cag cct gct ttc agt ata gca gcc acg tct cgc tgc agg ctt cct agt ggg cac atg tgg ggc
E Q P A F S I A A T S R C R L P S G H M W G

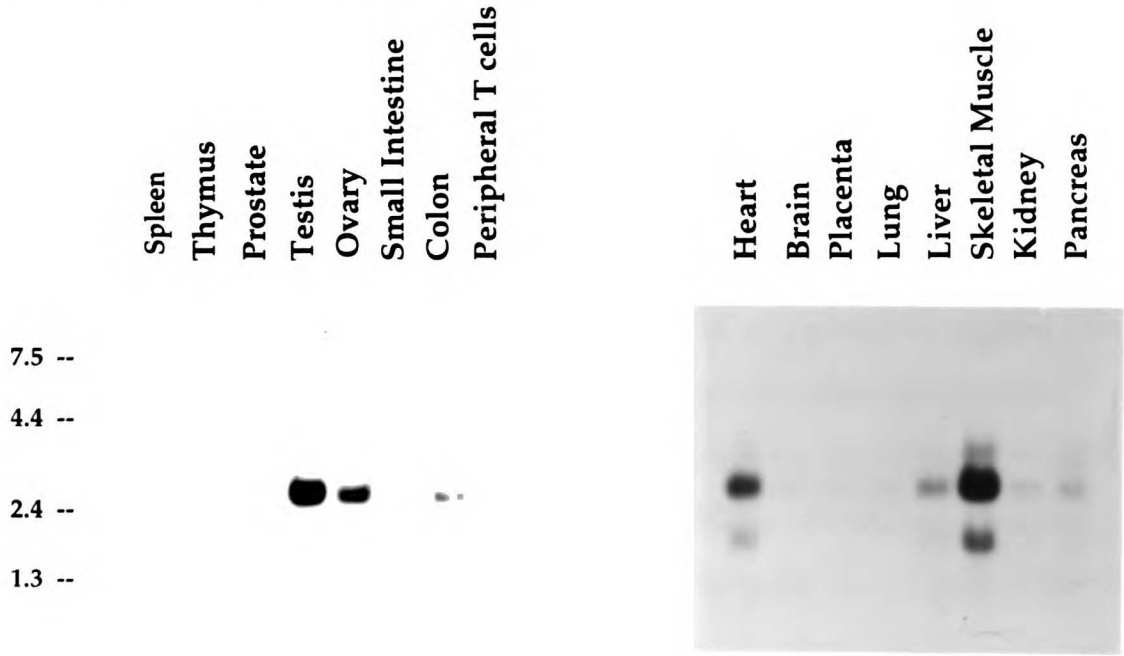
acg ttc cgc ttt gaa aca cct gat ggc tcc cac ttt gat gtt cgg att cct ccc ttc tcc ctg gaa agc
T F R F E T P D G S H F D V R I P P F S L E S

aat aaa gat gag aag aca cca ccc tca ggc ctt cac tgg tag gcc agc tga gg
N K D E K T P P S G L H W * A S *

Figure 6. **Tissue expression of p36.**

p36 is widely expressed, with highest levels of expression in heart, skeletal muscle, ovary and testes. A 500 bp fragment from p36 was used as a probe to blot an adult human tissue northern (Clonetech). The northerns were exposed 2 days.

Tissue expression of p36



in Figure 5. Interestingly, the region of homology to the protein in C. elegans seem to be restricted to the released 320 amino acid fragment.

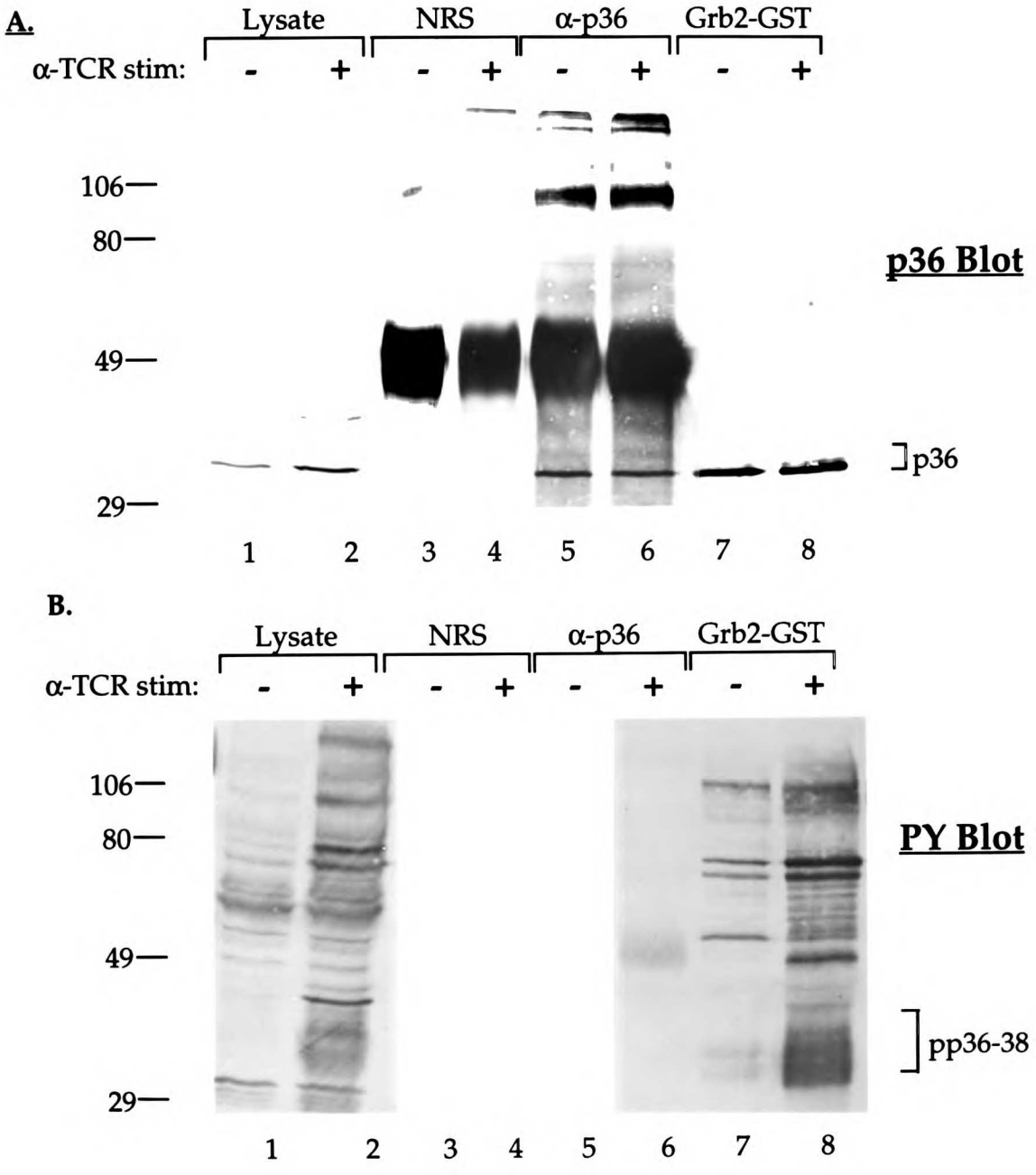
To determine if this cDNA encoded pp36-38, rabbit antisera against the amino- or the carboxy- terminus of p36 were generated. As shown in Figure 7A, the antibody raised against the amino terminus of p36 immunoprecipitated a doublet of 36 kDa and 38 kDa (lanes 5 and 6) that was not present in immunoprecipitates with the control pre-immune sera (lanes 3 and 4), demonstrating that the anti-p36 antibody was specific. Surprisingly, the same amount of p36 bound to Grb2-GST from both unstimulated and stimulated Jurkat cells (lanes 7 and 8) even though there was a significant increase in tyrosine phosphorylated pp36-38 bound to Grb2-GST fusion protein following TCR stimulation as detected by anti-phosphotyrosine blot (Figure 7B, lanes 7 and 8). Furthermore, p36 was not inducibly tyrosine phosphorylated following TCR stimulation (lanes 5 and 6). These results show that although p36 can bind to the Grb2-GST fusion protein, it is not inducibly tyrosine phosphorylated. Therefore, the gene cloned probably does not encode pp36-38.

To determine the specificity of the *in vitro* association of Grb2-GST with the cloned p36, several GST fusion proteins containing SH2 or SH3 domains were tested for their ability to bind p36 from Jurkat lysate. p36 specifically associated with the Grb2-GST fusion protein but not with several other GST fusion proteins containing one or two intact SH2 domains (Figure 8, lanes 3 thru 10). Interestingly, p36 did not associate with the Grb2-GST fusion protein that contained a point mutation in the SH3 (P49L) or SH2 (R86K) domain (lanes 4 and 5, respectively), a surprising finding given that p36 was initially purified using the P49LGrb2-GST fusion protein. The only difference between

Figure 7. **p36 constitutively associates with Grb2-GST and is not tyrosine phosphorylated.**

The equivalent of 50 x 10^6 Jurkat T cells were left unstimulated or stimulated with anti-TCR antibody. The lysates were precipitated with pre-immune sera (lanes 3 and 4), anti-p36 antibody (lanes 5 and 6) or Grb2-GST (lanes 7 and 8). The bound proteins were resolved by SDS-PAGE and analysed by western blotting with anti-p36 antiserum (panel A). This blot was stripped and reprobed with anti-phosphotyrosine antibody (panel B). Control lysates from unstimulated or stimulated cells are shown in lanes 1 and 2, respectively.

p36 constitutively associates with Grb2-GST but it is not tyrosine phosphorylated.



the P49LGrb2-GST fusion protein used in this experiment and the one used during the purification protocol, was that the latter was covalently crosslinked to beads with dimethylpimelimidate (DMP). Therefore, it was possible that covalently crosslinking the P49LGrb2-GST fusion protein to beads with DMP altered its structure and increased its binding affinity for p36. To directly test this possibility, the P49LGrb2-GST and R86KGrb2-GST fusion proteins were either left uncrosslinked or covalently crosslinked to beads with DMP and used to precipitate p36. Consistent with the previous results, p36 did not bind to P49LGrb2-GST (Figure 9, lane 5) or to R86KGrb2-GST (lane 3). However, p36 did bind to the GST fusion proteins that had been covalently crosslinked to beads (lanes 2 and 4) to the same extent as seen with the wild type Grb2-GST (lane 1), providing an explanation as to how p36 purified with P49LGrb2-GST.

Since p36 could specifically bind to Grb2-GST *in vitro*, it was of interest to determine if it could also associate with Grb2 *in vivo*. For this purpose, Grb2 was immunoprecipitated from stimulated Jurkat lysates and the presence of coprecipitating p36 was verified by western blot. As shown in Figure 10, no p36 was detected in the Grb2 immunoprecipitations even though four different anti-Grb2 antibodies raised against the SH2 or SH3 domains in Grb2 were used (lanes 3, 4, 5 and 7). Additionally, there was no detectable Grb2 in anti-p36 immunoprecipitations (data not shown), further demonstrating that the association between p36 and Grb2 was an *in vitro* phenomenon.

New Purification protocol for pp36-38

Although it was clear how the contaminating p36 copurified with P49LGrb2, the fact that ten of the eleven p36 peptides microsequenced

Figure 8. **p36 specifically binds to Grb2-GST in unstimulated Jurkat T cells.** Lysate from the equivalent of 50 x 10^6 unstimulated Jurkat T cells was precipitated with different GST fusion proteins: Grb2-GST (lane 2), S80TGrb2-GST (lane 3), P49LGrb2-GST (lane 4), R86KGrb2-GST (lane 5); CD3ε-GST (lane 6); NH2Vav-GST (lane 7); SH2Vav-GST (lane 8); SH2ZAP-70-GST (lane 9); PTP1C-GST (lane 10). The bound proteins were resolved by SDS-PAGE and analyzed by western blotting with anti-p36 antiserum. The equivalent of 2 x 10^6 3T3 cells was loaded on lane 1.

p36 specifically binds to Grb2-GST in unstimulated Jurkat T cells

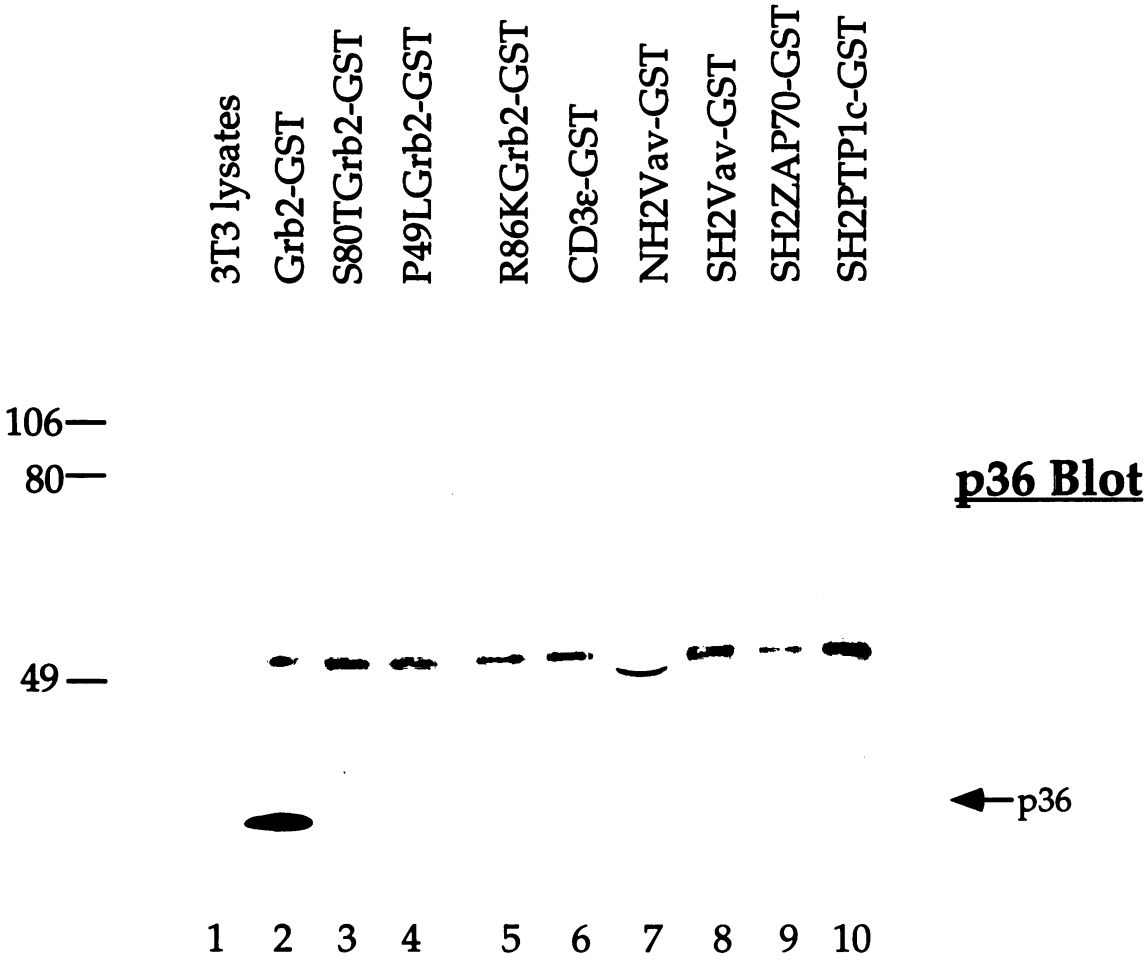


Figure 9. **Covalently crosslinking P49LGrb2-GST to beads alters its structure and promotes the binding of p36.**

Lysate from 50 x 10^6 unstimulated Jurkat T cells was incubated with different uncrosslinked Grb2-GST fusion proteins (lanes 1, 3 and 5), with fusion proteins covalently crosslinked to beads (lanes 2 and 4), with anti-p36 antiserum (lane 6) or control NRS (lane 7). The bound proteins were resolved by SDS-PAGE and analyzed by western blotting with anti-p36 antiserum. The GST fusion proteins used are as shown: wild type Grb2-GST (lane 1); R86KGrb2-GST (lanes 2 and 3) and P49LGrb2-GST (lanes 4 and 5).

Covalently crosslinking P49LGrb2-GST to beads alters its structure and allows binding of p36

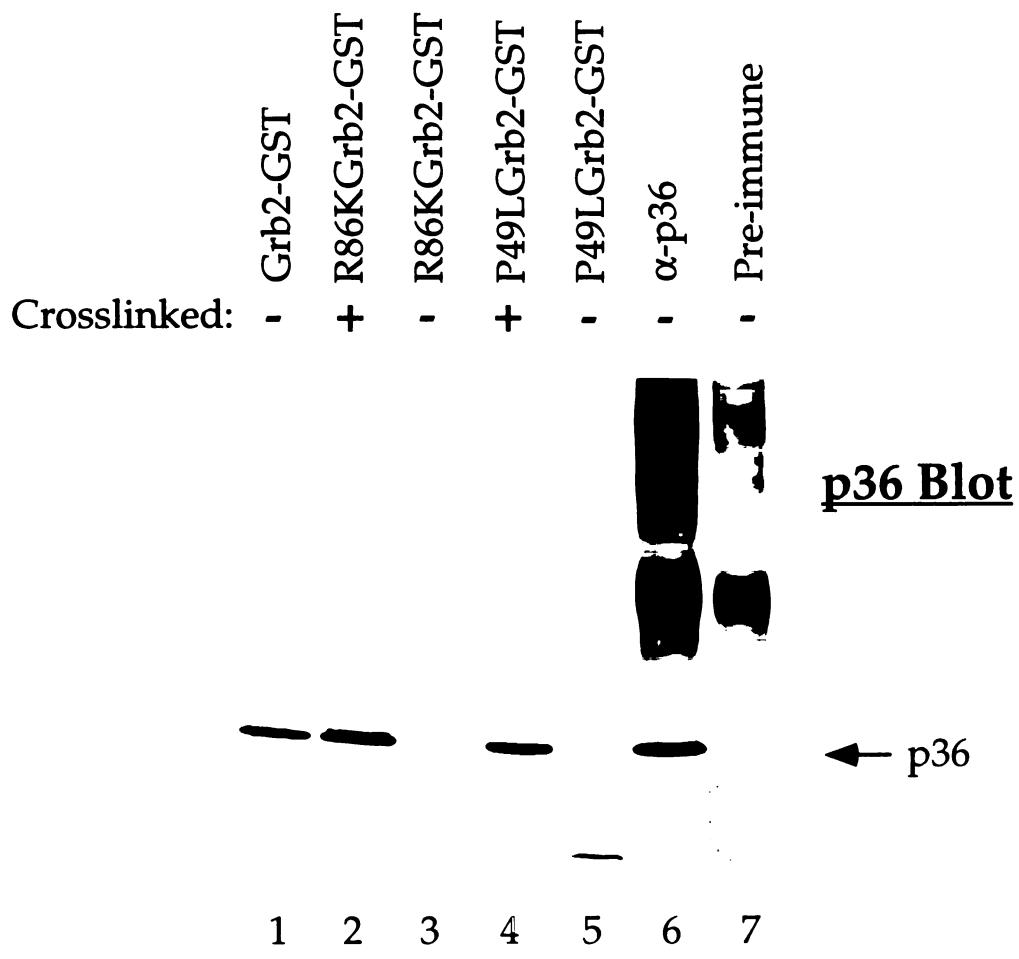
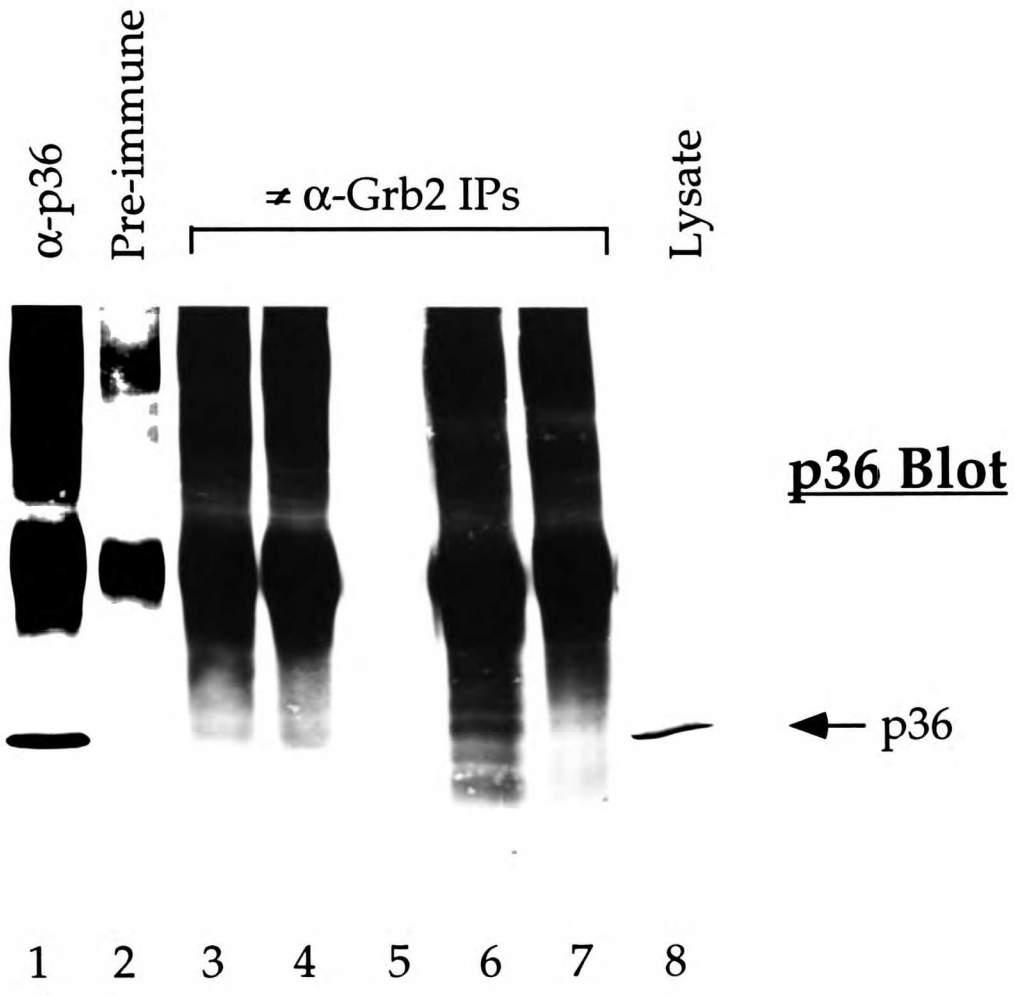


Figure 10. **p36 does not associate with Grb2 *in vivo*.**

Lysate from the equivalent of 50 x 10^6 unstimulated Jurkat cells was precipitated either with anti-Grb2 monoclonal antibody generated against the SH2 (lane 5), with several different (=) polyclonal antisera raised against the SH3 (lanes 3, 4 and 7) domains of Grb2, or with anti-p36 antiserum (lane 1). The bound proteins were resolved by SDS-PAGE, transferred to Immobilon membrane and western blotted with anti-p36 antisera. Normal rabbit serum immunoprecipitates are shown in lanes 2 and 6. Control lysate is shown in lane 8.

p36 does not associate with Grb2 in vivo



originated from the contaminant protein was a strong indication that very little, if any, pp36-38 was purified. Therefore, in an effort to optimize the amount of pp36-38 recovered and reduce the amount of contaminating proteins that bind to Grb2-GST, several changes were incorporated into the purification scheme presented above. The rationale for this new purification scheme as well as the results of a pilot study are presented below.

i. Optimizing the phosphorylation of p36-38 with the use of pervanadate. There were several possibilities that could explain why a greater amount of pp36-38 did not copurify with P49LGrb2, one of them being that optimal tyrosine phosphorylation of pp36-38 was not obtained due to the rapid kinetics of phosphorylation and dephosphorylation of pp36-38 (Sieh et al., 1994). The TCR complex in Jurkat cells is rapidly internalized following exposure to soluble anti-TCR monoclonal antibody (M. Graber, personal communication), which could contribute to the rapid dephosphorylation of pp36-38. Thus, it was reasoned that one could sustain the phosphorylation of pp36-38 by inhibiting the downregulation of the TCR. However, treatment with sodium azide as well as extensively crosslinking the TCR complex with a combination of anti-TCR antibodies such as Leu4, 235 and C305 did not affect the kinetics of pp36-38 dephosphorylation (data not shown). Since it was not clear how the dephosphorylation of pp36-38 could be prevented, pilot experiments were performed to assess whether pervanadate could enhance the phosphorylation of p36-38.

Pervanadate, a tyrosine phosphatase inhibitor, offers advantages over using stimulating anti-TCR antibodies. First, it bypasses the need for high TCR expression levels, an important consideration because Jurkat cells can lose expression of the surface TCR when kept in culture for long periods of

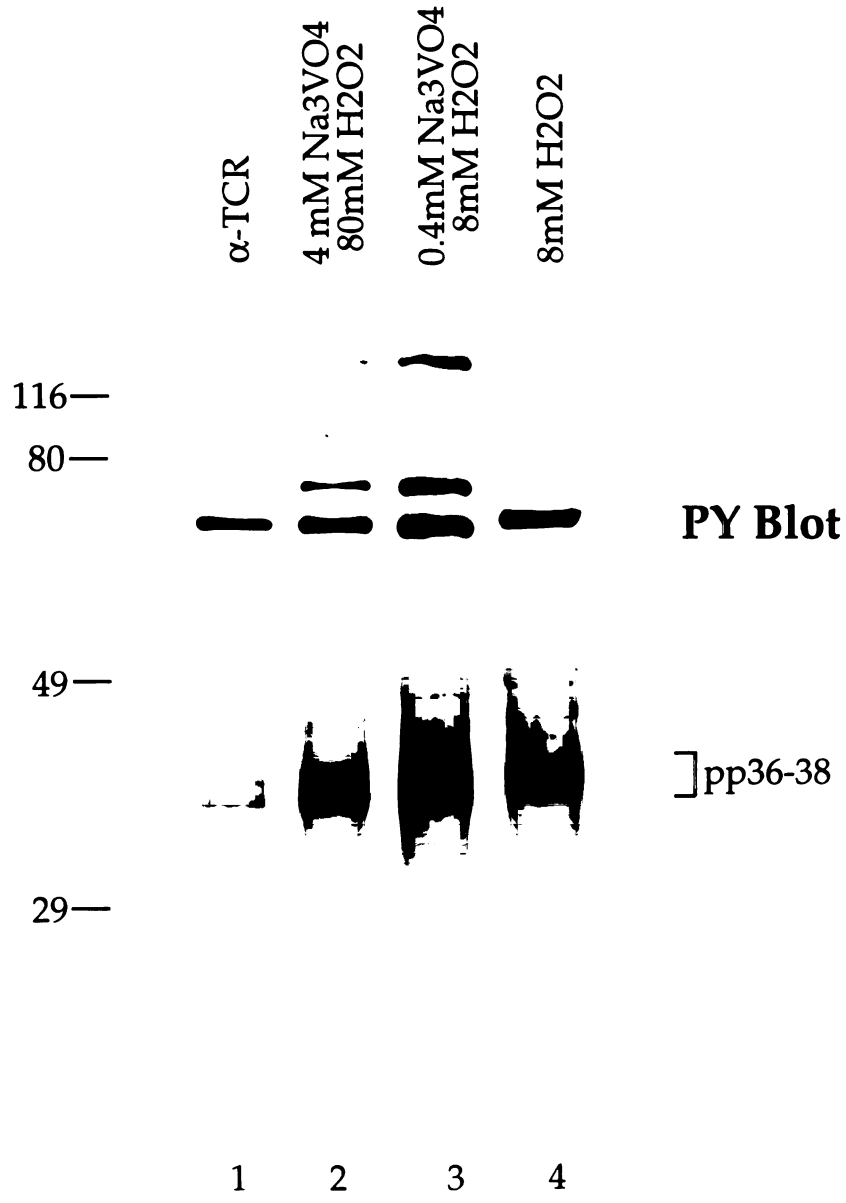
time (our unpublished observation). Second, pervanadate is fairly easy to generate compared to producing high quantities of stimulating anti-TCR antibody. Third, pervanadate would allow the purification of pp36-38 from thymic or splenic tissues. Fourth, cells could be uniformally stimulated in large 10 ml quantities with pervanadate, an important issue when attempting to purify pp36-38 from as much as 200 liters of Jurkat cells. However, despite these clear advantages, the actions of pervanadate stimulation are complex. Thus, there is a great risk of inducing artifactual tyrosine phosphorylation of irrelevant proteins. In order to determine whether pervanadate and TCR stimulation induced the same tyrosine phosphorylated proteins to bind to Grb2-GST, Jurkat cells were treated with different concentrations of pervanadate or with anti-TCR antibody. As shown in Figure 11, both pervandate stimulation and anti-TCR stimulation induced similar proteins, pp115, pp80, pp70 and pp36-38, to bind to Grb2-GST. pp36-38 isolated from pervanadate treated cells migrated as a significantly more diffuse band than pp36-38 from anti-TCR stimulated cells (Figure 11, lane 1 versus lanes 2, 3, and 4). These results suggest that a short treatment with pervanadate can mimic TCR stimulation in inducing the specific binding of pp36-38 to Grb2-GST.

ii. Eliminating contaminating proteins by immunoprecipitating proteins with anti-phosphotyrosine antibody. The purification and cloning of an irrelevant 36 kDa non-tyrosine phosphorylated protein underscored the importance of adding a second affinity purification step for isolating only the tyrosine phosphorylated proteins associated with Grb2-GST. Because preliminary experiments using NEPHGE suggested that pp36-38 was an

Figure 11. **Pervanadate treatment mimics TCR stimulation in inducing specific binding of pp36-38 to Grb2-GST**

The equivalent of 50 x 10^6 Jurkat cells were stimulated with anti-TCR antibody for 2 minutes (lane 1), with different concentrations of pervanadate (H2O2+ NA3VO4) (lanes 2 and 3) or with hydrogen peroxide (H2O2) lane 4) for 1 minute. Lysates were precipitated with Grb2-GST and the bound proteins were resolved by SDS-PAGE, transferred to membrane and analyzed by western blotting with an anti-phosphotyrosine antibody.

Pervanadate treatment mimics TCR stimulation in inducing specific binding of pp36-38 to Grb2-GST



extremely acidic protein (data not shown), an anion exchange was used as the first step in the purification. Only 10% of the total cellular protein co-eluted with pp36-38 as measured by absorbance at 280 nm, however, the amount of pp36-38 recovered was only 23% of the total pp36-38 in the lysate (data not shown). Therefore, this approach was unsatisfactory due to a significant loss of pp36-38.

In contrast, the introduction of an additional anti-phosphotyrosine affinity purification step following the purification with Grb2-GST, did not cause significant losses of pp36-38. Jurkat lysates from unstimulated or pervanadate-stimulated cells were precipitated with Grb2-GST and the bound proteins were then eluted by boiling in 0.5% SDS. After diluting the lysate to 0.1%SDS, the proteins were reprecipitated with anti-phosphotyrosine antibody. The bound tyrosine phosphorylated proteins were eluted with phenylphosphate as diagrammed in Figure 12. As shown in Figure 13, 90% of the phosphoproteins were efficiently eluted with 0.5% SDS (Compare lanes 3 and 4). Following reprecipitation with anti-phosphotyrosine antibody, at least 80% of pp36-38 was eluted with phenylphosphate (Compare lanes 5, 6 and 7) or about 60- 70% of the total precipitatable pp36-38 was recovered (Compare lanes 2 and 7). This data suggests that the addition of the anti-phosphotyrosine affinity purification step does not cause a significant loss of pp36-38.

This purification scheme was then scaled up so that quantitation of purified pp36-38 could be assessed using silver stain. The equivalent of 4 x 10^9 unstimulated or stimulated Jurkat cells were subjected to the purification protocol described above and the bound proteins were visualized either by silver stain or analyzed by western blotting with an anti-phosphotyrosine

Figure 12. **Diagram of new pilot purification scheme for pp36-38.**

5×10^9 Jurkat cells were stimulated with pervanadate for 1 minute. Following stimulation, the lysates were incubated with Grb2-GST for 2 hours, the bound proteins were extensively washed with lysis buffer, and eluted with 0.5% SDS with heating at 95°C for 3 minutes. The supernatant was diluted to 0.1% SDS with lysis buffer and subjected to re-immunoprecipitation with anti-phosphotyrosine antibody 4G10 for 2 hours at 4°C. Following extensive high salt washes, the bound proteins were eluted with phenyl phosphate. The supernatant containing the eluted proteins was resolved by SDS-PAGE and analysed by silver stain or by western blotting with anti-phosphotyrosine antibody.

Diagram of new purification scheme for pp36-38

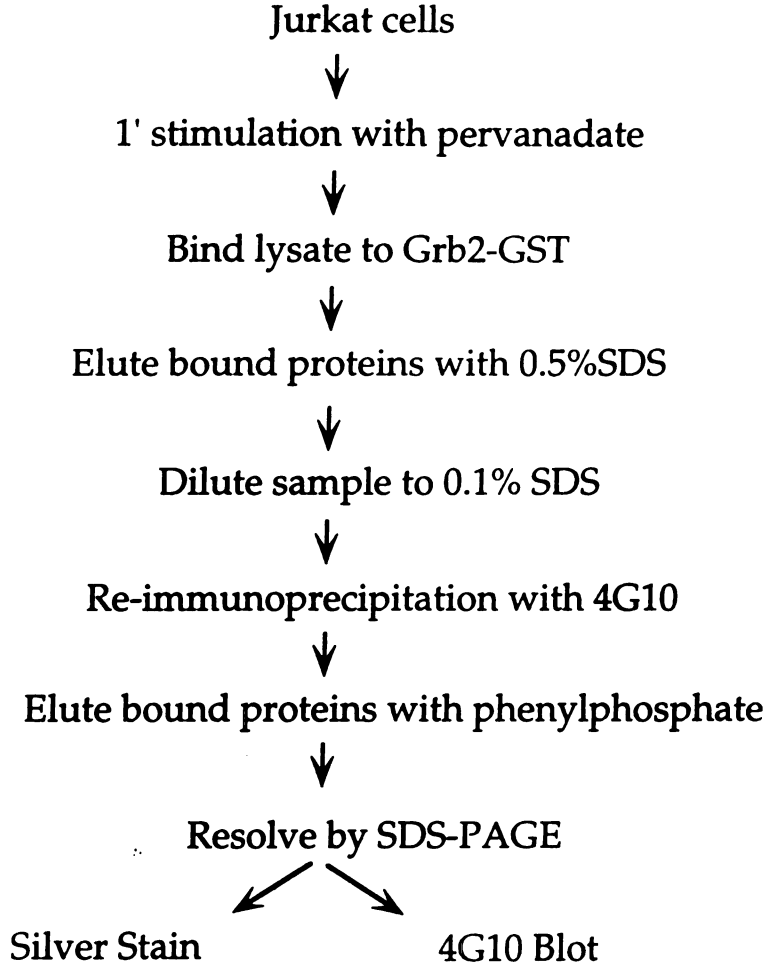
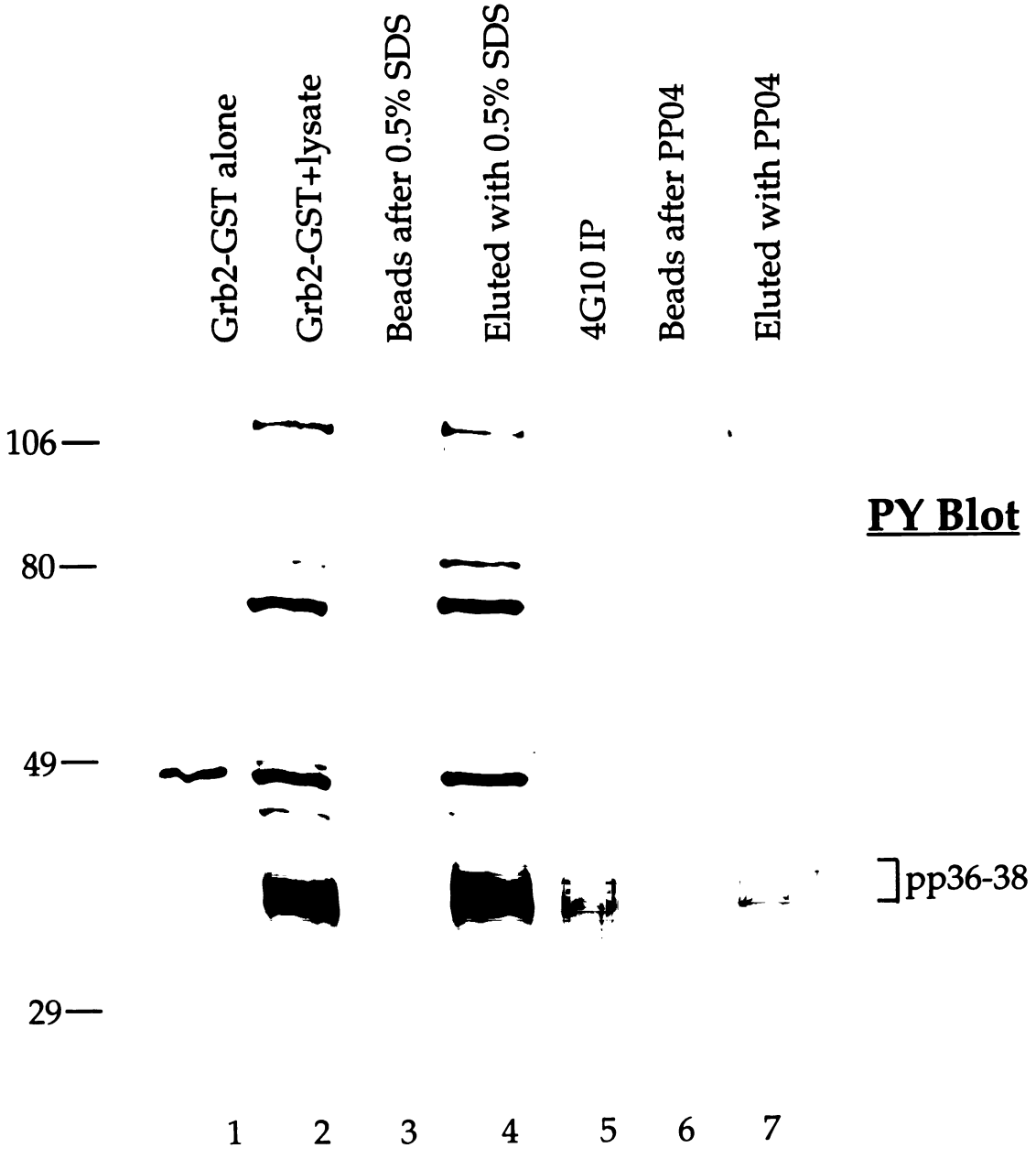


Figure 13. **Estimation of pp36-38 losses with the addition of an anti-phosphotyrosine affinity purification step.**

Jurkat lysate from the equivalent of 50 x 10^6 pervanadate stimulated cells was precipitated with Grb2-GST (lane 2). Following a 2 hour incubation, the proteins bound to Grb2-GST were eluted with 0.5% SDS (lane 4) and reprecipitated with anti-phosphotyrosine antibody 4G10 (lane 5). The tyrosine phosphorylated proteins were eluted off the 4G10 beads with phenylphosphate (PPO_4) (lane 7). The proteins that did not elute off the beads with 0.5% SDS (lane 3) or with phenylphosphate (lane 6) were solubilized with 2x sample buffer and loaded directly onto SDS-PAGE, transferred to membrane and western blotted with anti-phosphotyrosine antibody RC20. 2 ug of Grb2-GST was loaded in lane 1.

Estimation of pp36-38 loss with the addition of an anti-phosphotyrosine affinity purification step



antibody. While pp36-38 was the major phosphotyrosine protein that bound to Grb2-GST and that eluted with phenylphosphate (Figure 14, lane 4), it was barely detectable on the silver stained gel (an upper estimate of about 5 ng, data not shown). These results suggests that although pp36-38 is heavily tyrosine phosphorylated, it is not purified in large quantities using this protocol.

iii. Dephophorylation of pp36-38 with CD45. In addition to pp36-38 not being purified in large quantities, it migrates as a very diffuse band, further diluting the silver stain signal. Therefore, dephosphorylating pp36-38 after the last purification step could potentially compress the diffuse pp36-38 into a single tight band that could be more easily visualized by silver stain. In order to determine whether pp36-38 could be dephosphorylated by CD45, pp36-38 bound to Grb2-GST was incubated with purified CD45 for up to one hour. As shown in Figure 15, most of the pp36-38 was dephosphorylated by purified CD45 within 10 minutes (Compare lanes 1 and 2) and completely dephosphorylated within 20 minutes (lane 3). Although CD45-GST was also able to dephosphorylate pp36-38, albeit less efficiently, covalently crosslinking CD45-GST to beads with DMP completely inhibited its phosphatase activity (data not shown). Thus, a pilot experiment using purified CD45 to dephosphorylate pp36-38, was performed to determine whether the dephosphorylated pp36-38 was now visible by silver stain. Although pp36-38 isolated from 5×10^9 pervanadate treated Jurkat cells was efficiently dephosphorylated by purified CD45 there was no detectable dephosphorylated pp36-38 band on the silver stained gel (data not shown). These experiments suggest that pp36-38 cannot be easily purified in large quantities from Jurkat lysates using this methodology.

Figure 14. **Pilot experiment for pp36-38 purification.**

Lysates from 4 x 10^9 unstimulated (lanes 1, 3 and 5) or pervanadate stimulated (lanes 2, 4 and 6) Jurkat T cells were prepared as diagrammed in the previous figure. Following purification, 1/30 of the purified proteins eluted with phenylphosphate (PPO4) were loaded in lanes 3 and 4, while the remaining sample (29/30) was analysed by silver stain on a separate gel. As controls for this pilot experiment, 1/15 of the proteins that did not elute off the beads with 0.5% SDS (lane 1 and 2) or with phenylphosphate (PPO4)(lane 5 and 6) were also solubilized in 2XSDS sample buffer. The proteins were resolved by SDS-PAGE and analysed by western blotting with the anti-phosphotyrosine antibody RC20.

Pilot experiment for purification of pp36-38

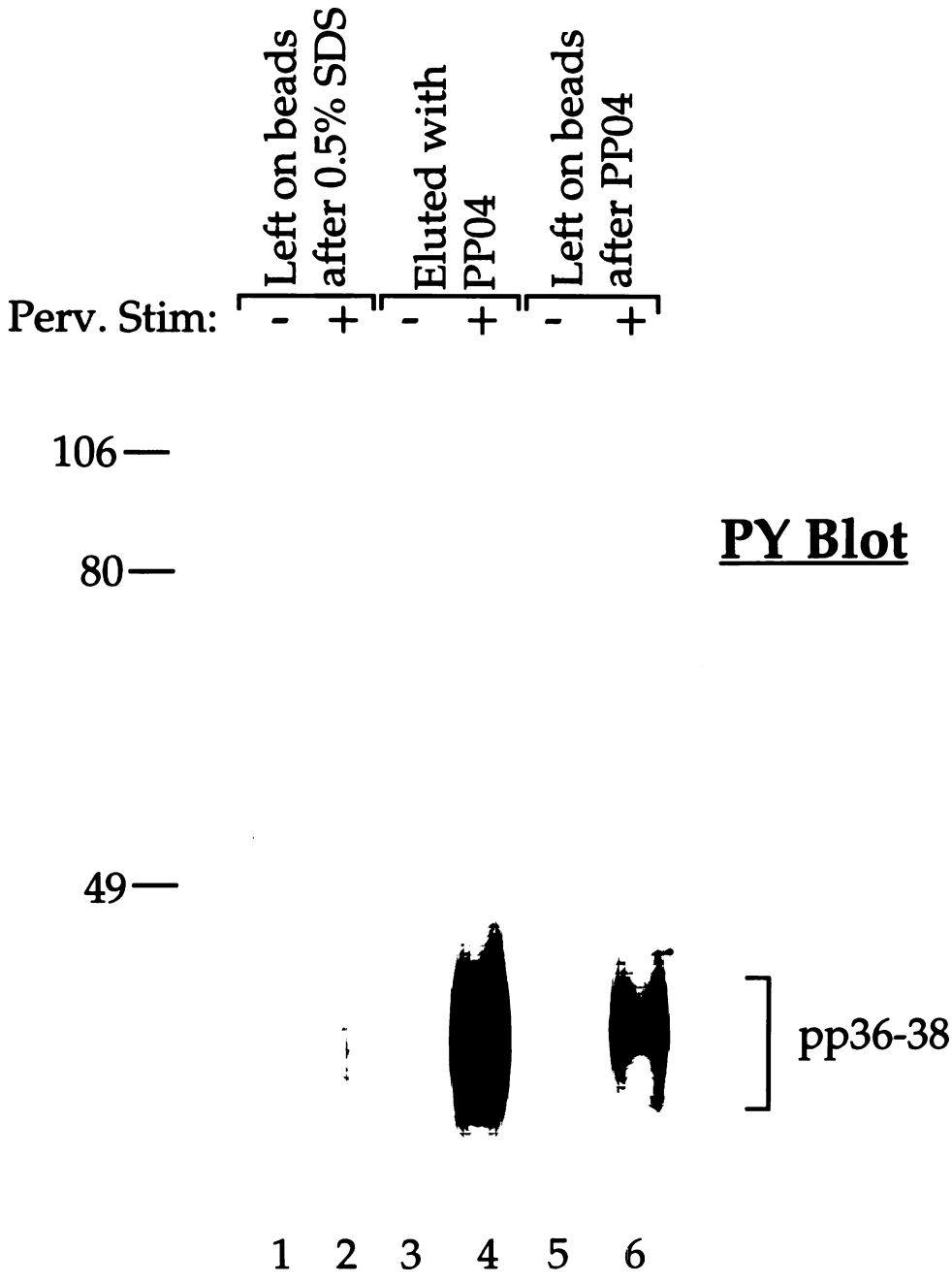
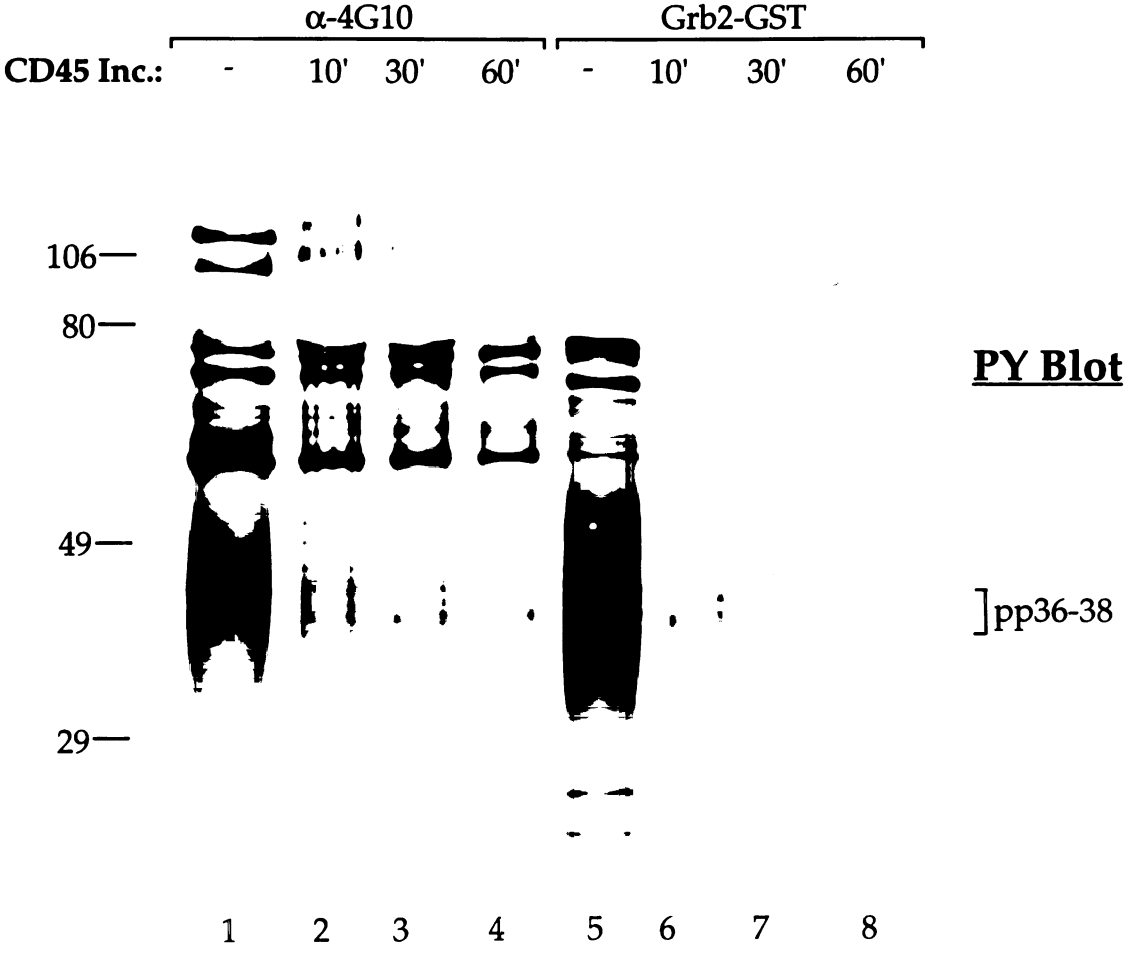


Figure 15. Dephosphorylation of pp36-38 by purified CD45.

Jurkat lysates from the equivalent of 50 x 10^6 pervanadate stimulated cells were precipitated with anti-phosphotyrosine antibody 4G10 (lanes 1 - 4) or with Grb2-GST (lanes 5 - 8). The proteins bound to the beads washed and incubated with purified CD45 (lanes 2 - 5 and 6 - 8) for the periods of time indicated. The proteins were resolved on SDS-PAGE and western blotted with anti-phosphotyrosine 4G10 mAb. Lanes 1 and 5 are the control immunoprecipitations without CD45.

Dephosphorylation of pp36-38 by CD45



Lnk is unlikely to be pp36-38

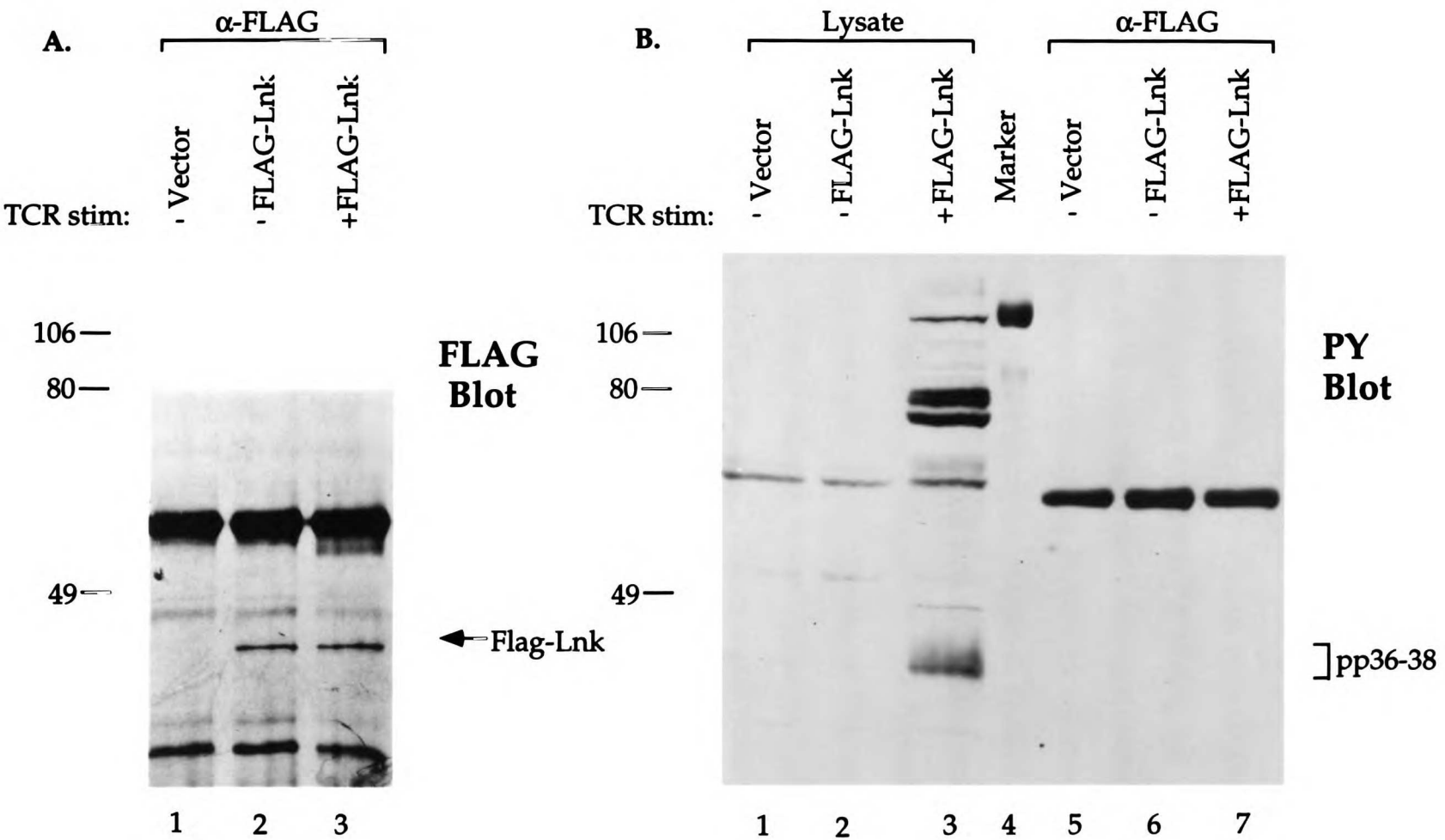
Recently, the cloning of a novel 34 kDa protein containing a single SH2 domain that was highly expressed in rat spleen, lymph nodes and thymus (129) raised the possibility that it was the rat homolog of pp36-38. Biochemical studies of Lnk showed that a phosphopeptide based on its most carboxyl-terminus tyrosine could associate with Grb2, PLC γ1 and the p85 of PI3 kinase, even though this tyrosine is not in a consensus motif for binding to Grb2, PLC γ1 or p85 (130, 131). Although Lnk was tyrosine phosphorylated following TCR and CD4 crosslinking, there was no detectable tyrosine phosphorylated PLC γ1 coprecipitating with Lnk (129). Additionaly, Grb2, P85 and PLC γ1 constitutively bound to Lnk with only a minimal increase in association following TCR crosslinking (129). Therefore, it is unclear whether Lnk represents the rat homolog of pp36-38 observed in Jurkat cells. Because anti-Lnk antiserum was not yet available, Lnk was epitope tagged in the amino-terminus with FLAG or Myc, and transiently transfected into Jurkat TAg cells. As shown in Figure 16A, the epitope-tagged Lnk was expressed in both unstimulated and stimulated Jurkat TAg cells (lanes 2 and 3) but not in vector transfected Jurkat TAg cells (lane 1). However, no basal or inducible tyrosine phosphorylation of FLAG-Lnk could be detected (Figure 16B, lane 5 and 6) even though pp36-38 could be easily detected in stimulated Jurkat lysates (lane 3), suggesting that Lnk is not the rat homolog of pp36-38. The acidity of the FLAG tag is unlikely to be preventing Lnk from becoming tyrosine phosphorylated because similar results were obtained with a Myc-Lnk construct (data not shown).

To address whether Lnk is capable of being tyrosine phosphorylated under other conditions, Jurkat TAg cells were transfected with FLAG-Lnk and

Figure 16. FLAG-tagged Lnk is not tyrosine phosphorylated following TCR engagement.

50×10^6 Jurkat TAg cells were transfected either with 200 ug of control vector (lanes 1 and 4) or with 200 ug of FLAG-Lnk (lanes 2, 3, 5 and 6). After 48 hours, the cells were either left unstimulated (lanes 2 and 5) or were stimulated with anti-TCR antibody for 2 minutes (lanes 3 and 6). The lysates were prepared and either directly loaded on SDS-PAGE (lanes 1, 2 and 3), or first immunoprecipitated with anti-FLAG antibody (lanes 4, 5 and 6). The proteins were transferred to membranes and western blotted with anti-phosphotyrosine antibody 4G10 (panel B). The piece of membrane containing lanes 5, 6 and 7 (panel B) was stripped and re-probed with anti-FLAG antibody (panel A, lanes 1, 2 and 3).

FLAG-tagged Lnk is not tyrosine phosphorylated following TCR engagement



stimulated with anti-TCR antibody or pervanadate. Consistent with previous results, FLAG-Lnk was not inducibly tyrosine phosphorylated following TCR stimulation, though it could be tyrosine phosphorylated with pervanadate (Figure 17A). Interestingly, the phosphorylated FLAG-Lnk appeared to migrate as a doublet reminiscent of pp36-38 (Figure 17A), raising the possibility that Lnk is indeed pp36-38, and that a much stronger stimulus is required to induce tyrosine phosphorylation of the FLAG-Lnk construct. In order to address this possibilitity, tyrosine phosphorylation of FLAG-Lnk was induced with pervanadate and the ability of FLAG-Lnk to associate with PLC γ1, SLP-76 and Grb2 was assessed. As shown in Figure 17, even though FLAG-Lnk was tyrosine phosphorylated with pervanadate treatment (Figure 17A, lane 7) it did not coprecipitate with PLC γ1(panel B, lane 7), nor with SLP-76 (panel C, lane 7), even though both PLC γ1and SLP-76 were easily detected in the lysates (panels B and C, respectively, lanes 1, 2 and 3). Furthermore, there was no detectable association between FLAG-Lnk and Grb2-GST (Figure 18A, lanes 4, 5, and 6), even though FLAG-Lnk was expressed (lanes 10, 11, 12) and inducibly tyrosine phosphorylated with pervanadate treatment (Figure 18B, lane 12). Of note, the tyrosine phosphorylated FLAG-Lnk has a slower mobility on SDS-PAGE and comigrates with the non-specific band (Figure 18A, lane 12). However, Grb2-GST still bound pp36-38 from these same lysates and could be easily detected following stimulation with anti-TCR antibody or with pervanadate (Figure 18B, lanes 2, 3, 5 and 6).

Taken together, the experiments above demonstrate that FLAG-lnk differs from pp36-38, in that the former is not inducibly tyrosine phosphorylated following TCR stimulation. Additionally, even when FLAG-Lnk is artificially tyrosine phosphorylated with pervanadate treatment, it

Figure 17. **FLAG-Lnk is phosphorylated with pervanadate treatment but does not associate with SLP-76 or PLC γ1.**

50×10^6 Jurkat TAg cells were transfected with 200ug of plasmid encoding FLAG-Lnk and harvested 48 hours later. Lysates from unstimulated (lanes 1 and 4), TCR stimulated (lanes 2 and 5) or pervanadate treated (lanes 3 and 6) cells were immunoprecipitated with anti-FLAG antibody (lanes 4, 5 and 6) or directly resolved by SDS-PAGE (lanes 1, 2 and 3). The proteins were transferred to membrane and analyzed by western blotting with anti-phosphotyrosine antibody 4G10 (A); anti-PLC γ1 mAb (B) or anti-SLP-76 antiserum (C). The doublet corresponding to FLAG-Lnk is indicated by the arrow.

FLAG-Lnk is phosphorylated with pervanadate but does not associate with SLP76 or PLCγ1

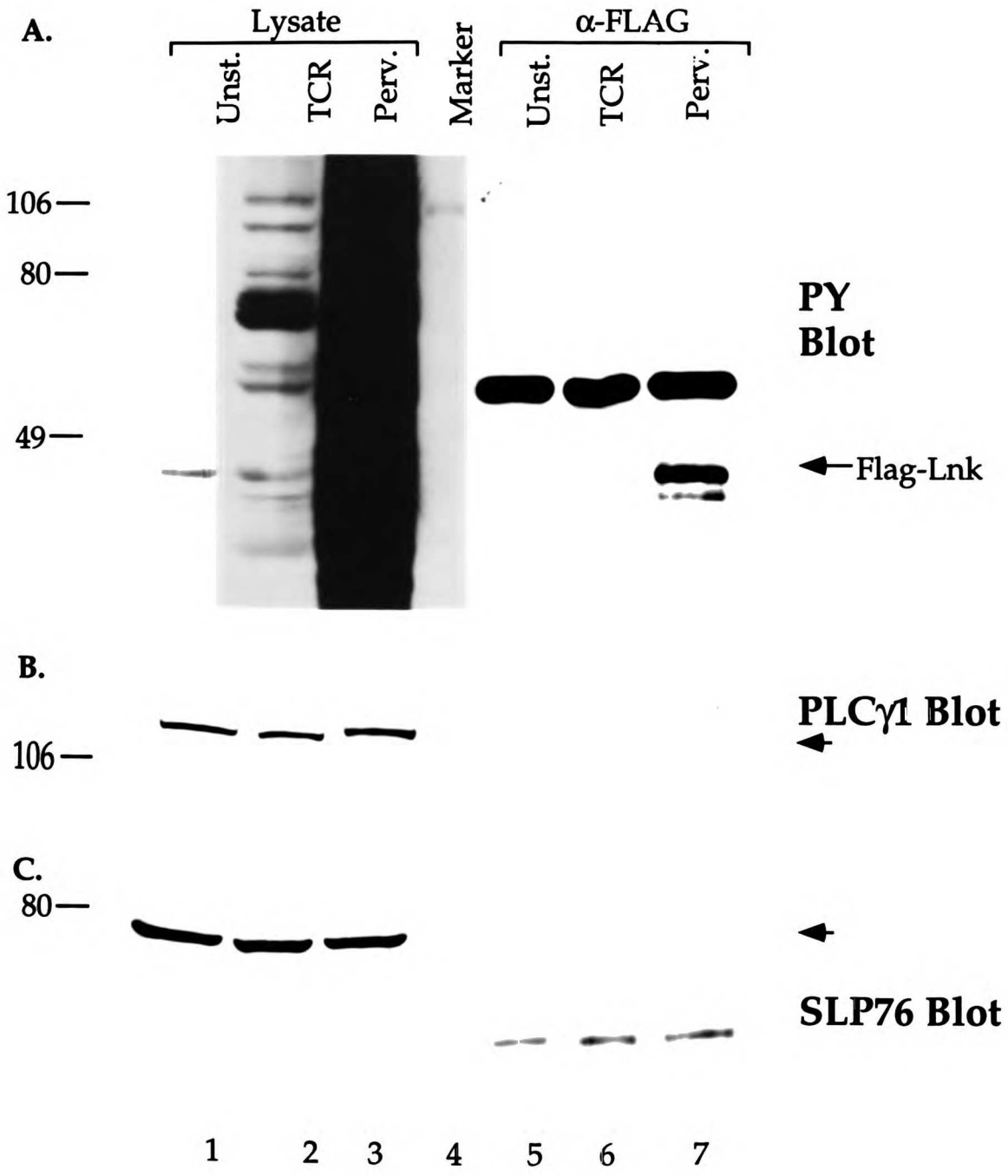
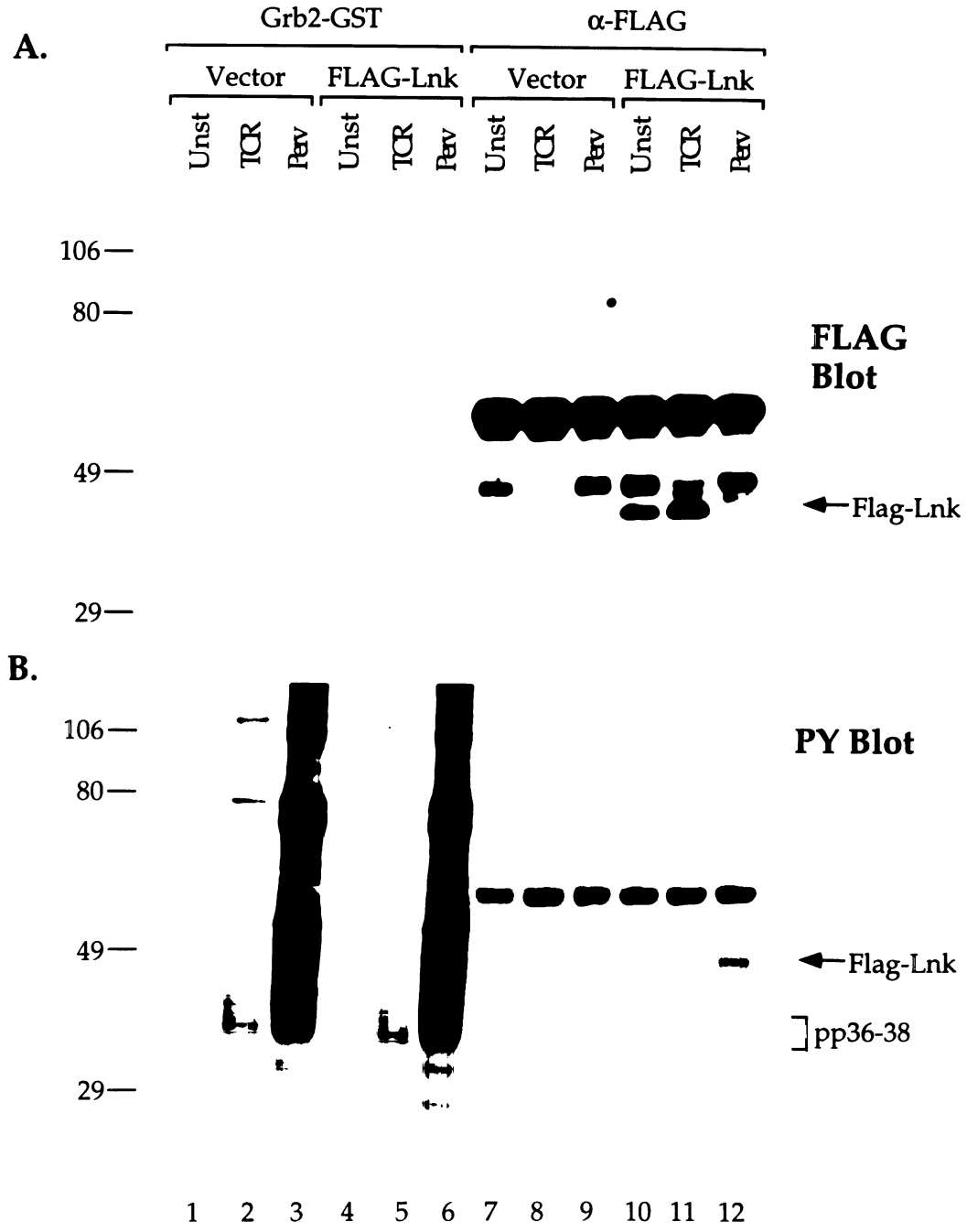


Figure 18. **Tyrosine phosphorylated FLAG-Lnk does not bind to Grb2-GST.** Jurkat TAg cells were transfected with either control vector (lanes 1 - 3, 7 - 9) or with FLAG-Lnk (lanes 4 - 6, 10 - 12) as described in Material and Methods. After 48 hours, the cells were harvested and either left unstimulated (lanes 1, 4, 7 and 10), stimulated with anti-TCR antibody (lanes 2, 5, 8 and 11) or pervanadate treated (lanes 3, 6, 9, and 12). The lysates were prepared and either precipitated with Grb2-GST (lanes 1 thru 6) or with anti-FLAG antibody (lanes 7 thru 12). The bound proteins were resolved by SDS-PAGE and western blotted with anti-FLAG antibody (panel A). The membrane was then stripped and re-probed with anti-phosphotyrosine 4G10 antibody (panel B).

Tyrosine phosphorylated FLAG-lnk does not bind to Grb2-GST



does not coprecipitate with the proteins known to associate with pp36-38, such as PLC γ1, Grb2 and SLP-76. Therefore, these results strongly argue that Lnk is unlikely to be the rat homolog of pp36-38. However, because the experiments described above rely on the overexpression of an epitope-tagged construct of Lnk, the possibility that the epitope destroys the function of Lnk, or that the overexpressed Lnk is not properly localized, still remains. Thus, the formal proof that Lnk is not the rat homolog of pp36-38, awaits the development of good antisera against Lnk.

p80

In the course of these studies, it was reported that a serine/threonine kinase was involved in the negative regulation of Grb2-SOS complexes. More specifically, phosphorylation of SOS by a MEK-dependent kinase results in the dissociation of SOS from Grb2 and deactivation of p21Ras (126-128). In order to determine if a serine/threonine kinase was associated with Grb2 in T cells, *in vitro* kinase assays were performed on Grb2 immunoprecipitates from unstimulated and stimulated Jurkat cells. As shown in Figure 19A, a single major 80 kDa *in vitro* phosphorylated band co-immunoprecipitated with Grb2 (lanes 2, 3 and 4) but not with control normal rabbit serum (lane 1). The *in vitro* phosphorylation of p80 did not depend on Lck, since p80 was equally phosphorylated in JCaM1.6 cells. Phospho-amino acid analysis of p80 revealed that it was phosphorylated on serine but not on tyrosine or threonine residues (Figure 19B), suggesting that either p80 itself is a serine kinase or that Grb2 is constitutively associated with a serine kinase. In order to determine if this serine kinase activity could be mapped to either the SH2 or SH3 domain of Grb2, Jurkat lysates were incubated with Grb2-GST fusion proteins containing only the SH2 domain (Figure 19C, lane 3), the amino-

terminal SH3 domain (lane 4) or the carboxyl-terminus SH3 domain (lane 5) of Grb2. Although the 80 kDa phosphoprotein was present in the *in vitro* kinase assay of Grb2 immunoprecipitates, it was not detected in the different Grb2-GST precipitates. Re-immunoprecipitation experiments with anti-ZAP-70 and anti-Raf antibodies demonstrated that p80 was not ZAP-70 nor Raf (data not shown). It is tempting to speculate that an intact Grb2 protein is required for association with p80 or for the phosphorylation of p80. Additional kinase assays using the full length Grb2-GST fusion protein should address this issue.

Interestingly, one of the proteins that copurified with the P49LGrb2-GST fusion protein was 80kDa (Figure 3). Microsequencing of the tryptic peptides revealed that one of the peptides had homology to a serine/threonine kinase involved in meiosis in yeast (191) (Figure 2). Although no other peptide showed any homology to a serine kinase or was found in the EST database, it is tempting to speculate that the 80 kDa protein purified may be the major 80 kDa protein seen in Grb2 *in vitro* kinase reaction.

Figure 19. **Grb2 constitutively associates with an 80kDa *in vitro* phosphorylated protein.**

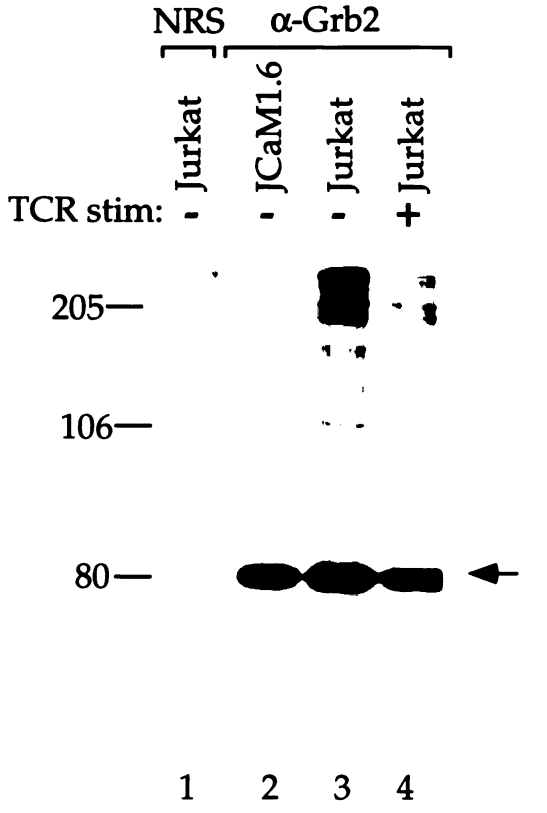
(A) Lysates from unstimulated (lanes 1 - 3) or stimulated (lane 4) Jurkat and JCaM1.6 (lane 2) cells were prepared and precipitated with anti-Grb2 antibody (lanes 2 thru 4) or with control NRS (lane 1). The bound proteins were subjected to an *in vitro* kinase assay and resolved by SDS-PAGE. The gel was dried and exposed overnight.

(B) The major 80 kDa phosphorylated band was excised from the gel, rehydrated, and digested with concentrated HCl before the phosphoaminoacids were resolved by 2 dimensional analysis. The spots corresponding to the standard phosphoaminoacids phosphoserine (S), phosphotyrosine (Y) and phosphothreonine (T) are shown. The film was exposed for three days.

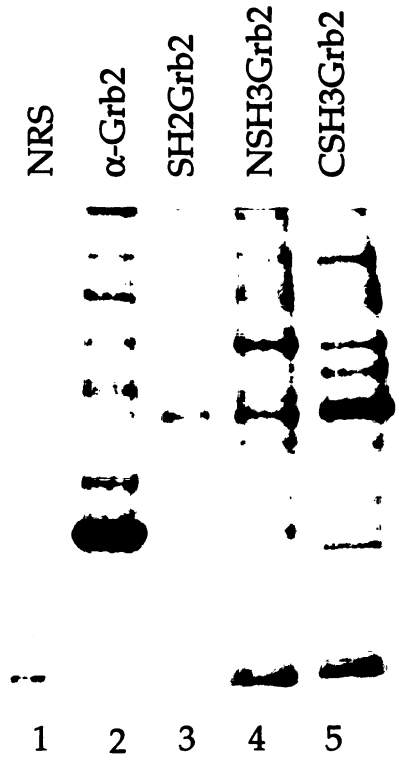
(C) Same as in (A), except that Grb2-GST fusion proteins containing the SH2 (lane 3) amino-terminal SH3 (lane 4) or carboxy-terminal SH3 (lane 5) domains were incubated with the lysates from unstimulated Jurkat cells. As a control, lysates were incubated with anti-Grb2 antiserum (lane 2) or with NRS (lane 1).

Grb2 constitutively associates with an 80 kDa protein

A. In vitro kinase assay



C. In vitro kinase assay



B. Phosphoamino acid analysis

Discussion

Purification of pp36-38

Using a covalently coupled Grb2-GST mutant containing a point mutation in the amino-terminal SH3 domain, P49LGrb2-GST, we have purified five proteins from stimulated Jurkat lysates. Three of these proteins, Cbl, dynamin and Sam68, have been previously shown to associate *in vivo* and/or *in vitro* with Grb2 (68-70, 118-120), indicating that the affinity purification had worked reasonably well. The other two proteins, p36 and p80, were novel proteins and p36 will be further discussed below.

Cloning of the gene encoding the purified p36 (Figure 5) followed by biochemical analysis of p36 demonstrated that it did not correspond to the pp36-38 associated with Grb2 (Figure 7, 9 and 10). Thus, the initial affinity purification protocol was modified in such a way as to include stimulation with pervanadate instead of with anti-TCR antibody, an additional anti-phosphotyrosine affinity purification step, as well as, a treatment with CD45. Although a pilot experiment using the equivalent of 5×10^9 Jurkat cells indicated that the revised purification protocol had worked well in that there was little loss of pp36-38 during the procedure (Figure 14), there was no detectable pp36-38 protein by silver stain. These results strongly indicate that the biochemical purification of pp36-38, though conceptually straightforward, is not trivial due to low amounts of purification of this phosphoprotein from the cell. Consistent with this notion, Motto et al., using a similar purification protocol as described above, could easily detect pp36-38 by anti-phosphotyrosine blots but not by silver stain (118). One way of overcoming prohibitively low amounts of the protein of interest is by resorting to the use of animal tissues that express this protein. In this way, NFAT was purified

and cloned from bovine thymus (132). However, although there is a tyrosine phosphorylated 36 kDa protein that associates with Grb2-GST in lysates from mouse thymus stimulated with anti-CD3 antibody (N. Van Oers, personal communication), it is still unknown whether it corresponds to pp36-38 because it migrates as a single band as opposed to the characteristic diffuse band seen for pp36-38 in Jurkat cells. Further experiments using anti-PLC γ1 antibody and p85-GST are needed to establish whether pp36-38 is expressed in the thymus. Alternatively, other cloning techniques that are based on the differential expression of pp36-38 mRNA, such as PCR-based subtractive hybridization, restriction differential analysis (RDA), and phage display, are possible but high risk approaches, especially in the case of JCaM2.5 cells.

There are two observations that suggest that pp36-38 is drastically reduced in JCaM2.5 cells. First, it is remarkable that the signaling defect of JCaM2.5 cells, i.e, no increase in intracellular calcium following TCR engagement, parallels the results of a study aimed to specifically dephosphorylate pp36-38 (Motto et al., in press). More specifically, Motto et al., have stably transfected a chimera containing the extracellular and transmembrane domains of Class I MHC, followed by the SH2 domain of Grb2 linked to the phosphatase domains of CD45, into Jurkat cells. Following engagement of the TCR of these stable transfectants, there is no pp36-38 and no increase in intracellular calcium. This experiment is consistent with the notion that tyrosine phosphorylation of pp36-38 is required for increase in intracellular calcium and that JCam2.5 cells have drastically reduced amounts of pp36-38. Second, there was barely any detectable pp36-38 in JCaM2.5 cells even when these cells were stimulated with pervanadate (Figures 1 and 2), suggesting that JCaM2.5 cells are deficient either in pp36-38 or in the kinase responsible for phosphorylating p36-38.

There are several indications that both Lck and ZAP-70 are functional in JCaM2.5 cells. First, in complementation assays, heterokaryons derived from the fusion of JCaM2.5 to JCaM1.6 cells are able to signal. Since JCaM1.6 cells are deficient in Lck, this suggests that the Lck provided by JCaM2.5 cells is functionally competent. Second, the tyrosine phosphorylation of the TCR ζ chain, an event thought to be mediated by Lck (22), is normal (A. Weiss and T. Kadleck, personal communication). Third, the expression level as well as the molecular weight of ZAP-70 and Lck in JCaM2.5 cells are equivalent to that in Jurkat cells. Fourth, transfection of Syk, a kinase homologous to ZAP-70, did not rescue the signaling defect of JCaM2.5 cells but did rescue the defect of JCaM1.6 cells (D. Chu, personal communication). These results suggest that the signaling defect(s) of JCaM2.5 cells most likely lie downstream of the proximal PTKs. Taken together, it is likely that pp36-38 is not expressed in JCaM2.5 cells. Thus, reconstitution of the increase in intracellular calcium induced by TCR activation in JCaM2.5 cells could be used as a readout for expression cloning of pp36-38.

Potential role of pp36-38 in T cell signaling

pp36-38 is very likely to play a key role in T cell signal transduction. It is the most prominently tyrosine phosphorylated protein that has been shown to be associated with all the major signaling complexes in *vivo*, i.e., PLC γ1, Grb2-SOS, PI3 kinase as well as ZAP-70. It is tempting to speculate that p36-38 is constitutively anchored to the plasma membrane in close proximity to the TCR complex, via a transmembrane domain or a lipid modification, and becomes phosphorylated by ZAP-70 and/or Lck following TCR engagement. Tyrosine phosphorylated p36-38 could then serve as a docking protein to recruit signaling complexes from the cytosol to the plasma membrane where

these complexes would then become tyrosine phosphorylated and activated (PLC γ1) or be in close proximity to its substrates (Grb2-SOS). Alternatively, pp36-38 itself may contain an SH2 domain, and translocate from the cytosol to the plasma membrane by binding to a tyrosine phosphorylated protein in the membrane. So far, we have been unable to detect any pp36-38 associated using fusion proteins containing either the cytoplasmic domains of CD3ε or ζ chains, or anti-Lck antisera (unpublished observations), making it more likely that pp36-38 is constitutively present in the plasma membrane. On the other hand, treatment of intact Jurkat cells with biotin failed to label pp36-38, suggesting that pp36-38 does not contain an extracellular domain (data not shown). Further experiments that dissociate ionic but not hydrophobic interactions are necessary to determine whether p36-38 contains an SH2 domain or whether it is constitutively in the plasma membrane via a lipid modification. Clarification of this issue, along with a more detailed cellular fractionation analysis confirming that pp36-38 is in the plasma membrane, may shed some light on the function of pp36-38.

In general, tyrosine phosphorylation serves to recruit signaling complexes to a specific cellular compartment or to induce a conformational change that ultimately leads to the activation of these complexes. In the case of the Grb2-SOS complex, autophosphorylation of the epidermal growth factor receptor creates a binding site for the Grb2-SOS complex. This allows the Grb2-SOS to translocate from the cytosol to the plasma membrane, placing SOS in close proximity to its substrate Ras (reviewed in Ref. 116). Thus, the binding of the Grb2-SOS complex to the receptor is thought to regulate substrate accessibility rather than the intrinsic exchange activity of SOS. Consistent with this notion, phosphopeptides based on the autophosphorylation site of the EGFR did bind to the SH2 domain of Grb2 without affecting the ability of the SH3

domain of Grb2 to bind proline-rich peptides (133, 134) These studies suggest that the SH2 and SH3 domains of Grb2 functions as independent modular units and that binding of a phosphoprotein to the SH2 domain of Grb2 is unlikely to affect the exchange activity of SOS. Indeed, the crystal structure of Grb2 revealed that its SH2 and SH3 domains are not in contact with each other, making it unlikely that a conformational change induced by binding of a phosphoprotein to the SH2 domain could be transmitted to the SH3 domain and vice-versa (135). However, these results differ from a study demonstrating that there is an increase in Grb2-SOS association following TCR stimulation and that this increase in association could be mimicked by incubating *in vitro* tyrosine phosphorylated Shc-GST with unstimulated T cell lysates (136). It is likely that the use of proline-rich peptides and bacterially expressed Grb2 proteins (133, 134) instead of the native mSOS and Grb2 protein (136) may account for this difference. Alternatively, the association between Grb2-SOS may be differentially regulated in T cells and fibroblasts. Thus, pp36-38 may not only have a role in translocating the Grb2-SOS complex to the membrane but it may also induce a conformational change in Grb2 that could affect its association with SOS.

In the case of PLC γ1, tyrosine phosphorylation has been demonstrated to be required for stimulation of its phospholipase activity (63-65). However, there is an increasing amount of evidence suggesting that the activity of PLC γ1 can be modulated by other mechanisms as well. First, phosphatidic acid has been shown to activate PLC γ1 by acting as an allosteric modifier (137). Second, *in vitro* experiments suggest that a catalytically inactive EGFR can increase PLC γ1 activity perhaps by inducing a conformational change in PLC γ1 (138). Third, intriguing studies using peptides based on the phospholipase C inhibitory (PCI) region could inhibit the phospholipase activity (139),

suggesting that PLC γ1 is an autoregulated enzyme (140). Consistent with this notion, partial V8 protease treatment suggests that PLC γ1 folds back on itself, inhibiting its own enzymatic activity (141). Although none of these experiments are without shortcomings, taken together they raise the intriguing possibility that PLC γ1 contains an autoinhibitory region that could be released upon tyrosine phosphorylation, or by binding of a phosphoprotein to either/both of its SH2 domain(s). Indeed, there is correlative evidence indicating that tyrosine phosphorylation of PLC γ1 may not be sufficient for its activation in T cells. First, PLC γ1 is inducibly tyrosine phosphorylated in JCaM2.5 cells, albeit to a lower level than in Jurkat cells, but there is no increase in intracellular calcium. Second, engagement of the TCR in a Jurkat cell stably expressing a chimera containing the extracellular domain of Class I MHC, the SH2 domain of Grb2 and the phosphatase domain of CD45, induced the tyrosine phosphorylation of PLC γ1 but there was no calcium influx or phosphatidylinositol turnover (Motto et al., in press). Although it is possible that in both cases PLC γ1 was not tyrosine phosphorylated at the appropriate sites, it is intriguing that the amount of associated or detectable pp36-38 was drastically reduced in both cases. Thus, it is tempting to speculate that although tyrosine phosphorylation contributes to the activation of PLC γ1, binding of pp36-38 may be required for its maximal activation.

Recently, pp36-38 has been shown to directly bind the SH2 domains of the 85 kDa subunit of PI3 kinase *in vivo* (120). Studies with growth factors and degenerate peptide libraries have demonstrated that the two SH2 domains in p85 subunit preferentially binds to phosphorylated YxxM motif (reviewed in Ref. 142); (131). Binding of various phosphopeptides containing this YxxM consensus sequence enhances the phosphatidylinositide kinase activity 2-3 fold *in vitro* (143, 144), raising the possibility that binding of pp36-38 to p85

may have a similar effect in T cells. Interestingly, the only peptide obtained during the purification scheme for pp36-38 that was not accounted for in the p36 cDNA, had a YMXM motif (VAQIPIYMEM; Figure 4). Although there are no acidic residues upstream of the tyrosine that would make it a likely phosphorylation site, it is possible that the presence of a glutamic acid at the +2 position is sufficient to ensure phosphorylation of the tyrosine. Unfortunately no matches were found in the EST database. However, it would be interesting to use this peptide to screen an antibody phage display library or to generate an antiserum.

In conclusion, there is suggestive evidence indicating that pp36-38 may be (1) a docking protein, recruiting signaling complexes from the cytosol to the membrane; (2) a protein coupling cytoplasmic tyrosine kinases, such as ZAP-70 and/or Lck, to downstream signaling complexes; (3) required for the proper activation of these complexes; or (4) all the above. It is clear that elucidation of its role in TCR signaling awaits its molecular characterization.

Experimental Procedures

Cell lines and Transfections

The human leukemic T cell line Jurkat and its derivatives JCaM1.6 and JCaM2.5 cells (121, 145) were maintained in RPMI 1640 supplemented with 5% fetal bovine serum (FBS), glutamine, penicillin and streptomycin (Irvine Scientific). Jurkat TAg (gift from G. Crabtree) and DNAX Jurkat were maintained in the same media containing 10% FBS. Electroporation of FLAG-Lnk into Jurkat TAg cells was performed in a Bio-rad Gene Pulser using a voltage of 250V and a capacitance of 960uF with 40 ug of plasmid per 10^7 cells. After transfection, cells were grown for two days in RPMI before harvesting.

Antibodies

C305 and Leu4 mAbs recognize the Jurkat Tiβ chain and an extracellular determinant of CD3ε, respectively. The anti-phosphotyrosine mAb 4G10 was a generous gift of Drs. D. Morrison, B. Druker, and T. Roberts. RC20, a recombinant anti-phosphotyrosine antibody directly conjugated to HRP, was purchased from Transduction Laboratory. The anti-FLAG mAb was purchased from Kodak, PLC γ1 mAb purchased from Upstate Biotechnology Institute (Lake Placid, NY); anti-Grb2 antisera was a generous gift from J. Schlessinger. Polyclonal antisera against p36 was generated against the peptide PEGKVLETVGVGFEVPK and GREPVLSKEQPAFSIAATS coupled to KLH, by Animal Services, (La Jolla, CA), according to their standard immunization protocol.

GST constructs

Grb2GST constructs containing only the SH2 domain (SH2Grb2), only the amino-terminus SH3 domain (N-SH3Grb2), only the carboxyl-terminus SH3 domain (C-SH3Grb2), a point mutation in the SH2 domain (R86KGrb2), a point mutation in the amino-terminus SH3 domain (P49LGrb2), a point mutation in the SH2 domain corresponding to a loss of funtion in Sem5 (S80TGrb2), and wild type Grb2 (Grb2GST) were kindly provided by A. Batzer and J. Schlessinger. PTP1C-GST containing the two SH2 domains of PTP1C and CD3εGST containing the cytoplasmic domain of CD3ε were provided from N.Van Oers. The fusion proteins SH2Vav-GST and NH2Vav-GST, containing only the SH2 domain of Vav or the first 200 amino acids of Vav, respectively, were provided from J. Wu.

Immunoprecipitations

Cells were lysed at 1 x 10^7 cells/100ul in 1% NP40 (Nonidet P40), 150 mM NaCl, and 10 mM Tris pH 7.8, 1 mM Na_3V0_4, 1 mM PMSF, 10 ug/ml aprotinin, pepstatin and leupeptin and 1 mM sodium molybdate. After 15 minutes on ice, lysates were clarified by centrifugation at 14,000 rpm for 15 minutes and incubated for 2 hours at 4°C with 20 ul of protein A sepharose CL-4B beads and the immunoprecipitating antibody. Washed immunoprecipitates were resuspended in SDS sample buffer and boiled for 5 minutes prior to electrophoresis on 8 to 10% polyacrylamide gels. Alternatively, protein G was used whenever the immunoprecipitating antibody was of the IgG1 isotype.

Stimulation of Jurkat cells

Cells were washed once with room temperature PBS, resuspended at 1 x 10^8 cell/ml, and incubated at 37°C for 15-20 minutes before stimulating with 1:500

dilution of C305 ascitic fluid. After 2 minutes at 37°C, the cells were microcentrifuged for 5 seconds, the medium was aspirated, and the cells lysed at 1 x 10^8/ml of lysis buffer. For pervanadate stimulation, Jurkat cells were washed with room temperature PBS, resuspended at 1 x 10^8 cells/ml and stimulated with pervanadate (0.2 mM Na_3VO_4, 8 mM H_2O_2) for 1 minute.

***In vitro* kinase assays**

The immunoprecipitates were washed 5 times with lysis buffer, twice with 50 mM LiCl and once with kinase buffer (10 mM $MnCl_2$/20 mM Tris pH 7.6). The kinase reaction was performed by incubating the washed immunoprecipitates with 30 ul of the kinase buffer and 10 uCi of ^{32}P-gATP for 10 minutes at room temperature. The immunoprecipitates were washed twice with lysis buffer and resolved by SDS-PAGE.

Cloning of p36 using degenerate oligonucleotides

Degenerate oligonucleotides (sense: 5'CA(G/A)ACTTA(T/C)TATCAAGT and sense: 5'TT/CTT(A/G)GCiAA(T/C)CA(T/C)GA) derived from tryptic peptides QTYYQV and TFLANHD (shown in Figure 2) were synthesized in a Millipore Expedite DNA synthesizer. Total cellular RNA was prepared from Jurkat cells using a standard protocol (146). Briefly, 1 x 10^8 Jurkat cells were lysed in guanidinium isothiocyanate and layered on a CsCl gradient, and mRNA was isolated using the Magnesphere PolyATract mRNA isolation system (Promega). First strand cDNA was synthesized with an oligo$(dT)_{12}$ primer using Copy Kit (Invitrogen). The cDNA was diluted 1:50 and 1 ul was used in a thirty-five cycle PCR amplification with a denaturing temperature of 94°C for 1 min, an annealing temperature of 37°C for 2 min, and an extension temperature of 72°C for 2 min along with the degenerate oligonucleotide

5'CA(G/A)ACTTA(T/C)TATCAAGT and 5'GGATCC$(dT)_{12}$. 1 ul of the PCR product was then used to set up a second nested PCR with the degenerate oligo 5'TT(T/C)TT(A/G)GCIAA(T/C)CA(T/C)GA and 5'GGATTC$(dT)_{12}$ using the same conditions described above. In this manner, a specific 870 bp fragment was amplified, labeled with (^{32}P)dCTP, and used as a probe to screen a human leukemic T cell cDNA library under high stringency conditions. Nine positive phage plaques were isolated, and their insert was subcloned into Bluescript SK (+) phagemid (Stratagene), sequenced and shown to encode for the same cDNA. The longest clone contained an 1171 kb insertion and had an open reading frame of 391 amino acids. To obtain the 5' end of the p36 cDNA, PCR amplifications were performed using the 5'-Ampli-FINDER RACE [rapid amplification of cDNA ends) systems (Clonetech) using anchor primers supplied by the manufacturer and two gene-specific primers.

Purification protocol for pp36

The lysates from the equivalent of 6x 10^{10} Jurkat cells were stimulated with C305 ascitic fluid and prepared as described above. The lysates were then precleared with 20 ug of GST bound to glutathione-sepharose beads for one hour at 4°C, followed by another preclearing step using 30 ug of PTP1C- GST coupled to glutathione-sepharose beads. The beads were collected and the supernatant was subjected to another preclearing step with 20 ug of R86KGrb2-GST for 2 hours at 4°C. The beads were collected again and the supernatant was precipitated with P49LGrb2-GST coupled to glutathione-sepharose beads for 2 hours at 4°C. The bound proteins were extensively washed 5 times with lysis buffer and three times with high salt buffer (500 mM NaCl, 1% NP40, 20 mM Tris pH7.8). A small fraction (1/50) of the proteins that bound to the glutathione-sepharose beads, was resolved by SDS

PAGE, transferred to polyvinylidene difluoride membrane (Immobilon) and western blotted with anti-phosphotyrosine antibody. Alternatively, the rest of the bound proteins were resolved by SDS-PAGE, transferred to Immobilon membrane, visualized with Ponceau S and individual bands were subjected to trypsin digestion. Tryptic peptides were then separated by reverse phase high performance liquid chromatography and individual peaks sequenced with a model 475A Protein Sequencer (Applied Biosystems).

Further purification of pp36-38

Jurkat lysates from unstimulated cells or pervandate stimulated cells were precipitated with Grb2-GST coupled to glutathione-sepharose beads for 2 hours at 4 C. The bound proteins were eluted by boiling the samples in 100 ul of 0.5% SDS for 3 minutes. After diluting the lysate to 0.1% SDS with lysis buffer containing protease and phosphatase inhibitors, the proteins were reprecipitated with antiphosphotyrosine 4G10 mAb coupled to protein A-sepharose for 2 hours at 4°C. The immunoprecipitates were washed extensively with lysis buffer and the bound proteins were eluted with 30 ul of 0.25 M phenylphosphate vigorously shaking for 45 minutes at 4°C. The supernatant containing the eluted material, as well as the proteins bound to the pellet were resolved by SDS-PAGE and analyzed by western blotting with anti-phosphotyrosine antibody.

Epitope tagging Lnk

Lnk cDNA, kindly provided from J. Hayashi, was digested with Xho and XbaI and directly subcloned into pCDNA3. This construct was then digested with Xho and EcoRI so that the Myc or FLAG tag could be inserted at the amino-terminus. Myc and FLAG tag containing an XhoI site-a Kozak consensus

sequence-ATG-FLAG/Myc tag-EcoRI were synthesized as described. FLAG: sense 5'AATTCCACCATGGACTACAAGGACGACGATGACAAGC and antisense 5'GGTGGTACCTGATGTTCCTGCTGCTACTGTTCGAGCT. Myc: sense 5'AATTCCACCATGGAACAGAAGCTTATTTCCGAAGAGGATCTGA AGC and antisense 5'GGTGGTACCTTGTCTTCGAATAAAGGCTTCTCCTA GACTTCGAGCT .

Northern blots

Nitrocellulose membranes containing poly(A) enriched RNA from adult human tissues were purchased from Clonetech and probed with a 500 bp ^{32}P-labeled cDNA fragment of p36 according to manufacture's protocol using QuickHyb (Clonetech).

Covalently crosslinking beads

P49LGrb2GST was covalently crosslinked to beads using the standard protocol described elsewhere (192). Briefly, 100 ug of P49LGrb2GST was bound to 50 ul of CL-4B glutathione-sepharose beads (Pharmacia), washed once with 0.2 M sodium borate pH 9.0 and crosslinked by adding 5 mg of dimethylpimelimidate (Sigma) for 30 minutes at room temperature. The reactive sites were blocked with ethanolamine, pH 8.0 for two hours and extensively washed with PBS before being used in the purification protocol described above.

CD45 dephosphorylation assay

Immunoprecipitates containing tyrosine phosphorylated proteins were washed five times with lysis buffer (no phosphatase inhibitors), twice with phosphatase buffer (2 mM Hepes pH 7.3, 5mM EDTA and 10mM DTT) and

dephosphorylated by incubating it in 30 ul of phosphatase buffer with 15 ug of purified CD45 (gift from R. Fletterick) for up to 1 hour at 37°C. The efficiency of dephosphorylation was determined by solubilizing the untreated and treated CD45 immunoprecipitates in sample buffer, resolving by SDS-PAGE and western blotting with anti-phosphotyrosine antibody. In the case where pp36-38 was to be quantified using silver stain, the 4G10 immunoprecipitate was incubated with 30 ug of CD45 for 30 minutes at 37°C. Following dephosphorylation, the beads were collected and the supernatant was directly loaded onto a polyacrylamide gel and silver stained.

CHAPTER 5

SAM68 IS INDUCIBLY TYROSINE PHOSPHORYLATED FOLLOWING TCR STIMULATION

Summary

Engagement of the T cell antigen receptor (TCR) leads to rapid tyrosine phosphorylation of many intracellular proteins. Sam68 is an RNA binding protein that has recently been found to be a mitotic substrate of Src. Though Sam68 contains five proline rich motifs that could potentially bind SH3 containing proteins, as well as a tyrosine rich carboxyl-terminus, very little is known about its function. In order to begin to understand its role in TCR signaling, a polyclonal antisera against one of the proline rich regions of Sam68 was generated. Sam68 was found to be inducibly tyrosine phosphorylated following TCR stimulation in DNAX Jurkat cells, a variant of Jurkat cells that expess high levels of Syk. Interestingly, Sam68 is not inducibly tyrosine phosphorylated in Jurkat cells that express low levels of Syk, strongly suggesting that Syk, but not ZAP-70, is directly or indirectly responsible for Sam68 phosphorylation folowing TCR engagement. Taken together, these results suggest that Syk and ZAP-70 may differ in their ability to phosphorylate downstream substrates.

Preface

Sam68, a ubiquitously expressed protein that was erroneously cloned as the p62 rasGAP associated protein (147), has been recently identified as a mitotic substrate for Src (148, 149). Studies overexpressing either c-src or activated F527src in fibroblasts have demonstrated that Sam68 is tyrosine phosphorylated in fibroblasts arrested in mitosis but not in asynchronously growing fibroblasts. Point mutations within the SH2 and SH3 domains of Src showed that these domains were required for maximal binding to Sam68 and optimal Sam68 tyrosine phosphorylation (148, 149).

In T cells, Sam68 has been reported to be constitutively associated with Fyn, a member of the Src-family of tyrosine kinases, via one of its proline-rich motifs (150). Thus, it has been proposed that Sam68 is constitutively associated with the SH3 domains of Fyn and that it is inducibly tyrosine phosphorylated following TCR stimulation. Tyrosine phosphorylated Sam68 could then serve as a docking site for binding to other proteins containing SH2 and SH3 domains such as PLC γ1, GAP and Grb2 (150). Although this model suggests that Sam68 plays a crucial role in T cell receptor (TCR) signaling, there is yet no evidence demonstrating that Sam68 is inducibly tyrosine phosphorylated following TCR stimulation or following treatment with a growth factor.

Another proposed function for Sam68 is that it may regulate RNA stability, localization or efficiency of translation because it contains an evolutionary conserved RNA binding domain (KH domain). Indeed, Sam68 selectively binds to ribonucleotide homopolymers (148) and it has been demonstrated that the KH domain of FMR1 and hnRNP K could bind RNA (151). Interestingly, the ability of Sam68 to bind to RNA *in vitro* is

dramatically impaired when it is tyrosine phosphorylated by Fyn (152). Furthermore, Src-induced tyrosine phosphorylation of an hnRNP protein from brine shrimp Artemia selectively impairs its RNA binding ability (153). Thus, tyrosine phosphorylation maybe a common mechanism for regulating the activity of some RNA binding proteins.

Engagement of the T cell receptor (TCR) leads to the activation of two classes of PTK: the Src family and the Syk/ZAP-70 family. Although ZAP-70 and Syk are homologous, there is increasing evidence that they may not be regulated in the same manner. First, Syk, but not ZAP-70, is able to reconstitute Jurkat variants that lack CD45 or Lck (Chu et al., submitted). Second, crosslinking of a CD16/Syk chimera, but not of a CD16/ZAP-70 chimera, triggered calcium mobilization and initiated cytolytic effector functions, although CD16/ZAP-70 could elicit these functions when co-aggregated with Fyn or Lck (154). Third, binding of Syk or ZAP-70 to an ITAM induced a marked increase in the catalytic activity of Syk (153, 155) but had a minimal effect on ZAP-70 (53, 156). Instead, the phosphorylation of the ZAP-70 kinase domain by Lck seems to regulate its catalytic activity (60). Taken together, these results suggest that Syk and ZAP-70 may not be regulated in the same manner, raising the possibility that they may perform different effector functions.

In order to understand the role of Sam68 in TCR signal transduction, an antiserum against the most amino-terminal proline-rich regions of Sam68 was generated. The studies described herein demonstrate that Sam68 is rapidly and inducibly tyrosine phosphorylated in a variant of Jurkat cells that expresses Syk (DNAX Jurkat). Interestingly, Sam68 is not tyrosine phosphorylated in Jurkat cells. In contrast to what has been previously reported in studies using fibroblasts, the tyrosine phosphorylation of Sam68

in T cells does not depend on mitosis since treatment with nocodazole had no effect on Sam68 phosphorylation in Jurkat or DNAX Jurkat cells. Aside from being the first report demonstrating that Sam68 can be inducibly tyrosine phosphorylated following TCR engagement, these results suggest that Syk and ZAP-70 may differ in their ability to directly or indirectly phosphorylate Sam68.

Results

Anti-Sam68 antibody is specific. In order to begin to understand the function of Sam68 in TCR-mediated signaling, we generated a rabbit polyclonal antibody against the first proline-rich sequence of Sam68 (Figure 1). Immunoprecipitation followed by immunoblotting with this antisera demonstrated that the antibody was specific and detected a single band with an apparent molecular mobility of 68 kDa in whole-cell lysates from Jurkat cells (Figure 2, lane 2). The 68 kDa band immunoprecipitated by this antibody was recognized by another anti-Sam68 antibody generated independently (147), confirming that our antibody indeed recognized Sam68 (data not shown). Because our anti-Sam68 antibody was raised against the proline rich region of Sam68, there was some concern that it would not be able to immunoprecipitate a pool of Sam68 that was interacting with an SH3 containing protein. Therefore, lysates from Jurkat cells were prepared and subjected to four sequential immunoprecipitations with the anti-Sam68 antibody. A preliminary experiments demonstrated that the anti-Sam68 antibody was found to fully deplete the cellular Sam68, even though there there was as much Sam68 in the fourth immunoprecipitate as the first three immunoprecipitates (Figure 2).

Sam68 is inducibly tyrosine phosphorylated following TCR engagement in DNAX Jurkat. Because Sam68 has been shown to be constitutively associated with Fyn and Lck in T lymphocytes (157), it was of interest to determine whether it could be inducibly tyrosine phosphorylated following engagement of the T cell receptor. Therefore, DNAX Jurkat cells, a variant of Jurkat cells expressing Syk (Chu et al, submitted) were stimulated with anti-TCR antibody

Figure 1. **Amino acid sequence of human Sam68.**

The five proline-rich motifs are designated P1 through P5; the tyrosine rich carboxyl-terminus is boxed; the peptide sequence used to generate a rabbit polyclonal antiserum is underlined with a bold dash. This sequence was reported by Richard et al. (150).

Amino acid sequence of human Sam68

MQRRDDPAARMSRSSGRSGSMDPSGAHPSVRQTPSRQPPLPHRSRGGGGG

P1 P2

SRGGARASPATQPPPLLPPSATGPDATVGGPAPTPLLPPSATASVKMEPEN

KYLPELMAEKDSLDPSFTHAMQLLTAEIEKIQKGDSKKDDEENYLDLPSHK

NMKLKERVLIPVKQYPKFNFVGKILGPQGNTIKRLQEETGAKISVLGKGSM

RDKAKEEELRKGGDPKYAHLNMDLHVFIEVFGPPCEAYALMAHAMEEV

P3

KKFLVPDMMDDICQEQFLELSYLNGVPEPSRGRGVPVRGRGAAPPPPPVP

P4

RGRGVGPPRGALVRGTPVRGAITRGATVTRGVPPPPTVRGAPAPRARTAG

P5

IQRIPLPPPPAPETYEEYGYDDTYAEQSYEGYEGYYSQSQGDSEYYDYGHG

EVQDSYEAYGQDDWNGTRPSLKAPPARPVKGAYREHPYGRY

Figure 2. **The anti-Sam68 antibody specifically recognizes a 68 kDa protein.** Lysates from the equivalent of 50 x 10^6 unstimulated Jurkat cells were immunoprecipitated with pre-immune sera (lane 1) or with anti-Sam68 antibody (lane 2, 5 - 8). Four sequential immunoprecipitations with anti-Sam68 antibody were performed (lanes 5 - 8). The bound proteins were resolved by SDS-PAGE, and the membranes were immunoblotted with anti-Sam68 antisera. Lysates containing the equivalent of 2 x 10^6 Jurkat cells were loaded before depletion (lane 3) or after the fourth depletion (lane 4).

α-Sam68 antibody is specific and capable of immunodepleting

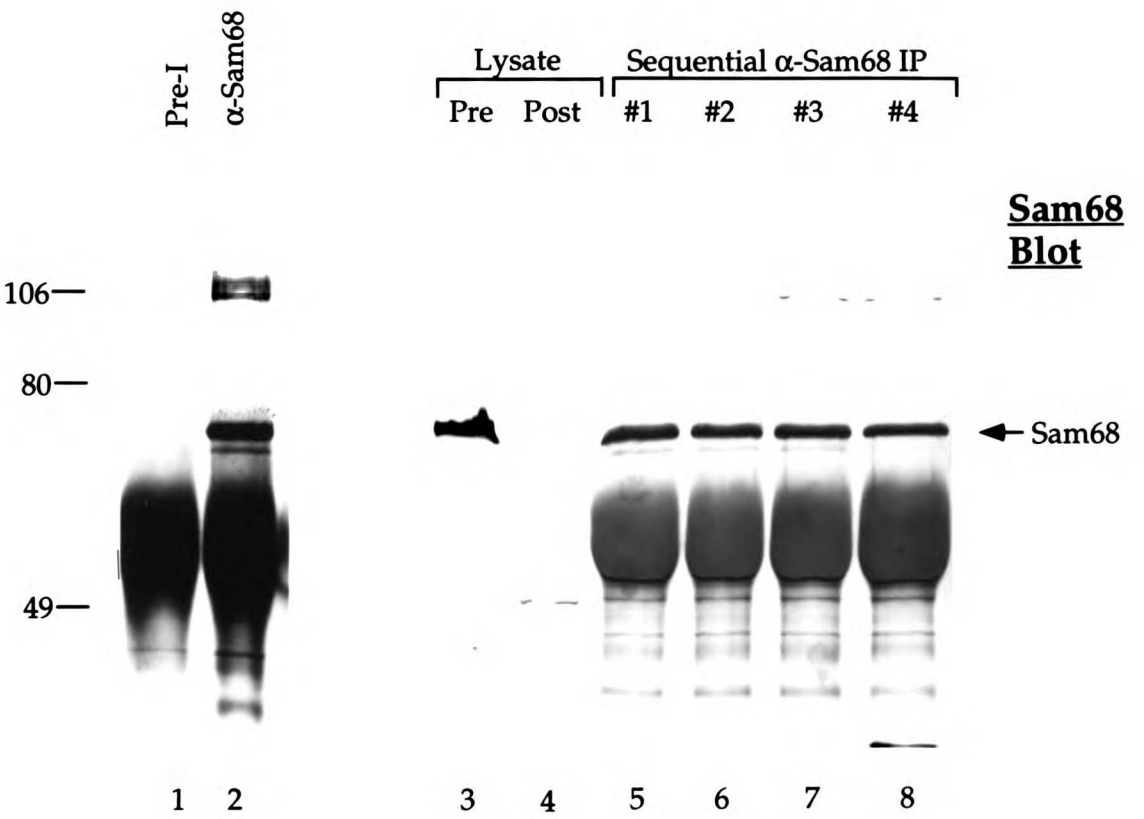
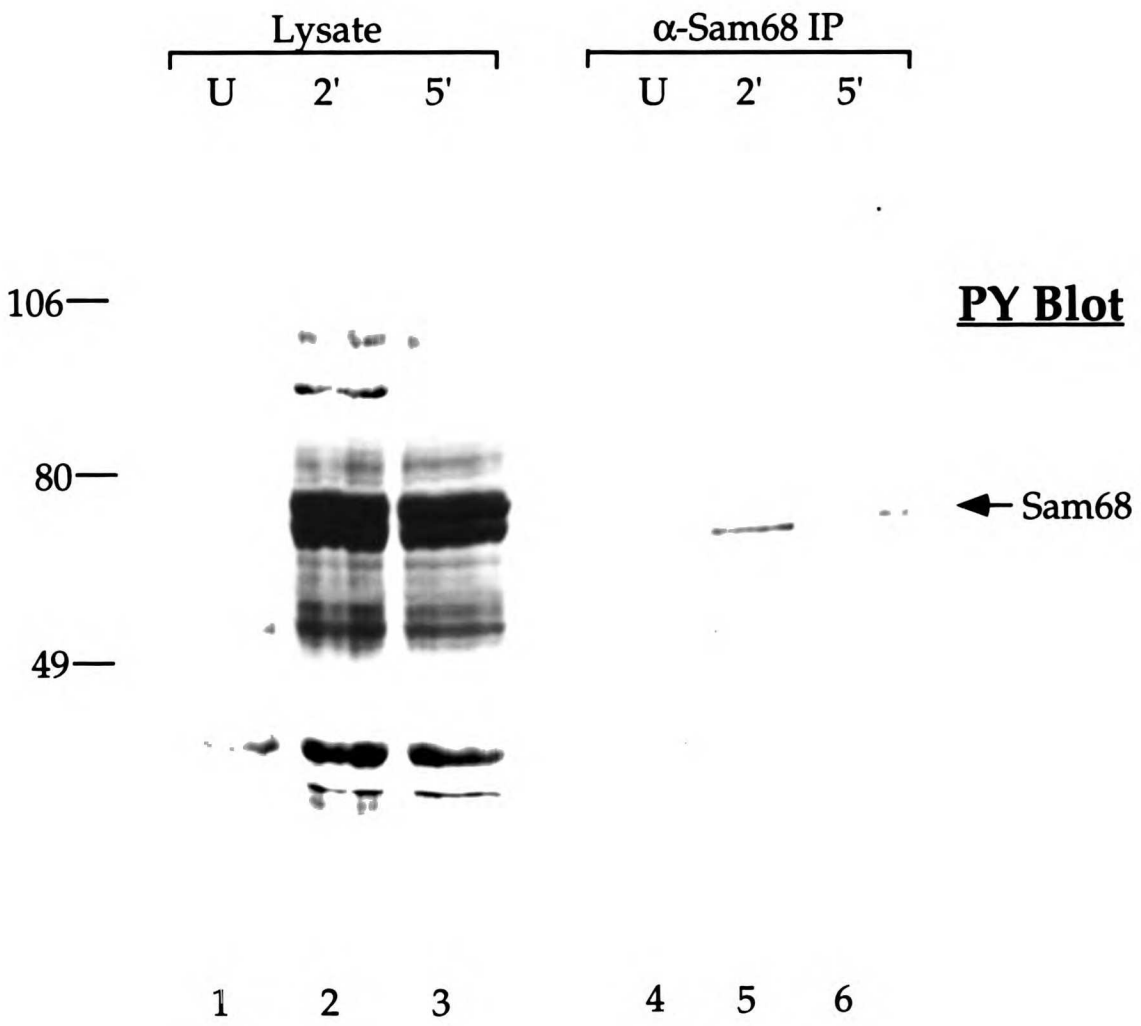


Figure 3. **Sam68 is inducibly tyrosine phosphorylated following TCR engagement in DNAX Jurkat cells.**

The equivalent of 50 x 10^6 DNAX Jurkat cells were either left unstimulated (lanes 1 and 4), stimulated with anti-TCR antibodies for 2 minutes (lanes 2 and 5) or for 5 minutes (lanes 3 and 6). The lysates were either first immunoprecipitated with anti-Sam68 antibody (lanes 4 - 6), or directly loaded on SDS-PAGE (lanes 1 - 3) and analsed by western blotting with 4G10 mAb.

Sam68 is inducibly phosphorylated following TCR stimulation in DNAX Jurkat cells



and the lysates were immunoprecipitated with the anti-Sam68 antibody. As shown in Figure 3, Sam68 was rapidly and inducibly tyrosine phosphorylated, though upon longer exposures low levels of basal phosphorylation could also be detected (data not shown). Interestingly, no other tyrosine phosphorylated protein was reproducibly seen associated with Sam68 following TCR stimulation.

Since the kinetics of phosphorylation was quite rapid, peaking within two minutes following TCR stimulation, it was unlikely that it was due to an arrest in mitosis as previously reported (148, 149). However, to determine whether mitosis contributed to its basal phosphorylation, DNAX Jurkat cells were either left growing asynchronously or arrested in mitosis with nocodazole treatment (149). The cells were then stimulated, and the lysates were immunoprecipitated with anti-Sam68 antibody. Consistent with previous results, Sam68 was inducibly tyrosine phosphorylated following TCR engagement (Figure 4A, lanes 4, 5 and 6) but nocodazole had no effect on the basal or inducible level of Sam68 phosphorylation when compared to untreated cells (lanes 7, 8 and 9 versus 4, 5 and 6). As a control, this blot was reprobed with anti-Sam68 antibody to ensure that equivalent amounts of Sam68 were immunoprecipitated (Figure 4B, lanes 4 through 9). Although these results suggest that, unlike what has been reported in fibroblasts (148, 149), mitotic activity is not required for Sam68 tyrosine phosphorylation in T cells, further experiments demonstrating that mitotic arrest had indeed occurred are necessary.

Sam68 is not inducibly tyrosine phosphorylated following TCR engagement in Jurkat cells. Surprisingly, Sam68 from Jurkat cells was not inducibly tyrosine phosphorylated to the same extent as seen in DNAX Jurkat cells

(Figure 4A, compare lanes 1, 2 and 3 with 4, 5 and 6). This result was not due to poor stimulation of Jurkat cells since both cells expressed equal levels of TCR on the surface and were equally well stimulated (data not shown), nor was it due to lower expression of Sam68 in Jurkat compared to DNAX Jurkat cells because equivalent amounts of Sam68 were immunoprecipitated (Figure 4B, compare lanes 1, 2 and 3 with 4, 5, and 6). As seen with DNAX Jurkat cells, nocodazole treatment arrested Jurkat cells in mitosis, as measured by the failure of these cells to divide overnight compared to untreated cells (data not shown), but did not induce a basal level of Sam68 tyrosine phosphorylation (Figure 5B, lanes 1 through 6). As previously shown, nocodazole treatment does not alter the ability of Sam68 to be immunoprecipitated since equivalent amounts of Sam68 were precipitated from untreated and treated Jurkat cells (Figure 5A, compare lanes 1 - 3 with lanes 4 - 6). Since one difference between Jurkat and DNAX Jurkat cells is that DNAX Jurkat cells express Syk, the data suggest that Syk, but not ZAP-70, is required for the tyrosine phosphorylation of Sam68.

Figure 4. **Sam68 is phosphorylated following TCR stimulation of DNAX Jurkat versus but not of Jurkat cells.**

The equivalent of 50 x 10^6 DNAX Jurkat (lanes 5 - 10) and Jurkat cells (lanes 1 - 3) were left untreated (lanes 1 - 7) or were treated with nocodazole overnight (lanes 8 - 10). The cells were left unstimulated (lanes 1, 5 and 8), stimulated with anti-TCR antibodies for 2 minutes (lanes 2, 6, and 9) or for 5 minutes (lanes 3, 7 and 10). The lysates were immunoprecipitated with anti-Sam68 antibody, loaded on SDS-PAGE and anaylsed by western blotting with 4G10 mAb (panel A). The membrane was then reprobed with anti-Sam68 antibody (panel B).

Sam68 is inducibly phosphorylated following TCR stimulation in DNAX Jurkat but not in Jurkat

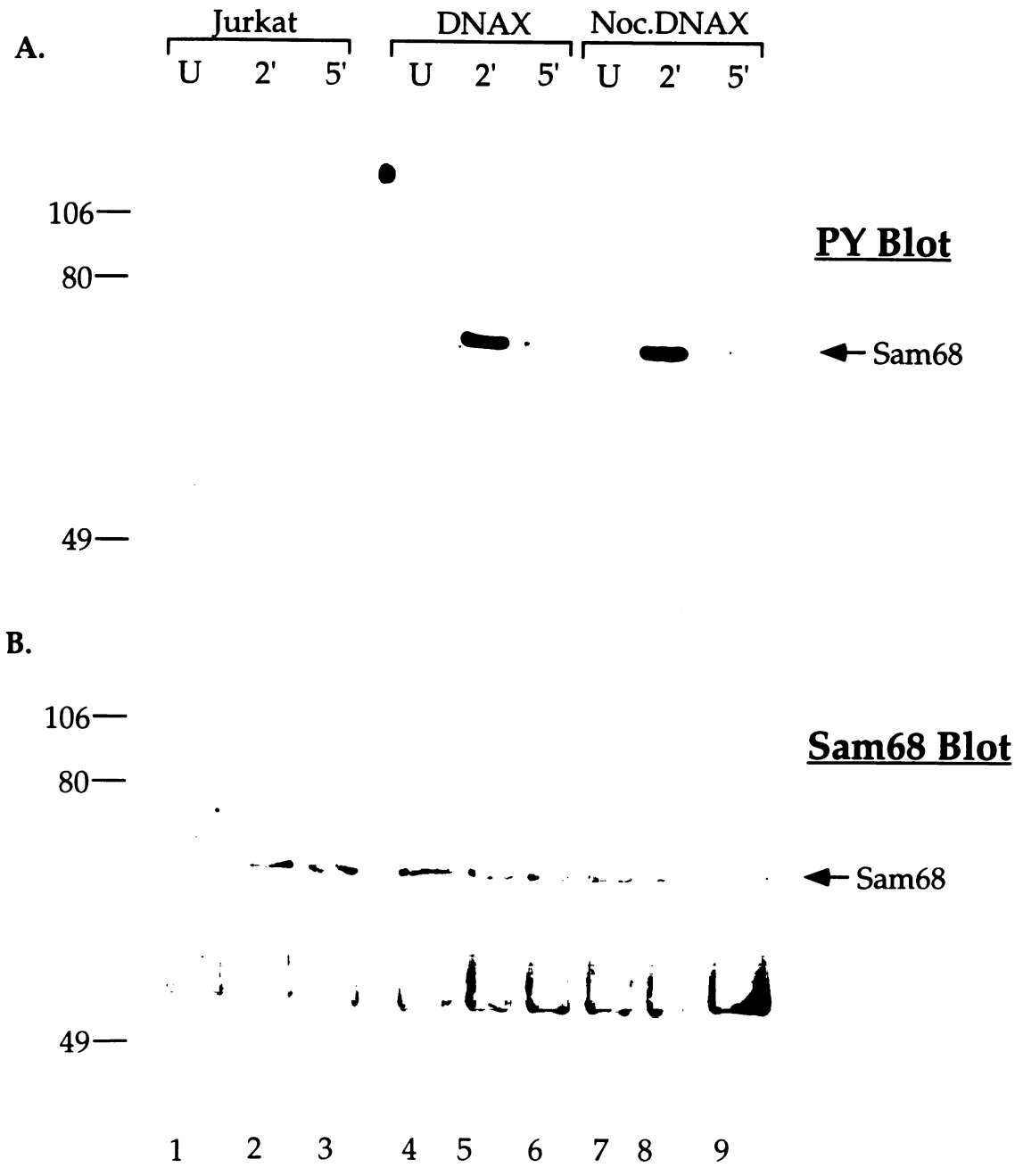
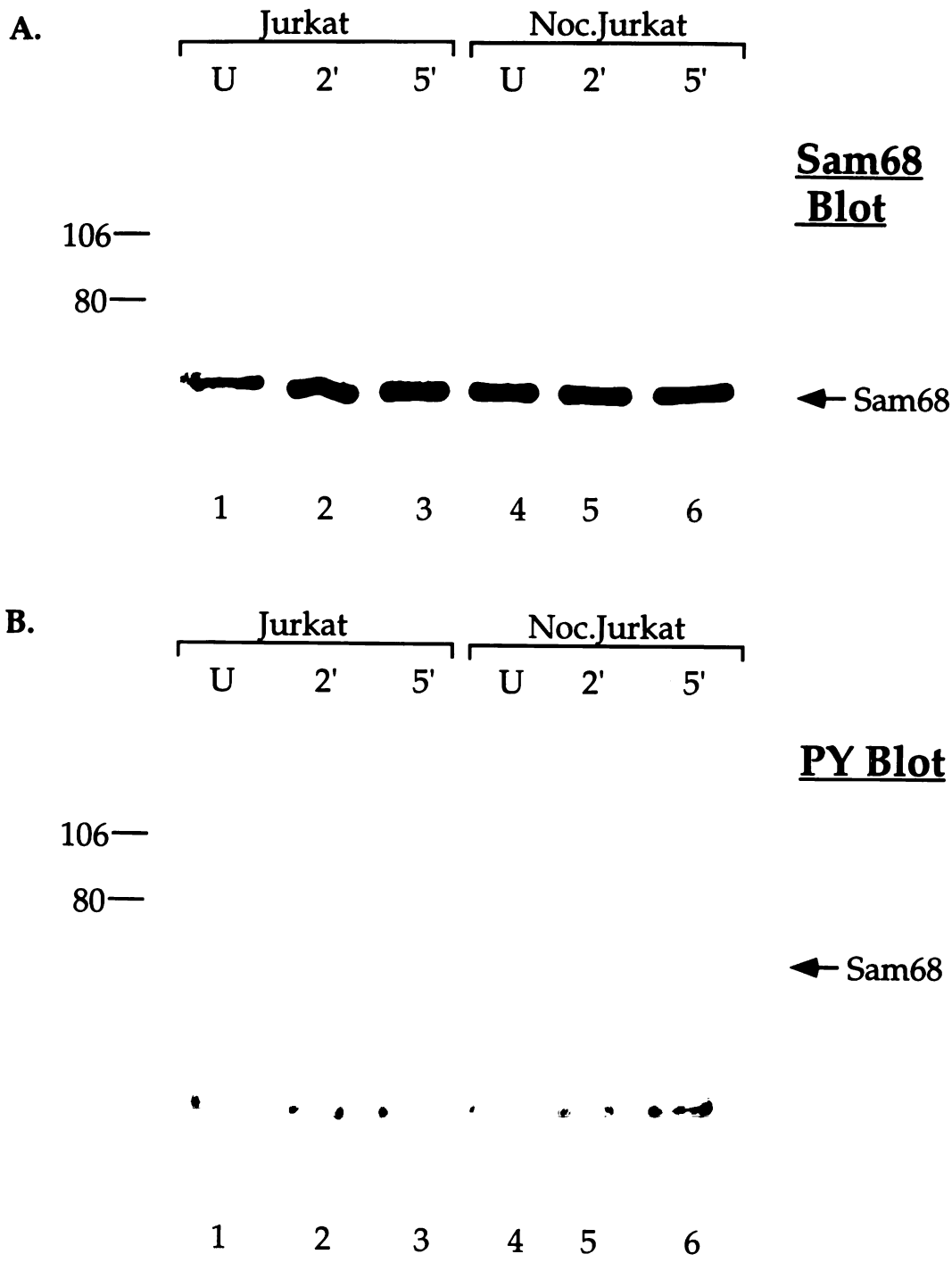


Figure 5. **Sam68 is not inducibly tyrosine phosphorylated in Jurkat cells treated with nocodazole.**

The equivalent of 50 x 10^6 Jurkat cells (lanes 1 - 6) were left untreated (lanes 1 - 3) or were treated with nocodazole overnight (lanes 4 - 6). The cells were left unstimulated (lanes 1 and 4), stimulated with anti-TCR antibodies for 2 minutes (lanes 2 and 5) or for 5 minutes (lanes 3 and 6). The lysates were immunoprecipitated with anti-Sam68 antibody, loaded on SDS-PAGE and anaylzed by western blotting with 4G10 mAb (panel B). The membrane was then reprobed with anti-Sam68 antibody (panel A).

Sam68 is not inducibly phosphorylated following TCR stimulation in Jurkat cells



Discussion

This report demonstrates that Sam68 is inducibly tyrosine phosphorylated following TCR engagement in DNAX Jurkat, a Jurkat variant expressing high levels of the protein tyrosine kinase Syk (Chu et al, submitted). The kinetics of phosphorylation was rapid, peaking within the first two minutes (Figure 3 and 4A), suggesting that Sam68 phosphorylation was a primary event caused by triggering of the TCR, rather than a secondary event caused by the induction of mitosis. Indeed, treatment of Jurkat cells with nocodazole, a reagent known to arrest cells during mitosis, was incapable of inducing Sam68 phosphorylation (Figure 4A). This differs from what has been previously reported in studies using fibroblasts, where Sam68 was shown to be associated and phosphorylated by Src during mitosis (148, 149) but not following growth factor stimulation (S. Courtneidge, personal communication). These results suggest that the regulation of Sam68 tyrosine phosphorylation in T cells is different from fibroblasts.

Further insight into the regulation of tyrosine phosphorylation of Sam68 comes from the surprising observation that Sam68 is not tyrosine phosphorylated following TCR engagement in Jurkat cells that do not express Syk (Figure 4A). Given that ZAP-70 is equally expressed in both DNAX Jurkat and Jurkat cells (Chu et al, submitted), and that Syk is the only known tyrosine kinase differentially expressed in these two cells, this implies that Syk, but not ZAP-70, is directly or indirectly responsible for Sam68 tyrosine phosphorylation.

The finding that Syk might be responsible for the tyrosine phosphorylation of Sam68 is quite surprising, especially in light of several experiments demonstrating that Sam68 is phosphorylated by a member of the

Src family of tyrosine kinase in both fibroblasts and T cells (148, 149, 150). First, Sam68 is constitutively associated with Fyn and Lck in T cells (150). Furthermore, using a heterologous system, co-transfection of Fyn with Sam68 into Hela cells, was shown to be sufficient to induce Sam68 tyrosine phosphorylation (150). Similarly, preliminary studies have demonstrated that Lck can associate with and phosphorylate Sam68, when Lck is transiently expressed in cos cells (our unpublished observations). Taken together, these experiments suggests that a member of the Src family PTK is responsible for phosphorylating Sam68.

There are three models which could explain how Syk might be involved in Sam68 phosphorylation. The first two models are based on the assumption that Lck, or a member of the Src family of PTK, directly or indirectly mediates Sam68 phosphorylation. More specifically, Syk could regulate Sam68 phosphorylation if Syk, but not ZAP-70, could modify Lck and render it capable of phosphorylating Sam68. Support for this model comes from *in vitro* studies showing that Syk can phosphorylate Y192 in Lck, potentially increasing its kinase activity (158) and ability to phosphorylate Sam68. The second model predicts that Lck functions as a bridging protein between Syk and Sam68, allowing Syk but not ZAP-70 to phosphorylate Sam68 following TCR stimulation. This model implies that Syk and ZAP-70 differ in their ability to regulate or associate with Lck. There is suggestive evidence supporting this notion. In a heterologous system, Sam68 associated with phosphorylated Syk but not with phosphorylated ZAP-70, in the presence of Lck (our unpublished observations). This suggests that Syk and ZAP-70 may differ in their ability to associate with Sam68 and Lck. The third model stipulates that Syk, but not ZAP-70, directly associates with and phosphorylates Sam68 without the need for Lck. Although this model is

more difficult to reconcile with the observations described above, it still remains a possibility.

One way of potentially distinguishing between these models is by transiently transfecting Syk into JCam1.6 cells, a derivative of Jurkat cells that lack Lck and Syk (D. Chu, personal communication). If one detects Sam68 phosphorylation following TCR engagement, it would argue that Syk can directly phosphorylate Sam68 without the need for Lck. If, however, Sam68 is not inducibly tyrosine phosphorylated, it would strongly argue that Lck is required for Sam68 phosphorylation. This could occur if the ability of Lck to phosphorylate Sam68 depended on the ability of Lck to associate with Syk. Alternatively, Lck could function as a bridging protein between Syk and Sam68. Regardless of which is the correct model, co-transfection experiments could be used to further dissect the region(s) in Lck or Syk required for Sam68 phosphorylation.

The importance of studying Sam68 tyrosine phosphorylation is underscored by a recent study demonstrating that tyrosine phosphorylation of Sam68 severely impairs its ability to bind to RNA (152), raising the possibility that Sam68 may be involved in targeting RNA to cytoskeletal structures. This is interesting given that results from a preliminary fractionation experiment, have demonstrated that 70% of Sam68 in unstimulated Jurkat cells was detected in the particulate fraction, which might contain cytoskeletal structures (our unpublished observation). Additional fractionation of lysates from unstimulated and stimulated DNAX Jurkat cells could address whether Sam68 tyrosine phosphorylation plays a role in its cellular localization.

Our data suggest that Syk and ZAP-70 may differ in their ability to phosphorylate downstream substrates, consistent with the recent observations of Chu et al., who demonstrated that Syk, but not ZAP-70, can

reconstitute TCR-mediated signaling in Lck or CD45 deficient cells (Chu et al, submitted). Importantly, preliminary experiments suggest that Sam68 is inducibly tyrosine phosphorylated in thymocytes following TCR stimulation (T. Kadlececk, personal communication), ruling out the possibility that the inducible phosphorylation of Sam68 is an artifact of the leukemic DNAX cells. Furthermore, it is intriguing that although Syk is expressed in T cells, it is most highly expressed in thymocytes (37). Additional experiments demonstrating that Sam68 phosphorylation correlates with Syk expression *in vivo* will help solidify the notion that there is a functional difference between ZAP-70 and Syk.

In conclusion, though we do not yet understand the function of Sam68 and the importance of its tyrosine phosphorylation in lymphocyte signal transduction, it is clear that it is differentially phosphorylated in DNAX Jurkat versus Jurkat cells. The biochemical basis for this difference is most likely due to the expression of Syk and the experiments discussed above could potentially demonstrate that Syk, though homologous to ZAP-70, is unique in its ability to modify Lck and/or directly phosphorylate downstream substrates.

Experimental Procedures

Cell lines and Transfections

The human leukemic T cell line Jurkat E6.1 was maintained in RPMI 1640 supplemented with 5% fetal bovine serum (FBS), glutamine, penicillin and streptomycin (Irvine Scientific). Jurkat TAg and DNAX Jurkat were maintained in the same media containing 10% FBS.

Antibodies

C305 mAb recognize the Jurkat Ti β chain. The anti-phosphotyrosine mAb 4G10, was generously provided by Drs. D. Morrison, B. Druker, and T. Roberts. Polyclonal antisera against the peptide ASPATQPPPLLPPSTPGPDATVVSA of Sam68 coupled to KLH was raised by Animal Services (La Jolla, CA) according to their standard immunization protocol. The antisera was affinity purified with the immunizing peptide.

Immunoprecipitations

Cells were lysed at 1 x 10^7 cells/100ul in 1% NP40 (Nonidet P40), 150 mM NaCl, and 10 mM Tris pH 7.8, 1 mM Na_3V_4, 1 mM PMSF, 10 ug/ml aprotinin, pepstatin and leupeptin and 1 mM sodium molybdate. After 15 minutes on ice, lysates were clarified by centrifugation at 14,000 rpm for 15 minutes and incubated for 2 hours at 4°C with 20 ul of protein A sepharose CL-4B beads and the immunoprecipitating antibody. Washed immunoprecipitates were resuspended in SDS sample buffer and boiled for 5 minutes prior to electrophoresis on 8 to 10% polyacrylamide gels. Alternatively, protein G was used whenever the immunoprecipitating antibody was of the IgG1 isotype.

Stimulation of Jurkat and DNAX Jurkat cells

Cells were washed once with room temperature PBS, ressuspended at 1 x 10^8 cell/ml and incubated at 37°C for 15-20 minutes before stimulating with 1:500 dilution of the ascitic fluid. After 2 or 5 minutes at 37°C, the medium was aspirated, and the cells lysed at 1 x 10^8/ml of lysis buffer.

Nocodazole treatment

Exponentially growing Jurkat or DNAX Jurkat cells were diluted to 0.7 x 10^6 cells/ml with regular media or with regular media containing 40 ug/ml nocodazole for 15 hours before harvesting. The efficacy of the nocodazole treatment was verified by counting the cells to make sure that there was no cell division. The cell count for untreated Jurkat or DNAX Jurkat cells was 1.2 x 10^6 cell/ml whereas nocodazole treated Jurkat or DNAX Jurkat cells was 0.7 x 10^6 cells/ml.

CHAPTER 6

CONCLUDING REMARKS

Concluding Remarks

As our knowledge of the signaling cascades involved in antigen receptor stimulation increases, it becomes more apparent that the simplistic notion of a linear pathway leading to the tight regulation of gene transcription is being replaced with a far more complex maze of parallel pathways that seem to synergize and cross-talk with each other. The complexity of these interacting pathways is very apparent in this thesis, where we have identified of a prominent 36-38 kDa tyrosine phosphoprotein (pp36-38) associated with both Grb2 and SOS complex as well as with PLCγ1 *in vivo*. Moreover, our studies demonstrate that pp36-38 is found exclusively in the particulate fraction, suggesting that it may serve as a docking protein to recruit several signaling complexes from the cytosol to the plasma membrane. While this work, and that of others, has enhanced our understanding of the mechanisms by which the TCR links to the Ras pathway, a number of issues remain unanswered. The following discussion will address some of these issues and examine future directions for research toward a more complete understanding of how the TCR links to Ras.

Role of PLCγ1 in leading to Ras activation in T cells

One of the first models proposed for linking the TCR complex to Ras involved the activation of PKC, followed by a concomitant decrease in GAP activity by an as yet poorly understood mechanism (102). Indirect support for this model came from a study in which the human muscarinic receptor subtype 1 (HM1), a member of the seven transmembrane domain receptors that activates the PI pathway via G-proteins (160), was transfected into Jurkat cells. Treatment of these transfected cells with the HM1 agonist carbachol

stimulated the phosphatidylinositol pathway and induced the activation of MAPK as well as IL-2 production (161), suggesting that the phosphatidylinositol pathway could lead to Ras activation and was sufficient for IL-2 production. However, a similar study in which the PDGFR was transfected into a T cell line also induced tyrosine phosphorylation of PLC γ1 and an increase in intracellular calcium, but failed to produce IL-2 (162). Since a sustained signal transduction by the TCR is required for cellular commitment to IL-2 production, the ability of the HM1 but not the PDGF receptor to induce substantial amounts of IL-2 may relate to the former's ability to generate a greater or a more sustained amount of second messengers. Alternatively, it is possible that the HM1 receptor activated a pathway(s) other than the phosphatidylinositol pathway which was required for the activation of MAP kinase and IL-2 production. Recently, an increasing number of serine/threonine (163) as well as tyrosine kinases (164) have been shown to become activated by G-protein coupled receptors. Although carbachol treatment did not activate a tyrosine kinase pathway (161), it is difficult to assert that the phosphatidylinositol pathway was the only pathway activated by carbachol treatment and is required for Ras activation and IL-2 production. Additionally, mutation of Y1009 and Y1021 in the PDGFR greatly reduced the tyrosine phosphorylation of PLC γ1 in fibroblasts following receptor stimulation but did not affect the phosphorylation of MAPK, suggesting that PLC γ1 activation is not essential for Ras stimulation by the PDGFR (165). Furthermore, in a heterologous cell system, engagement of the B cell receptor leads to MAP Kinase activation without generation of phosphoinositides, suggesting that the phosphatidylinositol pathway is not required for Ras activation (157).

Thus, it is not clear that activation of the phosphatidylinositol pathway induced by tyrosine phosphorylation of PLC γ1 is sufficient to cause Ras activation in T cells. Although studies with phorbol esters and ionomycin predict that activation of PLC γ1 should be sufficient to induce NFAT activation in Jurkat cells, this may not necessarily be the case. First, it is not known whether PLC γ1 and phorbol esters activate the same PKC isozymes *in vivo*. Similarly, phorbol esters may have a pronounced effect on NFAT because it binds and activates a number of proteins with cys-rich motifs (reviewed in Ref. 87), that may not be activated by PLC γ1 *in vivo*. Second, studies that attempt to inhibit the activity of PKC, either with the use of peptide pseudosubstrates or with prolonged treatment with PMA, support the notion that PKC does not play a major role in activating Ras (115).

The optimal way of addressing whether PLC γ1 plays a role in Ras activation in T cells is with a loss of function mutation of PLC γ1. In B cells, loss of PLC γ2 leads to an impairment in calcium influx following stimulation, but its effect on Ras has not been examined (166). Alternatively, given what is known about the putative function of pp36-38, it is likely that once pp36-38 is cloned, individually mutating the binding site for PLC γ1, Grb2, or PI3K in pp36-38 will allow us to dissect the contribution of each of these pathways in leading to Ras activation.

<u>Role of Grb2-SOS complex in leading to Ras activation in T cells</u>

Another model proposed for how the engagement of the TCR induces Ras activation involves the adaptor protein Grb2 and its ability to recruit SOS to the plasma membrane following receptor stimulation. Although there is substantial data indicating that artificially targeting SOS to the plasma membrane via a lipid modification is sufficient to induce Ras activation in

fibroblasts (167) and T cells (168), there is no data demonstrating that the Grb2-SOS complex translocates to the plasma membrane following TCR stimulation*in vivo*. Cellular fractionation experiments in T cells demonstrated that Grb2 and SOS partition to the cytosolic and particulate fraction in the unstimulated state (68, 169, 170), but there was no significant translocation to the particulate fraction following TCR stimulation, even though this assay has been shown to be sensitive enough to detect the translocation of PLC γ1 to the membrane (68) and the translocation of Grb2-SOS to fibroblasts membranes (169). Since SOS could associate via its pleckstrin homology domain with proteins localized in the plasma membrane (128), it is important to clarify whether membrane targetting of SOS is the primary event regulating its activity in T cells. Sensitive immunofluorescence techniques capable of examining engaged TCR complexes *in vivo* such as the one recently developed by Kupfer et al (personal communication), could help address this issue.

The failure to detect a translocation of SOS to the plasma membrane could be an indication that in T cells, the activity of SOS is regulated by alternative mechanisms, or that SOS does not play a major role in the activation of Ras in T lymphocytes. Evidence that SOS can be regulated by multiple mechanisms comes from studies demonstrating that deletion of the carboxyl-terminus accentuates SOS activity, suggesting that Grb2 binding may eliminate a negative regulatory region in SOS (171-173). However, it is possible that deletion of the C-terminal domain eliminated sites of phosphorylation by a mitogen-activated protein kinase family member, which may negatively regulate SOS (126-128) or that it induced an errant conformational change resulting in higher catalytic activity or accessiblity to

Ras. In either case, it is clear that the association between Grb2 and SOS is used as a mechanism to regulate Ras activity.

In T cells, the association between Grb2-SOS complexes seems to be regulated. Although Grb2-SOS complexes can be found in resting cells (68, 169), this association seems to increase following stimulation of T cells (136) but not of fibroblasts (169). Thus, it is tempting to speculate that in T cells, Grb2 is constitutively associated with an inhibitor that prevents the formation of Grb2-SOS complexes. Following TCR stimulation, this putative inhibitor could be displaced by a phosphoprotein such as pp36-38 or Shc (136), increasing the association of Grb2 and SOS. Indeed, there is suggestive evidence indicating that the proto-oncogene Cbl may be this inhibitory protein. First, Cbl can bind to Grb2 in unstimulated Jurkat T cells, an interaction mediated by the proline-rich sequences in Cbl and the SH3 domain in Grb2 (71, 174). Second, following stimulation with a TCR agonist, Cbl is transiently tyrosine phosphorylated (71, 174) and dissociates from Grb2 (174). Third, genetic studies in C. elegans suggest that Cbl functions as a negative regulator of the Ras pathway by binding to Grb2 (175). Thus, it is tempting to speculate that Cbl competes with SOS for binding to the SH3 domains of Grb2 in unstimulated T cells. Following TCR stimulation, tyrosine phosphorylation of Cbl and/or binding of a phosphoprotein to the SH2 domain of Grb2 would cause Cbl to dissociate from Grb2 and allow SOS to bind to Grb2. Therefore, it seems that in T cells, the association between Grb2-SOS complexes could be modulated by interactions with other proteins such as Cbl.

Although SOS is likely to be involved in the activation of Ras in T cells, it is becoming clear that other pathways can lead to Ras activation independently of SOS. Recently, Farnsworth et al (176) have shown that a

Ras-GRF exchange factor can activate Ras *in vivo*. Interestingly, the activity of the Ras-GRF was regulated by binding of calmodulin to an IQ motif in Ras-GRF since point mutations in this domain abrogated both calmodulin binding to Ras-GRF and Ras-GRF activation *in vivo* (176). Although the full-length Ras-GRF has been detected only in brain neurons, this finding raises the possibility that there may be other mechanisms for activating Ras in T cells.

Role of Shc in leading to Ras activation in T cells

There is substantial data indicating that the proto-oncogene Shc is involved in Ras activation The most convincing evidence comes from the observation that overexpression of Shc in PC12 pheochromocytoma cells leads to neurite outgrowth in a ras dependent manner (177). Further biochemical studies demonstrated that tyrosine phosphorylated Shc could associate *in vivo* with Grb2 in Src transformed cells (177, 178), or following growth factor stimulation (179-181), leading to the proposal that cytoplasmic tyrosine kinases activated Ras by inducing the tyrosine phosphorylation of Shc and the formation of a Shc-Grb2-SOS complex (117). Additionally, there is substantial data suggesting that Shc is involved in the regulation of Ras in cells stimulated with interleukin-2 (IL-2) (182, 183), interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF) and erythropoietin (184, 185) as well as through the BCR (186-188). However, it is peculiar that Shc is the only protein implicated in the Ras pathway that has not yet been isolated in sensitive genetic screens designed to identify suppressors or enhancers of the Ras pathway. Furthermore, although Drosophila Shc contains extensive homogy to its mammalian counterpart and is tyrosine phosphorylated by DER (Drosophila EGF-like Receptor), it

lacks the high affinity binding site for Grb2 suggesting that Shc may have other functions in addition to regulating the Ras pathway.

In T cells, there is suggestive evidence that Shc plays a role in IL-2R signaling (182, 183), but its function in TCR-mediated signal transduction is less clear. First, tyrosine phosphorylation of Shc following TCR stimulation is still controversial; with some groups detecting considerable (72, 136), little (68) or no Shc tyrosine phosphorylation (52, 170). Second, although Shc has been shown to associate with the TCRζ chain following TCR stimulation (136) and the ζITAM contains a consensus binding motif for the SH2 of Shc (131), the binding affinity of Shc to the phosphorylated ζITAM is in the micromolar range (52, 61). This is particularly low when compared to the nanomolar concentrations observed for ZAP-70 binding (52). Finally, although Grb2 could bind the phosphorylated ITAM, this binding was independent of Shc (52) and there was no formation of a ζITAM-Shc-Grb2-SOS complex in antigen receptor-activated cells (52 and our unpublished observation). Furthermore, overexpression of a dominant negative Shc construct containing a point mutation in the Grb2 binding site, does not inhibit NFAT induction in Jurkat cells (our preliminary results). Similarly, overexpression of the SH2 domain of Grb2, but not of Shc, inhibited TCR signaling (44). Thus, it is still unclear whether Shc is directly involved in the activation of Ras via Grb2-SOS complex or whether Shc is involved in other pathways that indirectly contribute to Ras activation in T cells. Elucidation of the role of Shc in TCR signaling awaits the functional analysis of the proteins known to associate with Shc, such as p145 (187, 189, 190).

In conclusion, it seems that the engagement of the TCR complex and the formation of several complexes such as pp36-38/PLC γ1, pp36-38/Grb2/SOS and Shc/Grb2 may contribute to the activation of Ras. The functional role of

these complexes awaits the molecular characterization of pp36-38 as well as the identification of other potential substrates for ZAP-70 and Lck/Fyn.

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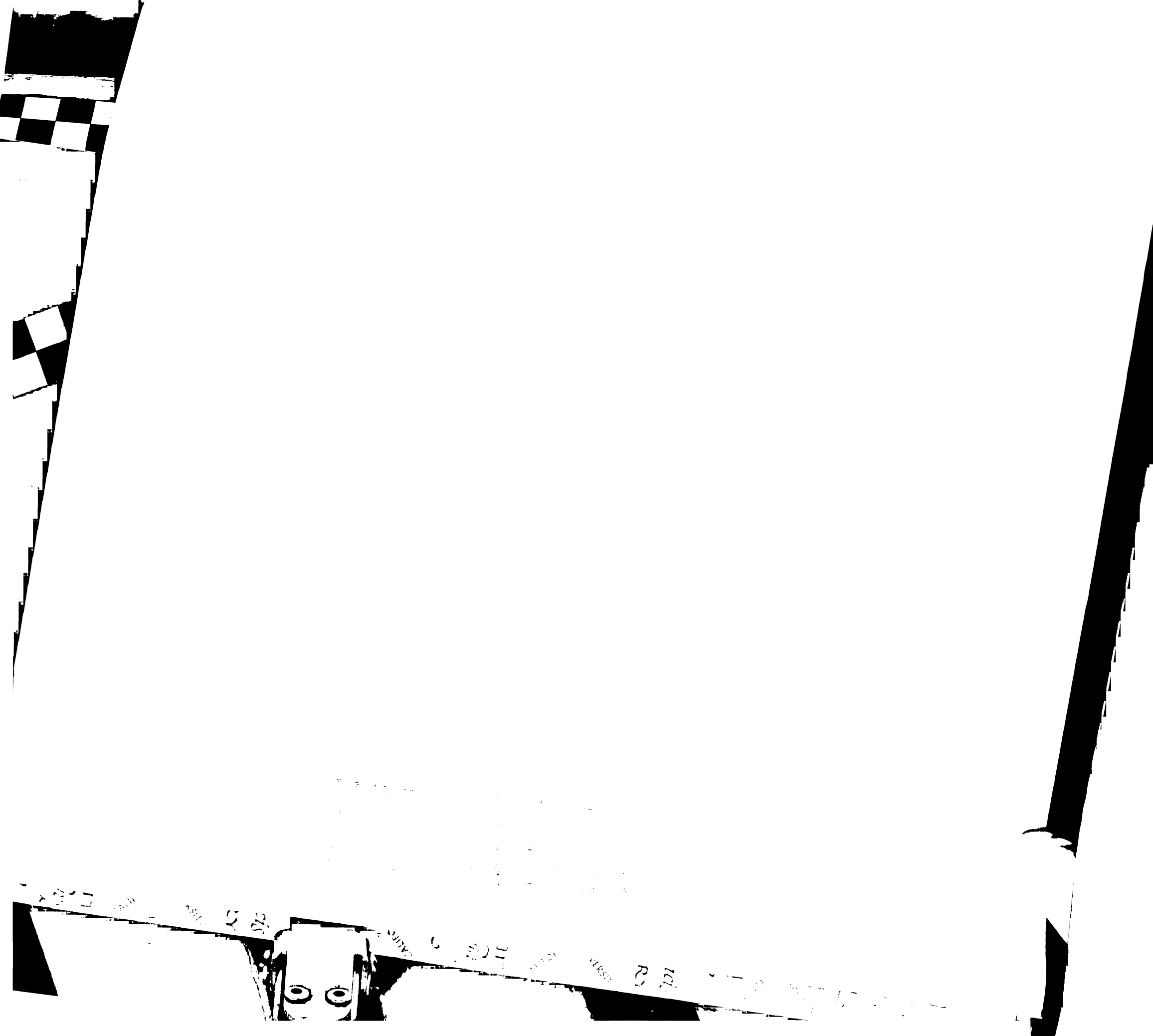

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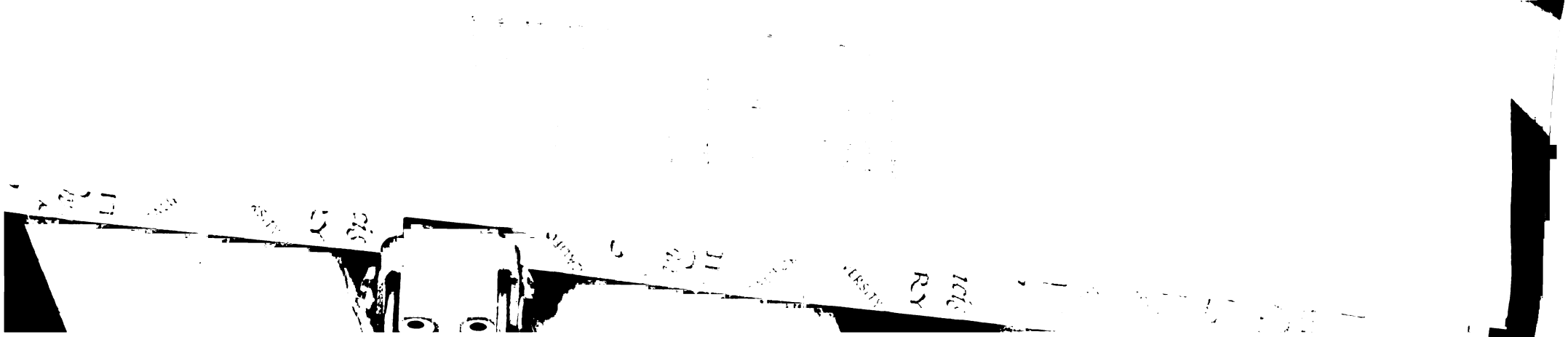

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