Immunity, Vol. 5, 197–205, September, 1996, Copyright ©1996 by Cell Press

Complex Complexes: Signaling at the TCR

Review

Ronald L. Wange and Lawrence E. Samelson
Cell Biology and Metabolism Branch
National Institute of Child Health
and Human Development
National Institutes of Health
Bethesda, Maryland 20892

Engagement of the T cell antigen receptor (TCR) on mature peripheral T cells initiates multiple intracellular signals that can lead to cellular proliferation and the acquisition of complex effector functions. The biochemical mechanisms that couple receptor binding to these intracellular events have been intensively investigated. Early events such as activation of tyrosine phosphorylation, elevation of intracellular calcium, activation of lipid-dependent kinases, and activation of Ras and its downstream kinase cascade are well known (Weiss and Littman, 1994; Cantrell, 1996). Moreover, there has been extensive analysis of the events involved in transcriptional induction of T cell–specific lymphokines. There remain, however, significant gaps in our understanding of TCR signaling, particularly in how the early tyrosine phosphorylation events couple receptor activation to later cellular events. Perhaps the most critical insight of the past decade in the study of signal transduction has been the recognition that activation of receptor tyrosine kinases results in the assembly of multimolecular complexes at the cytoplasmic domain of the receptor. Likewise, a major role of the tyrosine phosphorylation cascade initiated by TCR engagement is the assembly of multimolecular signaling complexes at and near the TCR itself. The identity and possible functions of the proteins that engage in such molecular interactions are the subjects of this review.

The T Cell Antigen Receptor

The complexity of molecular associations involved in TCR signaling begins with the TCR, which is composed of six different polypeptide chains thought to be organized into an eight-chain structure (Weissman, 1994). These polypeptides include a ligand-binding heterodimer ($\alpha\beta$ or $\gamma\delta$) and the nonpolymorphic CD3ϵ, CD3γ, CD3δ, and TCRζ chains. These nonpolymorphic chains are required for receptor assembly, cell-surface expression, and signaling (Ashwell and Klausner, 1990). Critical regions of the cytoplasmic domains of these chains are the immunoreceptor tyrosine-based activation motifs (ITAMs), three of which are in each of the TCRζ chains, and one in each of the CD3 chains (Cambier, 1995). Since each TCR can contain a TCRζ dimer and two CD3 dimers ($\epsilon\delta$ and $\epsilon\gamma$), each TCR can contain a total of ten ITAMs.

It has been well documented that these motifs are necessary and sufficient for coupling the TCR to the intracellular signaling machinery and function by binding key signaling molecules in resting and activated T cells. Upon TCR activation, the tyrosine residues within the ITAMs become phosphorylated, permitting the binding of SH2 domain–containing proteins as a consequence of the ability of SH2 domains to bind specific phosphotyrosine-containing polypeptides. Additional signaling molecules are subsequently recruited to these newly TCR-associated proteins via SH2 or other modular interaction domains.

While a similar requirement of ITAMs for initiating signaling by the B cell antigen receptor (BCR) and certain immunoglobulin receptors (IgR) has been shown, the TCR is notable for the sheer number of ITAMs present in a single receptor. Two different explanations for the presence of ten ITAMs within a single receptor have been proposed. The multiple ITAMs may provide the capacity to amplify the signal received by each TCR. Indeed, it has been shown that the intensity of the response to signaling by recombinant ITAM-containing polypeptide chains is dependent on the number of ITAMs present (Irving et al., 1993). As the T cell may be required to respond strongly in the presence of a low antigen concentration, this multiplicity of ITAMs is potentially quite important. However, recent studies offer an alternative explanation. A specificity of interaction with different ITAMs has been noted for a number of molecules including ZAP-70, phospholipase Cγ1 (PLCγ1), phosphatidylinositol 3-kinase (PI3K), and Shc, raising the possibility that ITAMs are bound differentially (Exley et al., 1994; Cambier and Johnson, 1995; Isakov et al., 1995; Osman et al., 1996). Of course, the multiplicity of ITAMs within the TCR may actually serve both purposes.

Another important function of ITAMs may be to mediate an activation-induced association of TCRζ and CD3ϵ with the actin cytoskeleton. There is increasing evidence to suggest that in resting T cells a fraction of the TCR is tightly associated with the actin cytoskeleton and that upon TCR stimulation the portion of TCRζ and CD3ϵ recoverable in the cytochalasin-disruptable, detergent-insoluble fraction increases (Caplan et al., 1995; Rozdzial et al., 1995). This association with the actin cytoskeleton requires the intact C-terminal ITAM of TCRζ. Further investigation is needed to determine how these interactions impinge on TCR recycling, trafficking, and signaling and to assess the role of TCR-cytoskeletal interactions in mediating the changes in cell shape and motility patterns that can accompany T cell activation.

TCR-Associated Protein-Tyrosine Kinases and Their Substrates

Immediately downstream of the TCR in the signaling pathway are the TCR-associated protein-tyrosine kinases (PTKs). Two families of PTKs have been shown to be involved in TCR signaling. Lck and Fyn are members of the Src family, while ZAP-70 and Syk make up another PTK family. Characteristics of these enzymes have been extensively reviewed (Bolen, 1993; Peri and Veillette, 1994). A primary function of the Src family kinases is to phosphorylate key tyrosine residues within the ITAMs (Iwashima et al., 1994; van Oers et al., 1996). An additional function of these kinases includes the

phosphorylation and concurrent activation of the ZAP-70 kinase (see below) (Iwashima et al., 1994; Wange et al., 1995a; van Oers et al., 1996; Guanghui et al., 1996). Other substrates for these enzymes remain to be defined, though some candidates such as the receptor for IP_3 (providing a mechanism for Fyn to regulate intracellular Ca^{2+} directly) have been identified (Jayaraman et al., 1996). It remains to be determined whether Lck and Fyn have any unique substrates, or whether their roles in signaling are redundant. Genetic experiments in which these enzymes are either deleted or overexpressed suggest unique roles for them during development, but these experiments do not address function in a mature T cell that developed in a normal environment (Perlmutter et al., 1993; Peri and Veillette, 1994; Weiss and Littman, 1994).

The relatively recent discovery of the PTKs ZAP-70 and Syk has led to intense investigation of their function in lymphocytes. Syk is central to BCR and IgR function. Though possibly involved in T cell development and the function of certain subsets of $\gamma\delta$ T cells, its importance to TCR signaling in mature T cells has not been demonstrated (Cheng et al., 1995; Fargnoli et al., 1995; Turner et al., 1995). In contrast, study of patients with ZAP-70 deficiency and mice with a genetically engineered absence of ZAP-70 confirm the critical function of this enzyme for TCR-mediated signaling (Arpaia et al., 1994; Chan et al., 1994; Elder et al., 1994; Negishi et al., 1995). Currently, there is intense focus on understanding the mechanisms of ZAP-70 regulation, identifying ZAP-70 substrates, and characterizing this enzyme as a docking site for other critical signaling molecules.

Certain factors that regulate the activity of ZAP-70 have been identified. That activation of ZAP-70 requires a Src family kinase could be inferred from the initial description of this enzyme (Chan et al., 1992). Full enzymatic activity of ZAP-70 expressed in COS cells required coexpression of Lck or Fyn. These results, and the generally recognized ability of tyrosine phosphorylation to regulate PTK activity, lead to an investigation of the sites of tyrosine phosphorylation within activated ZAP-70. Recombinant ZAP-70, when analyzed in vitro, becomes tyrosine phosphorylated at a low level at Y126 and Y292 (Watts et al., 1994). However, only when assayed with added recombinant Lck does maximal tyrosine phosphorylation and maximal activity of the enzyme occur. Prominent sites of phosphorylation under these conditions are the adjacent tyrosines Y492 and Y493 in the kinase domain as well as Y69, Y126, Y178, and Y292. A similar experiment using kinase-dead ZAP-70 (K369R) and the isolated kinase domain of Lck found only a single prominent site of phosphorylation in ZAP-70 at Y493 (Guanghui et al., 1996). ZAP-70 isolated from intact, activated T cells contains phosphate on Y492 and Y493 as well as on Y292, which lies between the second SH2 domain and the kinase domain (Watts et al., 1994). Another critical observation was that isolated proteolytic phosphopeptides purified from activated ZAP-70 contained phosphate on Y493 alone or on both Y492 and Y493, but not on Y492 alone, suggesting that phosphorylation at Y493 precedes and is required for phosphorylation of Y492 (Chan et al., 1995). This has also been suggested by COS cell studies with ZAP-70 carrying tyrosine to phenylalanine mutations at either Y492 or Y493 (Wange et al., 1995a).

These observations have led to a model describing the sequential activation of PTKs following TCR engagement, which is depicted in Figure 1 (Weiss and Littman, 1994; Cantrell, 1996). Occupancy of the TCR causes the initial activation of Lck and/or Fyn leading to tyrosine phosphorylation of ITAMs. By virtue of its tandem SH2 domains, ZAP-70 then binds to these motifs (Wange et al., 1993; Iwashima et al., 1994). Recruitment of ZAP-70 to the ITAMs is required for activation of its kinase activity and for T cell activation, since agents that block recruitment prevent these events (Wange et al., 1995b; Qian et al., 1996). This sequence of events can be detected in cultured cell lines and in peripheral blood lymphocytes. However, in thymocytes and lymph node T cells, one can detect inactive, nonphosphorylated ZAP-70 bound to the basally tyrosine-phosphorylated TCR in the absence of activation (van Oers et al., 1993; Wiest et al., 1996). The phosphorylation of ZAP-70 on Y493 by a Src family kinase results in the activation of ZAP-70 kinase activity (Chan et al., 1995; Wange et al., 1995a). Although this site shares sequence similarity with the so-called autophosphorylation sites defined on other PTKs, for ZAP-70 it is more accurate to refer to this as a transphosphorylation/activation site, since ZAP-70 is unable to phosphorylate this site and since phosphorylation of this site is required for full kinase activity. The mechanism of subsequent tyrosine phosphorylation events within the kinase domain at Y492, outside this region at Y292, and perhaps at other sites is not defined, but is likely to be due to autophosphorylation or transphosphorylation by other activated ZAP-70 molecules on adjacent ITAMs, although an additional role for a heterologous kinase has not been ruled out.

Clearly, the role of phosphorylation of Y493 is to activate the kinase activity of ZAP-70; however, the roles played by phosphorylation of Y492 and Y292 are less clear. In contrast with what is seen in Y493F mutants, Y492F results in increased activity in ZAP-70 isolated from transfected COS cells and enhanced BCR signaling in $Syk^{-/-}$ B cells reconstituted with this ZAP-70 mutant (Wange et al., 1995a; Guanghui et al., 1996). Y492 may therefore serve a negative regulatory function in ZAP-70 when phosphorylated. Alternatively, the Y492F mutation may cause a loss of hydrogen bonding capacity that maintains the kinase in an inactive conformation when unphosphorylated (Wange et al., 1995a). Y292F, unlike the Y492F mutation, has no effect on the activity of purified ZAP-70, but like the Y492F mutation, results in a hyperactivated state upon BCR stimulation in the $Syk^{-/-}$ chicken B cells (Guanghui et al., 1996). This is consistent with phosphorylation of Y292 serving a role as a binding site for regulatory molecules, such as SH2 domain–bearing protein-tyrosine phosphatases (PTPs) (see below).

Recent reports have identified yet another class of PTKs, the Itk/Btk/Tec family, that may be involved in TCR signaling (reviewed by Desiderio and Siliciano, 1994). This family of PTKs is characterized by having an N-terminal pleckstrin homology (PH) domain, and an SH2 and SH3 domain, in addition to the C-terminal kinase domain. This family of PTKs was first shown to be

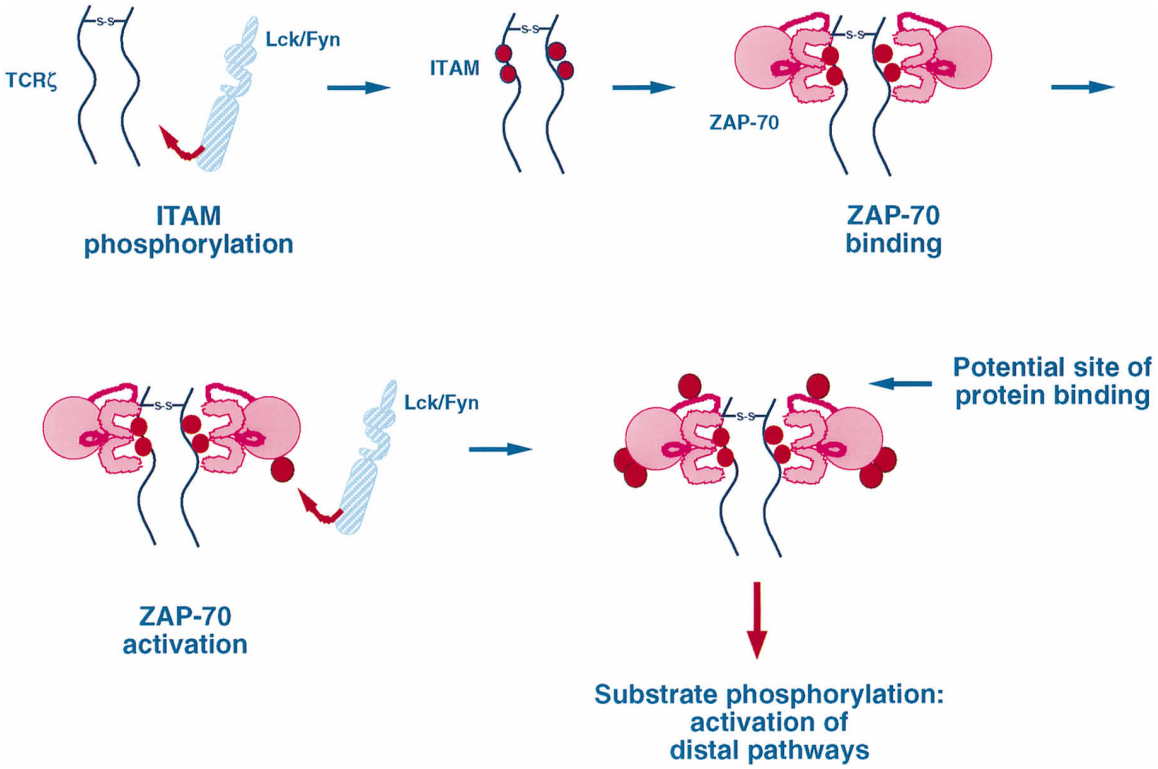

Figure 1. Multiple Events Are Required for ZAP-70 Activation

Recent studies have indicated that activation of this critical tyrosine kinase requires multiple steps. Lck and/or Fyn, two members of the Src family of protein tyrosine kinases, phosphorylate tyrosine residues on the cytoplasmic domains of the TCR depicted here only as dimeric TCRζ chains. These tyrosines are critical residues in the ITAMs. Two doubly phosphorylated ITAMs are shown in red circles. ZAP-70 binds these and other phosphorylated ITAMs via its tandem SH2 domains. This binding event is required for ZAP-70 activation. Likewise, activation of ZAP-70 requires phosphorylation at Y493 in the kinase domain by Lck or Fyn. Subsequently, additional sites of tyrosine phosphorylation can be detected on the enzyme. These are presumably due to transphosphorylation events as adjacent ZAP-70 molecules interact. Sites of tyrosine phosphorylation on ZAP-70, such as Y292, shown in the hinge region between the carboxy-SH2 and kinase domains, may serve as targets for binding to other SH2-containing molecules. The active ZAP-70 kinase phosphorylates multiple substrates involved in critical signaling events.

important in hematopoietic cell function when it was discovered that mutations in the *Btk* gene at the *XLA* locus cause X-linked agammaglobulinemia in humans. Thus Btk, which is expressed predominantly in B lymphoid and myelomonocytic lineages, is required for normal B cell function. Itk is predominantly expressed in T cells. Itk$^{-/-}$ mice produce fewer thymocytes, and mature T cells isolated from these mice proliferate poorly in response to TCR stimulation, but respond normally to phorbol ester plus ionomycin (Liao and Littman, 1995). In Jurkat T cells, TCR cross-linking rapidly and transiently tyrosine phosphorylates Itk, resulting in increased kinase activity in anti-Itk immunoprecipitates (Gibson et al., 1996). The precise role of Itk in TCR signaling remains to be determined.

Characterization of pathways downstream of the PTKs depends on identification of their substrates. Detection of protein-tyrosine phosphorylation by immunoblotting with specific anti-phosphotyrosine antibodies has revealed a number of kinase targets (Peri and Veillette, 1994). In this fashion, PLCγ1 was the first substrate, other than the TCR subunits, to be identified in T cells. Tyrosine phosphorylation of this enzyme is critical to its activation. Vav, a hematopoietic cell-specific proto-oncogene, and more recently, the proto-oncogene Cbl were also identified as being PTK substrates in T cells (Bustelo et al., 1992; Donovan et al., 1994). SH2 domain leukocyte protein (SLP-76), a prominent substrate, was recently isolated by virtue of an interaction with the Grb2 linker protein as described below (Jackman et al., 1995). More difficult than identifying the proteins that become tyrosine phosphorylated upon TCR stimulation is determining which PTKs are directly responsible for a given phosphorylation event. As stated above, it is clear that ITAM phosphorylation is a function of the Src family kinases, as is phosphorylation of Y493 of ZAP-70. ZAP-70, at least in vitro, does not phosphorylate these sites.

Substrates of ZAP-70 have been more difficult to identify. Based on previous study of Syk, it has been shown that ZAP-70 can phosphorylate the cytoplasmic domain of erythrocyte band 3 (cdb3) and tubulin in vitro (Wange et al., 1995b; Isakov et al., 1996). Evidence that cdb3 and tubulin are genuine in vivo substrates of Syk has also been presented (Harrison et al., 1994; Peters et al., 1996). Whether tubulin is an in vivo substrate of ZAP-70 remains to be determined; however, it is interesting that tubulin is tyrosine phosphorylated in activated T

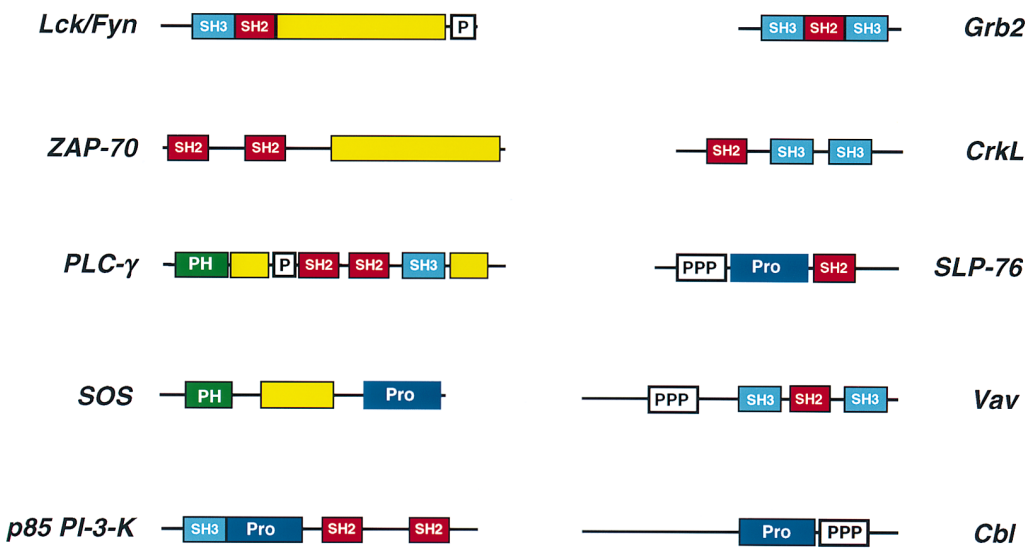

Figure 2. A Selection of Signaling Proteins Found in T Lymphocytes Depicted to Highlight Their Modular Structures

SH2, SH3, and PH domains are in red, blue, and green, respectively. Sites of tyrosine phosphorylation are indicated with P and proline-rich sites are indicated Pro. Domains with enzymatic function are in yellow.

cells, and both ZAP-70 and Vav have been found in association with tubulin (Ley et al., 1994; Huby et al., 1995). These findings further support the contention that cytoskeletal components may play an integral part in the early signal transduction steps. Of the molecules involved in TCR signal transduction that undergo tyrosine phosphorylation upon TCR engagement, only SLP-76 has been shown to be a substrate of ZAP-70 (Wardenburg et al., 1996). Tyrosine phosphorylation of SLP-76 mediates association with Vav (see below) (Wu et al., 1996). Of note is that sites likely to be phosphorylated in these three proteins, all share the sequence Asp–Tyr–Glu. Interestingly, the hematopoietic cell specific protein HS1, which has two Asp–Tyr–Glu sites, has been shown to be a substrate for ZAP-70 when HS1, ZAP-70, and Fyn are coexpressed in COS cells (Fusaki et al., 1996). The function of HS1 remains unknown.

Multimolecular Complexes

One of the most critical insights into mechanisms of receptor-mediated signal transduction is that receptor activation leads to assembly of multimolecular complexes on the cytoplasmic domain of platelet-derived and epidermal growth factor receptors (Ullrich and Schlessinger, 1990; Fantl et al., 1993). These complexes consist of multiple signaling molecules that often bind to the receptor by interaction at sites of tyrosine phosphorylation (reviewed by Pawson, 1995). Many of these signaling molecules are themselves modular, containing variable numbers of interaction domains such as SH2, PTB, SH3, and PH, which bind, respectively, to pY (in the context of specific residues C-terminal of pY), to pY (in the context of specific residues N-terminal of pY), to proline-rich regions, and to phosphotidylinositol (4,5)bisphosphate, inositol phosphates, and certain proteins such as G protein $\beta\gamma$ subunits. PH domains are thus thought to target some proteins to the plasma membrane. This modular architecture permits the assembly of large multimolecular complexes, as each signaling protein may bind to several different signaling proteins, which may themselves associate with a whole host of additional signaling molecules. Examples of modular proteins involved in T lymphocyte function are depicted in Figure 2. Recent advances in our understanding of TCR signaling (discussed below) suggest just how complex interactions among these proteins can become.

Proteins with the potential to become involved in multimolecular signaling complexes at the TCR include the receptor-proximal kinases themselves. The Src family kinases contain an SH2, an SH3, and a unique N-terminal domain, all of which can be involved in protein–protein interactions (Bolen, 1993; Peri and Veillette, 1994). In addition, lipid modification of consensus N-terminal sites stabilizes association of these PTKs with cellular membranes. The unique N-terminal domain of Lck binds to the coreceptor molecules CD4 or CD8, while the comparable region in Fyn binds to nonphosphorylated ITAMs. The SH2 and SH3 domains of Src family PTKs form an intramolecular association with the tyrosine-phosphorylated C-terminal tail, inhibiting their kinase activity. The phosphorylation status of this negative regulatory tyrosine is controlled by the competing activities of the Csk PTK and the CD45 PTP. In addition to activating the kinase activity, dephosphorylation of this residue by CD45 also increases the availability of the SH2 and SH3 domains for interaction with other proteins (e.g., Cbl, PI3K, and ZAP-70) (Fujita, 1993; Donovan et al., 1994; Vogel and Fujita, 1993; Thome et al., 1995).

ZAP-70 is also capable of forming multimolecular complexes through its two SH2 domains and via tyrosine-phosphorylated SH2 domain acceptor sites. The only known function of the SH2 domains of ZAP-70 is to target the kinase to the two phosphorylated tyrosines

of the ITAM (Wange et al., 1993; Iwashima et al., 1994). It is possible, however, that these SH2 domains have other targets when not engaged to the activated TCR, but this seems unlikely given the unique structure of the tandem SH2 domain of ZAP-70, which only permits high affinity binding to polypeptides possessing two phosphotyrosines within a prescribed distance, as is found in ITAMs (Hatada et al., 1995). It is important to note that, unlike Syk, ZAP-70 is not activated by the binding of its tandem SH2 domains to ITAMs (Shiue et al., 1995; Neumeister et al., 1995; Isakov et al., 1996).

In addition to its role as an enzyme responsible for protein-tyrosine phosphorylation, ZAP-70 appears also to function as an adaptor protein or scaffold upon which other signaling molecules assemble (Neumeister et al., 1995). Interestingly, Lck, Vav, and Cbl have all been found in association with ZAP-70 (Thome et al., 1995; Katzav et al., 1994; Fournel et al., 1996), and at least for Lck and Vav, this association appears to involve the SH2 domains of these two proteins binding to phosphorylated tyrosines on ZAP-70. As mentioned previously, Y69, Y126, Y178, Y292, Y492, and Y493 have all been shown to be capable of accepting phosphate in an in vitro kinase reaction with Lck (Watts et al., 1994). It has been suggested that two other tyrosines (Y315 and Y319) may be phosphorylatable, and phosphorylated peptides cognate for this region can disrupt the association of Vav with ZAP-70 (Katzav et al., 1994). While the functional consequences of the ZAP-70 association with these signaling molecules remains to be determined, one can speculate that these associations ensure their efficient phosphorylation by ZAP-70 or associated PTKs, such as Lck. In addition, the association of Lck with tyrosine-phosphorylated ZAP-70 has been suggested to be required for recruitment of Lck–CD4 to the TCR (Thome et al., 1995), thereby enhancing the assembly of a fully effective signaling complex.

The adaptor protein Grb2 is an example of a modular, so-called linker protein, consisting entirely of a central SH2 domain flanked by two SH3 domains. In a number of growth factor receptor tyrosine kinase systems, Grb2 has been shown to couple these receptors to the Ras activator protein SOS (Downward, 1994). As the Ras pathway was known to be required for lymphokine gene activation, a number of investigators sought to determine whether Grb2 was linked to this pathway in T cells. The Grb2–SOS interaction was indeed documented, and SOS has been shown to be involved in Ras activation in T cells (Downward, 1994; Holsinger et al., 1995). While Shc, another linker protein, has been shown to link Grb2 to certain growth factor receptors, its role in TCR signaling remains controversial (Ravichandran et al., 1995; Osman et al., 1995).

In addition to the anticipated role of Grb2 in Ras activation, Grb2 was also found to bind several of the most prominent substrates of the TCR-associated PTKs (Motto et al., 1994; Buday et al., 1994; Reif et al., 1994). A 120 kDa protein that binds to Grb2 has been shown to be the proto-oncogene Cbl (Donovan et al., 1994; Fukazawa et al., 1995; Meisner et al., 1995). The Grb2–Cbl association is mediated via the N-terminal SH3 domain of Grb2 and is observed in resting and activated T cells. Affinity purification with a Grb2 fusion protein was used to isolate a 76 kDa protein, SLP-76, which binds to the C-terminal SH3 domain of Grb2 (Jackman et al., 1995). The identity of pp36, a 36 kDa substrate, which binds to the SH2 domain of Grb2, remains in doubt. This protein has the potential to play a critical role in TCR signaling, as in addition to binding Grb2, it has also been found in association with PLCγ1 and PI3K (Cantrell, 1996). The tight membrane localization of pp36 provides a possible mechanism for recruitment of Grb2, PLCγ1, and PI3K to the plasma membrane. Grb2–SOS activates the Ras pathway when localized to the plasma membrane (Downward, 1994; Cantrell, 1996), while membrane localization of PLCγ1 and PI3K permits phosphorylation of these proteins by membrane-associated PTKs and provides access to lipid substrates.

The binding of SOS and Cbl to Grb2 is mutually exclusive, as proline-rich regions in these proteins compete for the same binding site (Meisner et al., 1995). An interesting area of investigation is whether this competition plays a role in TCR signaling. It is also possible that Grb2 could mediate a complex between either pp36 and/or SLP-76 with Cbl or SOS. Whether such complexes form or have a functional significance remains to be established. An additional consequence of formation of the Grb2–Cbl complex could depend on the ability of Cbl, itself, to serve as an adaptor protein. Recent studies demonstrate that Cbl can be found in complex with PI3K, ZAP-70, 14.3.3τ, and CrkL (Meisner et al., 1995; Fukazawa et al., 1995; Fournel et al., 1996; Liu et al., 1996; Reedquist et al., 1996). CrkL is another adapter protein that, in turn, interacts with C3G, a nucleotide exchange factor for members of the Rac/Rho family of small G proteins.

Study of Vav and SLP-76 has led to the most significant recent insights into TCR signal transduction mechanisms. Vav, first identified as a transforming oncogene when truncated, consists of a number of interaction modules, two SH3 domains, one SH2 domain, and one PH domain, along with regions sharing homology with Rac/Rho guanine nucleotide exchange proteins (Bustelo et al., 1992). Overexpression of this protein in Jurkat T cells results in enhanced basal activation of interleukin-2 (IL-2) promoters and further enhances the response to TCR signaling (Wu et al., 1995). Its proximal position in the TCR pathway was established by the observation that dominant negative Ras or Raf could block the Vav-mediated enhancement of TCR signaling. The position of Vav in the signaling cascade has been further delineated by the observation that the activity of Vav requires active PTKs and is not functional in a T cell line lacking Lck. Similar studies with SLP-76 overexpression also demonstrated an enhanced response to TCR engagement, although without an increase in basal levels of activation (Motto et al., 1996). Overexpression of both Vav and SLP-76 causes a synergistic induction of basal and TCR-stimulated NFAT and IL-2 promoter activation (Wu et al., 1996).

How these two signaling molecules ultimately affect transcriptional activity is unknown, but it has been shown that a specific interaction between Vav and SLP-76 is required for this activity (Wu et al., 1996). This association requires the SH2 domain of Vav and tyrosine phosphorylation of SLP-76. This association is required

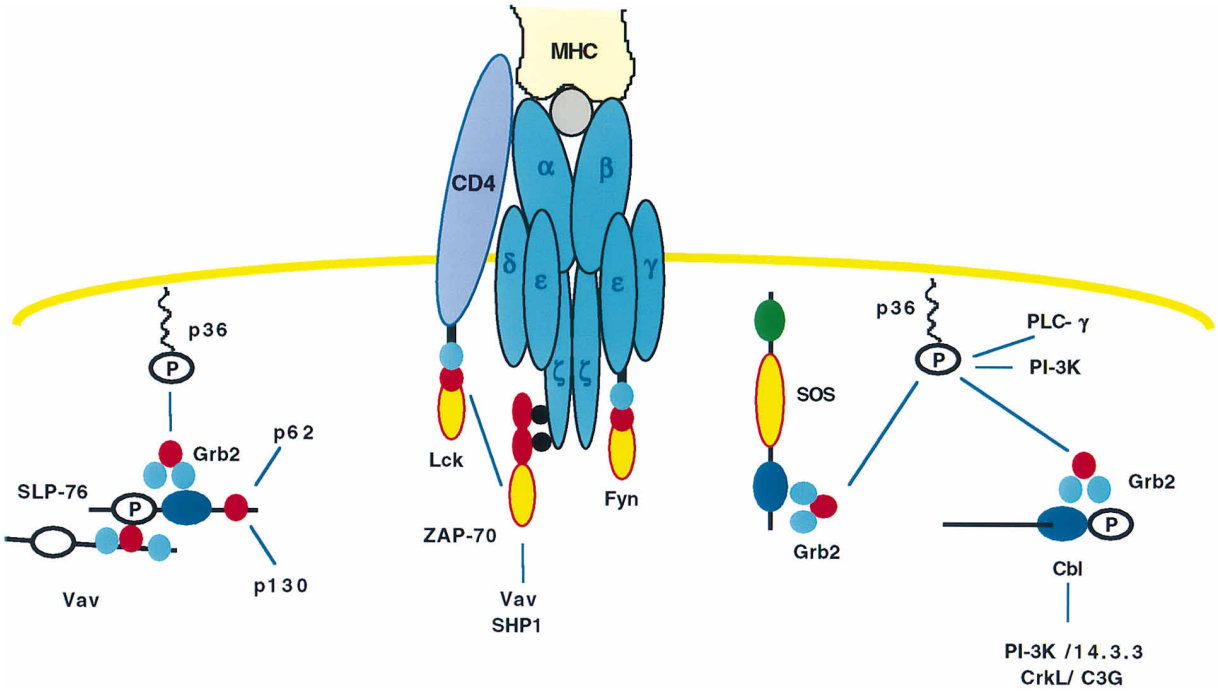

Figure 3. Signaling Complexes at the T Cell Membrane

The multisubunit TCR and coreceptor CD4 are shown engaged to a specific antigen–MHC ligand. Associated enzymes and critical substrates are shown. Color-coding, where present, reflects domains depicted in Figure 1. Associations of proteins are shown directly or are indicated by lines. Only a few known complexes are depicted.

for the subsequent tyrosine phosphorylation of Vav, as crippling the SH2 domain of Vav prevents its tyrosine phosphorylation. Similarly, enhanced signaling does not occur if critical tyrosine residues (Y112, Y128, and Y145) in SLP-76, presumably the binding sites for the Vav SH2 domain, are mutated to phenylalanine. SLP-76, in turn, binds two as yet undefined tyrosine-phosphorylated proteins of 130 and 62 kDa. An inactivating mutation within the SLP-76 SH2 domain prevents association of these proteins and significantly blocks the stimulatory effect of SLP-76 overexpression. Clearly, Vav and SLP-76 have critical functional effects and the complex of these two proteins with others (e.g., pp130 and pp62) is central to TCR function. Possible arrangements of these proteins with the activated TCR are shown in Figure 3. How this complex interacts with downstream effectors is actively under investigation.

Tyrosine Phosphatases

The addition of phosphate to tyrosine residues catalyzed by tyrosine kinases induces many binding interactions as described. Regulation of these events by tyrosine phosphatases has equal significance both in initiating and quenching TCR signaling pathways (reviewed by McFarland et al., 1994). Activation of Src family kinases by CD45 has already been mentioned. Recent evidence for interaction of the SHP-1 PTP with ZAP-70 suggests that regulation of this kinase or its substrates by SHP-1 may also be significant (Plas et al., 1996). This association is mediated by the SH2 domains of SHP-1, which bind to tyrosine phosphorylated ZAP-70 after TCR engagement. Engagement of the SH2 domains of SHP-1 by ZAP-70 stimulates the phosphatase activity with a consequent decrease in net ZAP-70 kinase activity. The functional significance of this interaction can be demonstrated by overexpression of wild-type SHP-1, which decreases IL-2 production in response to TCR stimulation, or conversely by overexpression of catalytically inactive SHP-1 (C453S), which has the opposite effect of enhancing IL-2 production. Other as yet unidentified PTPs are likely to have functional significance, and substrates of known PTPs remain to be defined. It is also interesting that SHP-1 has been found to associate with Vav in activated splenocytes and the EL4 T cell lymphoma (Kon-Kozlowski et al., 1996). This association appears to involve the SH2 and flanking SH3 domains of Vav. As Vav and ZAP-70, and ZAP-70 and SHP-1 have also been shown to associate, determining the actual nature of the associations between these proteins will be required to more fully understand these signaling events. Another PTP that probably plays a role in down-regulating the TCR signal is SHP-2, which has been found to associate with the activation-upregulated T cell surface protein CTLA-4. Fyn, Lck, and ZAP-70 are all hyperphosphorylated in CTLA-4$^{-/-}$ mice (Marengere et al., 1996).

Implications

The focus of this review has been on the complexity of biochemical events at a very proximal position in the TCR signaling cascade. A number of proteins and protein–protein interactions have been described. It should be noted that the function of many of these proteins (e.g., Vav, Cbl, PI3K, and SLP-76) remains to be defined. Likewise, a full accounting of all of the proteins involved

in signaling complexes remains to be performed. The reader should know that extensive analysis of the pathways further downstream in the T cell and in other systems suggests that complexity exists at these levels too (Cantrell, 1996). For example, Ras is likely to be only one of the many small G proteins involved in transduction events. Similarly, the well-known ERK kinase, which is most certainly involved in TCR-coupled events, is only one of several related serine/threonine kinases involved in signaling via TCR and other surface receptors. The complexity of the transcriptional machinery assembled at each lymphokine promoter is also well known. We also acknowledge that we have focused almost entirely on TCR-mediated signaling. Biochemical signals arising from engagement of coreceptors, costimulatory receptors, and adhesion molecules are also integrated to give the complex cellular response. Moreover, complex protein–protein interactions driven by these receptors are likely to be found. T cell activation can no longer be described only in terms of simple linear or even branching biochemical steps, but instead must also be thought of as a complex web of associated proteins.

Several observations indicate that careful attention to nuances in the T cell signaling pathways is warranted. Activation of T cells with peptides or MHC molecules bearing subtle changes in sequence, so-called altered peptide ligands, results in dramatic differences in TCRζ and ZAP-70 tyrosine phosphorylation and ZAP-70 activity (Sloan-Lancaster et al., 1995; Madrenas et al., 1995). Similarly, T cell activation schemes that result in anergic T cells fail to induce Ras and ERK activation (Li et al., 1996; Fields et al., 1996). These examples are likely the first of many instances of variable biochemical responses to receptor engagement. Subtle changes in biochemical signals and in the composition of signaling complexes may have profound effects on cellular physiology. The existence of highly regulated signaling pathways offers the possibility of great flexibility of response to the T cell.

References

Arpaia, E., Shahar, M., Dadi, H., Cohen, A., and Roifman, C.M. (1994). Defective T cell receptor signaling and $CD8^+$ thymic selection in humans lacking ZAP-70 kinase. Cell *76*, 947–958.

Ashwell, J.D., and Klausner, R.D. (1990). Genetic and mutational analysis of the T-cell antigen receptor. Annu. Rev. Immunol. *8*, 139–167.

Bolen, J.B. (1993). Nonreceptor tyrosine protein kinases. Oncogene *8*, 2025–2031.

Buday, L., Egan, S.E., Rodriguez Viciana, P., Cantrell, D.A., and Downward, J. (1994). A complex of Grb2 adaptor protein, Sos exchange factor, and a 36-kDa membrane-bound tyrosine phosphoprotein is implicated in ras activation in T cells. J. Biol. Chem. *269*, 9019–9023.

Bustelo, X.R., Ledbetter, J.A., and Barbacid, M. (1992). Product of the Vav protooncogene defines a new class of tyrosine protein kinase substrates. Nature *356*, 68–74.

Cambier, J.C. (1995). Antigen and Fc receptor signaling: the awesome power of the immunoreceptor tyrosine-based activation motif (ITAM). J. Immunol. *155*, 3281–3285.

Cambier, J.C., and Johnson, S.A. (1995). Differential binding activity of ARH1/TAM motifs. Immunol. Lett. *44*, 77–80.

Cantrell, D. (1996). T cell antigen receptor signal transduction pathways. Annu. Rev. Immunol. *14*, 259–274.

Caplan, S., Zeliger, S., Wang, L., and Baniyash, M. (1995). Cell-surface expressed T-cell antigen-receptor ζ chain is associated with the cytoskeleton. Proc. Natl. Acad. Sci. USA *92*, 4768–4772.

Chan, A.C., Iwashima, M., Turck, C.W., and Weiss, A. (1992). ZAP-70: a 70 kd protein-tyrosine kinase that associates with the TCRζ chain. Cell *71*, 649–662.

Chan, A.C., Kadlecek, T.A., Elder, M.E., Filipovich, A.H., Kuo, W. L., Iwashima, M., Parslow, T.G., and Weiss, A. (1994). ZAP-70 deficiency in an autosomal recessive form of severe combined immunodeficiency. Science *264*, 1599–1601.

Chan, A.C., Dalton, M., Johnson, R., Kong, G.H., Wang, T., Thoma, R., and Kurosaki, T. (1995). Activation of ZAP-70 kinase activity by phosphorylation of tyrosine 493 is required for lymphocyte antigen receptor function. EMBO J. *14*, 2499–2508.

Cheng, A.M., Rowley, B., Pao, W., Hayday, A., Bolen, J.B., and Pawson, T. (1995). Syk tyrosine kinase required for mouse viability and B-cell development. Nature *378*, 303–306.

Desiderio, S., and Siliciano, J.D. (1994). The Itk/Btk/Tec family of protein-tyrosine kinases. Chem. Immunol. *59*, 191–208.

Donovan, J.A., Wange, R.L., Langdon, W.Y., and Samelson, L.E. (1994). The protein product of the c-cbl protooncogene is the 120-kDa tyrosine-phosphorylated protein in Jurkat cells activated via the T cell antigen receptor. J. Biol. Chem. *269*, 22921–22924.

Downward, J. (1994). The GRB2/Sem-5 adaptor protein. FEBS Lett. *338*, 113–117.

Elder, M.E., Lin, D., Clever, J., Chan, A.C., Hope, T.J., Weiss, A., and Parslow, T.G. (1994). Human severe combined immunodeficiency due to a defect in ZAP-70, a T cell tyrosine kinase. Science *264*, 1596–1599.

Exley, M., Varticovsky, L., Peter, M., Sancho, J., and Terhorst, C. (1994). Association of phosphatidylinositol-3-kinase with a specific sequence of the T cell receptor ζ chain is dependent on T cell activation. J. Biol. Chem. *269*, 15140–15146.

Fantl, W.J., Johnson, D.E., and Williams, L.T. (1993). Signalling by receptor tyrosine kinases. Annu. Rev. Biochem. *62*, 453–481.

Fargnoli, J., Burkhardt, A.L., Laverty, M., Kut, S.A., van Oers, N.S., Weiss, A., and Bolen, J.B. (1995). Syk mutation in Jurkat E6-derived clones results in lack of p72*syk* expression. J Biol. Chem. *270*, 26533–26537.

Fields, P.E., Gajewski, T.F., and Fitch, F.W. (1996). Blocked Ras activation in anergic $CD4^+$ T cells. Science *271*, 1276–1278.

Fournel, M., Davidson, D., Weil, R., and Veillette, A. (1996). Association of tyrosine protein kinase ZAP-70 with the protooncogene product $p120^{c\text{-}cbl}$ in T lymphocytes. J. Exp. Med. *183*, 301–306.

Fukazawa, T., Reedquist, K.A., Trub, T., Soltoff, S., Panchamoorthy, G., Druker, B., Cantley, L., Shoelson, S.E., and Band, H. (1995). The SH3 domain-binding T cell tyrosyl phosphoprotein p120. J. Biol. Chem. *270*, 19141–19150.

Fusaki, N., Matsuda, S., Nishizumi, H., Umemori, H., and Yamamoto, T. (1996). Physical and functional interactions of protein tyrosine kinases, p59*fyn* and ZAP-70, in T cell signaling. J. Immunol. *156*, 1369–1377.

Gibson, S., August, A., Kawakami, Y., Kawakami, T., Dupont, B., and Mills, G.B. (1996). The EMT/ITK/TSK (EMT) tyrosine kinase is activated during TCR signaling. J. Immunol. *156*, 2716–2722.

Guanghui, K., Dalton, M., Wardenburg, J.B., Straus, D., Kurosaki, T., and Chan, A.C. (1996). Distinct tyrosine phosphorylation sites within ZAP-70 mediate activation and negative regulation of antigen receptor function. Mol. Cell. Biol., in press.

Harrison, M.L., Isaacson, C.C., Burg, D.L., Geahlen, R.L., and Low, P.S. (1994). Phosphorylation of human erythrocyte band 3 by endogenous p72*syk*. J. Biol. Chem. *269*, 955–959.

Hatada, M.H., Lu, X., E.R., L., Green, J., Morgenstern, J.P., Lou, M., Marr, C.S., Phillips, T.B., Ram, M.K., Theriault, K., Zoller, M., and Karas, J.L. (1995). Molecular basis for interaction of the protein tyrosine kinase ZAP-70 with the T-cell receptor. Nature *377*, 32–38.

Holsinger, L.J., Spencer, D.M., Austin, D.J., Schreiber, S.L., and

Crabtree, G.R. (1995). Signal transduction in T lymphocytes using a conditional allele of Sos. Proc. Natl. Acad. Sci. USA *92*, 9810–9814.

Huby, R.D.J., Carlile, G.W., and Ley, S.C. (1995). Interactions between the protein-tyrosine kinase ZAP-70, the proto-oncoprotein Vav, and tubulin in Jurkat T cells. J. Biol. Chem. *270*, 30241–30244.

Irving, B.A., Chan, A.C., and Weiss, A. (1993). Functional characterization of a signal transducing motif present in the T cell antigen receptor ζ chain. J. Exp. Med. *177*, 1093–1103.

Isakov, N., Wange, R.L., Burgess, W.H., Watts, J.D., Aebersold, R., and Samelson, L.E. (1995). ZAP-70 binding specificity to T cell receptor tyrosine-based activation motifs: the tandem SH2 domains of ZAP-70 bind distinct tyrosine-based activation motifs with varying affinity. J. Exp. Med. *181*, 375–380.

Isakov, N., Wange, R.L., Watts, J.D., Aebersold, R., and Samelson, L.E. (1996). Purification and characterization of human ZAP-70 protein tyrosine kinase from a baculovirus expression system. J. Biol. Chem. *271*, 15753–15761.

Iwashima, M., Irving, B.A., van Oers, N.S., Chan, A.C., and Weiss, A. (1994). Sequential interactions of the TCR with two distinct cytoplasmic tyrosine kinases. Science *263*, 1136–1139.

Jackman, J.K., Motto, D.G., Sun, Q., Tanemoto, M., Turck, C.W., Peltz, G.A., Koretzky, G.A., and Findell, P.R. (1995). Molecular cloning of SLP-76, a 76-kDa tyrosine phosphoprotein associated with Grb2 in T cells. J. Biol. Chem. *270*, 7029–7032.

Jayaraman, T., Ondrias, K., Ondriasova, E., and Marks, A.R. (1996). Regulation of the inositol 1,4,5-trisphosphate receptor by tyrosine phosphorylation. Science *272*, 1492–1494.

Katzav, S., Sutherland, M., Packham, G., Yi, T., and Weiss, A. (1994). The protein tyrosine kinase ZAP-70 can associate with the SH2 domain of proto-vav. J. Biol. Chem. *269*, 32579–32585.

Kon-Kozlowski, M., Pani, G., Pawson, T., and Siminovitch, K.A. (1996). The tyrosine phosphatase PTP1C associates with Vav, Grb2, and mSos1 in hematopoietic cells. J. Biol. Chem. *271*, 3856–3862.

Ley, S.C., Verbi, W., Pappin, D.J.C., Druker, B., Davies, A.S., and Crumpton, M.J. (1994). Tyrosine phosphorylation of a tubulin in human T lymphocytes. Eur. J. Immunol. *24*, 99–106.

Li, W., Whaley, C.D., Mondino, A., and Mueller, D.L. (1996). Blocked signal transduction to the ERK and JNK protein kinases in anergic $CD4^+$ T cells. Science *271*, 1272–1276.

Liao, X.C., and Littman, D. (1995). Altered T cell receptor signaling and disrupted T cell development in mice lacking Itk. Immunity *3*, 757–769.

Liu, Y.-C., Elly, C., Yoshida, H., Bonnefoy-Berard, N., and Altman, A. (1996). Activation-modulated association of 14–3–3 proteins with Cbl in T cells. J. Biol. Chem. *271*, 14591–14595.

Madrenas, J., Wange, R.L., Wang, J.L., Isakov, N., Samelson, L.E., and Germain, R.N. (1995). ζ phosphorylation without ZAP-70 activation induced by TCR antagonists or partial agonists. Science *267*, 515–518.

Marengere, L.E., Waterhouse, P., Duncan, G.S., Mittrucker, H.W., Feng, G.S., and Mak, T.W. (1996). Regulation of T cell receptor signaling by tyrosine phosphatase SYP association with CTLA-4. Science *272*, 1170–1173.

McFarland, E.C., Flores, E., Matthews, R.J., and Thomas, M.L. (1994). Protein tyrosine phosphatases involved in lymphocyte signal transduction. Chem. Immunol. *59*, 40–61.

Meisner, H., Conway, B.R., Hartley, D., and Czech, M.P. (1995). Interaction of Cbl with Grb2 and phosphatidylinositol-3′-kinase in activated Jurkat cells. Mol. Cell. Biol. *15*, 3571–3578.

Motto, D.G., Ross, S.E., Jackman, J.K., Sun, Q., Olson, A.L., Findell, P.R., and Koretzky, G.A. (1994). *In vivo* association of Grb2 with pp116, a substrate of the T cell antigen receptor-activated protein tyrosine kinase. J. Biol. Chem. *269*, 21608–21613.

Motto, D.G., Ross, S.E., Wu, J., Hendricks-Taylor, L.R., and Koretzky, G.A. (1996). Implications of the GRB2-associated phosphoprotein SLP-76 in T cell receptor-mediated interleukin 2 production. J. Exp. Med. *183*, 1937–1943.

Negishi, I., Motoyama, N., Nakayama, K., Nakayama, K., Senju, S., Hatakeyama, S., Zhang, Q., Chan, A.C., and Loh, D.Y. (1995). Essential role for ZAP-70 in both positive and negative selection of thymocytes. Nature *376*, 435–438.

Neumeister, E.N., Zhu, Y., Richard, S., Terhorst, C., Chan, A.C., and Shaw, A.S. (1995). Binding of ZAP-70 to phosphorylated T-cell receptor ζ and η enhances its autophosphorylation and generates specific binding sites for SH2 domain-containing proteins. Mol. Cell. Biol. *15*, 3171–3178.

Osman, N., Lucas, S.C., Turner, H., and Cantrell, D. (1995). A comparison of the interaction of Shc and the tyrosine kinase ZAP-70 with the T cell antigen receptor ζ chain tyrosine-based activation motif. J. Biol. Chem. *270*, 13981–13986.

Osman, N., Turner, H., Lucas, S., Reif, K., and Cantrell, D.A. (1996). The protein interactions of the immunoglobulin receptor family tyrosine-based activation motifs present in the T cell receptor ζ subunits and the CD3 γ, δ and ε chains. Eur. J. Immunol. *26*, 1063–1068

Pawson, T. (1995). Protein modules and signaling networks. Nature *373*, 573–580.

Peri, K.G., and Veillette, A. (1994). Tyrosine protein kinases in T lymphocytes. Chem. Immunol. *59*, 19–39.

Perlmutter, R.M., Levin, S.D., Appleby, M.W., Anderson, S.J., and Alberola-Ila, J. (1993). Regulation of lymphocyte function by protein phosphorylation. Annu. Rev. Immunol. *11*, 451–499.

Peters, J.D., Furlong, M.T., Asai, D.J., Harrison, M.L., and Geahlen, R.L. (1996). Syk, activated by cross-linking the B-cell antigen receptor, localizes to the cytosol where it interacts with and phosphorylates α-tubulin on tyrosine. J. Biol. Chem. *271*, 4755–4762.

Plas, D.R., Johnson, R., Pingel, J.T., Matthews, R.J., Dalton, M., Roy, G., Chan, A.C., and Thomas, M.L. (1996). Direct regulation of ZAP-70 by SHP-1 in T cell antigen receptor signaling. Science *272*, 1173–1176.

Qian, D., Mollenaver, M.N., and Weiss, A. (1996). Dominant-negative ζ-associated protein 70 inhibits T cell antigen receptor signaling. J. Exp. Med. *183*, 611–620.

Ravichandran, K.S., Lorenz, U., Shoelson, S.E., and Burakoff, S.J. (1995). Interaction of Shc with Grb2 regulates association of Grb2 with mSOS. Mol. Cell. Biol. *15*, 593–600.

Reedquist, K.A., Fukazawa, T., Panchamoorthy, G., Langdon, W.Y., Shoelson, S.E., Druker, B.J., and Band, H. (1996). Stimulation through the T cell receptor induces Cbl association with Crk proteins and guanine nucleotide exchange protein C3G. J. Biol. Chem. *271*, 8435–8442.

Reif, K., Buday, L., Downward, J., and Cantrell, D.A. (1994) SH3 domains of the adapter molecule Grb2 compete with two proteins in T cells: the guanine nucleotide exchange protein SOS and a 75-kDa protein that is a substrate for T cell antigen receptor-activated tyrosine kinases. J. Biol. Chem. *269*, 14081–14087.

Rozdzial, M.M., Malissen, B., and Finkel, T.H. (1995). Tyrosine-phosphorylated T cell receptor ζ chain associates with actin cytoskeleton upon activation of mature T lymphocytes. Immunity *3*, 623–633.

Shiue, L., Zoller, M.J., and Brugge, J.S. (1995). Syk is activated by phosphotyrosine-containing peptides representing the tyrosine-based activation motifs of the high affinity receptor for IgE. J. Biol. Chem. *270*, 10498–10502.

Sloan-Lancaster, J., Shaw, A.S., Rothbard, J.B., and Allen, P.M. (1995). Partial T cell signaling: altered phospho-ζ and lack of ZAP-70 recruitment in APL-induced T cell anergy. Cell *79*, 913–922.

Thome, M., Duplay, P., Guttinger, M., and Acuto, O. (1995). Syk and ZAP-70 mediate recruitment of p56*lck*/CD4 to the activated T cell receptor/CD3/ζ complex. J. Exp. Med. *181*, 1997–2006.

Turner, M., Mee, P.J., Costello, P.S., Williams, O., Price, A.A., Duddy, L.P., Furlong, M.T., Geahlen, R.L., and Tybulewicz, V.L. (1995). Perinatal lethality and blocked B-cell development in mice lacking the tyrosine kinase Syk. Nature *378*, 298–302.

Ullrich, A., and Schlessinger, J. (1990). Signal transduction by receptors with tyrosine kinase activity. Cell *61*, 203–212

van Oers, N.S., Tao, W., Watts, J.D., Johnson, P., Aebersold, R., and Teh, H.S. (1993). Constitutive tyrosine phosphorylation of the

T-cell receptor (TCR) ζ subunit: regulation of TCR-associated protein tyrosine kinase activity by TCRζ. Mol. Cell. Biol. *13*, 5771–5780.

van Oers, N.S., Killeen, N., and Weiss, A. (1996). Lck regulates the tyrosine phosphorylation of the T cell receptor subunits and ZAP-70 in murine thymocytes. J. Exp. Med. *183*, 1053–1062.

Vogel, L.B., and Fujita, D.J. (1993). The SH3 domain of p56*lck* is involved in binding to phosphatidylinositol 3′-kinase from T lymphocytes. Mol. Cell. Biol. *13*, 7408–7417.

Wange, R.L., Malek, S.N., Desiderio, S., and Samelson, L.E. (1993). Tandem SH2 domains of ZAP-70 bind to T cell antigen receptor ζ and CD3ε from activated Jurkat T cells. J. Biol. Chem. *268*, 19797–19801.

Wange, R.L., Guitian, R., Isakov, N., Watts, J.D., Aebersold, R., and Samelson, L.E. (1995a). Activating and inhibitory mutations in adjacent tyrosines in the kinase domain of ZAP-70. J. Biol. Chem. *270*, 18730–18733.

Wange, R.L., Isakov, N., Burke, T., Jr., Otaka, A., Roller, P.P., Watts, J.D., Aebersold, R., and Samelson, L.E. (1995b). $F_2(Pmp)_2$-TAMζ$_3$, a novel competitive inhibitor of the binding of ZAP-70 to the T cell antigen receptor, blocks early T cell signaling. J. Biol. Chem. *270*, 944–948.

Wardenburg, J.B., Fu, C., Jackman, J.K., Flotow, H., Wilkinson, S.E., Williams, D.H., Johnson, R., Kong, G., and Chan, A.C. (1996). Phosphorylation of SLP-76 by the ZAP-70 protein tyrosine kinase is required for T cell receptor function. J. Biol. Chem., in press.

Watts, J.D., Affolter, M., Krebs, D.L., Wange, R.L., Samelson, L.E., and Aebersold, R. (1994). Identification by electrospray ionization mass spectrometry of the sites of tyrosine phosphorylation induced in activated Jurkat T cells on the protein tyrosine kinase ZAP-70. J. Biol. Chem. *269*, 29520–29529.

Weiss, A., and Littman, D.R. (1994). Signal transduction by lymphocyte antigen receptors. Cell *76*, 263–274.

Weissman, A.M. (1994). The T-cell antigen receptor: a multisubunit signaling complex. Chem. Immunol. *59*, 1–18.

Wiest, D.L., Ashe, J.M., Abe, R., Bolen, J.B., and Singer, A. (1996) TCR activation of ZAP-70 is impaired in $CD4^+CD8^+$ thymocytes as a consequence of intrathymic interactions that diminish available p56lck. Immunity *4*, 495–504.

Wu, J., Katzav, S., and Weiss, A. (1995). A functional T-cell receptor signaling pathway is required for p95*vav* activity. Mol. Cell. Biol. *15*, 4337–4346.

Wu, J., Motto, D.G., Koretzky, G.A., and Weiss, A. (1996). Vav and SLP-76 interact and functionally cooperate in IL-2 gene activation. Immunity *4*, 593–602.