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SIGNAL TRANSDUCTION MEDIATED BY THE T CELL ANTIGEN RECEPTOR: The Role of Adapter Proteins*

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■ **Abstract** Engagement of the T cell antigen receptor (TCR) leads to a complex series of molecular changes at the plasma membrane, in the cytoplasm, and at the nucleus that lead ultimately to T cell effector function. Activation at the TCR of a set of protein tyrosine kinases (PTKs) is an early event in this process. This chapter reviews some of the critical substrates of these PTKs, the adapter proteins that, following phosphorylation on tyrosine residues, serve as binding sites for many of the critical effector enzymes and other adapter proteins required for T cell activation. The role of these adapters in binding various proteins, the interaction of adapters with plasma membrane microdomains, and the function of adapter proteins in control of the cytoskeleton are discussed.

INTRODUCTION

Signal transduction refers to the process by which extracellular events or cues are transmitted via a receptor or multiple receptors to the interior of the cell. Many of the current principles in the study of signal transduction have arisen from the study of various growth factor receptors. In these systems a transmembrane receptor binds ligand and then undergoes a conformational change or aggregation that has intracellular consequences. In many systems the receptor either is itself a protein kinase or is linked to one (1). The change in the receptor induced by binding results in kinase activation. The protein kinase(s) then phosphorylates a number

of proteins, some of which may be effector enzymes, and this phosphorylation may result in their activation. In many cases these critical kinase substrates also include adapter molecules (2, 3). Adapter or linker molecules are proteins lacking enzymatic activity and are comprised of multiple binding domains and sequence motifs to which such domains bind. Phosphorylation of adapter molecules alters the surface of these molecules and allows additional enzymes or adapters to bind, which results in formation of multiprotein complexes. The associated and activated enzymes can be at the proximal end of a series of subsequent activation events, and multiple enzymes and enzyme pathways can be involved. The general theme of these processes is the transfer of information (4), that is, the transmission of an event on the exterior of the cell (receptor engagement) to activation and regulation of multiple intracellular events occurring at the plasma membrane, cytosol, and nucleus.

Several principles leading to development of the above scheme represent some of the greatest advances in the field of signal transduction over the past decades. First, receptor molecules have both extracellular and intracellular functions. Ligands engage specific extracellular domains, thereby inducing conformational changes or aggregation that are transmitted intracellularly. The cytoplasmic component of growth factor receptors was recognized to contain multiple sites to which various signaling molecules could bind. In many cases the cytosolic tails of these receptors contain tyrosine residues, which upon phosphorylation serve as docking sites for proteins containing specific phosphotyrosine recognition domains such as SH2 domains. The second and related theme is that most signaling molecules are modular. Many enzymes and a large variety of adapter proteins contain domains for phosphotyrosine, polyproline, or lipid interaction as well as motifs to which some of these domains bind (Figure 1). There is great variety in the way in which these domains are brought together in a form of combinatorial diversity.

Over the past decade adapters have been increasingly recognized throughout biology as critical to cell function and, in particular, cellular signaling. Investigation of adapter molecules in cells of the immune system has also been intense. Numerous review articles contain surveys of the multiple adapter proteins found in lymphocytes (5–11). Many of these molecules are well studied, while others have only been recently described. Instead of providing a catalog of the multiple adapter molecules isolated from lymphocytes, the goal of this article is a combination of the general and the specific. The first intent is to put the study of adapter molecules in a broad context by reviewing certain issues common to various signaling systems and to relate these to the signaling pathways coupled to the T cell antigen receptor (TCR). The bulk of the discussion, in the second part of the review, focuses on certain topics now actively studied by those interested in the role of adapters in TCR-mediated activation. One particular adapter, LAT (linker for activation of T cells), is extensively discussed throughout the review.

SIGNALING VIA THE T CELL ANTIGEN RECEPTOR—BACKGROUND

The most obvious difference between growth factor receptors and the TCR is the added complexity of the latter both in terms of receptor structure and in the molecules most closely coupled to the receptor. The ligand recognition component of the TCR, the α and β chains, which engage the complex of peptide and MHC molecule, have been extensively studied for over two decades (12, 13). Their complexity lies in the genetic and cellular mechanisms involved in creating millions of different clonotypic receptors in any individual. A discussion of these processes—genetic rearrangement, the pairing of chains to create stable dimers, and then the intricate intrathymic selection of receptors—is outside the scope of this paper. The recognition components interact with the nonpolymorphic CD3, γ, δ, and ε, and the TCRζ chain dimer (14). These molecules are integral TCR components and as such are required for TCR expression. The cytosolic components of these molecules contain a unique motif, the ITAM (immunoreceptor-based tyrosine activation motif), which has a consensus sequence of YxxI/L $x_{(6\text{-}8)}$YxxI/L (15, 16). Each of the CD3 chains contains one such motif, whereas each TCRζ chain contains three. The actual arrangement and stoichiometry of CD3 and TCRζ chains within a TCR are unknown. However, for a working model of the TCR, one can consider each $\alpha\beta$ to associate with a $\delta\varepsilon$ dimer, a $\gamma\varepsilon$ dimer, and a TCRζ dimer. Each TCR in this configuration would thus contain ten ITAMs.

ITAMs are necessary and sufficient for TCR-mediated activation (17, 18). The tyrosine residues within each ITAM become rapidly phosphorylated upon optimal TCR engagement. The phosphorylated ITAMs become subject to binding by additional molecules. In this fashion the CD3 and TCRζ chains themselves behave like adapter proteins containing motifs that are modifiable by phosphorylation and serve as binding sites for critical proteins. ITAM phosphorylation is mediated by two members of the Src family of PTKs found in T cells. Lck is the predominant enzyme involved in ITAM phosphorylation, while Fyn also has this capacity (19, 20). The most important consequence of ITAM tyrosine phosphorylation is the binding of ZAP-70, a member of a second family of PTKs involved in TCR signaling (21). The two phosphorylated tyrosines of each ITAM are bound by the tandem SH2 domains of ZAP-70 in a highly specific and cooperative fashion (22, 23). ZAP-70, once bound to the TCR in this fashion is activated by phosphorylation of the kinase domain activation loop mediated by the Src PTKs (24, 25). Other phosphorylations of ZAP-70 allow additional proteins to bind, giving ZAP-70 itself the role of a scaffold (26). The activated TCR is thus characterized by phosphorylated ITAMs associated with phosphorylated, activated ZAP-70.

These activated PTKs then phosphorylate a large number of protein substrates. Over the past decade these proteins were first identified by detection of the subset of proteins phosphorylated on tyrosine residues after TCR engagement. Some of these proteins, such as the enzyme phospholipase Cγ1 (PLC-γ1), were known from

other systems and were rapidly identified (27, 28). Others were isolated because of their phosphorylation. These include other enzymes and, importantly, several of the adapter molecules such as LAT and SLP-76, discussed extensively in this review. The recognition that adapter molecules, both those subject to phosphorylation and others that are not, are critical in TCR signaling creates a number of analogies to other signaling systems in which adapters are of central importance in the signal transduction process (Figure 2).

Among the most prominent PTK substrates detected after TCR engagement is a protein of 36–38 kDa (29). Early studies demonstrated that this protein could be detected in a complex with a number of other PTK substrates including PLC-γ1 and the small linker molecule Grb2 (see below) (30–32). Despite both the ease of detection and its association with known proteins, the protein known then as pp36 proved difficult to isolate. Ultimately modified protein purification conditions enabled Zhang et al. to obtain amino acid sequence and clone the cDNA encoding this protein, which was named LAT (linker for activation of T cells) (33). Sequence analysis demonstrated that LAT is a member of a relatively unusual class of transmembrane adapter molecules. It is a class III–type protein, lacking a signal sequence. It contains a short extracellular sequence, a transmembrane domain, and a long cytosolic component containing nine tyrosine residues conserved between mouse and human LAT. Early studies also revealed that two cysteine residues (C26 and C29) are subject to posttranslational palmitoylation, which is responsible for specific localization within the plasma membrane (see below) (34).

The central role of LAT in TCR-mediated signaling has been revealed in studies of the Jurkat T cell and Jurkat variants that lack LAT (35, 36). Jurkat cells, activated by cross-linking the TCR with monoclonal antibodies directed at either TCRβ or CD3ε show activation of multiple intracellular biochemical pathways and transcriptional elements leading to induction of interleukin 2 synthesis. These events include calcium elevation and ERK, AP-1, and NFAT activation. They do not occur in the Jurkat variants that lack LAT, but all activation events can be observed if LAT expression is restored following transfection of LAT cDNA. LAT function is also required for intrathymic development of normal T cells (37). In animals that are genetically modified to lack the LAT gene, intrathymic development of T cells is blocked at an early stage, and thus no T cells are found in the lymph nodes or spleens.

LAT is rapidly phosphorylated on tyrosine residues following TCR engagement. Overexpression studies in fibroblastoid lines revealed that ZAP-70 and Syk are the PTKs most likely to be responsible for these phosphorylations. This conclusion is supported by the observation that LAT is very poorly phosphorylated in T cells lacking ZAP-70 (38). Nonetheless low levels of LAT phosphorylation are induced by activated forms of Lck, and it is possible that some of the multiple tyrosines in the LAT can be phosphorylated by PTKs other than ZAP-70. The consequence of these multiple phosphorylations on LAT in T cells is that a number of signaling proteins bind at these sites following TCR engagement.

The proteins that interact with LAT fit into two categories, enzymes and adapter proteins. A full description of all these proteins is outside the scope of this manuscript. It is also likely that additional proteins that bind LAT remain to be described. One set of LAT-binding adapter proteins, the Grb2 family, contains Grb2, Grap, and Gads. All are of very simple design, consisting of a central SH2 domain flanked by two SH3 domains. These domains show considerable sequence homology between the three proteins. The Gads protein, known also by a series of other names [Grpl, MONA, Grf40, reviewed in (9)] also contains an internal proline-rich region of unknown function between the SH2 and the C-terminal SH3 domains (39). These simple linker proteins bind to tyrosine phosphorylated LAT via their SH2 domains. LAT structure-function studies have shown that three distal LAT tyrosine residues, Tyr 171, 191, and 226 (human sequence), following phosphorylation bind Grb2, while Gads binds only phosphorylated 171 and 191 (40).

Grb2, Grap, and Gads are bifunctional molecules in the sense that their SH2 domains bind one protein, in this case phosphorylated LAT, while their SH3 domains bind other proteins, which contain the relevant proline-rich sequences. Grb2 and the related proteins are cytosolic and their binding to phosphorylated LAT translocates them and their SH3-associated proteins to a different cellular compartment, the plasma membrane. Thus, a consequence of LAT phosphorylation is the association of a number of Grb2-binding proteins at the membrane.

Grb2 is a ubiquitously expressed protein, and its function in many systems is to shuttle associated signaling proteins to tyrosine phosphorylated receptors or adapter molecules following ligand activation (41). The list of proteins that bind Grb2 SH3 domains is vast. In T cells several proteins have been prominently described (42). SOS is well known from many studies in nonlymphoid tissues as a critical activator of the small G protein Ras (43). Grb2-mediated translocation of SOS from the cytosol to the plasma membrane brings it to the site of Ras localization. The Grb2-SOS complex has been detected in T cells, and SOS has been found in association with LAT following TCR engagement (33, 44). Recently there has been the identification of another Ras activator, in T cells, Ras-GRP, and thus the relative importance and function of each of these effector molecules remains unclear (45, 46). Cbl is another Grb2-associated protein found in T cells, bound to phosphorylated LAT. Cbl too has many domains and interactions. Recently Cbl has been shown to be part of a ubiquitin ligase assembly, and its function in that capacity in T cells also needs much additional investigation (47). Another small linker molecule, Shc, was originally demonstrated to bind to growth factor receptors and simultaneously to Grb2, which in turn in these studies was bound to SOS. Thus, in these systems an additional molecule was interposed between the receptor and the effector enzyme, SOS. Shc interactions with Grb2-SOS complexes have been studied in T cells (48).

Another member of this simple adapter family is Grap, which also binds LAT following LAT phosphorylation (49). Grap-associated proteins include SOS, dynamin, and Sam68. Currently there is no good explanation for why T cells need two

adapter proteins, Grb2 and Grap, with similar SH3 binding specificities. The third member of the family is Gads, which differs from Grb2 in its tissue distribution. While Grb2 is ubiquitously expressed, Gads is found only in hematopoietic cells. Gads also has unique SH3 specificity. While Grb2 does not bind to SLP-76 in T cells, Gads specifically interacts with SLP-76. Thus, Gads brings another critical linker molecule to phosphorylated LAT (50). Recently Gads has been shown to interact with the serine-threonine kinase HPK (51). Certainly, the full inventory of Gads-associated proteins remains to be described.

SLP-76 was first identified as a tyrosine kinase substrate that could be affinity purified in vitro by binding to Grb2 (52). It is a 76-kDa protein expressed exclusively in hematopoietic cells. SLP-76 lacks enzymatic activity and structurally can be divided into three domains. At the amino-terminal end, multiple tyrosines become phosphorylated on TCR engagement. The central domain is rich in proline residues including those that interact with the SH3 domains of Gads. The carboxy-terminal end of the protein contains an SH2 domain. Overexpression of SLP-76 in Jurkat cells led to an increase in TCR-mediated activation of NFAT and IL-2 promoters (53). No increase in calcium mobilization was seen in such experiments, though ERK activation was enhanced. All three SLP-76 domains are required for this augmentation of function (54). Study of a Jurkat mutant lacking SLP-76 revealed that following TCR engagement these cells show decreased calcium flux and no ERK activation (55). Not unexpectedly in these cells, IL-2 promoter activity was not increased in response to TCR cross-linking. SLP-76 also has a critical role in normal thymocyte development. Mice lacking SLP-76 fail to generate normal, peripheral T cells (56, 57). An intrathymic block occurs in T cell development at an early stage. The T cell phenotype of these mice resembles that of mice that lack the LAT gene, as described above. Both of these mice demonstrate the same developmental block and absence of mature T cells.

SLP-76 functions as an adapter protein that binds multiple effector molecules, which can be brought to LAT via its association with Gads (50). A recent study demonstrated that a LAT-SLP-76 chimera containing only the transmembrane domain and palmitoylation sites of LAT, i.e., the raft targeting region (see below), suffices to reconsitute LAT-deficient variants of Jurkat. Although overexpression of such constructs in Jurkat variants does not fully mimic all the complex interactions that might occur in more physiologic T cell systems, the result does emphasize the significance of SLP-76 recruitment (58). Upon TCR engagement, activated ZAP-70 phosphorylates multiple tyrosine residues in the amino-terminal end of SLP-76. These phosphorylated residues serve as binding sites for a series of proteins containing SH2 domains. These include Vav, a guanine nucleotide exchange factor for the G-proteins of the Rac family; Nck, itself an adapter protein that interacts with the serine-threonine kinase Pak1; and the PTK Itk (59–61). These associated molecules have been implicated in the regulation of a number of pathways critical to T cell activation. Vav and Nck may integrate the activation of a number of pathways involved in both gene transcription and cytoskeletal rearrangement as discussed below. The association of Itk with SLP-76 brings this PTK into close contact with

PLC-γ. Recent studies suggest that Itk activation is required for optimal PLC-γ1 phosphorylation and activation (62). SLP-76 SH2 domain interacts with a 130-kDa protein named both SLAP (SLP-76-associated phosphoprotein) and Fyb (Fyn binding protein). This protein is also a multidomain adapter protein containing a proline-rich region, a tyrosine-rich region, and an SH3-like domain. The gene encoding this molecule has been genetically deleted by two groups (62a, 62b). The new proposed name for the protein is ADAP (adhesion and degranulation promoting adaptor protein).

LAT AND THE RAFT MODEL OF T CELL ACTIVATION

The classic lipid bilayer model describing the molecular organization of the plasma membrane has been modified over the past decade because of the realization that the plasma membrane is not a homogenous array of glycerophospholipids (63–65). Instead there is considerable heterogeneity of lipids in the membrane. Glycosphingolipids and cholesterol were shown to self-associate in model membrane systems, and similar phenomena were then observed in plasma membranes isolated from cells. The aggregation of glycosphingolipids and cholesterol is thought to induce the formation of microdomains in the membrane that are distinct from the more abundant and diffuse glycerolipids. These domains are known by a large number of acronyms: GEMs (glycolipid enriched microdomains), DIGs (detergent insoluble glycolipid-enriched membranes, DRMs (detergent-resistant membranes), or rafts. Many of these names reflect the standard method used to isolate such domains, which is the inability of non-ionic detergents such as Triton X-100 to solubilize these domains from plasma membranes in the cold. Such insoluble material can be separated from solubilized cellular material by sucrose gradient centrifugation.

These microdomains or rafts are also enriched in a number of molecules relevant to receptor-mediated signaling. These include the lipid substrates of PLC-γ and GPI (glycosylphosphatidylinositol)-anchored proteins, including such molecules expressed on T cells as Thy1 and Ly6. Additionally Ras, various G proteins, and members of the Src PTK family are enriched in these domains (66). For the proteins in rafts that are not GPI-anchored, one shared characteristic is posttranslational modification by several lipids. For example, Ras is both palmitoylated and farnesylated. Most of the Src PTKs (except for Src itself) are modified by myristoylation and palmitoylation (67, 68). Evidence obtained from study of the T cell–specific Src family PTK, Lck indicates that both of these lipid modifications are necessary for raft localization targeting and phosphorylation of the TCRζ chain (69).

Studies on the FcεRI receptor were the first to demonstrate that immunoreceptor activation involves interactions with rafts. These investigators showed that this receptor associated rapidly with rafts upon activation (70, 71). The very early tyrosine phosphorylation of receptor subunits by the Src family PTK, Lyn depended on this localization. Several groups subsequently made similar observations about TCR activation in cell lines and thymocytes (72, 73). Receptor engagement increased

the level of TCR association with rafts, and TCR subunits demonstrated enhanced tyrosine phosphorylation. Phosphorylated, and thus activated, ZAP-70 was found associated with these activated receptors. All of these studies depend essentially on a negative result, the inability of detergent extraction of the receptor at certain conditions of activation, cell number, temperature, and detergent type. Despite these results, several investigators at the time did not observe enhanced TCR association with membrane rafts. A possible explanation for the conflicting results was proposed by investigators who took an independent approach to the question (74). They visualized rafts using fluorescent cholera toxin B subunit, which binds glycosphingolipids. Antibodies to the subunit induced cross-linking of these lipids forming patches that are visible microscopically. With this technique they were able to observe colocalization in rafts of Lck and TCR subunits. Interestingly TCR subunits patched in this fashion were more sensitive to detergent extraction than were other raft-associated proteins. The authors speculated that this result indicated that the TCR is more weakly associated with rafts than are other molecules, which might constitutively localize in these domains.

The study of the LAT protein contributed to an understanding of raft localization and T cell activation. Examination of the LAT amino acid sequence revealed the presence of two cysteine residues (positions 26 and 29) adjacent to the putative transmembrane domain of the protein. Since juxtamembrane cysteines are likely to be targets of the membrane-associated palmitoylation machinery, T cells were labeled with [^{3}H]-palmitate and incorporation was demonstrated (34). Mutation of these two cysteines to alanine blocked this incorporation. These mutations and especially mutation of Cys 26 alone also had dramatic effect on LAT localization to rafts, as determined by the standard biochemical extraction assay. Though mutation of the cysteine residues did not affect membrane localization of LAT, mutation of Cys 29, partially, and Cys 26 fully inhibited LAT localization to rafts. LAT with these mutations were also expressed in T cells and examined following TCR engagement. Mutation of cysteine 26 had a dramatic effect, and no LAT tyrosine phosphorylation was detected. From these studies it was concluded that LAT had to be in rafts for it to be phosphorylated.

The majority of LAT molecules localize in rafts as determined by the sucrose gradient analysis. As expected in samples from nonactivated T cells, essentially none of the LAT substrates are detected in these fractions. However, upon activation and LAT phosphorylation, one observes a translocation of LAT binding proteins to the raft fraction. The fraction of such LAT binding proteins as PLC-γ, Cbl, or Grb2 that shifts in this way is small, but this is presumably the fraction that is functionally active. The consequences of the absence of LAT localization to rafts and the failure of LAT tyrosine phosphorylation were thought likely to be dramatic, and this prediction was confirmed in two subsequent studies (36, 75). In both, independently isolated Jurkat mutants lacking the LAT molecule were used. The absence of LAT had no effect on TCR-induced receptor phosphorylation or ZAP-70 activation, but all steps distal to this were inhibited. There was no activation of PLC-γ1, and thus there was minimal calcium flux or ERK activation. Several

transcription factors were not activated. In both of these studies, reintroduction of wild-type LAT restored all function. Introduction of LAT deficient in one residue, Cys 26, had the same effect as though no LAT was present in the cell. Thus, without LAT localization to rafts, there is no LAT tyrosine phosphorylation, no translocation of critical signaling molecules to the rafts, and no T cell activation.

If LAT is in membrane rafts and the TCR in the resting state is not, how do these structures interact, and how does the TCR become raft-associated, albeit weakly? It is possible that receptor engagement alters some biophysical properties of the TCR. A common model for TCR activation requires some level of TCR-TCR interaction, which at the most extreme would be TCR oligomerization or aggregation (76). Hypothetically this process could expose different regions of TCR subunits, which might enhance interactions with different membrane components. Similarly, receptor aggregates might also be more likely to trap rafts. Interactions of other TCR and LAT associated proteins have been described. Some or all of these interactions might be involved in bringing the activated TCR and associated PTKs to LAT molecules. The Lck PTK is located in rafts, and its SH2 domain can bind a phosphorylated tyrosine residue in the activated ZAP-70 PTK (77, 78). This intermolecular bridge may bring TCRs bearing activated ZAP-70 to rafts. Additionally, a subset of CD4 interacts with LAT (79). Other CD4 molecules may interact with Lck. Since CD4 interacts with MHC class II molecules and since some CD4 molecules exist as dimers (80), one can construct a model by which TCR and CD4 engage the same MHC, and the CD4-associated Lck and LAT, both in rafts, are brought to the TCR.

Several additional molecules have been proposed to bind both TCR and LAT, thus potentially linking these molecules. PLC-γ1 contains two SH2 domains, and it is well documented that the N-SH2 domain interacts with LAT. Williams et al., studying SH2 fusion proteins, proposed that the C-SH2 domain interacts with phosphorylated residues on activated ZAP-70 in a fashion similar to that described above for Lck (81). Deckert et al. have proposed that a molecule originally isolated as an Abl-SH3 interacting protein, 3BP2, can interact via its SH2 domain with both ZAP-70 and LAT (82). Though this interaction cannot be simultaneous because 3BP2 has only one SH2 domain, perhaps the molecule could multimerize. The authors of this study suggest that a functional coupling could occur, leading to the observed enhancement in T cell activation. Finally a small adapter protein known as Shb does contain two separate phosphotyrosine-binding domains (83). A classic SH2 domain in Shb was shown to bind TCRζ chain on phosphorylated tyrosine residues. A non-SH2 phosphotyrosine binding domain bound phosphorylated LAT. Expression of a mutant form of Shb with a defective SH2 domain inhibited LAT phosphorylation and distal signaling events. These data suggest that Shb links between the TCR and LAT have major functional significance.

A recent study provides further insight into TCR-LAT interactions. Harder & Kuhn incubated T cells with anti-TCR antibodies coupled to beads (84). Activation via the TCR was induced with warming to 37°C, and the cells were subjected to nitrogen cavitation. The material that associated with the TCR on the bead over

different times of warming was then analyzed biochemically. No detergent was used in these preparations, and what was isolated, presumably, were membrane patches. In addition to the TCR subunits and the associated ZAP-70, the investigators showed that over time LAT was detected in these complexes. Some of the proteins that bind activated LAT such as PLC-γ1, Grb2, and Cbl were also found in these patches. In contrast what they did not see associated with the TCR were the raft-associated PTKs, Lck or Fyn. The association of TCR and LAT in these membrane fragments depended on tyrosine kinase activity, and mutant LAT, which lacked the cysteines required for palmitoylation, did not co-isolate with the TCR. The conclusion from these studies is that TCR-LAT interactions do not represent an interaction of the receptor with LAT via large lipid aggregations in which Lck and Fyn would also be expected to be found. Instead the study supports the idea that protein-protein interactions induced by TCR activation and dependent on tyrosine phosphorylation control the critical TCR-LAT association.

Interest in the raft model of immunoreceptor signaling has been intense, and numerous studies have expanded an understanding of the interactions between signaling receptors and the plasma membrane. However, it is wise to recognize that there are still a number of problems with this model that investigators in this field must acknowledge. The main concern is methodologic. Nearly all studies of raft function rely on the crudest of preparations, material that fails to be solubilized by certain detergents. Moreover, the preparations are made from cells that have been chilled to near freezing temperature. In so doing one may force interactions of proteins and lipids that might not normally occur under physiologic conditions. Efforts to reproduce raft isolation without detergent or chilling are rare, but, as described above, some investigators have begun this process.

A similar criticism can be made about methodologies in which raft components are visualized after heavy cross-linking induced by multivalent toxins and antibodies. These studies demonstrate clustering of molecules shown to colocalize by biochemical analysis, but clearly the system is being forced. Ideally, imaging techniques could be used to demonstrate membrane heterogeneity. Investigators have attempted to use fluorescence energy transfer (FRET) techniques to visually demonstrate clustering of GPI-linked proteins. Two groups have reported contradictory results, with one obtaining results consistent with microdomains of less than 70 nM containing just a few molecules, whereas the other group saw no such structures (85, 86). The discrepancy could be resolved if only a few molecules were clustered over a minor fraction of the surface, or if such structures were short-lived.

There is certainly strong evidence that membrane heterogeneity exists in model systems and in cells. There is much evidence that membrane microdomains are relevant for signaling in lymphocytes. The dramatic effect on T cell signaling of the cysteine LAT mutations is an example of such an experiment. Frequently however one senses that rafts have come to imply long-lived, well-defined membrane substructures. The conclusion that membrane heterogeneity is a dynamic process and that transient interactions of lipids and proteins are likely is much more reasonable and cautious.

SIGNALING COMPLEXES: THE LAT-PHOSPHOLIPASE Cγ1 EXAMPLE

PLC-γ1 was one of the first enzymes demonstrated to be a PTK substrate in T lymphocytes, following its identification as a PTK substrate in growth factor receptor tyrosine kinase systems. Phosphorylation of PLC-γ1 on multiple tyrosine residues is required for its activation (87). PLC-γ1 is a central signaling molecule in T cells as well as other cells activated by PTKs. Activation of this enzyme leads to hydrolysis of phosphatidyl inositol (4,5)-bisphosphate to inositol (1,4,5)-trisphosphate and diacylglycerol (88). The former regulates intracellular calcium mobilization, and the latter regulates protein kinase C activation. Recent studies in T cells demonstrate that calcium and diacylglycerol regulate RasGRP, a newly described activator of Ras. By contributing to Ras activation, PLC-γ1 thus indirectly can control PI3 kinase and MAP kinase cascades (45, 46). The multiple binding interactions that engage PLC-γ1 and molecules that regulate this enzyme are now under intensive scrutiny, and the results of these studies serve as an excellent example of signaling complexes containing adapter molecules involved in TCR activation.

An interaction of PLC-γ1 and a 36–38 kDa protein was described long before LAT was isolated and characterized. The association was seen after T cell activation and was dependent on the two PLC-γ1 SH2 domains (30, 89). This and a subsequent study showed that the N-terminal SH2 domain was more specific for phospho-LAT, but in the later study the C-terminal SH2 domain was shown to bind LAT as well as other proteins (90). Mutation of the N-SH2 resulted in depressed tyrosine phosphorylation of PLC-γ1 following TCR binding. The sites of PLC-γ1 interaction with LAT were addressed in a LAT structure-function study. TCR engagement of LAT-deficient variants of the Jurkat cell line (J.CaM2) failed to activate PLC-γ1 and thus failed to elevate intracellular calcium or activate ERK. Reconstitution with wild-type LAT restored these pathways. Zhang et al. created a series of stable lines in which J.CaM2 was reconstituted with LAT mutants containing one or more tyrosine-to-phenylalanine mutations (40). The residues adjacent to Tyr 132, YLVV, form a consensus binding sequence for PLC-γ1 SH2 domains. Mutation of tyrosine at this site abrogated PLC-γ1-LAT association and PLC-γ phosphorylation on tyrosine residues. Cell lines expressing this mutant showed altered calcium flux following TCR engagement. The rapid onset of calcium elevation was observed, but the sustained influx, normally seen following TCR engagement, did not occur. Two additional reports showed subtly different results. In one, the 132 mutations inhibited PLC-γ1 phosphorylation and calcium flux (91). In the other, phosphorylation of PLC-γ1 persisted, but calcium flux was sharply curtailed (92). The differences are likely due to slight variations in technique; nonetheless, all three studies point to the critical role of this site on LAT for PLC-γ1 function.

The three distal tyrosine residues of LAT (Tyr 171, 191, and 226) all are found within YXNX motifs. Phosphorylation of this motif defines sites for binding via the SH2 domains of Grb2 or related adapters. Interestingly, mutation of all three

of these sites also had a strong impact on PLC-γ1 (40). The PLC-γ1-LAT association was not detected; PLC-γ1 tyrosine phosphorylation was nearly completely inhibited, and calcium flux was partially inhibited. These sites might bind the C-terminal SH2 domain of PLC-γ1 directly or might bind PLC-γ1 indirectly as described below. These three sites were also tested for binding by Grb2 and the related protein Gads. Mutation of any one of the three distal tyrosine residues (171, 191, or 226) had no effect on Grb2 or Gads binding, which suggests a degree of redundancy in the system. Loss of both 171 and 191 decreased Grb2 binding, and only mutation of all three of these tyrosines blocked Grb2 binding. Gads binding proved more restricted because mutation of both 171 and 191 inhibited interaction. Since a major binding partner of Gads is SLP-76, which in turn interacts with PLC-γ1, these results account for the loss of SLP-76 binding in the double Tyr 171, 191 mutant.

The multidomain adapter SLP-76 is critical to T cell activation, and as mentioned above, cell lines deficient in this molecule have a significant defect in PLC-γ1 activation (55), a result that now can be explained. As noted above, many molecules bind to SLP-76 following phosphorylation of its N-tyrosines or via its C-terminal SH2 domain. Yablonski et al. demonstrated the significance of a proline rich region (157–223) that interacts with the PLC-γ1 SH3 domain (93). This stretch of prolines is distinct from the residues involved in Gads binding (224–265). An SLP-76-deficient variant of Jurkat was used as the recipient for SLP-76 mutants in a structure-function study. Constitutive association between PLC-γ1 and SLP-76 was dependent on the PLC-γ1 SH3 domain and an inducible increase in association that they attributed to direct and indirect interactions via LAT. They proposed that two previously defined complexes, LAT-Gads-SLP-76 and LAT-PLC-γ1, in fact interact via the binding of SLP-76 to PLC-γ1. They suggest, in other words, that a multiprotein complex nucleated at LAT contains Gads, SLP-76, and PLC-γ1, and in this complex both Gads and PLC-γ1 bind LAT. This conclusion is bolstered by another study in which they demonstrate that the functional complex of these molecules must be bound to the same LAT molecule (92).

How the individual phosphorylation sites on all PTK substrates are targeted by various PTKs in T cells is still under investigation. Nonetheless, it is clear that members of the Tec PTK family are required for full PLC-γ1 phosphorylation and activation. In support of this conclusion is the observation that deletion of the Tec PTK Itk or deletion of two Tec PTKs, Itk and Txk/Rlk, produces defects in sustained calcium elevation following TCR engagement (62, 94). Overexpression of Txk/Rlk in transgenic mice also showed enhanced PLC-γ1 phosphorylation and calcium flux (95). If regulation of PLC-γ1 activation at LAT is likely to involve Tec family PTKs, the next question is how these enzymes are targeted to this site. A full answer is not yet in hand, though the multiple domains of Itk interact with many molecules, and one or more of these interactions might be relevant to this question of targeting (61). The N-terminal PH domain of Itk or the palmitoylation of Txk/Rlk are likely to control plasma membrane localization. The TH (Tec homology) region contains a proline-rich region that interacts with a Grb2 SH3 domain. The Itk SH3 domain interacts with proline-rich regions

of PLC-γ, though the authors of the study on PLC-γ1-SLP76 interactions think that this interaction is not physiologically relevant (93). The Itk SH2 domain has been shown, by several investigators, to interact with SLP-76 (60, 61). Finally, Itk interactions with LAT have been reported, though it is not known whether this is a direct Itk SH2-mediated interaction or whether it is indirect (96). Thus, it is not now clear whether one predominant mode of Itk interaction with LAT-associated molecules predominates, or whether there are multiple mechanisms of interaction.

The consensus derived from many investigators is that a complex of LAT, Gads, SLP-76, PLC-γ1, and a Tec PTK, usually Itk, regulates PLC-γ1 activation in response to TCR signaling. These conclusions follow from characterization of cell lines and mice lacking expression of one of these proteins and from an extensive analysis of the fine specificity of multiple protein-protein interactions. This model has certain strikingly positive features, but it remains incomplete in a number of ways. Its greatest strength is the manner in which the strength of multiple protein-protein interactions is likely to be far greater than the sum of individual interactions. Ladbury and Arold have noted that the difference in affinity between specific and nonspecific SH2 and SH3-mediated individual interactions is usually less than two orders of magnitude (97). These authors note that the assembly of multimolecular complexes involving many such interactions ensures that a proper assembly must occur before signaling transpires. Such interactions can be defined as highly cooperative. Interactions of this sort are central to the generation of the multiprotein complex regulating PLC-γ1. The multiple individual interactions might zip together the functional PLC-γ1 machine. In addition such multistage events also offer great potential for regulation, as inhibition of any of the multiple steps might block assembly.

However, before defining such a putative entity as a signalosome, a term that might imply a far more stable structure of defined stoichiometry, a number of caveats and concerns must be discussed. The major issue is that LAT has been shown to bind a large number of different signaling molecules. Gads, SLP-76, and PLC-γ1, described above, are just a subset. Moreover, even these molecules have additional binding partners. Gads also binds the serine-threonine protein kinase HPK, and thus a different LAT-Gads complex may exist (51, 98). Similarly SLP-76, as described in detail below, has many additional partners. Competition for LAT can also occur. Grb2 binding to LAT is well defined, and Grb2 is capable of coordinating a number of LAT-based complexes including interactions with SOS and Cbl. The SH2 domains of Gads and Grb2 have similar binding characteristics, and both were shown to bind to two of the three distal tyrosine residues of LAT (171 and 191) following activation. It is not clear what the relative binding affinities between Gads and Grb2 are for these sites, nor has the relative stoichiometry of binding between LAT, Gads, and Grb2 been determined. Clearly, though, the issue of whether and how much Grb2 or Gads is bound to LAT would have a great impact on which other molecules are brought to LAT. It is unlikely that all possible interactions can occur at one LAT molecule at the same time because there are too many possibilities leading to competition at the same site. The competition for

interaction might direct formation of a particular complex via multiple cooperative interactions, so that the binding of Gad-SLP-76 might enhance the probability of PLC-γ1 binding. However, the presence of any particular complex might also depend on local concentrations of proteins, and these factors might depend as well on the state of the cell.

To date there has been little analysis of the heterogeneity of LAT-based complexes to address the sort of questions raised above. It should be possible to determine by immunodepletion which molecules can coexist in the same complex. The possibility that multiple, different LAT-based complexes exist is real. Tremendous variety in time and space might be observed. Such different LAT-based complexes might come together so that the sum of complexes would be the critical factor determining progression of a signal for activation. The role of complexes not mediated by LAT will likely receive much attention (99). Signaling events may require the generation of a variety of structures or complexes of complexes to coordinate the various events that occur following TCR engagement.

LAT AND THE CYTOSKELETON

To date, most of the studies of adapter molecules involved in lymphocyte signaling have focused on characterizing associated proteins and demonstrating how these interactions regulate classic biochemical signaling cascades. One such pathway involves Grb2, which brings the Ras activator SOS to receptors or to other adapter molecules such as LAT, which have been phosphorylated on tyrosine residues. Activation of Ras leads to subsequent activation in sequence of several serine-threonine kinases, which in turn are responsible for enhanced transcription of a number of genes. (4). Recently there has been increasing attention to another consequence of receptor-mediated signaling, the regulation of the cytoskeleton. Cytoskeletal changes are required for lymphocyte movement, and they accompany and control adhesive interactions that regulate cell-cell interactions (100). These issues have relevance to the early events leading to T cell activation. Of more immediate relevance for this review is the realization that T cell activation involves significant rearrangement of a number of receptors and intracellular molecules over a prolonged period following interaction of the T cell and the ligand-bearing antigen presenting cell (APC). These molecular movements create a supramolecular arrangement of receptors known as the synapse (101, 102). The dynamics of these molecular rearrangements are in part regulated by the cytoskeleton. Another significant and relevant recent breakthrough comes from the basic cell biological study of the cytoskeleton (103–106). Investigators studying several model systems have made a number of conceptual advances in understanding the dynamics of actin polymerization. Many of the molecules involved in this process are either identical to or related to molecules known to interact directly or indirectly with critical lymphocyte adapters during T cell activation.

A detailed description of the immune synapse is outside the scope of this manuscript, and the topic has been extensively reviewed. In brief, several groups

observed, using fluorescence microscopy, that TCR engagement induces a series of molecular rearrangements at the contact zone between a T cell and an antigen presenting cell (APC). Upon T cell contact with an APC bearing peptide-MHC, a central region containing T cell integrin receptors and APC integrin receptor ligands is surrounded by a ring of MHC-peptide complexes. Over minutes this pattern reverses such that the TCR-MHC contacts move to the center. This region is known as the cSMAC (the central supramolecular activation cluster). It is surrounded by the integrin receptors that define the pSMAC or peripheral supramolecular activation cluster (101, 102, 107). Additional studies have located a number of molecules within the context of the SMAC architecture. Thus, for example PKCΘ is found in the cSMAC, talin is found in the pSMAC, whereas CD43 is excluded entirely from both cSMAC and pSMAC (101, 108). More recently individual molecules have been shown to migrate in relation to the SMAC over time. The integral membrane tyrosine phosphatase CD45 is initially excluded from the cSMAC and later migrates back to it (109). These supramolecular structures are stable over hours. The importance of actin polymerization to the generation of these structures is confirmed by their disruption after blockade with cytochalasin D.

Actin polymerization can be observed using fluorescent phalloidin binding in a microscopic or flow cytometric assay. A ring of polymerized actin can be detected in T cell-APC conjugates or at the interface between T cells and beads coated with stimulatory anti-TCR antibodies. Actin polymerization has recently been observed in live cells using Jurkat T cells stably expressing EGFP-actin (110). In this assay the cells are dropped on to cover slips coated with stimulatory anti-TCR antibodies. Upon contact lamellipodial projections from the cells engage the cover slip and merge into a circumferential ring tightly adherent to the coverslip. This ring spreads outward over 3–5 min as the cell spreads on to the coverslip. The ring is formed of polymerized actin, and rapid polymerization-depolymerization reactions can be observed with fast microscopy systems. This system is also amenable to quantitation. An index of spreading can be calculated by measuring the ratio of actin clustering at the cover slip and the cell body. With this assay actin polymerization appears to be biphasic with an early peak at 3–5 min followed by a prolonged shoulder lasting 15–20 min. The assay can be used to test the effects of various inhibitors on actin polymerization.

The role of the LAT molecule can be demonstrated using this assay. Jurkat cells lacking LAT (J.CaM.2 cells) spread very poorly on coverslips coated with stimulatory antibodies, and the little actin polymerization that is seen is short lived. In parallel the assay was also used to evaluate the function of various LAT tyrosine residues and thereby the role of different pathways coupled to LAT. Interestingly no difference appeared in response by these various mutants. The same degree of inhibition of actin polymerization was observed regardless of whether LAT was absent or whether LAT lacked a PLC-γ binding site or lacked all the Grb2 and Gads binding sites. Inhibitors were used to demonstrate a calcium-sensitive component to the regulation of actin polymerization; the inhibition of actin polymerization due to lack of PLC-γ1 binding may be thus explained. The loss of Grb2 and Gads binding sites may have many consequences leading to problems with actin

polymerization. These include loss of optimal PLC-γ1 binding, as mentioned above, or failure of SLP-76 association, among many possibilities.

As described above SLP-76 is a multidomain adapter protein. The central region of SLP-76 contains a proline-rich region that mediates Gads binding. In addition to that interaction and its interaction with PLC-γ1, SLP-76 also makes contact with a number of proteins that have an impact on the cytoskeleton. The SLP-76 amino-terminus contains tyrosine residues, which after phosphorylation bind Vav and Nck (59, 111, 112). Vav too is a PTK substrate and multidomain protein consisting of PH, SH2, and SH3 binding domains and a Dbl-homology domain required for activation of Rac or cdc42, small G proteins of the Rho family (113–115). Targeted disruption of Vav produces a complex T cell deficit, including a partial block in calcium mobilization and a defect in IL-2 production. Two recent studies demonstrated that T cells from these mice also demonstrate a defect in cytoskeletal function (116, 117). Antibody cross-linking of T cells from these mice failed to produce antigen-receptor caps or patches. A failure of actin polymerization was also demonstrated in a phalloidin-binding assay. The pattern of inhibition was mimicked in these studies by treatment with cytochalasin D. More recently SMAC formation was also shown to be impaired in T cells deficient in Vav (118). The absence of Vav or, in the case of the LAT-deficient cells, the failure of Vav recruitment via Gads and SLP-76 would decrease the amount of activated Rac and cdc42 in the vicinity of the TCR and LAT. Consequences could include inadequate activation of phosphatidylinositol 4-phosphate 5-kinase, which is responsible for generating the PLC-γ1 substrate phosphatidylinositol 4,5-bisphosphate (PIP2) (119). More importantly for this discussion, lack of activated Rac could result in inadequate WASP activation.

WASP is known to bind the Nck adapter molecule, which in turn binds SLP-76 (120). WASP was first identified as the defective protein in patients with Wiskott-Aldrich syndrome (121). T cells from patients with that disease, and murine cells from animals with targeted deletion of the WAS gene, have a phenotype similar to that observed in the Vav $-/-$ animals, showing decreased calcium flux, IL-2 production, and notably, defective actin polymerization (122, 123). An explanation for this phenotype is now clear with an increased understanding of the protein WASP (104, 124). This multidomain protein contains regions capable of binding activated Rac, phospholipids such as PIP2, soluble actin-profilin complexes, and at the C-terminus, the Arp2/3 complex responsible for actin polymerization. In the resting state WASP exists in an autoinhibited state in which the GTPase binding domain interacts with the C-terminal region. Activated G proteins and phospholipids synergistically activate WASP, thus allowing the Arp2/3 complex to mediate actin polymerization. Thus LAT, by recruiting the Gads-SLP-76 complex, may bring together WASP and Vav, the enzyme needed to produce activators of WASP.

A third protein bound to SLP-76 and relevant to actin polymerization is SLAP130/Fyb (62a, 62b, 125, 126). This protein was independently isolated as a protein that binds the SLP-76 SH2 domain and as a Fyn-binding protein. A clue to the function of this protein was recently presented (127). These investigators were studying a family of WASP-related proteins and in particular were interested

in proteins that bound to a particular domain in these proteins, the EVH1 domain. Their studies revealed that SLAP-130/Fyb is one such protein. They then demonstrated that this protein colocalized to polymerized actin in the assay in which Jurkat was activated by anti-CD3 coated beads. In addition they found colocalization with WASP, Arp2/3, Vav, and the EVH1-bearing protein, Evl. Microinjection of peptides capable of blocking the interaction of SLAP130/Fyb with Evl blocked actin remodeling at the bead interface. Similarly, disruption of Arp2/3 localization blocked actin polymerization. The actual function of Evl and other VASP-related proteins in actin polymerization in T cells is not clear. However, these authors demonstrate in this study that SLAP130/Fyb is an additional required component of the actin polymerization machinery in T cells.

SLP-76 thus binds the central molecular machinery involved in actin polymerization. Phosphorylation of LAT might bring these SLP-76-bound molecules together with other enzymes such as PLC-γ1 and PI3K, which may bind LAT directly (91) to the site of TCR engagement and PTK activation. Many of these molecules themselves integrate multiple inputs as described above for WASP and previously for PLC-γ1. In turn colocalization of these molecules at LAT ensures a highly ordered process of activation. Thus a staggering amount of molecular integration is occurring at multiple levels. In this context it is worth re-asking the question of whether one LAT molecule or one SLP-76 molecule can itself bind all the possible proteins to which it could bind. At the level of the individual protein molecule this seems sterically unlikely. However, mixed populations of molecules and complexes may very well be colocalized at sites of activation. In this view, T cell activation represents the assembly of multiple and varied complexes over time and in particular locations. The challenge for future studies of adapters and T cell activation is to develop approaches and techniques capable of defining these complicated molecular interactions and dynamics.

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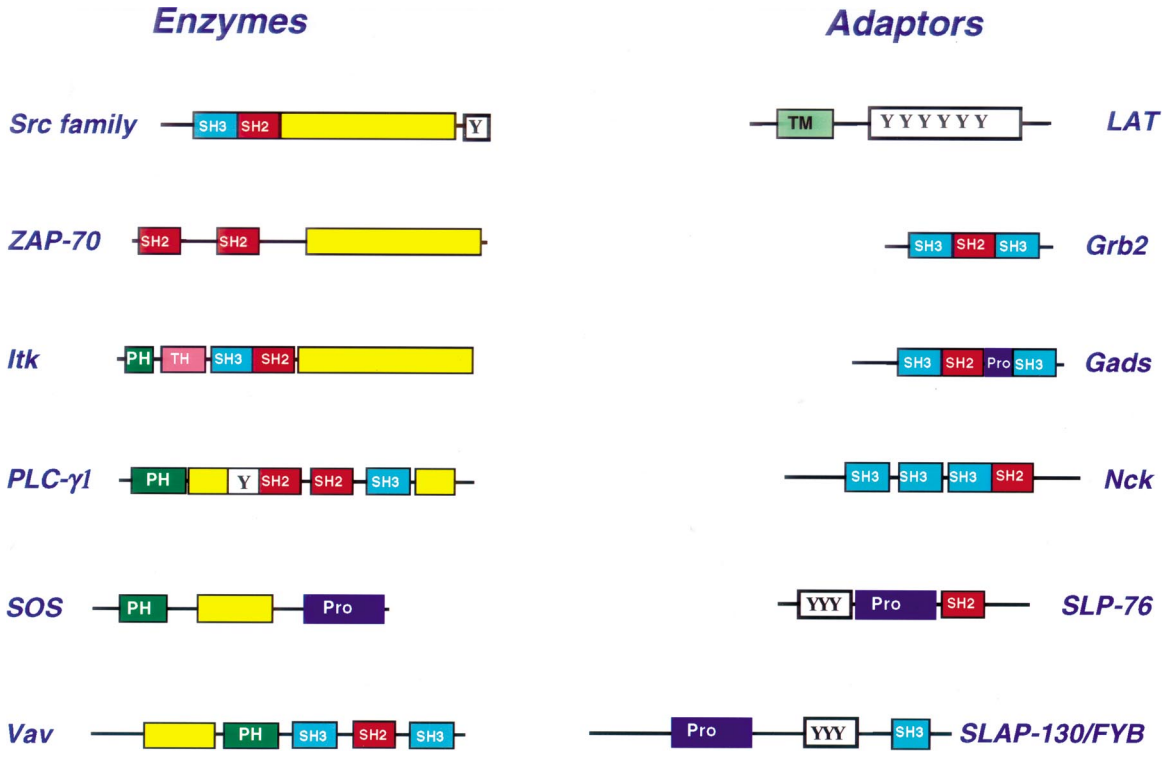

Figure 1 A selection of signaling proteins found in T lymphocytes depicted to highlight their modular structures. SH2, SH3 and PH domains are in *red*, *blue* and *green* respectively. A Tec homology domain is in *pink*, and a transmembrane domain is in *light green*. Sites of tyrosine phosphorylation are indicated with Y and proline-rich sites are indicated Pro. Domains with enzymatic function are in *yellow*.

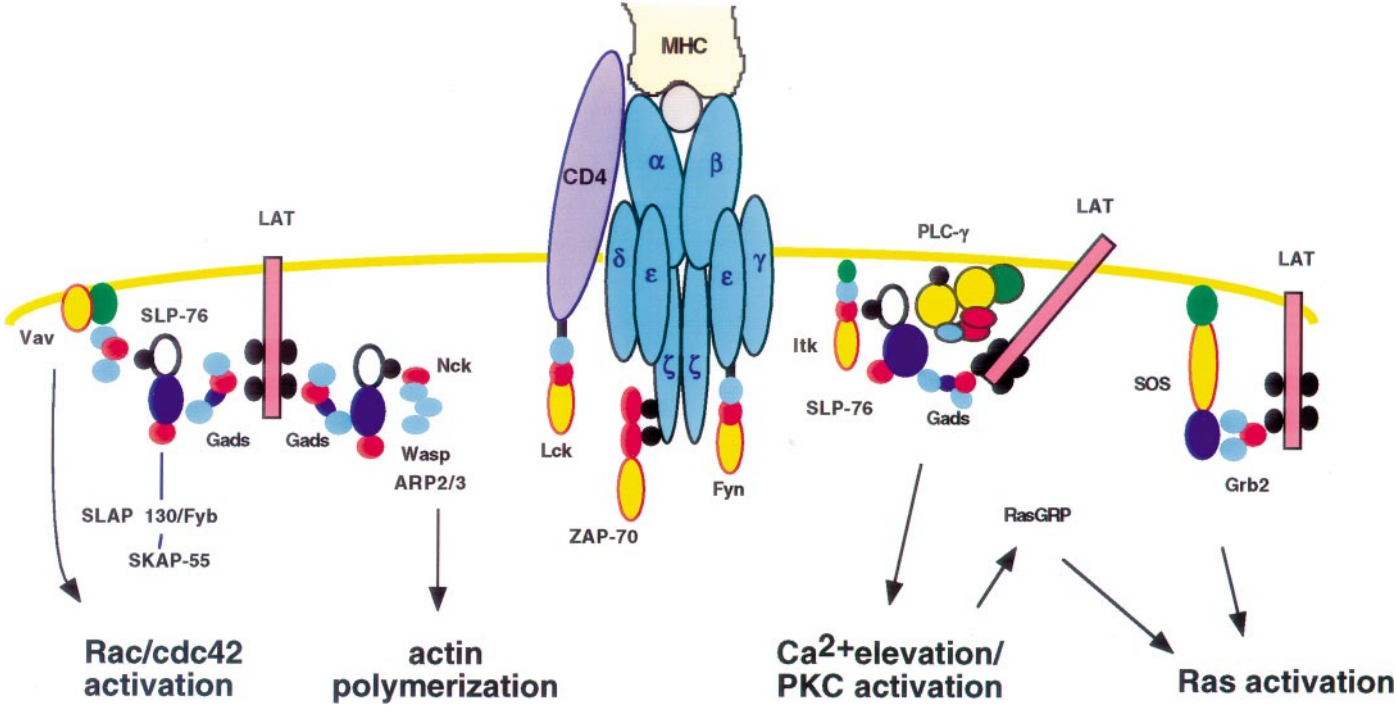

Figure 2 Signaling complexes at the T cell antigen receptor and at the LAT molecule. The TCR is depicted with its associated protein tyrosine kinases. Tyrosine phosphates are indicated with a *black ball* and the modular components of other linkers and enzymes are color coded with SH2 domains in *red*, SH3 in *light blue*, PH in *green*, proline-rich regions in *dark blue*, and enzymatic domains in *yellow*.

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