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The role of membrane-associated adaptors in T cell receptor signalling

*Weiguo Zhang† and Lawrence E. Samelson**

Engagement of the T cell receptor leads to activation of several tyrosine kinases and phosphorylation of many intracellular proteins. This is followed by Ca^{2+} mobilization and activation of multiple biochemical pathways, including the Ras/MAPK cascade, and several downstream serine/threonine kinases. Membrane-associated adaptor proteins play an important role in T cell activation by coupling TCR ligation at the membrane to distal signalling cascades. Several new membrane associated adaptors have been identified in recent years. LAT (linker for activation of T cells) is an adaptor molecule, which following its phosphorylation associates with Grb2, Gads, PLC-γ1, and other signalling molecules. The functional importance of this molecule has been demonstrated by the study of LAT-deficient cell lines and LAT-deficient mice. Two other recently identified adaptor proteins, TRIM (T cell receptor interacting molecule) and SIT (SHP2-interacting transmembrane adaptor protein), which constitutively associate with several surface molecules, bind to PI3K and SHP2, respectively, after T cell activation and might also function in the TCR signalling pathway.

Key words: Gads / Grb2 / PLC-γ1

Introduction

ADAPTOR PROTEINS ARE A family of proteins that lack enzymatic activity or a transcriptional activation domain. Many of these adaptor proteins have multiple binding domains, such as SH2, SH3, and PTB, while some adaptors have no obvious motifs, but simply contain multiple tyrosines or prolines within specific sequence contexts. The tyrosines in these adaptors, upon phosphorylation, act as docking sites for SH2 or PTB-containing proteins, while prolines serve as recognition sites for SH3 domains. Some adaptor proteins localize to the cytosol and are recruited to the plasma membrane after activation of various receptors (see accompanying review by Norian and Koretzky). Other adaptors are constitutively associated with the plasma membrane. For example, CD19 can be considered as a transmembrane adaptor protein and is involved in B cell activation.[1] CD19 has a cytosolic tail of 240 residues containing nine conserved tyrosines. Following B cell activation, these tyrosines are phosphorylated and interact with Vav, phosphatidylinositol-3 kinase (PI-3K), and Src family kinases Lyn, Lck, and Fyn. Another membrane-associated adaptor molecule, FRS2, a myristoylated protein associated with the membrane, is tyrosine phosphorylated upon activation via FGF, associates with the Grb2/Sos complex, and links FGF receptor activation to the Ras/MAPK signalling pathway.[2] This review will focus on the function of the membrane-associated adaptor protein LAT in TCR signalling. Since TRIM and SIT have not yet been extensively studied, they will only be discussed briefly here.

Early events during T cell activation

Activation via the T cell antigen receptor (TCR) triggers a cascade of intracellular biochemical events eventually leading to T cell proliferation and effector functions.[3–5] One of the earliest events is the activation of the Src family tyrosine kinases Fyn and Lck. These activated Src family kinases phosphorylate the paired tyrosines within the immunoreceptor tyrosine-based activation motifs (ITAMs) of the CD3 subunits and TCRζ chains. ZAP-70 is recruited to the activated antigen receptors via the interaction of its

*From the Laboratory of Cellular and Molecular Biology, Division of Basic Sciences, National Cancer Institute, National Institutes of Health, Bethesda MD 20892, USA; †Current address: Department of Immunology, Duke University Medical Center, Durham, NC 27710, USA. * Corresponding author.*

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tandem SH2 domains with the paired phosphotyrosines within the ITAMs. ZAP-70 is then tyrosine phosphorylated by these Src family kinases and thus activated. These activated tyrosine kinases phosphorylate a number of intracellular proteins and activate downstream signalling pathways including the Ras-MAPK pathway and Ca^{2+} mobilization. Activation of these two pathways is required for AP-1 and NFAT-mediated transcription and IL-2 production. One important question is how these two pathways are coupled to the T cell receptor during T cell activation. LAT, previously described as p36–38, is an ideal candidate to perform this coupling function.

Initial studies on p36–38

A 36–38-kD protein, p36–38, is one of the most prominently tyrosine phosphorylated proteins detected in T cells after anti-CD3 or anti-TCR antibody stimulation. Initial studies from several groups show that p36–38, a protein tightly associated with the plasma membrane, binds Grb2, PLC-γ1, and the p85 subunit of PI-3K upon T cell activation.[6–9] These molecules all have a critical function in the complex events that follow TCR engagement. Grb2, a small linker molecule containing an SH2 domain surrounded by two SH3 domains, is involved in activation of the Ras-MAPK pathway. The guanine nucleotide exchange factor and Ras activation protein, Sos, binds the Grb2 SH3 domains. In growth factor tyrosine kinase systems, the Grb2 SH2 domain binds activated and phosphorylated receptors, thus, bringing the SOS molecule to the membrane.[10] PLC-γ1 hydrolyzes phosphatidylinositol-4,5 bisphosphate to generate diacylglycerol (DAG) and inositol-1,4,5 triphosphate (IP3). These small molecules function as second messengers. IP3, via interaction with the IP3 receptor, induces intracellular Ca^{2+} mobilization and DAG activates protein kinase C[11]. The function of PI-3 kinase has just been elucidated in the past few years. It is necessary for the synthesis of the phospholipids required for targeting PH domain-containing proteins to the membrane and for activation of proteins such as the Akt kinase.[12] Interaction with these key signalling proteins suggested that p36–38 might be a critical molecule in TCR signalling. Because of this potentially important function, much effort was made to clone the cDNA for this protein. However, p36–38 proved difficult to purify, probably due to its low abundance and partial detergent-insolubility.

Cloning of LAT

We purified p36–38 from Jurkat cells activated by anti-CD3 antibodies. Membrane fractions were prepared and extracted with detergent. Phosphorylated p36–38 was then purified from those extracted membrane proteins using anti-phosphotyrosine antibodies. The p36–38 protein was subjected to microsequencing, which allowed us to clone the cDNA for p36–38. We named this protein LAT, linker for activation of T cells.[13] Sequencing of the LAT cDNA revealed that human LAT contains 233 amino acids with a very short extracellular domain, a putative transmembrane domain, and a long cytosolic tail. LAT lacks any known structural domains.[13,14] The cytosolic tail contains many negatively charged residues, explaining the difference between the apparent molecular weight of 36–38 kD and the calculated molecular weight of approximately 25 kD. One intriguing feature of this protein is the lack of a signal peptide for membrane insertion. The LAT transmembrane domain is preceded by negatively charged residues and followed by positively charged residues. This charge difference across the transmembrane domain might determine the orientation of LAT at the membrane. There are two conserved cysteines immediately after the transmembrane domain. As discussed below, these two cysteines are palmitoylated and this is required for LAT function. The cytosolic domain of LAT contains nine tyrosines conserved between humans and mice. Five of these nine tyrosines are within Grb2 binding motifs (YXN). One of these tyrosines (Y191 in human LAT) is known to be tyrosine phosphorylated based on the data from the initial microsequencing. There is also one likely PLC-γ1 binding site (Y132). Although LAT has been shown to bind p85 directly,[9] it contains no consensus p85 binding motif (YXXM).

LAT is a substrate of ZAP-70

LAT is heavily tyrosine phosphorylated upon T cell activation. In 293 cells, LAT can be tyrosine phosphorylated when Syk is co-expressed or when ZAP-70 and Lck/Fyn are co-expressed.[15] This can be explained by the fact that Syk has higher intrinsic kinase activity than ZAP-70,[16] while the activation of ZAP-70 requires phosphorylation by Lck.[17,18] ZAP-70 isolated from activated Jurkat cells can also phosphorylate LAT *in vitro* (unpublished observation). These results

suggest that ZAP-70 is likely to be one of the tyrosine kinases responsible for phosphorylation of LAT *in vivo*. This conclusion is supported by the observation that in a ZAP-70 deficient cell line, LAT phosphorylation is greatly reduced.[19] However, there is still some LAT phosphorylation in those deficient cells, suggesting that Src family kinases, and/or other tyrosine kinases, such as Itk, might also phosphorylate LAT.

Association of Grb2, PLC-γ1, and p85 with LAT

As mentioned above, the cytosolic domain of LAT contains five Grb2-binding motifs. Two of these tyrosines (Y171 and Y191) fall within the same Grb2-binding sequence (YVNV). The SH2 domain of Grb2 has been shown to bind several activated receptor tyrosine kinases, thereby coupling them to Ras activation, because the Grb2 N-terminal SH3 domain interacts with the Ras exchange factor, Sos. In T cells activated via the TCR, Sos can be coimmunoprecipitated with LAT,[15] most likely through the interaction with Grb2. Cbl is also found in the LAT complex, again likely via binding of Grb2.

Our initial studies indicate that a 76-kD phosphoprotein, likely to be SLP-76, can also be found in LAT complexes. It has been shown that the C-terminal SH3 domain of Grb2 can bind SLP-76 *in vitro*. It was thought that Grb2 might mediate the interaction between SLP-76 and LAT. However, no significant amount of SLP-76 is found to bind Grb2 *in vivo*.[20] Instead, a newly identified adaptor protein Gads (also called Mona, GrpL, or Grf40), which is homologous to Grb2, might perform this function.[21–23] Gads constitutively associates with the proline rich region of SLP-76 via its C-terminal SH3 domain. Gads also interacts with LAT via its SH2 domain. Overexpression of Gads and SLP-76 in Jurkat cells results in a synergistic augmentation of NFAT activation, demonstrating the functional importance of this interaction.[20] However, Gads might function in a different way from Grb2. The Sos exchange factor predominantly binds Grb2, not Gads.[20] The interaction of LAT with Grb2 and Gads might both be required for TCR signalling. LAT, as a linker protein, might recruit Sos to the plasma membrane via Grb2 and bring SLP-76 to the plasma membrane via Gads. It is not yet clear whether Grb2 and Gads bind the same tyrosine phosphorylated site(s). The function of SLP-76 in TCR signalling warrants further investigation (see review by Norian and Koretzky for a more detailed discussion on this adaptor protein).

The p85 subunit of PI-3K can be coimmunoprecipitated with LAT, although there is no consensus p85 binding motif (YxxM) present in LAT. Grb2 was reported to directly associate with the p85 subunit of PI-3 kinase via the interaction between the SH3 domains of Grb2 and the proline-rich sequence of p85.[24] Interaction of p85 with LAT, therefore, could be mediated via Grb2. This possibility is supported by the observation that mutating the tyrosines within two Grb2 binding motifs diminishes the binding of p85.[15] It is still possible, however, that the SH2 domain of the p85 subunit of PI3 kinase binds directly to a phosphorylated tyrosine of LAT in a motif other than the YxxM motif, such as Y171 and/or Y191.

In LAT, Y132 is in a potential PLC-γ1 SH2 binding motif. PLC-γ1 has two SH2 domains. Both SH2 domains, in the form of GST fusion proteins, bind LAT from activated Jurkat cells. *In vivo*, only the N-terminal SH2 domain associates with LAT. When the N-terminal domain of PLC-γ1 is mutated, PLC-γ1 fails to associate with LAT and PLC-γ1 tyrosine phosphorylation is significantly reduced.[25] In LAT-deficient cells, PLC-γ1 tyrosine phosphorylation is dramatically reduced, indicating that LAT is required for PLC-γ1 tyrosine phosphorylation and its function in Ca^{2+} mobilization.[26,27]

Interaction of LAT with other signalling proteins

Besides Grb2, PLC-γ1, p85 of PI-3 kinase, Cbl, SLP-76, and Gads, other signalling proteins such as Grap, Shb, and 3BP2 can also associate with LAT.[28–31] Grap is an adaptor protein also homologous to Grb2.[28,29] It is prominently expressed in hematopoietic tissues like Gads and binds Sos, LAT, Shc, and other proteins like Grb2. However, SLP-76 and Cbl, which are two GST-Grb2 interacting proteins, do not bind GST-Grap,[28] suggesting that Grap might function differently from Grb2. It is not clear whether Grb2 and Grap have a redundant role in TCR-dependent signalling.

Shb is an adaptor protein that associates with LAT and the phosphorylated TCRζ chain.[30] The interaction of Shb with TCRζ chain is mediated by the Shb SH2 domain and the interaction of Shb with LAT is mediated by the Shb PTB domain. Overexpression of wild type Shb in Jurkat cells results in increased basal

tyrosine phosphorylation of LAT and a 70-kD molecule, which is likely to be ZAP-70; whereas, overexpression of a Shb SH2 domain mutant reduces the tyrosine phosphorylation of several proteins after CD3 cross-linking. These results suggest that Shb might act as an adaptor protein to link TCR engagement with ZAP-70 activation and signalling events dependent on LAT. 3BP2 is another protein recently reported to associate with LAT via its SH2 domain.[31] Its functional importance was demonstrated by the fact that overexpression of 3BP2 in Jurkat cells induces transcriptional activation of AP-1 and NFAT. However, the role of 3BP2 in TCR signalling, at least in human T cells, still needs to be further examined as 3BP2 mRNA was not detected in human thymus by PCR, and the 3BP2 protein was not detected in Jurkat cells by Western blotting.

Function of LAT in TCR signalling

The function of LAT was initially demonstrated by overexpression of a dominant negative form with mutations at Y171 and Y191, two tyrosines likely to bind Grb2 upon phosphorylation.[15] The binding of this mutant LAT to Grb2, PLC-γ1, p85, and other signalling molecules was almost completely abolished when it was expressed in wild-type Jurkat cells. Expression of this form of LAT inhibited AP-1 and NFAT activation following TCR engagement. LAT function in TCR signalling was further demonstrated by using two LAT-deficient Jurkat cell lines.[26,27] These cell lines were generated by mutagenesis of Jurkat cells, and mutant cells defective in Ca^{2+} flux after TCR cross-linking or pervanadate stimulation were screened for LAT expression. Two of these lines, J.CaM2.5 and ANJ3, deficient in LAT expression, are defective in Ca^{2+} flux, Ras-MAPK activation, and upregulation of CD69 upon TCR engagement. Phosphorylation of PLC-γ1 is dramatically reduced compared with wild type Jurkat cells, and phosphorylation of Vav and SLP-76 is also reduced to some extent. Importantly, transfection of LAT into these cells corrects all the defects. These results clearly show that LAT is necessary for optimal phosphorylation of Vav, SLP-76, PLC-γ1 and demonstrate that LAT is essential for activation of the Ras-MAPK pathway and Ca^{2+} flux by the TCR. Our studies with LAT-deficient mice further indicate that LAT plays an important role in pre-TCR signalling and is required for T cell development[32]. Without LAT, T cell development fails to proceed past the $CD25^{+}CD44^{-}$ subset of the $CD4^{-}CD8^{-}$ stage where pre-TCR signalling is crucial.

Palmitoylation of LAT and its effect on LAT function

LAT has two conserved cysteines near the transmembrane domain. Similarly located cysteines are present in other membrane proteins known to be palmitoylated.[33] The enzyme responsible for this modification, palmitoyl acyl transferase (PAT), is membrane bound and the sequence recognition motif by PAT is not yet well defined. The palmitoylation of Lck has been shown to be important for TCR signalling.[34] In view of these data, we determined that LAT is palmitoylated when it is expressed in 293 cells or Jurkat cells.[13] When one of these cysteines is mutated, LAT is still palmitoylated, but at a reduced level. Mutation of both cysteines totally abolishes LAT palmitoylation. Deletion of the transmembrane domain of LAT also abolishes LAT palmitoylation, suggesting that this transmembrane domain might target newly synthesized LAT to the membrane where PAT is localized.

Like many palmitoylated proteins, such as the Src family kinases Lck and Fyn, LAT is enriched in the glycolipid and cholesterol-enriched microdomains, termed lipid rafts[35] (see also the accompanying review by Janes *et al*). These have also been referred to as GEMs[13] and DRMs.[36] Lipid rafts are implicated in many cellular processes, such as membrane trafficking and signal transduction. A function for rafts in T cell signalling is suggested by experiments in which lipid rafts have been disrupted by cholesterol depletion, which results in reduced protein tyrosine phosphorylation and Ca^{2+} flux after TCR ligation.[37]

The distribution of LAT in lipid rafts is not changed upon T cell activation. Although both cysteines (C26 and C29) are palmitoylated, they are not equally important. Thus, mutation of C29 leads to reduced palmitoylation but targeting of the mutant LAT to the lipid rafts and its signalling function are not severely affected. In contrast, the C26 mutation has a dramatic effect on LAT localization and function. LAT containing the C26A mutation is no longer enriched in lipid rafts. More importantly, this mutant LAT fails to be tyrosine phosphorylated upon T cell activation. When this mutant molecule is reintroduced into LAT-deficient cells, it fails to restore Ca^{2+} flux, MAPK activation, and AP-1 and NFAT activation

in these cells. These results indicate that palmitoylation and targeting of LAT into lipid rafts are necessary for LAT function. The fact that most tyrosine phosphorylated LAT is present in rafts suggests that it might be phosphorylated in this location by tyrosine kinases. TCR subunits are found in lipid rafts upon activation,[38] and it is very possible that tyrosine kinases, such as ZAP-70, are recruited to the rafts where they phosphorylate LAT. Because the majority of phosphorylated LAT is in lipid rafts, PLC-γ1, Grb2, and other signalling molecules are recruited to these microdomains by binding to LAT. Since the substrate for PLC-γ1, PIP2, is also enriched in lipid rafts,[39] this would bring PLC-γ1 to the site where it can both be activated by Lck or ZAP-70 and subsequently hydrolyze PIP2. Similarly, binding of Grb2 to LAT recruits Sos into lipid rafts where it might activate Ras.

TRIM and SIT in TCR signalling

The novel molecule TRIM is a 29/30 kD disulfide linked dimer initially identified as a protein associated with several surface molecules, such as CD2, CD3, CD4, CD5, and CD8.[40] Phosphorylated TRIM binds the SH2 domain of Lck and Fyn *in vitro*. Sequencing this protein by tandem mass spectrometry and further cloning of the cDNA encoding this molecule revealed that TRIM has a structure similar to LAT.[41] It has a short extracellular domain of eight

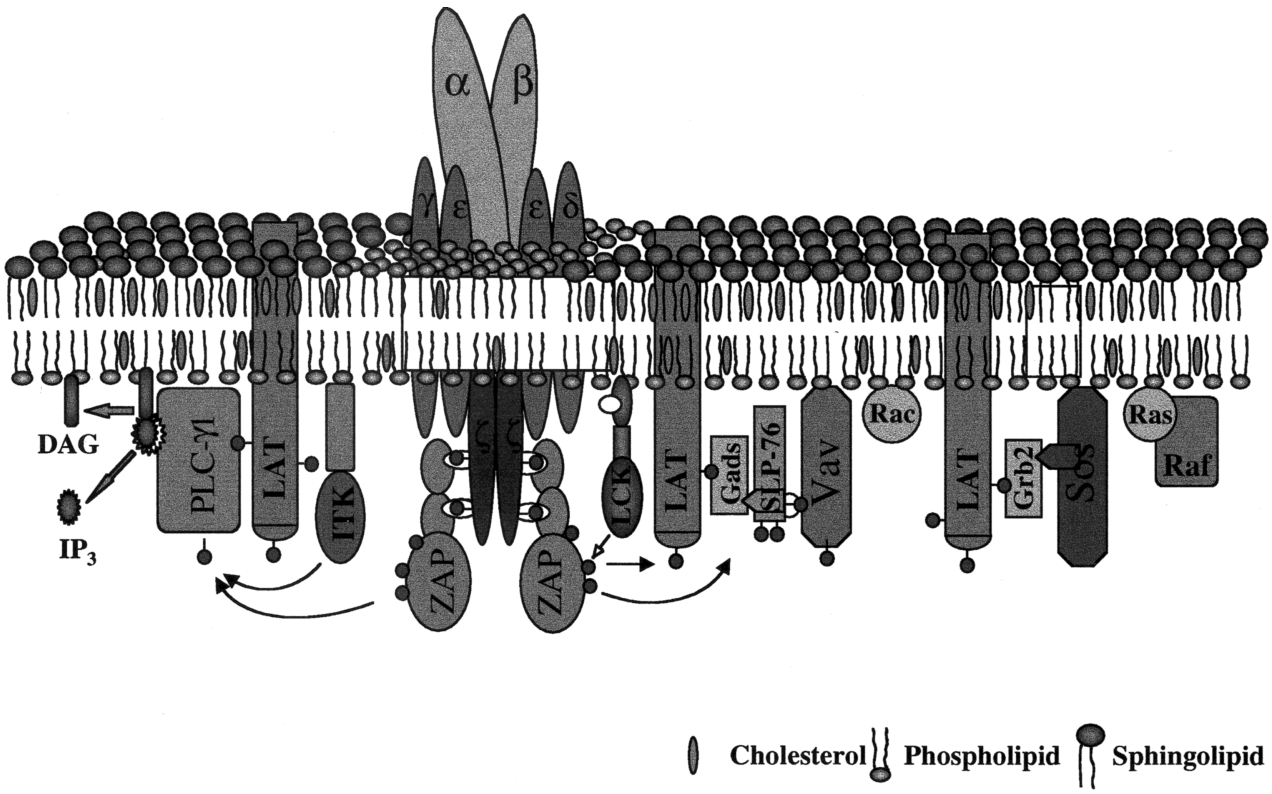

Figure 1. LAT medicated signalling complexes following T cell activation. The TCR and CD3 complex localize to the Triton-soluble fraction of the plasma membrane indicated as phospholipids. LAT, together with the Src family kinase Lck/Fyn, localizes to the glycolipid-enriched microdomains of the membrane (enriched in sphingolipids and cholesterol). Upon T cell activation, the TCR is recruited to the GEMs. Lck phosphorylates CD3 subunits and TCRζ chains (phosphate group is indicated as a red circle). ZAP-70 is recruited to the TCR and is activated by Lck/Fyn. ZAP-70 subsequently phosphorylates LAT and other signalling molecules, such as SLP-76 and PLC-γ1. Through binding to LAT, Grb2 recruits Sos to the membrane to activate Ras and Gads brings SLP-76 and possibly Vav to the membrane. Binding of LAT to PLC-γ1 brings PLC-γ1 to the GEMs where it can be activated by ZAP-70 or Itk. Note that Ras and Rac, as well as phosphatidylinositol-4,5 bisphosphate are also enriched in the GEMs.

residues, a 19-residue transmembrane domain, and a 159-amino acid cytoplasmic tail. The cytosolic domain contains several tyrosine-based signal motifs including a p85-binding YxxM motif. Its expression is restricted to hematopoietic tissues. TRIM can be phosphorylated by Fyn or Lck when it is co-expressed with these kinases in COS cells. In activated T cells, TRIM binds to p85 via its YxxM motif and also binds to Grb2 weakly. The function of TRIM is not clear. Overexpression of this molecule has no effect on TCR-mediated NFAT activation or the transcriptional activity of the IL-2 promoter, suggesting that this molecule might not be involved in the pathways leading to IL-2 production.

SIT is a glycosylated transmembrane adaptor protein. It was discovered as a protein associated with TRIM.[42] It is also a disulfide-linked homodimer like TRIM. SIT has an extracellular domain of 18 amino acids, a 20-aa transmembrane domain, and a 136-aa cytoplasmic tail which contains five potential tyrosine based signal motifs for binding of Grb2 and Src family kinases. SIT is most likely tyrosine phosphorylated by Src family kinases. There is also one immunoreceptor tyrosine based inhibition motif (ITIM) responsible for binding of the tyrosine phosphatase SHP2. Mutation of the tyrosine in this ITIM abolishes the binding of SHP2 to SIT. Overexpression of SIT in Jurkat cells inhibits TCR or PHA mediated induction of NFAT. However, overexpression of a mutant form of SIT lacking the SHP2 binding site also inhibits induction of NFAT, suggesting that the association of SIT and SHP2 is not required for this effect. The function of SIT-SHP2 interaction and the mechanism for SIT-mediated inhibition of TCR signalling require further investigation.

Concluding remarks

Membrane associated adaptor proteins are critical in the early stage of signalling since they couple the membrane-proximal signals to distal events in many receptor systems. LAT, one such membrane adaptor, upon phosphorylation by protein tyrosine kinases associated with the T cell antigen receptor, recruits critical signalling molecules to the plasma membrane, and in particular, to lipid microdomains. In this manner these signalling molecules are concentrated near the antigen receptor and near protein and lipid substrates that are present in the membrane and the rafts. LAT is required for Ras-MAPK activation and Ca^{2+} flux in TCR signalling and is also indispensable for T cell development. A model depicting TCR activation and LAT association with other signalling molecules is shown in Figure 1. Further studies will include the identification of tyrosine phosphorylation sites in LAT for binding Grb2, Gads, and PLC-γ1 and the effects of mutations of these tyrosines on signalling and development. The identification of other molecules which interact with LAT and couple its to the TCR is also an important area of future research. Two other adaptors, TRIM and SIT, might complement LAT function by directly binding p85 and SHP2, respectively. Elucidation of the function of SIT and TRIM in T cell signalling, however, will require additional experimentation. These further studies should lead to a better understanding of the mechanism of TCR signal transduction in the near future.

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