[Frontiers in Bioscience 7, d918-969, April 1, 2002]

PROTEIN TYROSINE PHOSPHORYLATION IN T CELL SIGNALING

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1. ABSTRACT

This review discusses in considerable detail the tyrosine phosphorylation events that couple the T cell antigen receptor to downstream signaling pathways. First, the protein kinases that catalyze these tyrosine phosphorylation are introduced, then the phosphatases that mediate removal of phosphate from tyrosine residues. Next, we discuss the molecular clock work by which these enzymes initiated T cell activation. Finally, we briefly review the key substrates for tyrosine phosphorylation and their role in coupling the kinases and phosphatases to gene transcription and other aspects of the T lymphocyte response to antigen.

2. INTRODUCTION

The principal function of the mature T lymphocyte is to recognize foreign antigen and respond to it by a complex activation process (reviewed in 1), which involves the transition to a metabolically active state and either secretion of immunoregulatory lymphokines or a targeted destruction of infected cells. Usually the triggered T cells undergo clonal expansion into a population of cells with identical specificity, some of which survive long-term as memory cells. T cell activation plays a crucial role in the adaptive immune response and is largely responsible for the recruitment and activation of many other cell types.

On the molecular level, recognition of antigenic peptides bound to MHC molecules on the surface of antigen-presenting cells is mediated by a unique, clonotypic T cell antigen receptor (TCR). If appropriate co-receptors (e.g. CD4 or CD8 and CD28) and accessory molecules are present, the resulting multiple receptor triggering events result in a highly organized cascade of biochemical events that are relayed from the contact site through the plasma membrane and the cytoplasm, and into the nucleus (reviewed in 2-5). This cascade causes multiple morphological and metabolic effects, including cytoskeletal reorganization, increased cell volume and, importantly, sequential transcriptional activation of previously silent genes.

On most mature T cells the TCR consists of two polymorphic disulfide-linked proteins, TCRalpha and TCRbeta, which are members of the immunoglobulin superfamily, in a complex with 4 invariant CD3 polypeptides: one gamma, one delta, and two epsilon, plus a homo- or heterodimer containing TCR-zeta, its splice-variant eta, or the gamma chain, which is shared with the FcepsilonRI. In most T cells, TCR-zeta is found as a homodimer. The antigen-recognizing alpha/betaTCR polypeptides each contain a single membrane spanning region, but very short cytoplasmic domains, while the other subunits, notably zeta, have large cytoplasmic tails that participate in signal generation. A conserved immune receptor tyrosine-containing activation motif (ITAM) is found once in each CD3 chains, in TCR-eta and in the gamma chain, and in three tandem copies in the TCR-zeta. As described below, these motifs are important for signal generation and transmission.

Many of the early signaling events initiated by the TCR occupancy have been uncovered during the past ten years (6-10). The identification of a number of protein tyrosine kinases (PTKs) and many of their substrates, constitutes a major advance in our understanding of TCR signaling (11,12). We have also learned that many receptors of the immunoglobulin superfamily employ a similar mechanism of signal transduction, which involves PTKs of the Src, Syk, Csk and Tec families, adapter proteins and effector enzymes in a highly organized tyrosine phosphorylation cascade. It is important to note, however, that this cascade is also equally dependent on the class of enzymes that remove phosphate from the PTK substrates (including from the PTKs themselves), the protein tyrosine phosphatases (PTPases)(reviewed in 13-17). In intact cells, tyrosine phosphorylation is rapidly reversible and generally of a very low stoichiometry even under induced conditions. It follows that a relatively minor change in the PTK - PTPase balance can have a major impact on the growth of T cells (18-20). Our understanding of the PTPases clearly lags behind the current state-of-the-art insights into the PTKs. This review will summarize the role of PTKs, PTPases and substrates in TCR signaling.

2.1. Inositol phospholipid hydrolysis and calcium mobilization

In the early 1980s it was found that antigen receptor-mediated activation of lymphocytes was associated with rapid hydrolysis of inositol phospholipids, mainly phosphatidylinositol-4,5-bisphosphate, by a phospholipase C (PLC) activity (1,21-24). For many years, this response was thought to be the key consequence of TCR ligation and all subsequent events were assumed to be regulated by either or both of the produced second messengers: diacylglycerol, which activates the serine/threonine-specific protein kinase C (PKC; 25-30), and inositol-1,4,5-trisphosphate (IP_3), which liberates Ca^{2+} from certain intracellular stores (31-37). This conclusion was largely based on the finding that increased inositol phospholipid hydrolysis was detected as early as 20 seconds after receptor triggering (31,32) and that $[Ca^{2+}]_i$ started to rise within 30-60 seconds (38-41), together with the observation that the combination of a Ca^{2+} ionophore plus a phorbol ester (which activates PKC) causes many responses characteristic of lymphocyte activation (42,43), such as lymphokine secretion, morphological changes and proliferation. Today we know that many other biochemical events, notably tyrosine phosphorylation, take place before or in parallel with the activation of phospholipase C, and that phorbol esters not only mimic the diacylglycerol-induced activation of protein kinase C, but also affects many other important enzymes. Although no longer considered the *sine qua non* of TCR signaling, protein kinase C and $[Ca^{2+}]_i$ are still regarded as important parts of TCR signal transduction.

2.2. TCR-induced tyrosine phosphorylation

The involvement of tyrosine phosphorylation as a mechanism of lymphocyte signal transduction was recognized in the late 1980s (44-53). The first polypeptide found to be tyrosine phosphorylated upon receptor triggering was the zeta-chain of the TCR complex itself

(44). The list of known substrates grown quite long since then and now includes numerous proteins with known enzymatic activities or other functions, such as the gamma isoform of phospholipase C (PLC-gamma1; 54-56), lipid kinases, protein kinases, nucleotide exchange factors, GTPase stimulating factors, adapter proteins, and cytoskeletal components. Importantly, protein tyrosine kinase (PTK) inhibitors (57,58), which prevent phosphorylation of all these substrates, also block all examined early and late parameters of cell activation, including increased phosphatidylinositol turnover, an increase in intracellular calcium, interleukin-2 (IL-2)-receptor expression, cytotoxicity, blast transformation and DNA synthesis (59-64).

The crucial tyrosine phosphorylation events that drive T cell activation by the TCR are catalyzed by a number of PTKs, which are expressed in T cells. These include the Src family PTKs Lck and Fyn, the Syk family kinases Zap-70 and Syk, the Csk kinase and, the Tec family kinases Itk (=Emt) and Txk (=Rlk). The biology of all these kinases will be discussed first.

3. SRC-RELATED KINASES IN T CELLS

The Src family of nonreceptor PTKs consists of nine members (reviewed in 68), of which three are expressed in T cells, namely Lck, Fyn and c-Yes. NK cells also express c-Fgr, while B cells have Blk, Lyn and Fyn.

3.1. Lck - a co-receptor associated PTK

Lck (65,66) was the first PTK to be implicated in T cell activation (67). This enzyme is a member of the Src family of nonreceptor PTKs and is expressed at high levels only in T lymphocytes, thymocytes and NK cells. It is located at the inner surface of the plasma membrane, largely due to the covalent attachment of both myristic acid at Gly-2 (69), a feature of all Src-family PTKs, and palmitic acid at Cys-3 and Cys-5 (70-73). This short first N-terminal region (sometimes termed the SH4 domain) is followed by a 80-amino acid long region, which is unique to Lck and which mediates a specific, interaction between Lck and the cytoplasmic domains of the CD4 and CD8 (74-76). The expression of these two glycoproteins on the surface of T lymphocytes correlates with their ability to recognize antigen in the context of class I and II major histocompatibility complex (MHC) molecules, respectively. CD4+ cells are usually of a helper/inducer phenotype, while CD8+ cells predominantly exhibit cytotoxic and suppressor properties. CD4 and CD8 play important accessory roles in T cell activation because they can bind to constant regions of the MHC molecules on the antigen-presenting cell (APC). Thus they participate in the formation of a multimeric complex that includes the TCR/CD3, MHC class I or class II molecules, CD4 or CD8, and Lck (67). This complex apparently serves both to stabilize the interaction between the T cell and the APC, and to facilitate transmembrane signaling. The formation of this multimeric complex is also important for thymic education, and for selection of the T cell repertoire.

The high-stoichiometry association of Lck with CD4 and CD8 is important for its function in T cells. On one hand, CD4 and CD8 restrict the random movement of Lck in the membrane (77), presumably limiting the ability of Lck to phosphorylate TCR-associated substrate proteins, such as the TCR-zeta chain. On the other hand, when an antigenic peptide presented on MHC molecules is recognized and bound by the TCR, CD4 or CD8 are brought to the immediate vicinity of this complex (78) due to their binding to invariable parts of the MHC molecule. As a result, CD4/CD8-associated Lck is juxtaposed to a previously inaccessible set of substrates, *e.g*. the TCR-zeta chain (67) and PLC-gamma1. However, recent studies have shown that it is not CD4 or CD8 that provide the driving force for the recruitment of Lck to the triggered TCRs, but that Lck itself is required for this translocation. More specifically, it seems that the SH2 domain of Lck is involved in bringing both Lck and the attached CD4 or CD8 to the site of TCR engagement (79). The properties of the various domains of Lck and the key mechanisms of Lck regulation are discussed below.

3.2. Fyn - a Src-family PTK associated with the TCR complex

The simple model of Lck being *the* initiator kinase for TCR signaling was complicated by the finding that another Src family PTK, Fyn, is directly associated with the receptor complex (80,81). Further support for an important role for Fyn came from the observations that thymocytes from mice expressing a *fyn* transgene were more readily triggered by TCR stimulation and produced higher levels of IL-2 than controls (82). Conversely, T cells from mutant mice lacking Fyn displayed somewhat diminished responses (83,84). It is still not clear to what extent Lck and Fyn have overlapping roles in TCR signaling, but there is ample evidence that they also have unique functions. For example, lack of Lck can not be compensated for by the normal expression of Fyn during early stages of thymocyte development (85). Conversely, both of them can phosphorylate TCR-zeta (although it is not known if they target the same tyrosines). Fyn also has a number of unique targets, such as FYB, which will be discussed below (see Chapter 7.4.).

3.3. c-Yes, the third and least understood Src-related kinase in T cells

In addition to Lck and Fyn, T cells contain a third Src-related kinase, c-Yes, the cellular homologue of the v-Yes oncogene from Yamaguchi's 73/Esh avian sarcoma virus (86). The role of this PTK in T cells, if any, remains unknown. As c-Yes is related to Lck and, in particular, Fyn, it may well participate in TCR signaling in a redundant manner. Its presence may also explain the less than striking phenotype of T cells from *fyn*$^{-/-}$ animals.

3.4. Structure and Regulation of Src family PTKs

The structure of Src family kinases, as well as the other key tyrosine kinases involved in T cell activation, are shown in **Figure 1**. For a detailed review of all Src family PTKs (Src, Fyn, Yes, Fgr, Lyn, Hck, Lck, Blk and Yrk), their structure, regulation and biology, we refer the reader to a recent book (68). The following will concentrate on the three members of the family that are present in T cells.

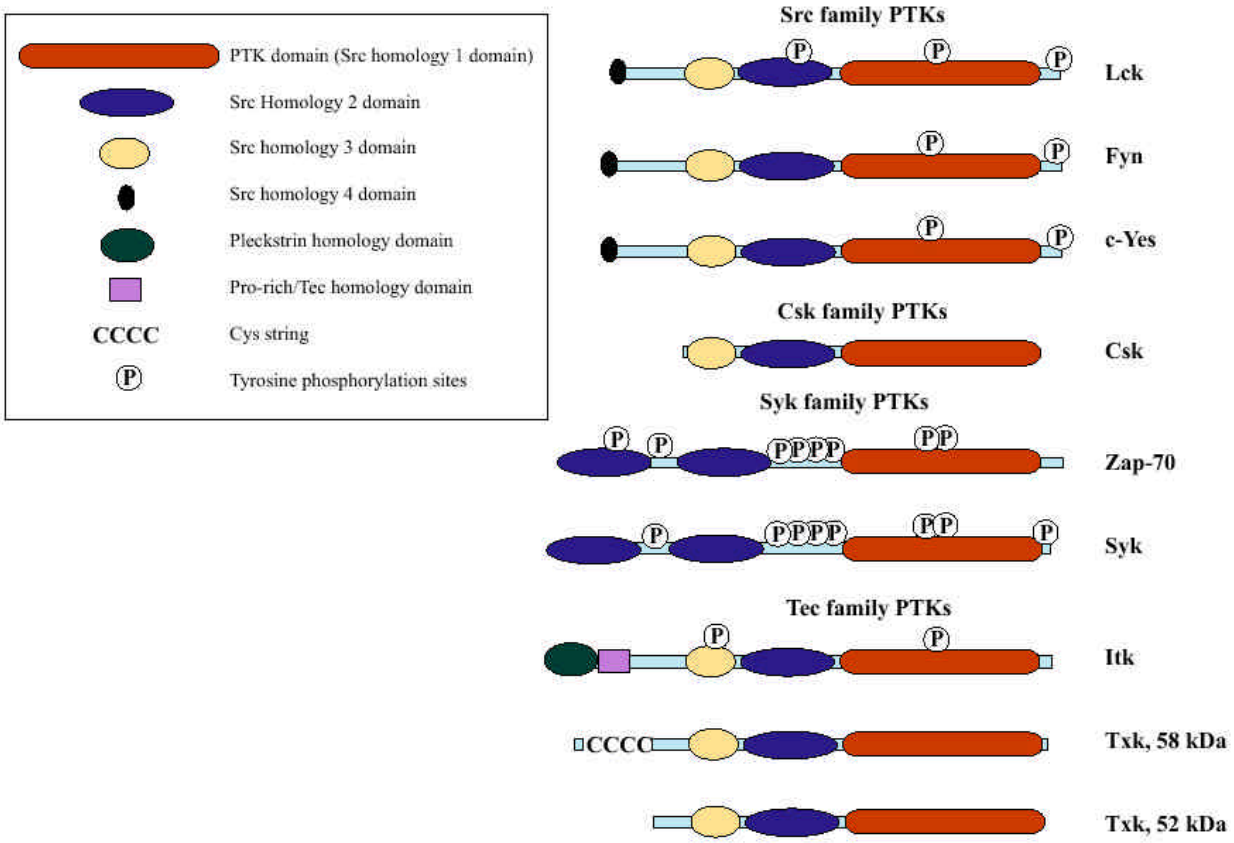

Figure 1. Structure of key PTKs involved in T cell activation. The location of domains and tyrosine phosphorylation sites are approximate only, and not all reported sites of phosphorylation are indicated.

3.4.1. Lipid modification and lipid raft association of Lck and Fyn

All Src family members have a glycine at position 2, which becomes covalently attached in an amide linkage to a myristyl moiety with the simultaneous removal of Met-1, in a reaction catalyzed by a endoplasmic myristyl transferase (68). Some Src-like kinases (e.g. Lck and Fyn) also have one or two cysteines within their first 6 amino acids, which become modified by palmityl groups in a thioester bond (70-73). Mutation of Gly-2 prevents membrane localization of Lck, and therefore binding to CD4 or CD8. Single mutations of Lck at Cys-3 and Cys-5 still allows some membrane localization, but a double mutation prevents it completely (72,73). Since membrane localization is required for the biological activity of Lck, these mutants also become functionally inactive (73). Fyn is also modified with myristate at Gly2 and palmitate at Cys-3, and these two acyl chains target the kinase to the plasma membrane and, perhaps even more importantly, to plasma membrane microdomains enriched in glycolipids and cholesterol, termed lipid rafts (87). This was initially observed as a co-immunoprecipitation of Lck with glycolipid-anchored proteins (88,89). During the last few years, intense focus on these lipid rafts has made it clear that they play an important role in T cell activation by being enriched in signaling molecules and by acting as platforms for the assembly of signaling complexes (90-95).

3.4.2. Unique N-Terminal Region

The nine Src kinases differ markedly from each other within the 80 - 100 amino acid region that follows the extreme N-terminal myristylation and acylation sequence (68). This region presumably confers unique properties on the different family members, including their interaction with various surface receptors (e.g. CD4, CD8 and TCR) and substrates. In many cases, the unique N-terminus also contains sites for phosphorylation by other kinases, such as protein kinases A and C, and a proline-directed kinase such as Erk or Cdc2.

In the case of Lck, the part of the unique N-terminus needed for binding to CD4 and CD8 has been mapped to a region surrounding cysteines 20 and 23 (96,97). The cytoplasmic tails of CD4 and CD8 contain similarly spaced cysteines, leading to the suggestion that binding may involve a metal ion and the formation of a tetrahedral cage-like configuration between the two proteins. Although the binding is tight, it can be disrupted in intact cells by serine phosphorylation of the cytoplasmic tail of CD4 (98). This phosphorylation can be induced by phorbol ester, suggesting an involvement of PKC, and results in release of Lck from CD4 and an enhanced internalization of CD4 from the cell surface (99). It thus seems that this regulation serves to remove CD4, decrease the availability of CD4-Lck co-receptors and downmodulate T cell activation.

The N-terminus of Lck can also be phosphorylated at several serine and threonine residues (100-104), including Ser-42 and Ser-59 (105,106). Phosphorylation of peptides corresponding to these latter sites suggests that they may be substrates for protein kinase C and a proline-directed kinase, perhaps Erk, respectively. Phosphorylation at Ser-194 in the SH2 domain was also reported by Soula and coworkers (107). Although serine phosphorylation has been speculated to act as a negative feedback and to reduce Lck's participation in signaling, the consequences of this modification have remained unclear.

The N-terminal region of Fyn is needed for binding to TCR-zeta (108-110). However, the mechanism of interaction seems to be quite different and involves binding to unphosphorylated ITAM motifs. The exact molecular mechanism is not understood, although this binding mode is shared with both Lyn and Fyn binding to the B cell receptor complex (111). By contrast, neither Lck nor Src can bind to TCR-zeta in this fashion. Mutation of four residues (Gly-2, Cys-3, Lys-7 and Lys-9) within the first 10 amino acids of Fyn inhibits its binding to TCR-zeta (110). These mutations all interfere with lipid modification and membrane localization of Fyn, the two latter by preventing recognition by the myristyl transferase.

Like Lck, Fyn can also be phosphorylated at N-terminal serine residues, although the sites of phosphorylation are unknown (112) and the physiological consequences, if any, remain unexplored.

3.4.3. SH3 Domain

Next from the N-terminal region reside the conserved SH3 and SH2 domains. The SH3 domain consists of 50-60 amino acids folded into a sandwich of five anti-parallel beta-strands that also create a hydrophobic pocket (113,114). The ligand binding surface contains a cluster of hydrophobic residues that is flanked by so called "RT" and "n-Src" loops that connect the ba and bb, and bb and bc strands, respectively. Mutations in the RT loop occur in the activating form of v-Src (115). As first evident with Abl-SH3-domain binding to 3BP-1 (116), the barrel-like pocket binds to polyproline motifs defined by either the sequence RXPXXP (Type 1) or PXXPXR (Type 2). Binding affinities are in the micromolar range. In the case of Lck and Fyn SH3 domains, as many as 10 proteins can be precpitated from T-cell lysates (unpublished data) and include the p85 subunit of PI 3-kinase (117-120) and the cytoskeleton protein dynamin (121,122). The physiological significance of many of these interactions remain to be defined.

The crystal structures of the Src family PTKs Hck and c-Src reveal that the SH3 domain also plays an important role in the suppression of the catalytic domain by binding to a linker region between the SH2 and kinase domain (123,124). This binding helps maintain the kinase in an inactive conformation. The SH3 domain binds to the proline-containing linker, while the RT and nSrc loops extend on either side of it to contact the catalytic domain. Although the linker sequence contains only one proline residue, it forms a left-handed PPII helix that binds in a characteristic type 2 orientation. The proline packs in a way similar to the first proline of a typical PXXPXR motif, while a Gln occupies the other site that normally binds to a proline. The connection between the SH3 and SH2 domains appears to be flexible with only minimal interaction between the SH3 and SH2 domains themselves. These structural insights explain why the SH3 domain is necessary for suppression of Src family PTKs (125-128)

3.4.4. SH2 Domain

SH2 domains are protein-protein interaction modules comprised of about 100 amino acids and bind PTyr in the context of specific adjacent amino acid sequences (129). SH2 domains are well conserved between different Src family members and are likely to have similar, although not identical, ligand binding specificities. Nuclear magnetic resonance (NMR) and crystal analysis show that the SH2 domain consists of two alpha-helices that pack at either side of a central four-stranded beta-sheet (123,124,130,131). PTyr-containing peptides bind in an extended fashion across the surface of the domain. In Src family SH2 domains, PTyr is bound in a pocket on one side of the central sheet, while an additional hydrophobic pocket recognizes a leucine or isoleucine at position PTyr- + 3. In its pocket, PTyr interacts with a key conserved Arg residue, forming an ion pair with the phosphate. Other residues stabilize the interaction by hydrogen bonding and amino-aromatic group interactions. The optimal binding motifs initially derived by screening peptide libraries were found to favor PTyr-Glu-Glu-IIe (PTyr + 1-3 = Tyr-EEI) (132). The dissociation constant for binding is in the 1 nM range. It should be noted, however, that physiological (or proposed) ligands do not have exactly this sequence, and it is likely that multiple ligands with a whole range of affinities from 1 to several tens of nanomolar exist in cells. Such a spectrum of ligands could play a role in hierarchical or sequential interaction events. In addition, the peptide binding analyses carried out so far have not (for technical reasons) included peptides with cysteine and tryptophan, or with PSer, PThr or multiple PTyr residues. In fact, peptides with PTyr at positions +1 or +2 (in addition to position 0) are very high affinity ligands for Src family SH2 domains (L. Cantley, personal communication).

The SH2 domain not only binds other proteins, but also participates in the folding of the inactive conformation of the kinase. This is accomplished by binding of the tyrosine phosphorylated (at Tyr-505 in Lck) short C-terminal tail (123,124). The interaction is of relative low affinity (133), apparently because the interaction is limited to recognition of Gln-PTyr-Gln-Pro motif without the usual interactions with a side chain occupying the PTyr- + 3 pocket. It also appears that the binding of the SH2 to the C-terminal tail must be accompanied by SH3 domain binding to the linker region for the holoenzyme to remain in an inactive state (123,124). A logical consequence of this notion is that alternative ligands with a high affinity for the SH3 or SH2 domains may break up the suppressed conformation by a competitive mechanism (68). Examples of this type of activation include the interaction of Hck with the HIV Nef protein (134) and the interaction of Lck with Tip (135-140), a transforming protein of *Herpesvirus saimiri*.

The importance of the SH2 domain both in interaction with other proteins (which can be regulators or substrates) and in intramolecular regulation of kinase activity, is also well illustrated by the effects of point-mutations or deletions within this region. Generally, Src family PTKs without functional SH2 domain are catalytically active, but have a reduced capacity to perform their biological functions (141-145). The SH2 domain of Lck is also subject to an incompletely understood mechanism of regulation through phosphorylation at Tyr-192 (146). This residue lies near the PTyr- +3 binding pocket and its phosphorylation results in a decreased affinity for ligand. The modification seems to occur in intact T cells as a somewhat later event of negative consequence for the participation of Lck in TCR signaling (146). *In vitro* and in co-transfected COS cells, both Syk and Zap-70 are able to catalyze the phosphorylation, which may be part of a negative feedback loop to limit the TCR signaling cascade.

3.4.5. Kinase Domain

Following the SH2 domain is a highly conserved kinase domain of about 300 amino acids, also called the Src homology domain 1 (SH1), showing some 80 percent homology amongst Src kinases family members. Structural motifs include a glycine-rich ATP-binding loop followed by a crucial lysine residue (Lys-273 in Lck) that interacts with ATP and is involved in phosphotransfer to the substrate (147), and a flexible so-called activation loop (148,149). The catalytic domain has an overall fold comprising a smaller N-terminal lobe connected by a flexible hinge to a larger C-terminal lobe (123,124). The N-terminal lobe is a five stranded antiparallel beta-sheet with a single helix (termed helix C) that connects with a mostly alpha-helical C-terminal lobe. In a restrained state, helix C contributes to the displacement of several catalytic residues from their optimal positions. In the case of cell cycle kinase Cdk2 binding to cyclin A, helix C is involved in inducing an active conformation (150). A similar mechanism involving helix C presumably operates in activation of Src family kinases.

3.5. Regulation of Src-family PTKs

The function of Src-family PTKs is tightly regulated at all available levels: transcription of the genes, processing of the resulting mRNA, translation, and several covalent modifications and protein-protein interactions (reviewed in 68). Best characterized are the post-translational mechanisms, which include acylation of the N-terminus, reversible phosphorylation of tyrosine and serine residues, intramolecular interactions, association with transmembrane receptors and cytoskeletal elements and complex formation with regulatory proteins or substrates. Different Src-family PTKs seem to be regulated principally by similar mechanisms, but may associate with different regulatory proteins or substrates. These associations are largely dictated by the unique N-termini and by the SH2 and SH3 domains.

3.5.1. Positive regulation by tyrosine phosphorylation in the kinase domain

A tyrosine residue in the activation loop of the kinase domain, Tyr-394 in Lck and Tyr-417 in the hematopoietic form of Fyn, is crucial for the catalytic function of the domain. The crystal structure of the activate conformation of the Lck kinase domain shows that the phosphoryl group at Tyr-394 is involved in generating a competent active site (152). The activation loop of the activated Lck domain has a radically different conformation when compared to the unphosphorylated loop of other kinases. This residue forms salt bridges with Arg-363 and Arg-387. Arg-363 interacts through a water molecule, while Arg-387 makes two hydrogen bonds. A comparison of activate and inactive kinases shows that Arg-387 is expelled from a pocket that it occupies in the inactive structure resulting in a re-ordering of the activation segment of the kinase. In this scenario, the side chain of Arg-363 becomes re-oriented freeing the helix C to lift back to a position needed for an active catalytic site.

Although Tyr-394 of Lck and Tyr-417 of Fyn are normally phosphorylated to very low stoichiometry *in vivo* (153-157), a higher level phosphorylation at these sites correlates with situations in which Lck and Fyn are activated. Particularly, C-terminally mutated Lck or Fyn have considerably higher levels of Tyr-394 and Ty-417 phosphorylation, respectively (158-160). Mutation of Tyr-394 to a phenylalanine in Lck abrogates the capacity of the kinase to phosphorylate substrates or transform cells (157). Thus, phosphorylation at Tyr-394 is crucial for catalytic activity of Lck, suggesting that the normally low level of phosphate at Tyr-394 detected in intact T cells indicates that very few Lck molecules are catalytically active at any given time.

It has long been taken for granted that phosphorylation of Lck at Tyr-394 (and at the corresponding site in all Src family PTKs) is solely the result of autophosphorylation (65,66,100,161). However, since the reaction most likely is catalyzed in *trans* between two kinase molecules, there is no particular reason to assume that it can only occur as a result of homotypic interaction. Rather, it is likely that two different Src family PTKs can transactivate each other. In addition, there is some data that suggests the existence of a cytosolic non-Src family PTK that can phosphorylate Tyr-394 of Lck in response to hydrogen peroxide (162). As this putative positive regulator of Lck (the "Tyr-394-kinase") has not been identified, the issue remains speculative.

3.5.2. Dephosphorylation of the activation loop tyrosine

It is still unclear how phosphate is removed from the positive regulatory tyrosine phosphorylation site in the activation loop of Src family PTKs, but studies with PTPase inhibitors (163), suggest that the turnover is rapid. Given the importance of phosphate at Tyr-394 for the catalytic function of Lck, a PTPase acting on this site would be expected to reduce tyrosine phosphorylation by Lck. Conversely, inhibition of this PTPase would probably cause a strong and unopposed activation of Lck with catastrophic consequences for the T cell and the host organism. It was recently shown that isolated T cell membranes contain a PTPase activity that maintains the TCR machinery in a resting state (164). Inhibition of this activity caused the same set of tyrosine phosphorylation

events as receptor ligation, a response that depended on Lck (i.e. it was absent in JCaM1 cell membranes), but was unaffected by the absence of SHP1, SHP2 or CD45 (164).

It has been proposed that CD45, the PTPase that removes phosphate from the negative regulatory site (see below), also dephosphorylates the tyrosine residue in the activation loop of Lck (and perhaps Fyn). While this certainly remains fully possible, there is evidence to suggest that another PTPase carries the main responsibility for this dephosphorylation. Perhaps this crucial and potentially dangerous task is shared by several PTPases.

Based on findings *in vitro* and in thymocytes from the SHP1-deficient *motheaten* mice, it has been suggested that SHP1 dephosphorylates Lck at Tyr-394 (165-168). This may well be true under circumstances where SHP1 is recruited to an ITIM-containing inhibitory receptor (such as LAIR-1 in T cells) and juxtaposed to the TCR/CD4, but it does not seem to occur in resting T cells or following triggering of T cells by monoclonal antibodies to the TCR complex alone (169).

We have found that the PEP PTPase can negatively regulate TCR-induced signaling events in a manner that suggests a very receptor-proximal site of action (170). As the TCR-induced tyrosine phosphorylation of Lck is decreased in the presence of active PEP, it is possible that Tyr-394 is a target for this PTPase. Another study concluded that PEP dephosphorylates Fyn at the corresponding Tyr-417 (171). Both possibilities are compatible with the physical association of PEP with Csk (172), the kinase that negatively regulates Lck and Fyn (173). These issues are discussed in more detail in section 5.6.

3.5.3. Regulatory C-terminus

The C-terminus of Src family PTKs plays an important role in suppression of the kinase domain (68). Within this short tail resides a conserved tyrosine residue (Tyr-505 in Lck and Tyr-528 for $Fyn^{(T)}$), which is phosphorylated to a relatively high stoichiometry in intact cells and subsequently binds to the SH2 domain within the same molecule and thereby stabilizes the kinase in a restrained inhibitory conformation (123,124). As mentioned above, the SH3 domain is also required to stabilize this conformation of the enzyme. The three-dimensional structure of the suppressed conformation reveals that these intramolecular interactions push the two lobes of the kinase domain together reducing access to the catalytic cleft and forcing the helix C to move outward (123,124). It has been estimated that ~50% of Lck is in this suppressed state in T cells (174). It appears that the percentage is somewhat higher for Fyn.

In particular, mutation of the conserved carboxy-terminal tyrosine residue to phenylalanine leads to a constitutively increased PTK activity of the enzyme and confers transforming potential to these proteins when expressed in NIH 3T3 cells (158-160). The effects of active Src-family PTKs in these cells are not, however, directly applicable to lymphoid cells. Transfection of Tyr-505-to-Phe mutated Lck into a $CD4^-8^-$ T cell line did not alter its level of basal tyrosine phosphorylation, but nevertheless augmented TCR-induced tyrosine phosphorylation and IL-2 production. We have observed the same effect of activated Lck expressed in the Lck-deficient JCaM1 variant of Jurkat. This indicates that the level of tyrosine phosphorylation in T cells does not simply reflect the catalytic activity of Lck, but that other mechanisms, such as Lck location, substrate accessibility, and PTPases play major roles.

3.5.4. Suppression of Src family kinases by Csk

Two important opposing enzymes regulate the phosphorylation status of the C-terminal negative regulatory tyrosine in Src family PTKs, namely the Csk PTK (173,175) and the CD45 PTPase (11,13). The Csk kinase seems to be the only PTK that efficiently phosphorylates this site, thus acting as a general negative regulator of all Src family PTK-mediated events. Csk is a small (only 50 kDa) cytosolic nonreceptor PTK expressed in all examined cell types. Structurally, it resembles the Src family kinases in having SH3, SH2 and kinase domains (Figure 1), but differs in lacking an N-terminal membrane attachment motif, a tyrosine autophosphorylation site and a C-terminal regulatory sequence typical of Src-family kinases. Csk seems to be highly specific for the C-terminus of Src-family PTKs, having only one additional putative substrate, namely CD45 (176).

Posttranslational regulation of Csk has been observed by several groups and two important mechanisms of Csk regulation were recently uncovered. Csk is an exception from the general rule that PTKs are regulated by tyrosine phosphorylation. Nevertheless, a posttranslational modulation of the catalytic activity of Csk has been observed in three instances. Oetken and co-workers (177) observed that the specific activity of Csk undergoes a transient activation after TCR triggering. Csk also became physically associated to a 72-75 kDa tyrosine phosphorylated protein, but with considerably slower kinetics. Another study by Fargnoli and colleagues (178) found that malignant T cells from patients with cutaneous T cell leukemia (Sezary syndrome) had an increased fraction of membrane-bound, but mainly inactive Csk. Finally, Vang et al (179) may have uncovered the operative mechanism in these settings with the discovery that cAMP-dependent protein kinase (PKA) phosphorylates a serine residue (S364) in the catalytic domain of Csk and thereby activates the kinase 2-3 fold. This cAMP-dependent activation mechanism may well explain the long-term inhibition of T cell activation by agents that elevate cAMP levels. In support of this notion, prostaglandin E and a membrane-permeable cAMP analogue caused the phosphorylation of Csk at Ser-364, activated Csk, suppressed Lck and the phosphorylation of TCR-zeta and Zap-70, and the TCR-induced transactivation of the IL-2 gene (179). All these effects were abrogated by introduction of a Csk-S364C mutant and all effects on Lck were abrogated by mutation of Tyr-505 to a Phe residue. Thus, Csk may be the most important target for cAMP-induced immune suppression.

Another important advance in our understanding of Csk regulation was the recent discovery of a

transmembrane molecule, termed PAG (180) or Cbp (181), which specifically binds Csk through its SH2 domain. PAG/Cbp is anchored to lipid rafts and is phosphorylated on tyrosine in resting T cells (181), thus anchoring Csk in the subcellular compartment that is enriched in Src family kinases. Upon TCR triggering, PAG/Cbp is rapidly dephosphorylated by an unknown PTPase and Csk dissociates (181). This apparently allows lipid raft-located Lck and Fyn to remain active and phosphorylate ITAMs and other molecules. After some 5 min, however, PAG/Cbp is rephosphorylated and Csk returns to the lipid rafts. This coincides with the downturn of tyrosine phosphorylation. The importance of this mechanism is perhaps best illustrated by the consequences of expression of a Csk-SH3-SH2 protein (lacking kinase domain), which will compete with endogenous Csk for binding to PAG/Cbp (181). This truncated protein caused a striking increase in basal and induced levels of tyrosine phosphorylation, which also lasted longer than in controls. The protein also augmented NFAT/AP-1 reporter gene activation (181). Interestingly, the dominant negative Csk-SH3-SH2 construct became even more potent when its SH3 domain was debilitated by a point mutation that prevented association with PEP (see Chapter 5.6).

3.5.5. Csk is opposed by CD45

Apparently, the catalytic activity of Src-family PTKs depends on the balance between the counteracting effects of Csk and CD45 (11,13). This balance is probably different in lymphocyte types and subpopulations expressing different types and amounts of Src-family PTKs and distinct isoforms of CD45.

CD45 is a large (180-220 kDa) transmembrane receptor-like PTPase with a highly glycosylated and variable extracellular portion and an invariable cytoplasmic part consisting of two PTPase domains in tandem (see chapter 5.2). CD45 appears to be essential for TCR-induced T cell activation, as indicated by the findings that CD45-negative T cell mutants failed to respond to TCR triggering with proliferation (183), early tyrosine phosphorylation and inositol phospholipid hydrolysis (184). The response to antigen was regained in a revertant $CD45^+$ clone (185). Although some controversies remain regarding the preference for specific Src-family members, data from several laboratories agree with the notion that at least Lck and Fyn are physiologically relevant substrates for CD45 *in vivo* (186-194). Dephosphorylation of these kinases by CD45 seems to correlate with responsiveness of the T cells to TCR stimulation, suggesting that the role of CD45 is, at least partly, to keep Src-family PTKs sufficiently active to participate in signal transmission. Findings with CD45-negative B lymphocytes suggest that B cell-specific kinases are similarly regulated by CD45 (195). It should also be noted that other PTPases seem to perform the same type of task. For example, expression of the CD45-related receptor-like PTPase, PTP-alpha in fibroblasts activated the transforming potential of c-Src by dephosphorylating its negative regulatory Tyr-527 residue (196).

3.5.5. Additional phosphorylation events

Src family PTKs can be phosphorylated at additional tyrosine residues and, as mentioned above, serine residues. Most of these modifications are poorly understood and their physiological significance has not been well documented yet. Known tyrosine phosphorylation sites include Tyr-192 in the SH2 domain of Lck (146,197). Phosphorylation of this residue (which is conserved in all Src family PTKs) by Syk or Zap-70 results in reduced ligand binding of the SH2 domain (146). Thus, this event may play a role in a negative feedback loop designed to limit the activation cascade initiated by TCR ligation. The corresponding residue in c-Src has also been reported to be phosphorylated (198), and we have found that the C-terminal SH2 domain of the p85 subunit of phosphatidylinositol-3-kinase (PI3K) can be phosphorylated by the Abl PTK at the corresponding Tyr-688 (199), with the same ligand affinity reducing consequences. Thus, this may be a more widespread mechanism for negative feedback of SH2 domains.

Another functionally similar modification is the tyrosine phosphorylation of the c-Src SH3 domain at Tyr-138 (200), which results in a reduced binding of Pro-rich ligands to this domain. It is not yet known if Lck or Fyn are phosphorylated at the corresponding site in their SH3 domains.

Mechanistically, the phosphorylation of Src family PTK SH3 and SH2 domains is intriguing, since it would be predicted to prevent the folding of the holoenzyme into the suppressed conformation, or, alternatively, open up this conformation. Consequently, these modifications should lead to activation, while at the same time preventing interaction with substrates that are ligands for the SH3 or SH2 domain.

3.5.6. Src family PTKs and viruses

An exciting development over the past few years has been the connection between viruses and their opportunistic use of Src kinases through viral proteins that have adapted to take advantage of the SH3 and SH2 domains of Lck and Fyn. The Nef protein of Human Immunodeficiency Virus -1 interacts with the SH3 domain of Lck and thereby inhibits T cell activation and production of lymphokines needed for a productive anti-viral response (201). Another example is the T lymphotropic virus of non-human primates *Herpesvirus saimiri*, which can immortalize human T lymphocytes *in vitro* (135-140). The protein of C-type strains of this virus, Tip (tyrosine kinase interacting protein), binds to the SH3 domain of Lck (135-140). This interaction activates Lck, but also interferes with the function of Lck in the cells. Tip even suppressed the transforming ability of oncogenic F505 Lck and anti-CD3 induced tyrosine phosphorylation. *Herpesvirus saimiri* strain 11 of subgroup A carries a gene termed StpA (saimiri transformation-associated protein, group A), which is required for the lymphoma-inducing potential of the virus (202). A highly conserved Tyr-Ala-Glu-Val/Ile motif binds to the SH2 domains of Src, Lck and Fyn (137).

The DNA tumor virus, hamster polyomavirus (HamT), causes lymphoid malignancies, and induces IL-2 transcription. Dominant negative Fyn, but not Lck, blocked this effect suggesting some kind of selective

interaction with Fyn (203). In another example, the $p40^{tax-1}$ oncoprotein of Human T cell leukemia virus type 1 (HTLV-1), the known etiologic agent of adult T cell leukemia can functionally cooperate with Lck (204). Tax can render the hematopoietic cell line BAF-B03 cytokine-independent when co-expressed with Lck-Y505F.

The envelope glycoprotein gp120 of Human Immunodeficiency Virus -1 binds to CD4. This binding has been reported to activate the CD4-associated Lck (205), a finding that has been contested (206). Binding of gp120 also activates phosphatidylinositol-3-kinase (PI3K) and phosphatidylinositol-4-kinases associated with CD4/Lck complex (118). This is probably related to the crosslinking effects of the capsid protein on the intact virus. In contrast, ligation of CD4 by soluble monovalent gp120 will inhibit TCR-induced use of Lck and subsequent Zap-70 recruitment (207,208) Membrane-associated HIVenv causes rapid T cell death and in the process activates Lck and Fyn, without activating Zap-70 (209). These effects may be due to the sequestering of Lck and Fyn from the TCR complex. A similar mechanism may apply to the stimulatory effects of antagonistic peptides on CD4-Lck in the absence of T-cell activation (210).

4. SYK FAMILY PTKS IN T CELLS

Unlike Src kinases, the Syk family PTKs, Zap-70 (211) and Syk (212) are comprised of two tandem SH2 domains linked to a kinase domain (Figure 1). The two SH2 domains are also spaced with considerable distance between them, and there is a long "linker" region between the second SH2 domain and the catalytic domain, which is similar to Src family kinases, but represents a separate evolutionary branch of PTKs. Both Zap-70 and Syk lack sites for myristylation, palmitylation or other recognized mechanisms for anchoring to the plasma membrane. Accordingly, they have been considered to be freely cytosolic enzymes. The use of confocal microscopy, however, has demonstrated that Zap-70 is preferentially located at the plasma membrane also in the resting T cell (213), albeit less tightly. Thus, the recruitment of Zap-70 can proceed more rapidly and efficiently.

Syk is expressed in all hematopoietic cell lineages (212), including platelets (214,215) and erythrocytes (216). It is also found in many nonhematopoietic cell types. Expression levels vary among leukocytes, with mature T cells in general having the lowest levels (217). Zap-70, on the other hand is exclusively found in T cells and NK cells (211).

A very important advance for our understanding of the importance of Syk and Zap in leukocyte signaling and development was the recent discovery of patients lacking a functional *zap* gene (218-220), as well as the generation of *zap* (221) and *syk* (222,223) null allele mutant mice. Lack of Zap in humans leads to a severe immunodeficiency characterized by the absence of $CD8^+$ T cells and TCR-unresponsive mature $CD4^+$ T cells. Mice lacking Zap are also deficient in the production of $CD4^+$ T cells, while the natural killer (NK) cells are unaffected. Mice lacking Syk, on the other hand, died *in utero* from massive hemorrhage, while $RAG2^{-/-}$ mice reconstituted with fetal liver cells from the $syk^{-/-}$ mice failed to develop B cells (222,223) and intraepithelial TCRgamma/$delta^+$ T cells (224). Most other T cells (as well as other leukocyte types) appear normal, although detailed signaling studies have not yet been carried out with them. Significantly, double knock-out mice (225) show a much more severe and early arrest in thymic development of T cells. Thus, it appears that Syk can compensate for Zap during thymocyte development, a notion that is supported by the higher level of Syk expression in thymocytes compared to mature T cells (226). These largely non-overlapping requirements for Syk and Zap clearly reflect differences in both expression pattern and differences in biological function. This question is particularly pertinent in cells expressing more equal levels of both PTKs, such as TCRgamma/$delta^+$ T cells and NK cells.

4.1. Regulatory mechanisms for Zap-70

Perhaps the most important feature of Zap-70 is its recruitment and high affinity association with the phosphorylated ITAMs of the TCR receptor complex (211,227-229). The crystal structure of the complex of the N-terminus of Zap-70 bound to a doubly phosphorylated ITAM peptide (230) shows that the second SH2 domain binds the first phosphorylated tyrosine of the peptide in the usual SH2 - ligand manner, while the second phosphorylated tyrosine interacts with both SH2 domains in a unique manner due to the presence of an incomplete PTyr-binding pocket in the N-terminal SH2 domain, which is made functional by the close proximity of the other SH2 domain. This feature of the solved structure explained the weak binding of singly phosphorylated peptides, the poor activity of the isolated SH2 domains, and the strongly synergistic binding of doubly phosphorylated ITAMs (231,232).

Binding of Zap-70 to ITAMs alone is insufficient to activate the kinase (233,234). This accounts for the fact that Zap-70 can be inactive when bound to TCR-zeta (as observed in thymocytes). Activation is dependent on the phosphorylation of a tyrosine residue in the activation loop of Zap-70, Tyr- 493 (235-238), which corresponds to Tyr-394 of Lck (see above) as well as the positive regulatory phosphorylation sites in nearly all other protein kinases. In contrast to Src family PTKs and Syk, Zap-70 is completely unable to autophosphorylate at Tyr-493 and is therefore under the control of PTKs that can, such as Lck and possibly Fyn (211,227,236,239,240). Phosphorylation by Lck can activate the kinase by as much as 10-fold. For catalytic activity, Zap-70 also requires a cation showing a preference for Mn^{2+} over Mg^{2+} *in vitro* (241). Zap-70 molecules with Tyr-493 substituted for by a phenylalanine residue show normal very low basal kinase activity in the test tube (and probably none in intact cells), but cannot be activated by Lck. Exactly how Lck interacts with Zap-70 to recognize Tyr-493 as a substrate is not clear, but it seems that the SH2 domain of Lck is involved (242,243), perhaps by binding to phosphorylated Tyr-319 of Zap-70 (244). This residue is crucial for proper function of Zap-70 in TCR signaling (245, 246), either because it is needed for interaction with Lck (244), or with PLC-gamma1 (246).

Although the binding of up to three Zap-70 molecules to each zeta-chain does not automatically lead to activation of Zap-70, it is thought to place Zap-70 in a favorable position and subcellular location for Lck-mediated phosphorylation at Tyr-493 and activation. In addition, it seems that the activated molecules of Zap-70 undergo enhanced autophosphorylation, probably in *trans*, at multiple additional sites (see below). Mutation of Tyr-493 reduces this phosphorylation at other sites (236).

While phosphorylation at Tyr-493 is needed to activate Zap-70, the role of the adjacent Tyr-492 is less clear. Lck does not phosphorylate Tyr-492, but once Zap-70 has been activated by Lck-mediated phosphorylation of Tyr-493, Zap-70 becomes able to autophosphorylate Tyr-492 (237,238). However, phosphorylation at Tyr-492 is not required for activation, and may even have some negative impact. Thus, a replacement of Tyr-492 with a phenylalanine resulted in a relatively normal Zap-70 protein with somewhat higher catalytic activity (237). It remains possible, however, that the mutant does not behave exactly as a normal Zap-70 without phosphate at Tyr-492 since the hydroxyl group at Tyr-492 may be involved in hydrogen bonding or other interactions in this regulatory region of the kinase. More phosphorylation sites in Zap-70 are discussed below (Chapter 3.3).

4.2. Regulation of Syk

Despite their high degree of structural similarity, Zap-70 and Syk show a surprising number of differences in their regulation and biology. Both seem to use their tandem SH2 domains to bind to doubly phosphorylated ITAMs in the signaling subunits of leukocyte surface receptors, although Syk also participates in signaling by receptors that lack these motifs, e.g. from the IL-2 receptor (247) and integrins on neutrophils (248). In all these cases, Syk becomes rapidly tyrosine phosphorylated in response to receptor crosslinking. In some of these papers, Syk was found to associate with the receptor prior to stimulation (249-251), while in others, Syk was reported to be recruited through its tandem SH2 domains to the receptor ITAMs upon their phosphorylation. This is perhaps best documented for the Fc-epsilonRI, in which the Fc-epsilonRI-gamma chain is a particularly good ligand for the SH2 domains of Syk (251,252). Some T cells have this gamma chain as part of their TCR complexes (253), as do NK cells as part of the CD16 receptor. The mode of binding to these receptors is important because the requirement for ITAM phosphorylation determines how dependent on Src family PTKs (assuming that they are the only PTKs phosphorylating the ITAMs) the utilization of Syk is. In the case of the TCR, it is not yet clear whether Syk associates with the TCR prior to receptor triggering and whether Syk binds through its SH2 domains to the ITAMs upon their phosphorylation (254).

Despite the relatively high degree of amino acid homology between their kinase domains, Zap-70 and Syk differ substantially in their substrate phosphorylation characteristics (255,256). It appears that Zap-70 has a much more narrow substrate spectrum. It has also been estimated that the specific activity of Syk is up to 100-fold higher than that of Zap-70 (257). For example, when Syk and Zap-70 are expressed in parallel in COS cells, Syk is highly phosphorylated and causes tyrosine phosphorylation of multiple cellular proteins, while Zap-70 remains mostly unphosphorylated and inactive. This situation changes for Zap-70 if Lck is co-expressed, but much less so for Syk. Direct measurements of the catalytic activity show that Zap-70 is activated by Lck, while the activity of Syk remains the same. Even under these circumstances, the activity of Zap-70 remains much lower than that of Syk (using traditional *in vitro* substrates). This difference in inherent kinase activity may be very important in T cells where Zap-70 is expressed at much higher levels than Syk. Nevertheless, we find approximately as much kinase activity in Syk immunoprecipitates as in Zap-70 immunoprecipitates from Jurkat T cells (146).

Unlike Zap-70, Syk binding alone to doubly phosphorylated ITAMs upregulates its catalytic activity by as much as 10-fold (252,258). This is probably because Syk lacks Zap-70's dependence on a Src family PTK for phosphorylation at the positive regulatory site in the activation loop (8,255,256,259). Syk autophosphorylates at both tyrosine residues in this loop, Tyr-518 and Tyr-519 (residues 520 and 521 in human Syk) both *in vitro* and in transfected mammalian, yeast or bacterial cells. For example, in a yeast two-hybrid system, Syk was catalytically active and contained phosphate at Tyr-518, Tyr-519, and several additional sites, while Zap-70 was completely unphosphorylated (260).

While Zap-70 activation is highly dependent on phosphorylation at Tyr-493 (but not Tyr-492) in its activation loop, Syk is positively regulated by phosphorylation at both Tyr-518 and Tyr-519, the latter perhaps being somewhat more important (232,259). In B cells, where Syk has been more widely studied, the autophosphorylation site of Syk is needed for BCR-mediated signaling (232,261,262). However, even a doubly phenylalanine-substituted mutant of Syk retains more than 60% of the catalytic activity of wild-type Syk and the single mutants are essentially as active as the wild-type enzyme (259). Nevertheless, all three mutants have severely reduced biological effects. This discrepancy between activity and signaling capacity is probably explained by the association of Lck through its SH2 domain to the doubly phosphorylated Tyr-518/Tyr-519 (263). This has been observed in the two hybrid system and in co-expression studies where the loss of these residues ablates Lck SH2 recognition. The sequence of this site is Tyr-Tyr-KAQ, in which the first phosphorylated tyrosine apparently fits in the PTyr binding pocket of the Lck-SH2 domain and the second is in position +1 and a hydrophobic ala in position +3 (263). Although this is not a classical SH2 ligand since there is a lysine at +2, it does have an acidic PTyr at +1 and a hydrophobic residue at +3, and direct binding studies indicate that it binds with a dissociation constant of approximately 10 nM. Based on these findings, we have speculated (263) that Lck or other Src family PTKs, in addition to being upstream activators, are also downstream effectors of Syk.

The C-terminal tail of Syk is some 20 amino acids shorter than that of Zap-70, and ends only three residues after a triple tyrosine motif, which apparently is phosphorylated in intact cells. Two different observations indicate that this site may be involved in regulation of Syk. First, the activity of Syk is reduced by binding of an antibody to the C-terminus, which also recognizes only a subpopulation of Syk molecules (252). Second, replacement of the 3 tyrosines with phenylalanines leads to activation of Syk (our unpublished observation).

Syk also differs from Zap-70 in its ability to phosphorylate ITAMs within the TCR-zeta chain (257). In transfected cells, Syk augmented TCR-induced ITAM phosphorylation. This was also observed in non-lymphoid cells in the absence of other lymphoid kinases, and in peptide phosphorylation studies. This observation suggests another mechanism by which Syk may bypass the need for Lck, essentially replacing both Lck and Zap-70. Since Syk is expressed at higher levels in thymocytes and some subsets of mature intraepithelial gamma/delta T cells, which often lack CD4 or CD8, Syk may be more important in these cells.

4.3. Both Zap-70 and Syk act as docking proteins

Despite their different expression, regulation and catalytic properties, Zap-70 and Syk share a common function as docking proteins that recruit a number of downstream signaling molecules in a manner similar to growth factor receptor PTKs. Assuming that all identified sites are correct, it seems that more than 10 tyrosines in Zap-70 and Syk can be phosphorylated, many of them located in the "linker" region of the kinases. Several identified phosphorylation sites resemble classical SH2 binding motifs and a number SH2 domain-containing proteins have been found to bind phosphorylated Zap-70 and Syk, including Lck (242,244,263), Fyn (242), Abl (234), PLC-gamma1 (246), Vav-1 (260,264), Ras-GAP (234), c-Cbl (265-267) In all these cases, it is believed that the physical proximity of these signaling proteins serves to facilitate their tyrosine phosphorylation by Zap-70/Syk, or by the Src family PTKs.

Of particular interest is the binding of the Lck and Fyn SH2 domains to Zap-70, which may provide a basis for the earlier observation that Lck is in physical association with the TCR complex (268), and provides a potential mechanism for the recruitment of Lck to the TCR and the close proximity with Zap-70. It is curious that binding of Lck to Zap-70 occurs at a different site in this kinase than in Syk, which attracts the Lck SH2 domain to the doubly phosphorylated Tyr-518/Tyr-519 motif in the activation loop (263). In the case of Zap-70, binding does not involve the corresponding Tyr-492/Tyr-493, but apparently occurs through Tyr-319 in the linker region (244). Despite this seeming difference, however, both sites are created through autophosphorylation. For Zap-70, Tyr-319 is an important site, since a Y319F mutant Zap-70 is unable to mediate TCR signaling and even acts as a dominant negative in reducing signaling in cells expressing endogenous Zap-70 (245,246). It remains unclear, however, if it is due to binding of Lck or PLC-gamma1 (or perhaps both) to phospho-Tyr-319. The corresponding residue in Syk, Tyr-345, is not nearly of the same importance for the function of Syk in signaling. An adjacent residue, Tyr-315 in Zap-70 and Tyr-341 in Syk (Tyr-352 in human Syk), binds the SH2 domain of Vav-1. These sites appear to be phosphorylated through autophosphorylation.

Finally, c-Cbl binds through its SH2 domain to Tyr-292 in Zap-70 and an unidentified tyrosine in Syk. This binding reduces the biological effects of Zap-70 and Syk via an unknown effect that may include ubiquitination of the kinase, steric hindrance of binding to the adjacent binding sites, or the Cbl-mediated juxtaposition of a PTPase.

4.4. Dephosphorylation of Zap-70 and Syk

Since the phosphorylation state of many different tyrosine residues on Zap-70 and Syk play important roles in the transmission of signals from the TCR, it is clear that one or, more likely, several PTPases participate in the regulation of these functions by dephosphorylating these phosphorylation sites. The rapid induction of hyperphosphorylation of Zap-70 and Syk following brief treatments of intact cells with PTPase inhibitors, supports a model in which the turnover of phosphate on these kinases is fast. Candidates for the PTPases that dephosphorylate Zap-70 and Syk include SHP1 (167-169) and PEP (171). At present, however, the available data do not demonstrate that these two PTPases play significant roles in intact cells, nor are other PTPases excluded. All of these enzymes are discussed more in Chapter 5.

5. STRUCTURE AND FUNCTION OF THE TEC-FAMILY PTKS IN T CELLS

Three non-receptor kinases of the Tec family are found in T cells, namely Tec (269-271), Itk (272) [also termed Emt (273) or Tsk (274)] and Txk (275) [also called Rlk (276)]. Like the Src-related kinases and Zap-70/Syk, these kinases contain a regulatory N-terminus with several protein-protein interaction regions linked to a C-terminal catalytic domain (277,278). The N-terminus of Tec and Itk begins with a pleckstrin-homology (PH) domain, followed by a proline-rich motif in a region shared with other Tec family PTKs and sometimes referred to as the Tec homology (TH) domain (Figure 1). This is followed by an SH3 domain, an SH2 domain, and a catalytic domain, as in Src family PTKs. Txk is unique in that it lacks a PH domain and instead comes in two different isoforms due to alternative initiation of translation (279). The smaller, 52-kDa, protein is nuclear, while the larger protein is 58 kDa and has a string of cysteine residues in its N-terminus. These residues are the target for palmitylation, which targets the proteins to the cytoplasmic face of the plasma membrane.

The presence of an N-terminal PH domain is a unique feature of the Tec family PTKs (except Txk). These domains are independently folded modules comprised of some 100 amino acid and bind phospholipids and some proteins. In many proteins, the PH domain mainly

functions to localize the protein to the phospholipid layer of the inner aspect of the plasma membrane. This seems to be the case for Itk, which binds phosphatidylinositol-3,4,5-trisphosphate produced by PI3K (280-282). Since this particular phosphoinositide normally is present in the plasma membrane at very low concentrations in resting T cells, Itk is mainly cytosolic. Upon TCR ligation and PI3K activation, Itk translocates to the plasma membrane. In Jurkat T leukemia cells, which lack the PTEN phosphatase that removes phosphatidylinositol-3,4,5-trisphosphate, the levels of this lipid are constitutively high and Itk is constitutively membrane bound (283).

Translocation of Itk is followed by phosphorylation of a tyrosine residue in its activation loop, catalyzed by Lck (284-286), which results in activation of the catalytic domain of Itk. Itk is also subject to an intramolecular folding mechanism reminiscent of, but different from, the suppression of Src family PTKs. In the case of Itk (and probably all Tec PTKs), the proline-rich region in the TH domain binds to the SH3 domain (287). This binding prevents the SH3 domain from binding to cellular ligands, which include Sam 68 and the Wiskott-Aldrich syndrome protein (WASP) (288), and also prevents the proline-rich motif from interacting with Grb-2.

The physiological roles of Tec family PTKs have been illuminated by several key discoveries during the past eight years. The first was the observation that Itk is physically and functionally associated with the CD28 co-receptor (289). However, mice lacking Itk (290-293) displayed a much more normal thymic development than anticipated and only mildly perturbed T cell immune responses. Mice lacking Txk (294) also had a minimal phenotype. Mice lacking both Itk and Txk (295), however, have severe defects in T cell function, notably a lack of PLC-gamma1 phosphorylation and activation. Itk has also been shown to form a physical complex with the PLC-gamma1 (296) and to be required for calcium mobilization (292). Both Itk and Txk also associate with and phosphorylate SLP-76 (297-299), LAT (298,300), and a novel adapter protein called RIBP (301)(also known as TSAd). Finally, at least Itk associates with WASP, a unique protein linked to Wiskott-Aldrich syndrome, an X-linked combined immunodeficiency affecting cells of several hematopoietic lineages, suggests a role in cytoskeletal responses (302). Thus, it seems that Itk and Txk are important for T cell activation, but have largely overlapping functions that include coupling the TCR to PLC-gamma1, Ca^{2+} and cytoskeletal reorganization (303). Itk appears to be involved particularly in signaling through CD28 and CD2 (304). A non-redundant function of Tec in CD28 signaling and phosphorylation of Dok has also been reported (305).

5.1. Other tyrosine kinases in T cells

T cells express several other PTKs, some of which may play various roles in TCR signaling. Among the kinases, the Ca^{2+}-dependent proline-rich kinase Pyk2 appears to be involved in a capacity that resembles that of focal adhesion kinase (Fak) in many other cell systems, namely to mediate integrin signaling and regulation of adhesion (306-308) and phosphorylation of paxillin (309). Pyk2 is activated by the TCR and CD28 (310-312), apparently downstream of Fyn (313), and seems to participate in pathways that lead to IL-2 production (314).

The Jak family PTKs Jak1 and Jak3 are expressed in T cells and mediate signaling from receptors for IL-2. T cells also contain c-Abl, Ack, Ttk, Ctk, and some receptor PTKs like the insulin receptor and the receptor for insulin-like growth factor 1.

6. PROTEIN TYROSINE PHOSPHATASES IN T CELL ACTIVATION

As we predicted already in 1990 (17), it has now become evident that several PTPases affect TCR signaling and T cell activation in a negative manner (13-16,169,170,315,316). It is clear that these PTPases require their catalytic activities for this inhibition and that they inhibit by different mechanisms and at different locations in the signaling cascades (Figure 2). In addition, several PTPases do not affect TCR signaling at all, supporting the notion that PTPases have a high degree of specificity. This specificity is the combined result of subcellular targeting, regulation, and intrinsic substrate specificity of the catalytic domain (reviewed in 14-16).

Subcellular targeting of PTPases is largely accomplished by the presence of protein-protein interaction domains or other modules and motifs in the non-catalytic N- or C-terminal extensions that most PTPases have. These domains include SH2, ERM, PDZ and Sec14p homology domains, as well as proline-rich motifs for binding SH3 domains, kinase interaction motifs or signals for retention in the endoplasmic reticulum. Through these domains, the PTPases are enriched in compartments that presumably also contain their physiological substrates. For TCR signaling, perhaps the most interesting compartment is the plasma membrane, where the TCR and the Src, Syk, and Tec family kinases reside when activated and most early tyrosine phosphorylation events occur. Recent findings have suggested that specialized domains within the plasma membrane, the so-called lipid rafts or glycolipid-enriched membrane domains, act as platforms for TCR signaling by concentrating key kinases and signaling molecules at the TCR. It is not yet known if specific PTPases reside in these domains, or are actively excluded from them, but it seems likely that such mechanisms add a new element of specificity and regulation to the PTPases.

The protein modules that direct PTPases to specific intracellular locations and juxtapose them to their substrates, also play an additional role, namely as tools for regulation of the catalytic activity. In isolation, several full-length PTPases adopt a suppressed conformation with a much lower phosphatase activity than their catalytic domains expressed alone. At least in the cases where this has been studied in some detail, it is clear that the non-catalytic regions fold onto the phosphatase domains and block their function. This intramolecular inhibition is relieved by engagement of the non-catalytic module by its cognate ligand (e.g. the ligands for the SH2 domains in

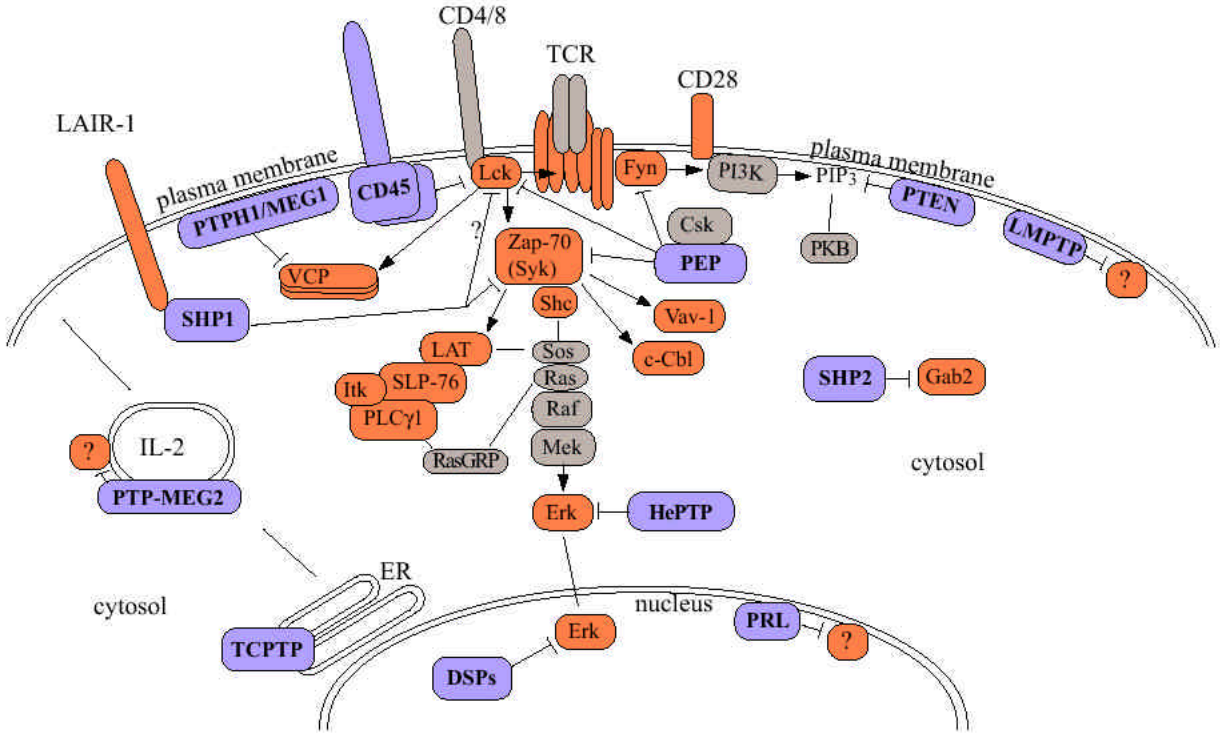

Figure 2. Schematic model for where PTPases are known or suspected to act in T cell activation. PTPases are in blue, tyrosine phosphorylated protein in red, and unphosphorylated (on tyrosine) proteins are in gray. Arrows denote phosphorylation, T-lines dephosphorylation, other interactions are indicated with simple lines. Note that most tyrosine phosphorylated proteins do not presently have a PTPase designated to them in the model, because the identity of the enzyme is unknown. However, all these proteins are dephosphorylated by some cellular PTPase in T cells.

SHP1 and SHP2). This mechanism ensures that the PTPase is only catalytically active when the enzyme is in its appropriate location and interacts with another protein. This clearly adds to the specificity of PTPases *in vivo*.

The past few years have also seen a tremendous increase in the interest in PTPases as drug targets. Particularly the lack of SHP1 in *motheaten* mice (317,318) and the demonstration that lack of PTP1B augments insulin receptor signaling (319), have opened many eyes to the potential of PTPase inhibitors as drugs for the treatment of human disease. The known crystal structure of many PTPases, a convenient colorimetric assay amenable to high-through-put screening, together with emerging information on substrate-specificity and regulation, make PTPases ideal targets for drug development. These efforts will undoubtedly benefit from, and synergize with, the advances in our understanding of the role of PTPases in physiological and pathophysiological processes, including the etiology and mechanisms of autoimmunity (13,320).

6.1. PTPases present in T lymphocytes

An important first step towards the goal of learning which PTPases are involved in T cell activation or other relevant aspects of T cell physiology, is the determination of which PTPases are present in these cells. We have found (315; unpublished) that T cells express at least three receptor-like PTPases (CD45, PTPepsilon and PTPalpha), some15 non-receptor PTPases (HePTP, TCPTP, SHP1, SHP2, PEP, PTP-PEST, PTP-MEG2, PTPH1, PTP-MEG1, PTP36, PTP-BAS, PRL-1, PRL-2, and LMPTP-A and -B) and at least nine dual-specific PTPases (Pac1, MKP1, MKP2, MKP3, MKP6, MKP-X, VHR, VHX, hYVH1). Most of these enzymes have not yet been studied much in T cells. They are expressed at different levels and are found in different compartments of the T cell. The subcellular locations of some of these PTPases are compatible with a role in signal transduction from the TCR or other surface receptors, while others presumably play entirely different roles in T cell physiology (315). The following sections will briefly review the properties and function of important PTPases in T cells.

6.2. Role of CD45 in T cell activation

The purification and cloning of the first PTPase, PTP1B, in 1990 (321,322), revealed that a well known glycoprotein expressed on all leukocytes, the leukocyte common antigen CD45, had a high degree of similarity to PTP1B (322). Indeed, it was shown soon thereafter that CD45 had a readily measurable PTPase activity (323). Around the same time, Jeanette Pingel and Matt Thomas found that CD45 was required for the activation of an antigen-specific T cell clone (183), and we discovered that

CD45 spontaneously dephosphorylated and activated Lck in T lymphocyte plasma membranes (186). These three findings together provided the basis for the current perception that CD45 is an important PTPase that maintains Src family PTKs in an unphosphorylated state, ready to be utilized for TCR signaling. Thus, CD45 sets the threshold for T cell activation and TCR sensitivity by determining the amount of Lck and Fyn available for TCR signal transduction. This concept has withstood the tests of a decade of experimentation although new details and some twists have been added.

CD45 is a transmembrane receptor-like glycoprotein with two catalytic domains in tandem in its cytoplasmic tail (324,325). The extracellular domain forms a highly glycosylated and slender rod, which is variable due to alternative splicing of exons 4, 5, and 6 during processing of the primary RNA transcript. CD45R0 lacks sequences encoded by exons 4-6, while the largest isoform, CD45-RABC, contains them all. Different T cell populations and subtypes have different expression patterns of CD45 isoforms, each individual cell expressing several isoforms, which presumably have somewhat different physiological functions. The functional differences may reflect variable interactions with other surface proteins, resulting in enhanced or reduced juxtaposition with other signaling molecules (e.g. CD4-Lck). Different isoforms have distinct effects on the tyrosine phosphorylation of some signaling molecules, like Vav-1 (326). However, our understanding of the alternative splicing of CD45 and its role in lymphocyte physiology is still rudimentary.

Expression of CD45 is required for TCR/CD3-induced early tyrosine phosphorylation and enhanced inositol phospholipid turnover (184) and calcium homeostasis (327). Although some controversy remains regarding the preference for specific Src family members, data from several laboratories agree with the notion that at least Lck and Fyn are physiologically relevant substrates for CD45 *in vivo* (186). It appears that Lck may be a better substrate than Fyn, perhaps because the N-terminus of Lck has a binding affinity for CD45 (328). The role of CD45 is also supported by the finding that chimeric receptors carrying a constitutively active Lck-Y505F mutant can restore signaling in CD45-negative cells (329), and the same Lck mutant can rescue the arrest in thymic development seen in CD45- mice (330).

This purely positive role of CD45 is apparently counterbalanced by a negative effect on cell adhesion (331) and the direct dephosphorylation of the zeta subunit of the TCR (332) and Jak family PTKs (333). The dephosphorylation of ITAMs would prevent the association with the Zap-70 PTK with the receptor complex and the subsequent activation of Zap-70 by Lck, and thereby reduce TCR signaling. This may, at least in part, explain the contradictory findings obtained with anti-CD45 mAbs, which can be either stimulatory or inhibitory for T cell activation. Similarly, the dephosphorylation of Jak kinases results in attenuated signaling from cytokine receptors (333). CD45 has also been proposed to dephosphorylate the activating site, Tyr-394, of Lck, but the evidence for this is not convincing.

The transmembrane nature of CD45, and its highly glycosylated extracellular domain, have prompted numerous investigators to ask how such a large molecule behaves during the contact of a T cell with an APC. It has been proposed that CD45 would provide steric hindrance to TCR - MHC interaction and therefore must be removed from the contact site (334). There is some evidence that may be the case (335). Furthermore, membrane-associated Lck is largely found in glycolipid-enriched membrane rafts. This fraction of Lck is hyperphosphorylated on tyrosine (presumably at Tyr-505) and has lower catalytic activity (336). CD45 is not located in these rafts, suggesting that only Lck molecules outside of rafts can interact with CD45 to be dephosphorylated at Tyr-505. The concept of PTPases being excluded form rafts and the contact area between the T cell and the APC is very intriguing and would provide a convenient mechanism for the enhanced tyrosine phosphorylation that occurs after TCR triggering despite the overwhelming overall PTPase activity. However, excluding CD45 does not achieve this objective, since it is primarily a positive regulator of Src family kinases and TCR signaling.

6.3. HePTP - in control of MAP kinases

The hematopoietic protein tyrosine phosphatase, HePTP (337), also called LCPTP (338), is a ~40-kDa PTPase expressed in all examined leukocyte populations and leukemic cell lines. The first hint of a physiological role in leukocyte biology was provided by the observation that the gene encoding HePTP is located on chromosome 1 at q32.1 (339), a site of frequent chromosomal deletions in leukemias and trisomy in myelodysplastic syndrome (340,341). Indeed, alterations in HePTP expression has been documented in myeloid leukemia (339). Recent insights into the function of HePTP fit well with this proposed role in the regulation of cell growth.

HePTP consists of PTPase domain with an N-terminal extension that contains a 15-amino acid motif (kinase interaction motif, KIM) that mediates a specific interaction with the MAP kinases Erk and p38 (342-344). Binding of these two kinases does not require phosphorylation or activation of either enzyme, but places the activation loop tyrosines of Erk/p38 close to the activating site of HePTP, leading to a very efficient dephosphorylation and inactivation of the bound MAP kinase. As these kinases play crucial roles in signaling pathways to IL-2 gene transactivation in T cells, it is not surprising that HePTP is able to suppress this transcriptional activation in response to TCR triggering of T cells (345). Thus, HePTP apparently is a negative regulator of T cell activation, and probably of many other aspects of T cell physiology that depend on MAP kinases, such as development in the thymus, survival and apoptosis. Surprisingly, however, mice lacking HePTP have a minimal phenotype and seem to develop thymocytes and immune responses with minimal problems, despite displaying a 2-3 fold hyperactivation of Erk (346).

The exact mechanism by which HePTP is targeted to the Erk and p38 MAP kinases merits a few words. Physical interaction occurs mainly through the KIM in the N-terminus

of HePTP and the back side (i.e. opposite the catalytic cleft side) of the MAP kinase molecule. A KIM is also found a two other PTPases, which also bind Erk and p38 (347). In the complex, the catalytic center of HePTP is juxtaposed to the activation loop of Erk, allowing for a very efficient dephosphorylation of Tyr-185 in the loop. This serves to maintain the bound Erk in an inactive state. Furthermore, the complex is apparently arranged so that Erk (if first activated) can phosphorylate a serine and a threonine in the N-terminus of HePTP adjacent to the KIM (342). This phosphorylation causes dissociation of the complex, allowing Erk to escape. In addition, a serine residue within the KIM can be phosphorylated by cAMP-dependent kinase, also leading to dissociation of the complex (342). In resting T cells, the complex of HePTP and Erk is cytosolic, but enriched near the plasma membrane (315). Upon TCR stimulation, some of the bound Erk molecules are activated by the upstream activator Mek, enabling them to phosphorylate HePTP, dissociate and translocate to the nucleus where most of Erk's substrates are located. HePTP remains cytosolic (315) and presumably serves as a trap for inactivated MAP molecules that return from the nucleus. Thus, a key function of HePTP is to sequester a pool of MAP kinases and to keep them handy for quick, but transient, activation.

Once active MAP kinase molecules have escaped HePTP and translocated to the nucleus, they become targets for another group of PTPases, the dual-specificity phosphatases, many of which are highly specialized for MAP kinases and efficiently remove phosphate from both Thr-183 and Tyr-185 in the activation loop (reviewed in 348). However, most of these enzymes are not expressed in resting lymphocytes, but are induced and appear some 30 - 60 min after TCR triggering. As a result, MAP kinases have a “window” of roughly 1 hour to accomplish their TCR-induced mission. We call this the "sequential phosphatase model". It is also interesting to note that there are many more phosphatases that counteract MAP kinase activation than there are MAP kinases or activators of MAP kinases. Thus, this may be the first example of regulation being mostly on the phosphatase side of the equilibrium.

6.4. SHP1 - mediator of inhibitory signaling

SHP1 (formerly called HCP, PTP1C or SHPTP1) is expressed at high levels in all cells of hematopoietic lineages, including T cells. The first insight into the importance of SHP1 came from the observation that the motheaten mutation in mice (349), which is characterized by spotty hairloss (hence the name) and a number of abnormalities in the function of the immune system and the phagocytic leukocyte types, is the result a homozygous loss of SHP1 (317,318). T lymphocytes from these mice are hyperresponsive to TCR stimulation (350), suggesting that some aspect of T cell activation is negatively regulates by SHP1. At least part of this function is mediated through a direct dephosphorylation of the Zap-70 and Syk (351), and perhaps also dephosphorylation of Lck (352,353).

It appears that all physiologically significant functions of SHP1 in hematopoietic cells are regulated by a class of inhibitory surface receptors, which possess the so called ITIM (immunoreceptor tyrosine-based inhibitory motif) (354-357). Upon ligation of these receptors by their ligands, which typically induces their juxtaposition to activating receptors, the ITIM motif is phosphorylated by Lck or Fyn, and then recruits the tandem SH2 domains of SHP1 in a manner that is very similar to the recruitment of ZAP-70 to ITAMs in the TCR complex. This mechanism not only serves to juxtapose SHP1 to its target molecules, but also activates SHP1 as much as 100-fold (358,359). At present, little is known about ITIM-containing receptors in T lymphocytes, while numerous such receptors are known in B cells and NK cells (reviewed in 14,356,357). The biology of ITIM-containing receptors is a new and promising field of study, which is likely to shed more light on the function of SHP1 and the dampening of immune responses. The autoimmune syndrome resulting from loss of SHP1 in the *motheaten* mouse, also sets a strong precedence for a possible role of disturbances in the ITIM-receptor/SHP1 system in human autoimmune conditions.

6.5. SHP2 in T cell activation

Despite their structural similarity, SHP1 and SHP2 have different, sometimes even opposite, physiological functions (reviewed in 14-16). In many systems, SHP2 seems to behave mainly as a positive regulator of signals leading to activation of the MAP kinase pathway (360,361). The mechanism of this positive role remains unclear, but seems to involve the dephosphorylation of a Gab2 associated negative regulator of the MAP kinase pathway. Initial reports suggested that tyrosine phosphorylation of SHP2 in its C-terminal tail was responsible for enhanced recruitment of Grb2 to the membrane and thereby translocation of Sos and activation of Ras (362,363). Although this mechanism may operate under some circumstances, there is compelling evidence that tyrosine phosphorylation of SHP2 is not important for the enhancement of MAP kinase activation (360; our unpublished observation).

In T lymphocytes, SHP2 has been reported to become phosphorylated on tyrosine upon TCR triggering (364) [although negative results have also been published (365)] as well as after addition of IL-2 (366). It was also recently shown that expression of SHP2 augments TCR-induced MAP kinase activation (367) albeit to a relatively small extent. An earlier report from the same group found a 110-120 kDa protein that was tyrosine phosphorylated upon TCR stimulation and co-immunoprecipitated with SHP2 from Jurkat cells (365). In our hands, SHP2 also affects the survival of Jurkat T cells, suggesting that the effects of SHP2 on the MAP kinase pathway in these cells may, at least in part, reflect differences in cell viability between transfectants.

Several papers have suggested that SHP2 plays a negative role in lymphoid cells by binding to ITIM sequences that also bind SHP1 and the inositol 5-phosphatase SHIP. SHP2 has also been proposed as the effector molecule for the inhibitory CTLA-4 receptor in mice (368). However, the loss of SHP2 is embryonically lethal and SHP2 is required for the development of hematopoietic and lymphoid cell lines (369). It appears that SHP2 is an important component of signaling from many growth factor receptors and integrins and that its

primary role is to augment MAP kinase activation. The physiologically relevant substrate(s) for SHP2, however, remain unknown.

6.6. PEP - a partner of Csk in suppression of TCR signaling

The proline-, glutamic acid-, serine-, and threonine-enriched PTPase PEP (370) is found primarily in hematopoietic cells, while the closely related enzyme PTP-PEST (371) is widely expressed. A third member of this group, PTP-HSCF, is present mostly in early hematopoietic stem cells and fetal liver (372-374). All three enzymes have been found to form complexes with Csk (172,375,376), the kinase that inhibits Src family PTKs by phosphorylating their C-termini (see Chapter 2.5.4), although their mode of binding varies. PEP and PTP-PEST bind to the SH3 domain of Csk through Pro-rich motifs in their C-terminal noncatalytic regions (172,375), while PTP-HSCF binds to the SH2 domain of Csk upon tyrosine phosphorylation (376). In T cells, most of PEP is found in complex with Csk (172).

Expression of PEP in Jurkat T cells results in strong inhibition of TCR-induced transactivation of the IL-2 gene, immediate early genes like c-*fos*, MAP kinase and JNK activation, TCR-zeta phosphorylation and increases phosphorylation of Lck at Tyr-394 (170). The catalytic activity of PEP was required for these effects and co-expression of Csk augmented them. In fact, expression of Csk even augmented the inhibitory effect of PEP in T cells expressing Lck-Y505F (170), which cannot be suppressed by Csk. Thus, in this system, Csk inhibited TCR signaling by strengthening the function of PEP, presumably by docking with PAG/Cbp. Taken together, it appears that PEP and Csk form a cooperative team that utilize PAG/Cbp to inhibit TCR signaling at a very receptor-proximal step, most likely at the level of Src family kinase activation loop phosphorylation (170-172). Dephosphorylation at this crucial regulatory point leads to global suppression of the whole T cell activation response. Thus, PEP would be an ideal candidate for a PTPase that may be altered in autoimmunity or other human diseases.

Although both PEP and PTP-PEST bind to Csk, they do not seem to have the same functions in cells. Unlike PEP, PTP-PEST binds to Cas (377,378) and paxillin (379) and is involved in the regulation of adhesion and migration of cells. PTP-PEST has been shown to directly dephosphorylate and inactivate the Pyk2 kinase (380). In lymphocytes, PTP-PEST dephosphorylates Shc, Pyk2, Fak and Cas and negatively regulates cell activation (381).

6.7. PTP-MEG2 - a regulator of secretory vesicles

The 68-kDa PTP-MEG2 (382) is unique among PTPases in that it contains the newly recognized phospholipid-binding Sec14p homology domain. This domain binds a highly phosphorylated inositol phospholipid in a manner that positively regulates the enzymatic activity of PTP-MEG2 *in vivo* (383; unpublished). The Sec14p homology domain is also responsible for targeting PTP-MEG2 to the enclosing membrane of secretory vesicles, which in T cells contain IL-2 and other lymphokines. The function of PTP-MEG2 on these vesicles is apparently to regulate their formation and size (384), which is important for proper secretion of their contents. Thus, PTP-MEG2 does not regulate TCR signaling events directly, but plays a role in the secretory response in already activated T cells. However, the importance of PTP-MEG2 is not yet clear and no studies have yet been conducted to address the possible role in PTP-MEG2 in human disease.

6.8. ERM-PTPases - gatekeepers at the membrane-cytoskeleton interface

The ERM domain-containing PTPases, often referred to as the "cytoskeletal" PTPases, are also newcomers in the T cell activation field. Four different enzymes of this group, PTPH1, PTP-MEG1, PTP36/PEZ, and PTP-BAS are present in T cells and all lymphoid organs (315). However, the expression these enzymes varies in different manners during hematopoiesis and thymocyte development. PTPH1 and PTP36/PEZ appear to be expressed early in development and then decline (315,385), while PTP-MEG1 is instead present at higher levels in more mature lymphoid cells (315).

The four enzymes share a common structure consisting of an N-terminal region with homology to band 4.1 and other cytoskeletal proteins (termed the ERM domain), a central region often with at least one PDZ domain, and a C-terminal PTPase domain. PTP-BAS is somewhat larger and contains several additional PDZ domains. The ERM domains are responsible for directing these enzymes to the actin cytoskeleton at the cytoplasmic face of the plasma membrane (316). PDZ domains typically bind to the cytoplasmic tails of several transmembrane proteins, as exemplified by the association of PTP-BAS with the cytoplasmic tail of Fas/CD95 (386), a receptor that induces programmed cell death. Similar associations may be relevant also for other ERM-PTPases in T cells, but have not yet been reported. Thus, the ERM-PTPases are ideally placed for involvement in transmembrane signaling events. Indeed, expression of PTPH1 and PTP-MEG1 in T cells, caused a marked decrease in TCR-induced gene activation and MAP kinase signaling (315,316). In contrast, PTP36/PEZ had no effect. The inhibition by PTPH1 and PTP-MEG1 required the presence of both catalytic activity and the N-terminal ERM domain (315), suggesting that these enzymes dephosphorylate one or several membrane-localized signaling molecules. Although it appears that TCR signaling was inhibited at a very receptor-proximal point, the substrates for these PTPases have not yet been found. Nevertheless, particularly PTPH1 is among the most efficient negative regulators of TCR signaling and it will be interesting to learn exactly how PTPH1 participates in TCR signaling.

6.9. PTEN - in control of lymphocyte life and death

The enzyme encoded by the *PTEN* tumor suppressor gene has the PTPase signature sequence, but was recently discovered to mainly dephosphorylate a non-protein substrate: the D3-position of the inositol ring of inositol phospholipids (387), including in T cells (388). Thus, PTEN is the direct antagonist of phosphatidylinositol

3-kinase (PI3K), an enzyme involved in many important aspects of T cell physiology, such as TCR signaling, co-receptor signaling, cytoskeletal rearrangements, motility, differentiation and cell survival. In T lymphocytes, PTEN is found in the cytoplasm, but is enriched at the plasma membrane (315) where these lipids mostly are located. Many lymphomas and leukemias have deletions or mutations in *PTEN* and typically display prolonged survival and resistance to receptor-induced cell death. Since antigen-induced cell death is an important part of the down-regulation of an immune response, alterations in PTEN expression or function would be expected to cause prolonged T cell activation. Indeed, the Jurkat T leukemia cell line, the most widely used model for TCR signaling, has debilitating mutations in both *PTEN* alleles and therefore completely lacks PTEN protein (283,389). Reintroduction of PTEN into Jurkat cells induces a rapid apoptosis (388), an effect that is counteracted by co-expression of constitutively active protein kinase B (also termed c-Akt). This kinase is normally activated by the phospholipids that PTEN removes by dephosphorylation. This and other similar findings suggest that PTEN directly controls the activity of protein kinase B, a key regulator of cell survival. It is likely that reduced PTEN function (e.g. by heterozygous mutations) will lead to prolonged or exaggerated immune responses and autoimmunity.

6.10. LMPTP - a small and peculiar enzyme

The low molecular weight PTPase, LMPTP, is different from other PTPases in many fundamental respects (reviewed in 16,390). Its amino acid sequence only shares the minimal essential catalytic core residues with other PTPases, and although the three-dimensional structure of the catalytic center is very similar to other PTPases, the rest of the enzyme is completely different. Unlike other PTPases, LMPTP is highly conserved through evolution from yeast to man. A highly homologous gene is even found in some prokaryotes. This suggests that LMPTP is likely to be involved in the regulation of an ancient and perhaps fundamental cellular function that predates the evolution of tyrosine phosphorylation as a mechanism of intracellular signal relay in single-cell organisms.

The primary transcript of the LMPTP gene undergoes alternative splicing to yield three mRNAs encoding different isoforms (390). The first two proteins (called fast and slow) are functional, while the C isoform encodes a smaller (15 kDa) protein devoid of PTPase activity and of unknown physiological significance (391). An interesting, and potentially very helpful, aspect of LMPTP is the marked correlation between allelic polymorphism in the LMPTP gene and many important allergic, autoimmune, inflammatory and degenerative diseases (reviewed in 390). There are three common alleles, termed A*, B* and C*, which affect mRNA splicing and result in different ratios of the two active isozymes and distinct total LMPTP activity. Thus each LMPTP-related disease can be correlated with levels of either isozyme or total LMPTP phosphatase activity. These studies also indicate that LMPTP has an important function in normal cells, including lymphocytes.

We have tried to elucidate the function of LMPTP in T cells, where it is found mostly at the cytoplasmic face of the plasma membrane (315). Despite this preferential location, LMPTP was recovered exclusively in the soluble fraction after ultracentrifugation, indicating that it does not associate stably with membrane proteins or cytoskeleton structures (315). Furthermore, LMPTP is phosphorylated on two tyrosine residues in resting T cells and is dephosphorylated (perhaps by itself) after TCR triggering (392). Expression of LMPTP also augmented TCR-induced reporter gene activation (315), an effect that may be mediated through dephosphorylation of the negative regulatory Tyr-292 of ZAP-70, resulting in hyperactivation of ZAP-70 and increased activation of MAP kinase (393). It is not yet clear if this is the principal function of LMPTP in T cells.

7. INITIATION OF THE TCR SIGNALING CASCADE

It is generally agreed that a key initiating event in T cell activation by antigen is the increased phosphorylation of ITAM tyrosines in TCR subunits by Src family kinases. How the TCR couples antigen recognition to this phosphorylation, however, is far from clear. Proposed models include juxtaposition of Lck to the TCR mediated by CD4 or CD8 (67,74-76,78), increased local concentration of kinases and substrates following receptor oligomerization, or a lipid raft-mediated enrichment of kinases and their substrates together with the exclusion of PTPases. Although many results agree with each of these models, there is ample evidence that none of these models is sufficient by itself. A central question is whether ligation of the TCR by MHC plus a peptide antigen induces a conformational change in the TCR/CD3 complex. No direct evidence exists to support this notion. Instead, most researchers believe that MHC-induced redistribution and clustering of TCRs in the plane of the membrane is the critical event that induces signaling. The molecular mechanisms, however, remain unclear. Most importantly, the functional connection between TCR ligation and the action of the Src family PTKs Lck and Fyn is unknown. Nevertheless, it is widely accepted that activation of these kinases is the key consequence of TCR triggering.

7.1. ITAM phosphorylation by Src family PTKs

In vitro, both Lck and Fyn can phosphorylate ITAM tyrosines of the zeta chain, but they do not phosphorylate all residues equally well (our unpublished observation). Fyn seems to phosphorylate only one site with high affinity, while Lck readily phosphorylates four or five of the sites. In Lck-deficient cell lines, such as the Jurkat T cell derivative JCaM1, TCR triggering results in a minimal (but detectable!) zeta chain phosphorylation, some Zap-70 recruitment, but no Zap-70 phosphorylation (256,394-396). A normal zeta phosphorylation and Zap-70 response is restored upon re-expression of Lck (394). Thus, it seems that Lck is the kinase responsible for much, if not all, of the ITAM phosphorylation that follows TCR triggering in T cells. Fyn may catalyze some ITAM phosphorylation in the absence of Lck, but is largely unable to compensate for the loss of Lck. It has been reported that

Syk also can phosphorylate ITAMs (257), but this is probably of relatively minor importance in most T cell populations due to their very low or undetectable levels of this kinase. It is not impossible that some other kinase(s) participate in ITAM phosphorylation in an Lck-dependent manner.

It was recently proposed that the tyrosines of the zeta-chain ITAMs are phosphorylated in a specific sequence: B1 - C2 - A2 - B2 - A1 - C1 (397), where A, B, and C refer to the three ITAMs from the N-terminus towards the C-terminus, and 1 and 2 are the first and second tyrosines within each ITAM. This order has been contested (398), but the paper does imply that some degree of organization and hierarchy does exist. A specific sequence of phosphorylation could be achieved by a combination of two mechanisms: first, if singly phosphorylated ITAMs serve as docking sites for the SH2 domains Src-related kinases, as has been proposed (399-401). Second, if more than one kinase is involved. Thus, when the first tyrosine of an ITAM is phosphorylated, a Src family PTK may bind via its SH2 domain to the phosphorylated site. This may place the kinase domain close to the next tyrosine and thereby facilitate its phosphorylation. Binding would also be expected to free the kinase from an auto-inhibitory conformation due to disruption of the association of its SH2 domain with the phosphorylated C-terminus. Alternatively, ITAMs may selectively bind only activated forms of Src kinases, where the SH2 domain is already free. Both tyrosines within each ITAM motif represent a reasonable Src SH2 domain-binding motif. It may important that these motifs do not match exactly the best possible binding sequence PTyr-Glu-Glu-Ile (192), and therefore would bind with somewhat lower affinities. Thus, it would be expected that Zap-70 would readily compete out any Src family PTKs from ITAMs as soon as the second tyrosine is phosphorylated.

It seems clear that TCR dimerization or oligomerization is important for initiation of signal transduction, but on a molecular level it is far from clear why a cluster of 20 ITAMs (carried by 2 TCRs) would initiate signaling where 10 ITAMs on one TCR does not. Similarly, chimeric molecules carrying the intracellular region of zeta (with three ITAMs) require crosslinking to signal even if these chimeras already exist as dimers. Perhaps the PTKs involved in the very first phosphorylation event can only phosphorylate another juxtaposed receptor, as in the case of most growth factor receptors. The association of $Fyn^{(T)}$ in T cells (and both Fyn and Lyn in B cells) with unphosphorylated ITAMs through a somewhat undefined mechanism (229) may imply that Fyn is involved in this initial event. This model, however, is insufficient since TCR function seems to be largely unperturbed in $Fyn^{-/-}$ mice (83,84). The notion that receptor oligomerization creates regions in the plasma membrane with a higher concentration of ITAMs that increase the efficiency of phosphorylation by Lck is also insufficient to explain the experimental data. In this case, signaling would be linearly correlated with the size of TCR surface caps and therefore much slower than what is observed.

7.2. Which PTPase dephosphorylates the ITAMs?

It appears that the ITAM phosphates are rapidly turned over in intact T cells since high levels of phosphate occupancy is rapidly induced by PTPase inhibitors like pervanadate (our unpublished observation). Thus, increased ITAM phosphorylation following TCR triggering could be achieved by elevated rates of phosphorylation, reduced dephosphorylation, or a combination of both. There is evidence that CD45 may be involved in zeta chain dephosphorylation (332). It has also been proposed that SHP1 catalyzes this reaction (350,353), but the evidence for a direct effect is lacking. It is likely that the main responsibility for zeta dephosphorylation is carried by another, unidentified PTPase.

7.3. Consequences of ITAM phosphorylation

It seems that the main purpose of ITAM phosphorylation is to create docking sites for the tandem SH2 domains of Zap-70 and perhaps also Syk. Since these motifs are sufficient to transduce seemingly complete activation signals by themselves when introduced into chimeric transmembrane proteins that can be oligomerized by extracellular stimuli, e.g. mAbs (402,403), it appears that their phosphorylation is sufficient for induction of a biological response. This notion is supported by experiments with cytotoxic T cells transfected with chimeric constructs bearing Src or Syk family kinases as their intracellular region and CD7 and CD16 as extracellular and transmembrane parts, respectively (8). Crosslinking of chimeras containing Syk, or co-crosslinking of a chimera of Zap-70 with a chimera having a Src family kinase, caused T cell activation and cytotoxicity. In contrast, crosslinking of Lck or Fyn chimeras had little effect (8). Thus, it seems that, by having Zap-70 and Syk already bound, these constructs completely bypassed the need for ITAM phosphorylation.

As shown by peptide binding and competition experiments, the binding affinity of the two tandem SH2 domains of Zap-70 for the doubly phosphorylated ITAMs is in the low nanomolar range (233,254), which is well within a physiologically relevant range. Currently, no other signaling molecule is known to bind to doubly phosphorylated ITAMs with affinities even close to those of Zap-70. In contrast, singly phosphorylated ITAMs have a low affinity for Zap-70 and cannot (at least by this mechanism alone) recruit Zap-70. In resting T cells, some of the ITAM tyrosines may be phosphorylated at an easily detectable level, but the ITAMs generally do not contain more than one phosphate. Thus, efficient Zap-70 recruitment occurs only after the later steps of sequential phosphorylation.

Even if most of the published work has focused on the zeta-chain, it seems that the solitary ITAMs of the CD3gamma, -delta, and -epsilon chains fulfill a very similar function; they become phosphorylated and recruit Zap-70 in what appears to be an identical manner. Nevertheless, it has also been proposed that these chains generate signals that are qualitatively distinct (404,405) and lead to the downstream tyrosine phosphorylation of a

distinct, but overlapping, spectrum of proteins. This finding has not been satisfactorily explained.

7.4. Multiple ITAMs - redundancy, amplification or sensitivity?

Since the most common form of the TCR complex is composed of two dimers of the CD3 subunits (epsilon-delta and epsilon-gamma) and a zeta-zeta dimer, there are 10 ITAMs in each receptor complex. Why does the receptor need so many functional motifs? At least two hypothesis have been proposed to answer this question. First, multiple ITAMs may serve to amplify the level of the signal. In support of this notion, all three doubly phosphorylated zeta ITAMs readily bind Zap-70 *in vitro* forming a 1:3 complex (406). Thus, the fully phosphorylated zeta-zeta dimer in a TCR complex may bind as many as six Zap-70 molecules, and the CD3 subunits four more. If the ITAMs simply serve to amplify the signal, it may well be that the motifs are functionally redundant and readily substitute for each other. Indeed, in chimeric molecules, the intensity of the signal generated by oligomerization correlates fairly well with the number of ITAMs present in the cytoplasmic tail (407). Peptide binding studies also demonstrate that all ITAMs bind Zap-70 with very similar affinities (233). ITAMs are also exchangeable in inducing apoptosis in double positive thymocytes or stimulation of single positive cells (408). Under physiological conditions T cells often encounter antigens of low affinity or abundance and it may be important for the TCR to be able to augment signaling by using multiple ITAMs and recruit up to ten Zap-70 molecules per receptor.

A second explanation for the presence of multiple ITAMs in the TCR complex is that they are functionally different in some way. The amino acid sequences of the 6 different ITAMs are far from identical. In fact, there are some potentially important differences in the nature of the two amino acids at positions +1 and +2 relative to the tyrosine and the leucine or isoleucine (at +3) in each ITAM. Such differences are likely to result in different affinities for various SH2 domains for these sites. In addition, there are differences in the number and placement of acidic residues, which have an important impact on the phosphorylation of adjacent tyrosine by PTKs, and some ITAMs contain proline residues that may kink the polypeptide backbone.

It is not entirely clear whether the ITAMs only bind Zap-70 in intact T cells. A number of other signaling molecules with SH2 domains, such as Grb-2, RasGAP, PI 3-kinase, PLC-gamma1, Shc, SHIP and Src family PTKs have been shown to bind (401,409-411), but in general with lower affinities. In addition, at least the zeta-chain associates with components of the cytoskeleton in a phosphorylation-dependent manner. Interestingly, some of these molecules bind reasonably well to singly phosphorylated ITAMs, which bind poorly to Zap-70, and some show more selectivity for the different ITAMs. For example, the binding of the TCR complex to a detergent insoluble cytoskeletal matrix is dependent on the most C-terminal ITAM of TCR-zeta (412,413). Shc may bind preferentially to the most N-terminal ITAM of zeta (414). In B cells, BCR ITAMs have also been reported to show different avidities for SH2 domains (401). In the case of the Fc-epsilonRI complex, Src family kinases show greater specificity in binding to the ITAM of the beta chain, while Syk preferentially binds the gamma chain (415). In general, it seems that doubly phosphorylated ITAMs bind Zap-70 (or Syk) with such a high affinity that other SH2 domain-containing signaling molecules are unable to compete for binding to any significant extent. However, the situation is likely to be quite different when ITAMs are monophosphorylated. Thus, if the phosphorylation of ITAM tyrosines in the TCR complex proceeds in specific sequence, it is possible that some molecules bind transiently at earlier steps, but are replaced by Zap-70 upon addition of a second phosphate within the ITAM. This notion begs the questions: To what stoichiometry are the individual ITAM tyrosines phosphorylated under physiological conditions? And, do alterations in this stoichiometry correlate with the biological outcomes of TCR triggering?

7.5. Partial TCR-zeta phosphorylation and T cell anergy

It has recently been observed that the degree to which ITAMs are phosphorylated does indeed vary and that this appears to correlate with different TCR signaling and radically different T cell responses. Unlike growth factor receptors, the TCR complex is not a simple binary on-off switch. Instead, the recognition of peptide in the groove of a MHC molecule is a delicate balancing act that can result in radically different responses ranging from survival in a resting state, to the induction of anergy, partial activation or full activation, which may lead to proliferation or cell suicide. The type of response depends on the avidity of peptide binding, on-off rates and degrees of receptor aggregation.

While unphosphorylated TCR-zeta is approximately 16 kDa, a one-dimensional SDS PAGE often resolves phospho-zeta into two Mr species of 21 and 23 kDa, the latter presumably corresponding to fully or nearly fully phosphorylated zeta. Under optimal conditions of TCR triggering, the 21-kDa form first increases rapidly and then the 23-kDa form appears, presumably reflecting the additive effects of increasing numbers of phosphate moieties. Under conditions that fail to cause T cell activation and instead induce a state of unresponsiveness referred to as T cell anergy, the 21-kDa form of zeta increases to a variable extent, but the most highly modified, 23-kDa form fails to appear (416-418). Since this resolution of phospho-zeta only into two Mr forms is far from actually visualizing the 6 different phosphorylation states (assuming that the location of the phosphate is irrelevant), it is not to be expected that this phenomenon will correlate perfectly with functional outcome.

The conditions that induce anergy generally represent stimulation with peptides that bind to MHC with good affinity, but are low to intermediate affinity ligands for the TCR. In molecular terms, this also means that the off-rate is relatively high and that each individual TCR is bound by peptide/MHC ligand for a shorter amount of time

following each binding event. Assuming that the tyrosines of the ITAMs of zeta are indeed phosphorylated in an ordered sequence (397), it is clear that this ordered phosphorylation must require a certain amount of time and that it must be rapidly reversible in the opposite order (otherwise it could not be observed!). If this assumption is correct, then engagement of an individual TCR by a high-affinity agonist results in the sequential and time-dependent transition of the zeta subunit from a low phosphorylation state through discrete intermediate steps to a fully hexaphosphorylated state. When the fourth phosphate is added, one ITAM becomes doubly phosphorylated and capable of recruiting one Zap-70 molecule. Similarly, when the fifth and sixth phosphates are added, the second and third Zap-70 can be recruited. We also assume that the recruitment of Zap-70 and, perhaps more importantly its phosphorylation at the activating site, Tyr-493, which perhaps must be preceded by phosphorylation at Tyr-319, also must require a certain amount of time. Importantly, in the case of TCR triggering by an agonist, the TCR remains ligated for a sufficiently long time for all these multiple steps to occur. In contrast, when the TCR is engaged by a somewhat lower affinity ligand, the average time that each TCR remains ligated is shorter than the time required for completion of the stepwise phosphorylation of the ITAMs, Zap-70 recruitment and activation. Such peptides will induce what seems like a partial signaling response with an increase in zeta phosphorylation and accumulation of the 21-kDa form of phospho-zeta, but fail to complete the phosphorylation sequence (and hence to generate 23-kDa phosphozeta). The recruitment of Zap-70 may proceed to some level, but there is no phosphorylation and activation of Zap-70. Importantly, however, an engagement of the TCR that lasts a shorter amount of time, can be repeated sooner than a longer lasting one. This can result in a higher accumulation of the 21-kDa form of zeta and create the impression of a qualitatively different signal.

Even if many details are uncertain at this time, the above model makes a number of predictions which are compatible with published observations. More importantly perhaps, many of these predictions can be tested experimentally. For example, the model predicts that there are intermediate affinity ligands or conditions that may induce a high level of zeta phosphorylation, but fail to induce sufficient Zap-70 phosphorylation for a full biological response. This has been observed for example in thymocytes where inactive Zap-70 constitutively associates with a highly phosphorylated zeta (419).

7.6. The role of CD4 and CD8 in the initiation of T cell activation

Under physiological conditions, T cell activation results from the contact of a T cell with an APC bearing antigenic peptide fragments complexed with self MHC molecules, as well as ligands for co-stimulatory receptors expressed on the responding T cell. While the recognition of antigen/MHC by the TCR is crucial for establishing a contact between the two cells, referred to as an immune synapse, the contact is stabilized by several other ligand - receptor pairs, such as the CD4 and CD8 co-receptors, which bind to non-polymorphic regions of the same MHC molecules. On the T cell, other important molecules include CD2, CD28, and several integrins. However, none of these interactions are productive unless the TCR is engaged.

It is generally believed that the juxtaposition of the TCR with the CD4 and CD8 co-receptors facilitates the initial event of TCR signaling, namely the phosphorylation of the tyrosines of the ITAMs of the TCR signaling subunits by the Lck bound to CD4 or CD8 (67,74-76,78). This notion is supported by a wealth of data, but there are also a few papers that hint at a somewhat more complicated reality. It is clear that antibody-induced CD4 co-ligation with the antigen-receptor complex potentiates the T cell responses and concordant zeta phosphorylation, that CD4 and CD8 function as co-receptors require their association with Lck (420-423), and that antibody-induced sequestering of CD4 or CD8 away from the TCR reduces TCR signaling (424). CD4 and CD8 are clearly of importance under physiologic conditions of T cell stimulation by low concentration of antigen (425). However, it is far from clear that CD4 and CD8 are the active partners that provide the driving force to pull Lck to the vicinity of the TCR. In fact, there is evidence that Lck mediates CD4 association with the TCR (426) and that the SH2 domain of Lck is required (427). Thus, it appears that Lck can be attracted to the TCR and brings CD4 or CD8 with it to strengthen the complex and cell-cell interaction.

Although most mature alpha/betaTCR^+ T cells require the presence of the CD4 and CD8 co-receptors for an optimal response to antigen, there are $CD4^-8^-$ T cells, which respond to antigen or mAbs against the TCR or CD3. A prime example is the most widely used model for TCR signaling, the Jurkat T leukemia cell line, which usually is CD8-negative and either has a very low level of CD4 or is CD4-negative. Nevertheless, these cells are among the most easily triggered T cells, perhaps due to their high expression of Lck. It is clear in these cells that Lck actively participates in TCR signaling in a manner that is completely independent of CD4 or CD8. Even if these cells are abnormal, they provide an undeniable contradiction to the dogma that CD4 or CD8 initiate T cell activation by bringing in Lck to the TCR. Thus, there must be an alternate mechanism for initiation of the tyrosine phosphorylation cascade. This other mechanism may naturally operate in normal T cells as well also when Lck is bound to CD4 or CD8.

7.7. Role of lipid rafts in TCR signaling

A new development that has met with great enthusiasm in the field, is the discovery of specialized aggregates within the plane of the plasma membrane, the so called lipid rafts or glycolipid-enriched microdomains (87,90-92). These detergent-insoluble regions consist of cholesterol, sphingolipids and proteins anchored by saturated fatty acids and glycosylinositol lipids. Some signaling molecules, like LAT, are almost exclusively located in these lipid rafts (93,95,428), while others, such as Lck and Fyn, are there to a variable degree or in a regulated manner. In a resting T cells, the TCR is not contained within lipid rafts, but upon TCR triggering the receptor becomes surrounded and apparently embedded in these rafts (429). During contact of a T cell with an APC

(335), the contact area (immune synapse) between the two cells becomes enriched in lipid rafts, which can be visualized by fluorescein-labeled cholera toxin. The molecular mechanism of lipid raft movement to the immune synapse is unknown, but appears to involve the actin myosin cytoskeleton (430).

The charm of lipid rafts is that their accumulation around the ligated TCRs provides a mechanism for a strong enrichment of Lck and Fyn and their immediate substrates (90-95). By extension, the model also predicts that inhibitory molecules, such as PTPases, are excluded from the 'hot spot' of TCR signaling (336,431). However, the dilemma of how TCR initiates ITAM phosphorylation is not solved by including lipid rafts into the model of TCR function. There is also very little evidence for exclusion of endogenous PTPases. Nevertheless, it seems that lipid rafts do play a positive role and may be very important for the stabilization and maintenance of an optimal TCR signaling machinery.

7.8. Nonredundant functions of Fyn(T)

A long-standing, and still unanswered, question in TCR signaling is the relative roles of Lck and Fyn. While Lck clearly has unique functions during thymic development (85), it is far from clear to what extent Lck and Fyn are responsible for overlapping or unique functions in mature T cells. The issue is complicated by the typically somewhat lower expression of Fyn in most T cell lines. The presence of c-Yes, which is closely related to Fyn, in most T cells also compromises the interpretation of findings in fyn-/- mice, in which TCR signaling was only modestly affected (83,84). Overexpression of Fyn correlated with enhanced Ca^{2+} influx and proliferation (82). By contrast, expression of the dominant negative form of kinase-inactive Fyn inhibited these events. However, it may well be that kinase-inactive Fyn also competed with endogenous Lck and it may well be that overexpression of any Src family kinase would enhance TCR signaling.

Perhaps the only direct comparison between Lck and Fyn under similar circumstances were experiments using the Lck-negative and Fyn-low Jurkat subline JCaM1 stably transfected with Lck or Fyn (432). In clones that expressed equal levels of either kinase, the TCR induced a very different set of tyrosine phosphorylation events. Interestingly, TCR-zeta phosphorylation was weaker and only reached the 21-kDa level in JCaM-Fyn cells, while the 23-kDa form readily appeared in the Lck-expressing cells. Fyn also failed to support Zap-70 phosphorylation and activation, but induced a normal Vav phosphorylation. Thus, Fyn was able to mediate a more restricted set of events similar to those seen in T cell challenged with altered peptide ligands (433). In agreement with this notion, such peptides induce activation of Fyn, but not Lck.

Another line of investigation that supports a nonredundant role for Fyn in TCR signaling is the identification and cloning of Fyn-specific substrates, such as FYB (434) and SKAP55 (435). The former protein was also cloned under the name SLAP-130 (436) and seems to be important for TCR signaling to integrins (437), suggesting a possible role for Fyn in this pathway.

7.9. Does Syk participate in T cell activation?

Although the model of T cell activation that begins with phosphorylation of the zeta chain by Lck (plus Fyn?), followed by Zap-70 recruitment and activation, is well established by numerous studies, the possible role of Syk is much less clear. Most mature T cells express Syk although the amount of Syk protein is much lower than the amount of Zap-70 protein. However, since the specific activity of Syk may be as much as 100-fold higher than that of Zap-70, the lower level expression of Syk may not exclude its participation as a first glance may suggest. It has also been debated whether Syk is redundant with Zap-70, or acts in a different manner either upstream of Lck, in parallel with Lck or in some combination of these. These issues are discussed above in section 3.2.

Zap-70 and Syk can substitute for one another in some cells, such as Syk-negative chicken B cells (231). On the other hand, in the Lck- and Syk-negative JCaM1 cells, function can be restored with either Lck or Syk, but not with Zap-70 (256). In certain $CD45^-$ cells, Syk can mediate TCR responses, while Zap-70 cannot (438). Thus, it seems likely that in $CD4^-8^-$ cells, particularly in some gamma/delta T cells, Syk may substitute for the combination of Lck and Zap-70 in TCR signaling. Since even low levels of Syk are easily activated by receptor crosslinking, these cells may have a low threshold for activation by antigen.

8. SUBSTRATES FOR KINASES AND PHOSPHATASES: ENZYMES, ADAPTERS AND DOWNSTREAM SIGNALING PATHWAYS

Numerous tyrosine phosphorylation events follow the triggering of the TCR and the mobilization of the PTKs discussed above. Some of these phosphorylation reactions are unique to T cells or lymphoid cells, while others are more or less generic to the activation of any mammalian cell. The increased phosphorylation of PTK substrates leads to the activation of many signaling pathways, morphological changes, and enhanced integrin function. Signaling pathways initiated by tyrosine phosphorylation include Ca^{2+} mobilization and influx, the activation of the small GTP-binding proteins Ras, Rac, and Rho, the activation of serine kinase cascades, activation of lipid kinases, cytoskeletal rearrangements and phosphorylation of integrins to enhance their avidity for extracellular ligands. Major challenges in T cell biology are to define the exact contribution of each signaling protein to the pleiotropic T cell activation response, as well as the spatiotemporal parameters that govern the dynamic interactions of individual proteins with multimolecular signaling complexes in activated T cells.

Over the past several years, considerable progress has been made in this area with the identification of an array of immune cell-restricted adapters (reviewed in 439,440). These adapters lack enzymatic or transcriptional domains, but have multiple motifs and domains

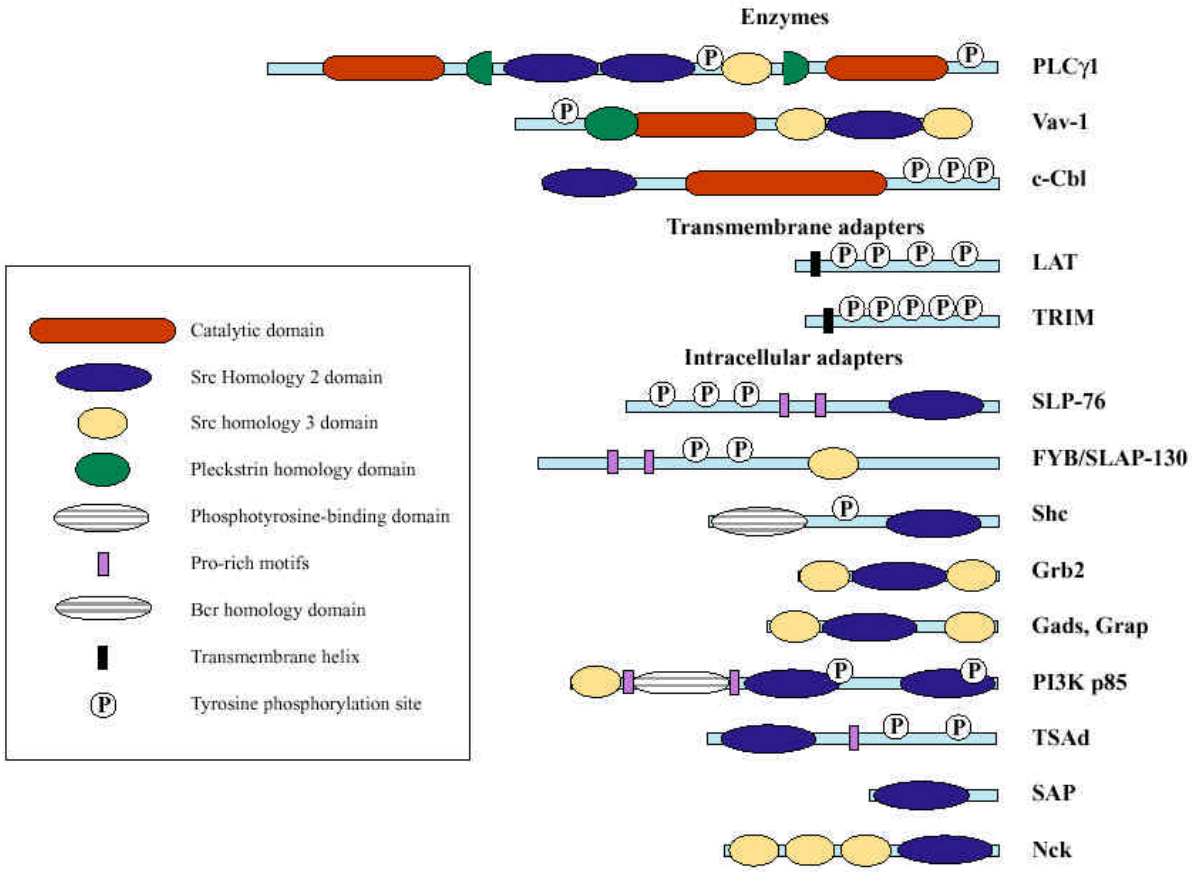

Figure 3. Domain structure of substrates for tyrosine phosphorylation and adapters that interact with them. The location of domains and tyrosine phosphorylation sites are approximate only, and not all reported sites of phosphorylation are indicated.

involved in association with other proteins. With the potential to create various combinations of multi-protein complexes, they appear to integrate signals and to coordinate the interplay between signaling molecules and pathways. Adapters include LAT (linker for activation of T cell), SLP-76 (SH2-domain-containing leukocyte protein of 76 kDa), FYB (Fyn$^{(T)}$-binding protein)/SLAP (for SLP-76-associated protein), SKAP55 (Src-kinase-associated protein of 55 kDa), the 3BP2 protein, Shc, Grb2 and others. In addition, some enzymes (e.g. PI3K) have adapter subunits that participate in orchestrating protein-protein interactions. The schematic structures of many PTK substrates and adapters are shown in Figure 3.

8.1. PLC-gamma1

A key substrate for the TCR-coupled PTKs is the gamma 1 isoform of phospholipase C (PLC-gamma1). TCR engagement provokes rapid increases in both the tyrosine phosphorylation and the catalytic activity of PLC-gamma1 (51,54-56). The activated enzyme hydrolyzes phosphatidylinositol-4,5-bisphosphate to IP_3 and diacylglycerol (see section 1.1). These metabolites act as second messengers to stimulate the release of Ca^{2+} from intracellular stores and activate PKC, respectively (33). The increase in the intracellular free Ca^{2+} concentration plays crucial roles in the induction of numerous T cell activation-associated responses (441,442). A pivotal target of the Ca^{2+} signaling pathway is NFAT, a transcription factor that regulates the expression of several T cell activation-associated genes, including the gene for IL-2 (443). The importance of the $[Ca^{2+}]_i$ increase during the early stages of T cell activation has raised considerable interest in the mechanism whereby the TCR activates PLC-gamma1, as well as the interactions of PLC-gamma1 with other components of the TCR-linked signaling machinery. Mammalian cells express at least 10 different PLC family members, which are grouped into three subfamilies, beta, gamma, and delta (444-446). The PLC-gamma subfamily contains two members, PLC-gamma1 and -gamma2, both of which bear structural motifs that confer regulation by PTKs. PLC-gamma1 is widely expressed in mammalian tissues, while PLC-gamma2 expression is largely restricted to hematopoietic and lymphoid lineage cells (447,448). Among lymphoid cells, T cells express predominantly PLC-gamma1, while NK and B cells express PLC-gamma2 in amounts similar to or greater than those of PLC-gamma1 (449). Although some evidence suggests that the two PLC-gamma isoforms are subject to different modes of regulation (450,451), the functional significance of PLC-gamma1 versus PLC-gamma2 activation in various lymphoid sub-populations remains unclear.

PLC-gamma1 is largely responsible for the increase in phosphatidylinositol hydrolysis induced by

stimulation of receptor tyrosine kinases (444), as well as multichain antigen receptors, which lack intrinsic PTK domains but employ nonreceptor PTKs as proximal signaling elements (51,55,56). Targeted disruptions of both *plcg*1 alleles in mice result in early embryonic lethality, indicating an essential role for this enzyme during development (452). The lethal consequences of *plcg*1 gene disruption have so far precluded analyses of the signaling functions of PLC-gamma1 in developing thymocytes or mature peripheral T cells *in vivo*. However, the availability of a *plcg*1-/-DT40 chicken B-cell line has allowed some valuable insights into the regulation and function role of PLC-gamma1 during B-cell antigen receptor (BCR) stimulation (453). A common structural feature of the PLCs is a split catalytic domain comprised of conserved X and Y subdomains. According to current models, PLC activation hinges, in part, on a conformational change that juxtaposes the X and Y subdomains to create a contiguous catalytic domain (445,446,454). In the PLC-gamma subfamily, the X and Y domains are separated by the SH region, a stretch of approximately 500 amino acids that encodes two SH2 domains and one SH3 domain. The dual SH2 domains permit PLC-gamma recruitment to specific phosphotyrosine-containing target sequences (132), which not only positions the enzyme for phosphorylation by membrane-associated PTKs, but also allows associations with other cytoplasmic signaling proteins. On the other hand, the SH3 domain may mediate associations between PLC-gamma1 and proline-rich motifs found in c-Cbl (455), as well as cytoskeletal proteins (456). In addition to its role in the regulation of PLC-gamma1 catalytic activity (457,458), the SH region may allow PLC-gamma1 to act as a scaffold for the assembly of multimolecular signaling complexes during TCR signaling.

Stimulation of the platelet-derived growth factor (PDGF) receptor (459) or TCR (54) triggers the rapid phosphorylation of PLC-gamma1 on at least three tyrosine residues. Studies performed with PDGF-responsive cells indicated that phosphorylation of Tyr-783 is essential for activation of PLC-gamma1, while phosphorylation at a second site, Tyr-1254, was needed for maximal stimulation of phosphoinositide breakdown by PDGF (459). Interestingly, a third Phe substitution, at Tyr-771, actually increased the PDGF-dependent activation of PLC-gamma1 in these cells. The mechanisms through which these phosphorylation events modulate the activities of the catalytic X and Y domains have not been defined.

The biochemical events that link TCR stimulation to the phosphorylation and catalytic activation of PLC-gamma1 are only partially understood. Results obtained with genetically deficient Jurkat T cell lines and gene-targeted mice indicate that optimal tyrosine phosphorylation of PLC-gamma1 requires the concerted activities of Lck, ZAP-70, and Itk (or Txk/Rlk) (246,295,394).

Moreover, studies performed with LAT- (95,460) or SLP-76- (461) deficient Jurkat T cell lines indicate that both of these adapter proteins are needed for optimal coupling of these upstream PTKs to PLC-gamma1. Although the sequence of events that leads to PLC-gamma1 activation remains unclear, both the amino- and carboxyl-terminal SH2 domains are required for phosphorylation and activation of the enzyme during PDGF receptor (462) or BCR stimulation (453). In addition to mediating associations with phosphotyrosine-containing proteins, the more C-terminal PLC-gamma1 SH2 domain binds to phosphatidylinositol-3,4,5-trisphosphate, which suggests that this region might also receive a regulatory input from phosphoinositide 3-kinase (463,464).

8.2. LAT

Following its activation by TCR engagement, Zap-70 phosphorylates LAT and SLP-76, two key adapter proteins for coupling the TCR to downstream signaling pathways. LAT (previously termed pp36) is a transmembrane type III surface protein with a small extracellular region attached to an extended cytoplasmic tail that contains nine tyrosine residues conserved between humans and mice (465,466). LAT contains two cysteine residues in the amino-terminal end that serve as palmitylation sites, targeting the protein to glycolipid-enriched microdomains in the cell membrane. LAT is expressed exclusively in hematopoietic cells, primarily in T cells, natural killer cells, mast cells, but not B cells.

The essential function of LAT was initially demonstrated by over-expressing LAT with mutations in Tyr-171 and Tyr-191 in Jurkat cells (465). Expression of this molecule inhibited AP-1 and NF-AT activation after TCR cross-linking. The availability of the LAT-deficient Jurkat cell lines JCaM2.5 and ANJ3 further supported this view (467,460). In these cell lines there is defective Ca^{2+} flux, Ras-Erk activation and there is also a reduction in the phosphorylation of PLC-gamma1, Vav and SLP-76. All of these defects were corrected after transfection of LAT.

The role of LAT *in vivo* has been probed with the generation of knockout mice by gene targeting (468). Disruption of the LAT gene produced healthy mice with a block in T cell development but not in NK cells or platelets. No mature T cells were present. Thymocytes in LAT-/- mice were arrested at the double-negative stage CD25+CD44-, indicating that LAT is essential already for pre-TCR signaling. Interestingly, mice deficient in SLP-76 also show a block in T cell development in the double negative stage.

The main function of LAT appears to be to create docking sites for SH2 containing proteins, including (directly or indirectly) PLC-gamma1, the 85 kDa regulatory subunit of PI3K, Vav, Cbl, Itk, Grb2, Grap, Gads and SLP-76. Although only one site, Tyr-191, has been directly shown to contain phosphate in intact T cells (465), site-directed mutagenesis experiments (467) along with knock-in mice (469) have shown that the four distal tyrosines are essential for LAT function. Knock-in mice, with the four distal tyrosines mutated to Phe, showed the same phenotype as the $LAT^{-/-}$ null mice: thymocyte maturation arrest at the CD4-CD8- stage and no mature T cells. The role of the four distal tyrosines has been studied in more detail in the LAT deficient cells, JCam2.5, reconstituted

with different LAT constructs mutated at Tyr-132, Tyr-171, Tyr-191 and Tyr-226 (467). The mutant lacking all four tyrosines was still tyrosine phosphorylated, indicating that at least one more site can be phosphorylated. Furthermore, this study showed that Tyr-132 is involved in PLC-gamma1 binding, while Tyr-171, Tyr-191 and Tyr-226 are responsible for Grb2 binding and Tyr-171 and Tyr-191 for Gads binding. Mutation of Tyr-132, which prevented PLC-gamma1 binding, also abrogated PLC-gamma1 tyrosine phosphorylation and Erk and NFAT activation, suggesting that PLC-gamma1 is involved in Ras activation. It remains unclear where PI3K, Vav, Cbl, Itk, and SLP-76 bind.

8.3. SLP-76

The pleiotropic signaling functions of LAT are intricately intertwined with those of a second adapter protein, SLP-76 (SH2 domain-containing leukocyte protein of 76 kDa). This adapter protein is expressed in T cells specifically, platelets, NK cells and myeloid cells of the monocyte and granulocyte lineage (470,471). The primary structure of this adapter molecule includes an NH2-terminal acidic domain containing three tyrosine phosphorylation sites, a central proline-rich region and an SH2 domain in its C terminus (439,472). The middle proline-rich region is constitutively associated with the SH3 domain of Gads, a hematopoietic-specific Grb2-related molecule (473-475). On the other hand, Gads, through its SH2 domain, binds to tyrosine phosphorylated LAT after TCR engagement, what leads to the recruitment of Gads/SLP-76 to the plasma membrane. Following TCR ligation SLP-76 becomes tyrosine phosphorylated by Zap-70 (476) generating binding sites for the SH2 domains of the guanine nucleotide exchange factor for the Rac/Rho family of GTPases, Vav (477,478), the adapter molecule, Nck (479) and the Tec family PTK, Itk (297).

Early studies performed in the Jurkat T cell line showed that SLP-76 over-expression augments TCR/CD3 induced IL-2 promoter activity and increases MAPK Erk activation (481,481). Characterization of a SLP-76 deficient T cell line (482) revealed that while phosphorylation of Zap-70, LAT and Vav remained unaltered in these cells after TCR stimulation, SLP-76 was required for optimal PLC-gamma1 phosphorylation and activation. In addition, Ca^{2+} mobilization and NF-AT activation were reduced, as well as CD69 expression, Erk2 phosphorylation and AP-1 transcriptional activity (482).

The physiological role that SLP-76 plays *in vivo* has been examined in mice deficient in SLP-76 by homologous recombination (483,484). These mice show the same phenotype than $LAT^{-/-}$ mice, absence of mature thymocytes and T cell development abrogation at the double negative stage $CD25^{+}CD44^{-}$. Although LAT and SLP-76 interact with the same molecules, the fact that $LAT^{-/-}$ or $SLP\text{-}76^{-/-}$ mice, along with deficient cell lines for these molecules, show the same phenotype points out that these molecules are not redundant in T cells, since they cannot substitute one another. Therefore, both are required for T cell development and for T cell activation after TCR cross-linking.

SLP-76 also acts as an adapter in the regulation of the lymphoid cytoskeleton. This is accomplished by the arrangement of a trimolecular complex between SLP-76, Vav and Nck (479). TCR ligation induces the Zap-70 phosphorylation of SLP-76 tyrosines, Tyr-113 and Tyr-128, which once phosphorylated provide docking sites for Vav-1 and Nck binding (477,479,485). Vav-1 is a hematopoietic protein, with GEF activity for the small GTP binding proteins Rho and Rac1 that regulates the assembly of filamentous actin into stress fibers and lamellipodia, respectively. On the other hand, Nck is an adapter protein with three SH3 domains and one SH2 domain that interacts with several proteins that include the serine/threonine kinase Pak (p21-activated kinases) (486) and the Wiskott-Aldrich syndrome protein (WASP) (487). Pak belongs to a family of serine/threonine kinases that are activated by binding to GTP bound Rac1 or Cdc42. Vav-1 and Nck ability to individually promote actin polymerization may be increased by their proximity on the SLP-76 scaffold. In this sense, Vav-1 activation of Rac1 generates Rac-GTP that could activate Pak1. Consistent with this, SLP-76, Nck and Vav-1 expression enhanced TCR induced actin polymerization, while dominant negative forms of these components inhibited polymerization.

Finally, it should be pointed out that SLP-76 is a member of a growing family of adapter proteins that also includes BLNK, BASH and MIST/CLNK. These proteins appear to have at least partly redundant functions, but are expressed at different levels in different subsets of hematopoietic cells and at different times of cellular activation or differentiation,

8.4. FYB/SLAP-130, now called ADAP

In initial studies on SLP-76, it was noted that the SLP-76 SH2 domain bound three proteins of 36, 55 and 120 kDa (480). Since the mutation of the SLP-76 SH2 domain blocks its ability to augment IL-2 (480), the identities of the binding partners was of interest. While the 36-kDa protein turned out to be LAT, the 120-130-kDa protein was novel and was given the name SLAP-130 (SLP-76 associated phosphoprotein of 130 kDa) (481). The same protein was found by C. Rudd's laboratory as a protein specifically associated with the SH2 domain of Fyn (488) and subsequently cloned under the name FYB ($Fyn^{(T)}$-binding protein) (489). FYB/SLAP-130 possesses a proline-rich region that can bind to the SH3 domains, several tyrosine phosphorylation sites, an SH3-like domain, an Enabled/vasodilator stimulated phosphoprotein homology 1 domain (EVH1) binding site, and two stretches of highly charged residues similar to nuclear localization sequences (481,489,490). Expression of FYB/SLAP-130 is restricted to T cells, monocytes, platelets and mast cells but not B-cells (489,491).

The physiological role of FYB/SLAP has been controversial since overexpression studies showed either an increase (489,492,493) or a reduction (93,481) in IL-2 production depending on the experimental system used. Recently, two different groups have reported the generation of knock-out mice for FYB/SLAP (494,495). T cells from these mice show defective T cell proliferation and cytokine production after TCR engagement. In addition, T cells lacking FYB/SLAP show no increase in integrin adhesion

after TCR ligation. Most signaling events, such as PLC-gamma1 and SLP-76 tyrosine phosphorylation, MAP kinase activation or Ca^{2+} were normal in FYB/SLAP$^{-/-}$ T cells. However, IL-2 production and CD25 or CD69 induction were lower compared to normal lymphocytes. This work indicates that FYB/SLAP is involved in coupling TCR induced cytoskeleton rearrangement with activation of integrin function, which is necessary for a complete antigen response. A role for FYB/SLAP in remodeling of the actin cytoskeleton upon TCR engagement was also suggested independently (490). Furthermore, in mast cells, upon stimulation by the Fc-epsilonRI, FYB/SLAP is involved in integrin-mediated adhesion to fibronectin and degranulation (491). Based on these new observations, FYB/SLAP-130 has been renamed ADAP for adhesion and degranulation-promoting adapter protein (491,494,495).

8.5. Ras activation

It has been known for a long time that TCR stimulation is followed by Ras activation (496). However, the mechanisms involved in this activation are not yet clear in T cells; there is data that supports the co-existence of at least two independent pathways for Ras activation in T cells. In the classical model, Ras activation is mediated by translocation of a Shc-Grb2-Sos complex to the plasma membrane after TCR stimulation. Shc is an adapter protein that is expressed in T cells as two isoforms of 46 and 52 kDa and is composed of an N-terminal phosphotyrosine binding domain (PTB), a central collagen homology domain (CH1), which contains tyrosine phosphorylation sites, and a C-terminal SH2 domain (497). Shc is rapidly phosphorylated on tyrosine residues in response to TCR engagement, and phosphorylated Shc subsequently binds Grb2 and Sos, in both T cell hybridomas and normal blood lymphocytes (498). A physical interaction between the SH2 domain of Shc and the TCR zeta-chain has been demonstrated (414), suggesting that a Shc-Grb2-Sos complex localized to the activated TCR would be one mechanism of Ras activation of T cells (414,499). However, this model has not been convincingly proven to be of relevance in T cells.

A variation of the first mechanism of Ras activation is that Shc-Grb2-Sos, or just Grb2-Sos, bind to LAT (467) instead of the TCR-zeta chain or CD3. However, to complicate matters, the ubiquitously expressed Grb2 (500) has company in T cells: Grap (Grb2-related adapter protein) (501,502) and Gads (Grb2-related adapter downstream of Shc) (503) are expressed preferentially in hematopoietic cells, and both share significant homology with Grb2. Like Grab2, they have a central SH2 domain and two SH3 domains at the ends. In addition, Gads, contains a proline- and glutamine-rich region between the SH2 domain and the C-terminal SH3 domain. Gads was cloned by five different labs and is also known as Mona, Grap2, GrpL, or Grf40 (503).

The second pathway for Ras activation has been delineated recently in T cells. This pathway involves a constitutive Gads/SLP-76 complex, PLC-gamma1 activation, and the guanine exchange factor RasGRP. First, Gads, through its SH2 domain, binds to tyrosine phosphorylated LAT after TCR engagement (473-475), leading to the recruitment of Gads/SLP-76 to the plasma membrane. This facilitates the interaction between a Tec family kinase associated with SLP-76 to phosphorylate PLC-gamma1 bound to LAT (504,505). PLC-gamma1 also associates directly with SLP-76 further facilitating the phosphorylation by Itk or Txk. Once activated, PLC-gamma1 hydrolyzes phosphatidylinositol 4,5 bisphosphate to produce diacylglycerol, which directly activates RasGRP, a recently characterized nucleotide exchange factor (506), which is a major Ras activator in T cells (507,508). This pathway may also explain the efficiency of phorbol esters, which mimic diacylglycerol, in activating the Ras - MAP kinase pathway in T cells.

Gads$^{-/-}$ mice have been generated by gene targeting (509). The mice exhibit less severe defects than do mice deficient in SLP-76. Gads knockout mice revealed the requirement for this molecule in T cell development, but not in the production of other lineages. Double-negative thymocytes showed a block in proliferation but they were still able to differentiate into mature T cells. Positive and negative selection was also impaired. In these thymocytes there is no interaction between LAT and SLP-76 and there is also a reduction in PLC-gamma1 phosphorylation and in Ca^{2+} mobilization. In addition, mice made transgenic for a dominant-negative form of Gads show similar effects on T cell development and T cell signaling (510). Gads$^{-/-}$ thymocytes presumably retain the Shc/Grb2/Sos or LAT/Grb2/Sos pathway, which might be enough for driving thymocyte differentiation, but not proliferation. Grb2 also plays an important and perhaps distinct role in thymocyte development, since its decreased expression, through haploid insufficiency, selectively reduces JNK and p38 but not Erk activation, and impairs negative but not positive selection (511).

8.6. Vav-1

The protooncogene product Vav-1 is expressed specifically in hematopoietic cells and trophoblastic cells (512). In T cells, this protein undergoes a rapid increase in tyrosine phosphorylation following TCR ligation, and this response is intensified by CD28 costimulation (513-515). Vav-1 functions as a guanine nucleotide exchange factor (GEF) for members of the Rac/Rho family of small GTPases, specifically Rac1 and RhoG (516,517). The relevance of Vav-1 to TCR-dependent responses was highlighted by the dramatic defects in T cell development and function displayed by *Vav-1*$^{-/-}$ mice (518-520). These mice exhibit a significant impairment in T cell maturation past the $CD4^+CD8^+$ double-positive stage. The single-positive T cells that do emerge from the thymus show severe defects in TCR-dependent IL-2 production, proliferation, and effector functions. In keeping with the GEF activity of Vav-1 toward Rac1/RhoG, loss of Vav-1 in T cells leads to dramatic defects in antigen-induced cytoskeletal reorganization and TCR clustering. However, the complex domain structure of Vav-1 suggests that this protein may have functions in addition to the Rac1/RhoG GEF activity, which is conferred by the pleckstrin homology (PH)- Dbl homology (DH) module found in other Rho family-directed GEFs. Unlike other Rho-GEFs, however, Vav-1 contains a calponin homology (CH)

domain, an acidic region containing tyrosine phosphorylation sites, a zinc finger (ZF) domain, and one SH2 domain flanked by two SH3 domains. The exact contributions of many of these domains to Vav1 function in T cells remain poorly understood, although Vav-1 does play a role downstream of the TCR in one or many pathways that lead to activation of the NFAT/AP-1 transcription factor complex (521).

The kinase that phosphorylates Vav-1 in T cells is not entirely clear, but there are good reasons to think that ZAP-70 is involved. First, SH2 domain of Vav-1 can bind to a Tyr-Glu-Ser-Pro motif at Tyr-315 in the 'linker' region of ZAP-70 (522,523). Second, both Zap-70 and Fyn are required for Vav-1 phosphorylation (524). Perhaps, Vav-1 needs to bind to phospho-Tyr-315 of Vav in order to be juxtaposed to Fyn for phosphorylation. On the other hand, Vav-1 was readily phosphorylated (together with SLP-76) in T cells stimulated with an antagonist peptide that failed to induce Zap-70 activation (525). In this system, Rac was activated, while the Ras - MAP kinase pathway was not. Perhaps, Vav-1 phosphorylation was carried out by Fyn without the help of Zap-70 under these circumstances.

8.7. PI3K

Although PI3K is not activated by direct tyrosine phosphorylation, this enzyme still participates in tyrosine phosphorylation-driven early signaling events in T cells (526-528). The p85 adapter subunit of PI3K contains a region with homology to the Bcr protein, Pro-rich motifs, one SH3 domain, two SH2 domains, and a coli-coil region involved in binding to the 110-kDa catalytic subunit. These domains provide multiple avenues for regulatory input and formation of multicomponent complexes, including binding to CrkL, c-Cbl (529), LAT (530), CD28 (531), and possibly with the TCR-zeta and CD3 chains (532), via the SH2 domains, and to Src family PTK SH3 domains via the Pro-rich motifs (117,118,533). Finally the catalytic subunit interacts directly with active GTP-associated Ras, an interaction that upregulates the enzymatic activity several fold (534). All these protein-protein interaction serve both to activate PI3K and to place the enzyme at the plasma membrane in the vicinity of the triggered TCR.

The importance of PI3K in TCR signaling seems to be two fold. First, the enzyme produces phosphatidylinositol-3,4,5-trisphosphate, which serves as a ligand for several PH domains, notably those of PLC-gamma1, Vav and Itk. This facilitates translocation of these proteins to the plasma membrane and explains why PI3K activity is required for their activation (535,536). The PI3K products also lead to the activation of numerous other signaling pathways, including the PDK - Akt kinases, which regulate cell survival (537). All these consequences are constitutively elevated (although still inducible) in the Jurkat T leukemia cell line due to their lack of the opposing PTEN phosphatase.

The second mode in which PI3K plays a role in TCR signaling is as an adapter or scaffolding protein that not only is recruited, but apparently helps recruit other protein to the TCR. Thus, overexpression of individual p85 domains will disturb and inhibit TCR signals in a manner that is unrelated to the phosphorylation of inositol phospholipids (538). This function, in contrast to the catalytic activity, appears to be regulated by tyrosine phosphorylation: Abl- or Lck-mediated phosphorylation at Tyr-688 in the C-terminal SH2 domain of p85 serves to turn off this domain (539). This may serve as a negative feedback regulation of SH2 domain engagement or as a step in a more dynamic signaling complex assembly.

8.8. c-Cbl

The multi-functional protooncogenic protein c-Cbl is rapidly phosphorylated on several tyrosine residues in response to a wide variety of extracellular stimuli, including T-cell receptor ligation (540-542). The 120-kDa protein was initially identified as a mammalian homologue to v-Cbl, an oncogenic gene product of the Cas NS-1 retrovirus. Since then, several additional vertebrate, as well as invertebrate, homologues have been discovered. c-Cbl is expressed in a number of tissues, including cells of hematopoietic and lymphoid origins (543-546).

The protein contains several highly conserved domains including an N-terminal tyrosine-kinase binding domain, a RING finger domain, C-terminal phosphorylation sites, a leucine zipper, as well as a UBA domain with homology to enzymes involved in ubiquitination and degradation. Since its discovery, a number of interesting functions of c-Cbl have been uncovered. These include the negative regulation of tyrosine kinases and gene expression, possible adapter functions, as well as being an E3 ubiquitin -ligase (547,548). c-Cbl has been shown to interact with several signaling proteins including the p85 regulatory subunit of PI3K and Vav-1 (528,545,549). Cbl is also a participant in interactions involving adapter proteins, CrkL, C3G, Grb-2 and SLP-76 (528,550). Moreover, the c-Cbl protein has been shown to interact with number of tyrosine kinases involved in TCR signaling. These kinases include Fyn, Zap-70, and Syk (265,548,551). In addition, Fyn and Zap-70 have been shown to be negatively regulated by c-Cbl, through mechanisms involving enhanced protein degradation, and reduced tyrosine phosphorylation of a key tyrosine residue, respectively (552,553).

An important step towards understanding the role of c-Cbl in T cells was the deletion of its gene through homologous recombination (554-556). The key feature of these mice is that Zap-70 is more phosphorylated and more active than in control mice. Another more recent advance was the discovery of Cbl-b, a related protein with opposite function, which is also tyrosine phosphorylated upon TCR triggering (557). It seems that c-Cbl and Cbl-b may balance each other by a competition mechanism.

8.9. TSAd

Another interesting molecule involved in T-cell signal transduction is TSAd (T-cell SH2 domain-containing adapter). The cDNA for this protein was cloned from an activated $CD8^+$ T-cell cDNA library (558). The mRNA was detected in several lymphoid tissues, preferentially in

activated $CD4^+$ and $CD8^+$ T-cells. However, TSAd expression was not detected in the Jurkat T cell leukemic line or in B-cells (558). The role of TSAd in T cell signaling remains unclear. One report suggests that TSAd is a negative regulator of activation induced responses to TCR, or TCR/CD28 triggering by interfering with the kinase activity of Lck (559). Another study suggests that TSAd is a positive regulator of IL-2 promoter gene activity and is localized predominantly in the nucleus through a mechanism involving its SH2 domain (560). TSAd was also found as a protein that associates with MEKK2 and that regulates the Mek5-Erk5 pathway (561), and as a protein that associates with Itk and Txk (301). At present, these results are difficult to reconcile and demonstrate the need for additional studies to determine the physiological role of TSAd in T cells.

8.10. SAP/SH2D1A

X-linked lymphoproliferative disease is a rare genetic immunodeficiency disorder characterized by extreme susceptibility to EBV infection, which often leads to early death as a result of uncontrolled responses to EBV infection (562). Recent evidence has implicated defects in SAP (SLAM-associated protein), a small 128 aa protein containing an SH2 domain and a short C-terminal tail, as the primary cause of this disease (563-566). The exact mechanisms by which defects in SAP cause XLP remain unclear. Characterization of several mutations identified in XLP patients suggests that the disease is associated with decreased SAP protein stability and mutations resulting in its inability to interact with its binding partners, leading to defective signaling from cell surface receptors. To date, SAP has been shown to interact with at least four CD2-related receptor proteins including CD150 (SLAM) and CD244 (2B4) and it is believed that disruption of signals transmitted through these receptors contributes to disease (564,566,567). A recent study has reported aberrant TCR signaling in CD4 Th cells from patients with XLP. T helper cells from these patients showed increased phosphorylation of several cellular proteins involved in TcR signaling, including hyperphosphorylation of c-Cbl, Zap-70, and the TCR-zeta chain. Furthermore, defects were also observed in the production of IFN-gamma and IL-2 by T helper cells from these patients (568). Though, it remains unclear which defects in SAP cause this deadly disease, it appears that this molecule is a versatile component of several signaling pathways involved in controlling lymphoproliferative responses. Evidence has shown that disruption of this subtle balance can lead to catastrophic consequences.

8.11. TRIM

While the TCR-zeta chain is the focus of early molecular changes, it is not the only member of the receptor complex with the potential the generate intracellular signals. Bruyns and coworkers (569) have recently identified a novel dimeric structure (termed TRIM for T-cell receptor interacting molecule) that loosely associates with the receptor complex. TRIM is member of a growing family of dimeric type III transmembrane proteins made of a short extracellular region attached to an extended cytoplasmic tail. As with TCR-zeta chain, the small extracellular region of TRIM is unlikely to directly bind to ligand. Instead, it may act as a cell surface adapter engaged by adjacent receptors. Interestingly, instead of possessing ITAMs, TRIM has six tyrosine residues that include a src-kinase (Tyr-XXL) and phosphatidylinositol 3-kinase (Tyr-XXM) SH2 domain binding motif. TRIM is therefore unlikely to be a simple homologue of TCR-zeta, and instead carries potential information linked to other functions.

8.12. Other PTK substrates

In addition to the substrates for tyrosine phosphorylation reviewed above, a large number of other proteins become phosphorylated on tyrosine upon TCR triggering. These proteins include additional transmembrane proteins and co-receptors, like CD5, CD6, CD28 and CTLA-4, many cytoskeletal proteins (e.g. ezrin), and proteins of unclear function or relevance, such as the AAA family ATPase VCP (570), the RNA binding protein Sam68 (571), LckBP1 (572), HS1 (573), Lnk (574), and Nck (575). Many more likely remain to be identified.

9. CONCLUDING REMARKS

During the last decade our understanding of the machinery of T cell activation has advanced tremendously. However, the wide-spread belief that we now know all the key players and pathways is probably an illusion. In fact, researchers in this field in the 1970s thought that they understood the biochemical basis for lymphocyte activation: it was simply a question of cAMP concentration. Later, it became clear that Ca^{2+} was the key to everything, then PKC entered the scene, then tyrosine phosphorylation, etc. At least, today we realize that all these pathways, and many more, operate in lymphocytes. All these pathways interact, synergize, counteract and control each other in a complicated spatial and temporal network of reactions that govern the behavior of T cells at all times. Despite all the progress, there is still limited understanding of how signaling interacts with general metabolism, the structural components and architecture of the cell, organelle homeostasis and replication, gene expression and DNA replication, the cell cycle, stimulus-induced secretion of bioactive peptides, and why one T cell responds in one way, while another T cell responds in a completely different manner to the same stimulus.

Since tyrosine phosphorylation and the first tyrosine kinase were discovered by Hunter and Sefton in 1980 (576), it has gradually become clear that this rare type of protein modification plays a fundamental role in numerous processes that have been acquired specifically by higher eukaryotes and multicellular organisms during the last 570 million years of evolution. In contrast to modification of serine and threonine residues, phosphorylation of tyrosine is typically involved in responses that are regulated with respect to the organism, rather than the cell itself. Thus, tyrosine phosphorylation is a key mechanism of cell-to-cell communication, signal transduction, cell growth and proliferation, cell cycle control, differentiation, malignant transformation, cell morphology, regulation of the cytoskeleton,

neurotransmission, adhesion, gene regulation and transcription, intracellular vesicle transport, endocytosis, exocytosis, embryogenesis and development, and a number of inherited or acquired human diseases from cancer and angiogenesis to immune function. Compared to protein phosphorylation in general, phosphorylation on tyrosine stands out as a unique feature of more highly evolved and complex multicellular life.

Tyrosine phosphorylation obviously plays an important role in the function of the immune system, a set of specialized cells that to a high extent combine most of the functions listed above for tyrosine phosphorylation. In many respects, the immune system competes only with the nervous system in complexity and extent of cell specialization and need for cell-to-cell communication. It is not surprising, therefore, that immune cells express numerous unique tyrosine kinases and phosphatases, many of which are also expressed at high levels in the brain. Compared to most cells, immune cells have much higher levels of basal tyrosine kinase activity, which is counterbalanced by a remarkably high tyrosine phosphatase activity. It appears that lymphocytes must spend a lot of ATP simply in adding and removing phosphate from tyrosine residues on cellular proteins. The advantage of this seemingly futile ATP consumption is that immune cells can respond rapidly and can monitor multiple changing inputs on a continuous basis and integrate them into a balanced and appropriate response. There are also risks involved: aberrant tyrosine phosphorylation, whether causative or not, is observed in many diseases of the immune system. Genetic damage that involves tyrosine kinases or phosphatases can result in serious or even fatal disorders that range from immunodeficiencies (218,220,577-579) to autoimmunity (13,320,390) and malignant diseases (178,339,580). Viruses have also evolved to take advantage of tyrosine phosphorylation in lymphoid cells (see section 2.5.7.).

The major challenges of the next decades of molecular T cell research will be to complete the schemes of signaling pathways and mechanisms, integrate them all into a working model that can accurately explain the behavior of T cells under all circumstances, and, most importantly, help us understand the biochemical and genetic basis for human diseases.

10. ACKNOWLEDGEMENTS

Work reported herein was supported by grants AI35603, AI40552, AI41481, and AI48032 from the National Institutes of Health (T.M.).

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Key words: Review, T cell activation, Lck, Fyn, c-Yes, Syk, ZAP-70, Itk, Txk, CD45, HePTP, PEP, PTP-MEG2, PTPH1, PTP-MEG1, PTP36, PTEN, LMPTP, PLCgamma1, SLP-76, LAT, FYB, SLAP-130, Vav-1, PI3K, c-Cbl, TSAd, SAP, SH2D1A, TRIM, Review

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