The adaptor protein LAT serves as an integration node for signaling pathways that drive T cell activation

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Abstract

T cells are essential for the adaptive immune response to pathogens. However, dysfunctional T cell activity has been implicated in numerous diseases, including the failure of organ transplants, allergic reactions, asthma, autoimmune disorders, and coronary artery disease. T cell responses to pathogens require the induction of the primary activating receptor, the T cell receptor (TCR), along with other costimulatory and adhesion receptors. Signal transduction pathways activated downstream of these receptors drive T cell responses required for the immune response and disease progression. A key question in our understanding of the mechanism of T cell activation is how signalling pathways emanating from multiple receptors integrate together to alter T cell effector functions. One integration node for intracellular signalling is the membrane-associated adaptor protein linker for the activation of T cells or LAT. Upon stimulation of the TCR and other receptors, LAT is phosphorylated at several tyrosines residues on its cytoplasmic tail. This leads to the binding of SH2-domain containing proteins and their associated molecules and the formation of large multiprotein complexes. These dynamic and highly regulated signalling complexes facilitate the production of second messengers, activate downstream pathways, induce actin cytoskeleton polymerization, and stimulate the activity of multiple transcription factors. Thus, signalling pathways from several receptors feed into LAT, which then integrates this information and selectively induces pathways critical for T cell activation and the adaptive immune response.

In multiple and diverse ways, T cells control the human immune response. Pathogen invasion triggers the innate immune response, leading to inflammation and the activation of antigen presenting cells (APCs) such as macrophages, dendritic cells, and B cells[1]. The activated APCs express peptide antigen-bound major histocompatibility complexes (MHC) on their surface, which then activate antigen-specific T cell receptors (TCR) present on the surface of $\alpha\beta$ T cells[1]. Activated T cells target specific pathogens by selectively differentiating into several unique functional subsets defined by their cytokine secretion, surface receptor expression, and transcription factor activation[2].

TCR stimulation by the peptide-MHC complex begins the process of communicating environmental information from the exterior to the interior of the T cell to alter its functional status[1]. In the cytoplasm, the activation signal is amplified through a combination of post-translational protein modifications, multi-protein interactions, and the production of second messengers. The first step in this signal transduction cascade is the phosphorylation of immune receptor tyrosine-based activation motifs (ITAMs), located on the cytoplasmic tails of the CD3 chains of the TCR complex, by the Src family kinases Lck and Fyn[3].

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Subsequently, Lck and Fyn phosphorylate and activate the Syk family kinase ZAP-70 when it is recruited to the phosphorylated ITAM motifs[3]. Active ZAP-70 then phosphorylates the downstream adaptor molecules SLP-76 and linker for activation of T cells (LAT), thus inducing the formation of the LAT and SLP-76 multi-protein complexes (Figure 1)[1]. Many of the SH2-domain containing molecules that interact with phosphorylated LAT also recruit other effectors. Structurally, these complexes form through the use of modular interaction domains to form a large, multi-protein complex that organizes and expedites signalling. Functionally, these interactions bring effector molecules close to both the membrane and other proteins where they are able to interact with their targets. The formation of a LAT-nucleated multi-protein complex leads to changes in cytoskeletal arrangement and gene expression, induces the production of second messengers, and elicits cellular responses specific to the environmental signal[1]. This results in the activation and coordination of the adaptive immune response that clears the body of the pathogen and leads to the development of a memory repertoire.

Besides the TCR, several other co-stimulatory and adhesion receptors also induce the activation of LAT. Ligation of human CD2 by its cognate ligand CD58 results in the phosphorylation of LAT and formation of LAT-mediated signalling clusters (Figure 1)[4–7]. CD2-induced LAT phosphorylation requires the activity of Lck and ZAP-70 and the activation of LAT is required for downstream functions of CD2[6]. In addition, CD5, CD9, and CD28 enhance TCR-mediated LAT phosphorylation (Figure 1)[7]. Induction of CD28 alone has also been suggested to induce LAT phosphorylation (Figure 1)[8]. The mechanism for the enhancement of LAT phosphorylation by CD5, CD9 and CD28 is unknown (Figure 1). Ultimately, signals from antigen, adhesion, and co-stimulatory receptors merge at LAT to drive downstream signalling critical for T cell effector functions. The correct spatial and temporal regulation of receptor-driven signalling complexes is vital for the ability of LAT to integrate signals from multiple receptors and then precisely control the activation of multiple downstream events. Thus, LAT serves as a critical and required integrator of activation signals that drive the adaptive immune response.

THE STRUCTURE OF THE LAT COMPLEX

SH2-domain containing proteins bind phosphorylated LAT

LAT has a very short external region, a transmembrane domain, and a long tail containing several tyrosines that are conserved among rats, mice, and humans[9]. LAT is required for T cell development; LAT $^{-/-}$ mice have an absolute block in T cell development at the DN3 stage of thymocytic development where functional TCR-mediated signaling is required for further development[10]. A recent study has examined the role of LAT in mature T cells using a tamoxifen sensitive floxed LAT allele. Deletion of LAT in mature CD8+ T cells resulted in impaired microtubule organizing center polarization and granule reorientation, resulting in unstable target cell interactions and reduced granule mediated killing[11]. Phosphorylated tyrosines 132, 171, 191, and 226 (mouse Y136, Y175, Y195 and Y235) on human LAT are required for normal T cell signalling and function. Human LAT is also phosphorylated LAT tyrosine 127 (mouse Y132), but the physiological function of the phosphoryaltion of this site is unknown[12]. Mice expressing a mutant form of LAT where all four distal tyrosines are mutated to phenylalanine have abnormal T cell development, similar to LAT$^{-/-}$ mice[13, 14]. Phosphorylated tyrosines 171, 191, and 226 bind to the SH2 domains of the Grb2 family of adaptor proteins and must be present for optimal signalling[12, 15, 16]. Mice with the three distal tyrosines mutated have very few αβ T cells and develop a lymphoproliferative disease involving γδ T cells[14]. Human tyrosine 132 phosphorylation is required for PLCγ1 association with LAT, and requires the phosphorylation of at least one of the distal three tyrosine residues in order to become phosphorylated[12, 16]. Mice expressing a tyrosine to phenylalanine point mutation at LAT Y136 show a severe but incomplete block in T cell

development early in life, and their T cells have severe signalling defects[17, 18]. In addition, later in life they develop signs of autoimmune disease and extraneous lymphocyte proliferation slightly later, potentially due to dysfunctional Tregs[17–19]. The phosphorylation of LAT Y132 is differentially regulated compared to other LAT tyrosines. The phosphorylation of LAT Y132 is slower than LAT Y191 and LAT Y132 requires the presence of at least one other LAT tyrosine for its in vivo phosphorylation[12, 20].

THE ROLE OF THE LAT MULTI-PROTEIN COMPLEX IN SIGNALING

The recruitment and activation of PLCγ1 and the Gads/SLP-76 complex

The importance of LAT's four distal tyrosine residues for recruitment of effector molecules and subsequent T cell function highlights how the structure of the LAT complex is critical in organizing signalling events. The activation of PLCγ1 is an excellent example of how the structure of the LAT complex organizes T cell signalling. PLCγ1 is phosphorylated downstream of TCR stimulation, and this activating event is dependent on both ZAP-70 and the Tec family kinase Itk[20, 21]. ZAP-70 phosphorylates LAT Y132, which then binds PLCγ1 via its SH2 domain and recruits it to the large LAT-nucleated signalling complex (Figure 2)[21]. Simultaneously, Gads, a member of the Grb2 family of adaptor proteins, binds to phosphorylated LAT Y171 and Y191 via its central SH2 domain[16, 22]. All Grb2 family members consist of a central SH2 domain with flanking SH3 domains connected by flexible linkers[23]. The primary SH3 domain ligand for Gads is SLP-76, which is a 76 KDa adaptor protein that is brought to the LAT complex via its interaction with Gads and PLCγ1[24]. SLP-76 uses its multiple domains to recruit effector molecules to the LAT complex and to stabilize the interaction of PLCγ1 with LAT (Figure 2)[24, 25]. Despite having no intrinsic enzymatic activity, SLP-76 recruitment to the LAT complex is essential for T cell function: mice with conditional SLP-76 deficiencies have no PLCγ1 phosphorylation or Ca^{2+} flux, and have defective proliferation[26]. SLP-76 also contains three tyrosines, tyrosines 113, 128 and 145, that are rapidly and transiently phosphorylated upon TCR stimulation[24]. SLP-76 knockout mice expressing a tyrosine to phenylalanine mutated version of SLP-76 produce mature T cells in drastically reduced numbers, and Jurkat E6.1 cells with the corresponding mutations are unable to proliferate or signal efficiently [24]. Itk binds to one of these phosphorylated tyrosine residues, a step that is required for Itk activation and its subsequent phosphorylation of PLCγ1 (Figure 2)[21]. SLP-76 is also phosphorylated on tyrosine 173 and this event is needed to facilitate the phosphorylation of PLCγ1 by Itk[27]. Itk has both kinase and adaptor functions: it is the kinase for PLCγ1, and it facilitates the recruitment of Vav1 to the LAT complex[21, 28]. Phosphorylated tyrosines on SLP-76 also directly interact with Vav1 and the adaptor protein Nck[24]. The association of Vav1 and Nck with SLP-76 and each otheris required for normal actin polymerization (Figure 2)[29]. SLP-76 and LAT are required for the induction of PI3 kinase activity upon TCR stimulation, although how the PI3 kinase regulatory and catalytic subunits interact with LAT and/or SLP-76 is unknown (Figure 2)[30]. The induction of PI3K increases the production of phosphoinositide 3,4,5 phosphate (PIP3) that induces the recruitment and activation of pleckstrin homology (PH) domain-containing proteins such as Vav1, PLCγ1 and Akt[31, 32].

Recruitment and function of Grb2 containing complexes

In addition to PLCγ1 and Gads/SLP-76 complexes, LAT also interacts with Grb2. The adaptor protein Grb2 binds to phosphorylated LAT tyrosines with its central SH2 domain, and the proline rich regions of various effector molecules via its N- and C-terminal SH3 domains (Figure 3)[12, 16, 22]. For example, SOS1 and SOS2 are large, multi-domain proteins brought to the LAT complex via the constitutive interaction of their proline-rich C-termini with the SH3 domains of Grb2[33, 34]. These Ras guanine nucleotide exchange factors (GEF) are widely expressed and have approximately 70% sequence identity, with the largest area

of difference occurring in the C-terminal proline rich regions (Figure 3)[34]. The current model of Ras signalling in T cell activation involves a digital signal relying on the GEF activity of both SOS1 and the DAG-activated guanine nucleotide exchange factor RasGRP1. Upon initial T cell stimulation, Ras activation is mediated by this Golgi-associated protein's GEF activity. This leads to the accumulation of GTP-bound Ras at the membrane, where the SOS1 REM domain can bind it and establish a positive feedback loop for Ras activation[35]. Activated Ras leads to induction of the MAP kinases Erk1/Erk2, which are essential for transmission of the signal from the TCR to the nucleus, and the induction of AP-1 transcription factors controlling T cell growth, differentiation, and development[36]. SOS1 and SOS2 appear to have unique roles in Ras signalling, as mice lacking SOS1 die before birth, while SOS2 deficient mice have normal growth, development, and reproduction[37]. In addition, SOS1 –but not SOS2— is required for thymocyte development [38]. The precise role of SOS2 in T cells is unclear.

The LAT complex also plays a role in the down-regulation of signalling

The LAT complex also plays a role in the negative regulation of TCR signalling. Mature CD4$^+$ T cells from mice with induced LAT deficiencies show signs of out-of-control lymphocyte proliferation[39]. This negative regulatory role is possible in several ways. First, LAT can recruit negative regulators to the multi-protein complex, such as the phosphatase SHP-1. Additionally, the E3 ubiquitin ligase c-Cbl and the inhibitory adaptor molecule Gab2 associate with LAT via Grb2 (Figure 3). Similarly to SOS1, the association of Grb2 with c-Cbl or Gab2 occurs via the interaction of the proteins' proline rich regions with Grb2's SH3 domains[40, 41]. c-Cbl mediates the ubiquitination and down regulation of stimulated TCRs, an event that occurs at the site of contact between the APC and activated T cell, and is known to ubiquitinate other proteins involved in the TCR signalling cascade, including LAT, PLCγ1, and Vav1 (Figure 3)[40, 42, 43]. Gab2 is a highly conserved adaptor protein that is able to bind multiple negative regulators in addition to Grb2 family members[44]. Following TCR stimulation, Gab2 is recruited to the LAT complex via its association with Grb2, a step required for its phosphorylation[41]. Phosphorylated Gab2 then binds the protein tyrosine phosphatase SHP-2, resulting in decreased NFAT and NFkB transcription[44]. Gab2 is also able to bind to Gads and this complex may compete with the Gads/SLP-76 complex for binding to LAT (Figure 3)[41]. By targeting LAT, this is an effective way for negative regulatory proteins to control an entire signalling node.

THE LAT COMPLEX OLIGOMERIZES INTO MICROCLUSTERS

In addition to its role in downstream signalling, Grb2 also has an essential function in the oligomerization of multiple LAT molecules into microclusters. The clustering of important signalling molecules is a phenomenon that is thought to be vital for the regulation of essential signalling events[45]. Clusters of TCR and costimulatory molecules, such as CD28 and CD2, form quickly following stimulation[4, 5, 46, 47]. LAT has also been shown to be nucleated into microclusters containing its binding partners following TCR and CD2 stimulation, and these microclusters intersect with the TCR clusters following stimulation by an actin-dependent, lipid raft-independent mechanism[5, 46–49]. The phosphorylation of LAT at the three distal tyrosine residues following TCR stimulation, and subsequent binding of Grb2, has been shown to be essential for LAT cluster formation (Figure 4)[33, 47, 49]. This requirement is likely due to the oligomerization of multiple LAT molecules, Grb2, and Grb2 SH3 domain-binding partners (Sidebar 1)[33]. One LAT molecule is capable of binding up to three Grb2 molecules at a time andtwo Grb2 molecules bind one SOS1 or c-Cbl proline rich domain (Figure 4)[33]. The ability of LAT and Grb2 to interact with multiple molecules facilitates the formation of LAT signaling clusters [33, 50]. Although thought to be vital for signal transduction, the precise role of LAT clusters in T cell signal transduction has eluded investigators for a decade. Houtman and colleagues observed a slight decrease in calcium

flux following a low level of TCR stimulation in cells unable to cluster LAT[33], but other biological functions for LAT clusters have not been identified. A major experimental focus in the future will be to uncover the role that the oligomerization of LAT plays in its ability to integrate receptor signals and properly activate multiple downstream signalling pathways.

SIGNALING DOWNSTREAM OF LAT CLUSTERS

The phosphorylation of LAT and the subsequent formation of multi-protein signalling complexes lead to the induction of several downstream events. LAT activation leads to the stimulation of proteins, such as SLP-76 and Vav1, that drives changes in the actin cytoskeleton (Figure 2). The actin cytoskeleton is important for many aspects of T cell signalling. TCR, costimulatory, and adhesion receptor stimulation leads to a rapid response that includes morphological changes to the cell. Actin cytoskeletal polymerization is important for T cell interaction with the APC, a process that requires the formation of lamellipodia through the polymerization of filamentous actin[58]. Additionally, following stimulation by the APC, microclusters of TCRs, co-receptors, or signalling molecules form due to the rearrangement of protein islands in the plasma membrane: this step is dependent on the rearrangement of the cortical actin mesh that supports the plasma membrane[1, 59]. At the site of T cell-APC contact, actin is also important for the down-regulation of signalling, and moves activated TCR microclusters into an area where they are endocytosed[60]. Defects in actin polymerization lead to severe immunodeficiency, as seen in Wiskott-Aldrich syndrome (WAS). WAS is a genetic disease where patients lack the actin nucleation protein WASp, and most die by 10 years of age due to infections or autoimmune disease[58].

The induction of LAT also controls proteins that drive second messenger production, spreading the signal from the cell surface through the cytoplasm. For example, LAT and SLP-76 are needed for the TCR-induced activation of PI3 kinase, which produces phosphoinositide 3,4,5 phosphate (PIP_3). PIP_3 interacts with molecules' PH domains, recruiting signalling molecules to the membrane and keeping signalling pathways primed[32]. In this way, the phosphoinositide pathway is important for signalling events such as regulating Ca^{2+} flux, Rac activation, and Erk1/Erk2 phosphorylation (Figure 2)[31]. Similarly, phosphoinositide 4,5 phosphate (PIP_2) is cleaved by PLCγ1 following TCR stimulation to form the second messengers IP_3 and DAG (Figure 2)[32]. DAG is membrane-associated and recruits proteins to the plasma membrane and organelle membranes via their cysteine-rich regions, leading to Ras and NFκB transcription factor activation[32]. IP_3 mediates store-operated calcium entry by binding to the IP_3 receptor on the endoplasmic reticulum, which triggers the release of calcium stores into the cytoplasm. This subsequently leads to calcium release-activated calcium channels opening in the plasma membrane and results in a sustained increase in Ca^{2+} concentration[32, 61]. Sustained, increased Ca^{2+} concentration is required for cytokine gene expression, motility, and immunological synapse formation in T cells[61].

Finally, signalling from LAT results in changes to gene expression by controlling the activation of important transcription factors such as NFκB, NFAT, and AP-1 (Figure 2 and Figure 3). The nearly ubiquitously expressed transcription factor NFκB regulates the expression of genes related to regulation of the immune response, production of cytokines, proliferation, and induction of apoptosis and cell survival pathways in T cells[62]. NFκB activation is induced when its bound inhibitors are phosphorylated and degraded in response to cell stimulation or other cell stresses, allowing NFκB to translocate to the nucleus[62]. Nuclear Factor of Activated T cells (NFAT) is induced by increased levels of calcium in the cell and regulates T cell activation, development, differentiation, and self-tolerance[63]. NFAT promotes or represses transcription of specific genes based on its binding with other transcription factors[63]. AP-1 transcription factors are the main partners of NFAT following

TCR stimulation-induced Ras activation[63]. By controlling the transcription of genes for cytokines such as IL-2, AP-1 and its partners control T cell proliferation and differentiation[62].

Conclusion

The ultimate effect of signalling cascades induced by the activation of the TCR, costimulatory and adhesion receptors is changes in cell morphology and gene expression, leading to specific outcomes for the T cell. These outcomes are critical for immune system homeostasis, the activation of the adaptive immune response and ultimately the clearance of pathogens. One large knowledge gap in our understanding of the molecular mechanism of T cell activation is where signals emanating from multiple receptors integrate together to alter T cell function. LAT is a key integration node for signalling pathways emanating from multiple receptors. Multiple receptors, including the TCR, CD2 and CD28 can all directly induce the activation of LAT. Similarly, CD5, CD9 or CD28 can further enhance TCR-induced LAT phosphorylation. The induction of LAT phosphorylation leads to the formation of dynamic signalling complexes containing PLCγ1, Grb2, Gads, SLP-76, and their ligands, which then drive downstream signalling to second messenger production, actin polymerization, and transcription factor activation. We have a basic knowledge of which receptors activate LAT, the mechanism of its phosphorylation, and which ligands interact at specific sites on LAT. However, we are just beginning to understand how the composition of specific LAT complexes is temporally and spatially regulated by the TCR and other receptors, and how LAT integrates this information to selectively drive specific downstream pathways. Examining the complexity of how LAT is activated and how LAT-mediated complexes are dynamically regulated will be an active area of research for the coming years.

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Sidebar 1: SH3 domain interactions

Grb2 is a ubiquitously expressed and evolutionarily conserved adaptor protein[51]. Although extensively studied, the exact mechanism for Grb2 binding to its full length SH3-domain partners is unclear. The interaction between Grb2 and SOS1 has been the subject of the most vigorous research. This has led to this interaction being considered a model SH3 domain-ligand interaction[52].

Four possible Grb2 SH3-domain binding sites with the consensus amino acid sequence of PXXPXR have been identified in the proline rich C-terminal tail of SOS1[52, 53]. Numerous studies have examined the relationship between Grb2 and this motif using short 10–15 amino acid long peptides and several different techniques, including isothermal titration calorimetry, surface plasmon resonance, and NMR. These studies indicated that the interaction of the whole Grb2 molecule with short peptides containing one PXXPXR motif has a low affinity, with reported dissociation constants (Kd) ranging from 2 μM to over 200 μM[54–56]. Interestingly, the interaction of full SOS1 with Grb2 appears be at least 100-fold stronger than these peptide-based studies would indicate, meaning that these studies may not be indicative of what is occurring in the cell[33, 57]. While informative, the use of short peptides and/or only one of the two Grb2 SH3 domains in these previous studies leaves the physiological relevance of the peptide-SH3 domain interaction ambiguous.
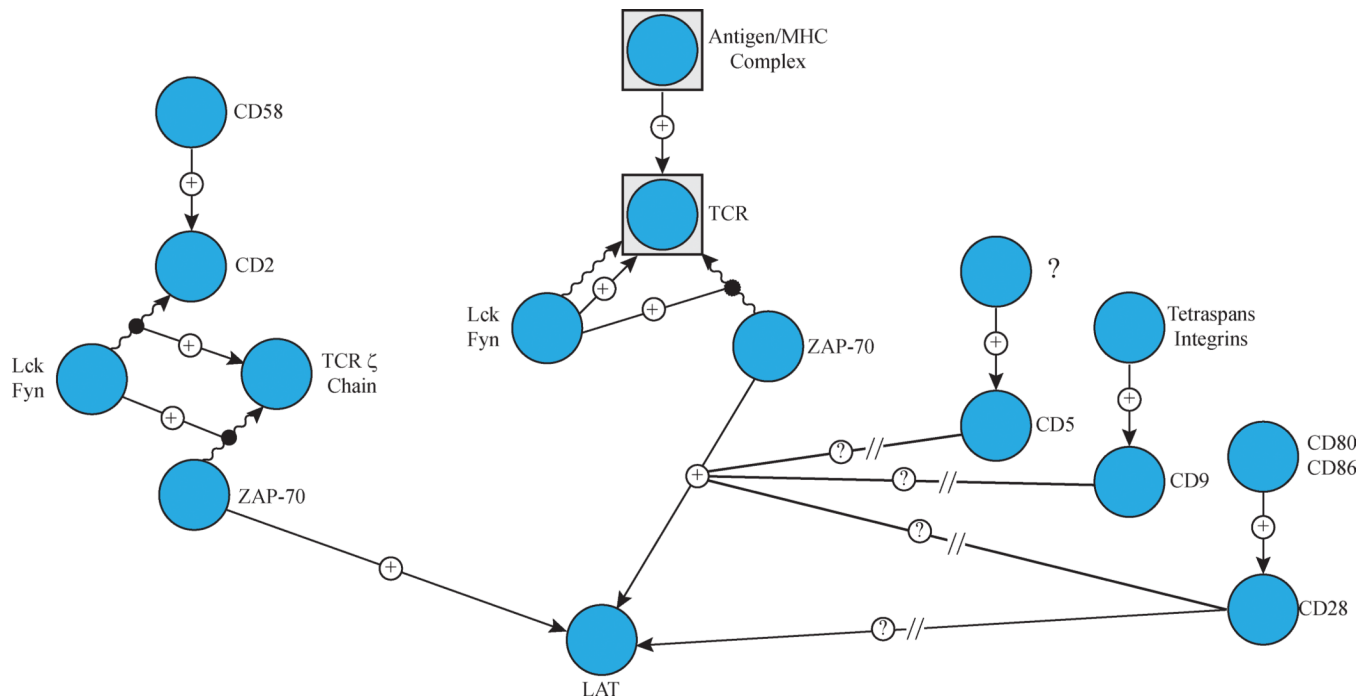
Figure 1. Receptor Mediated Induction of LAT Phosphorylation
TCR and CD2 induction results in the receptor localization and/or activation of the Src
family kinases Lck and Fyn. The kinases then phosphorylate ITAM motifs on the TCR. This
leads to the recruitment and activation of ZAP-70 and this kinase subsequently
phosphorylates LAT. Activation of CD5, CD9 and CD28 enhance TCR-mediated LAT
phosphorylation via an unknown mechanism. CD28 induction alone is also capable of
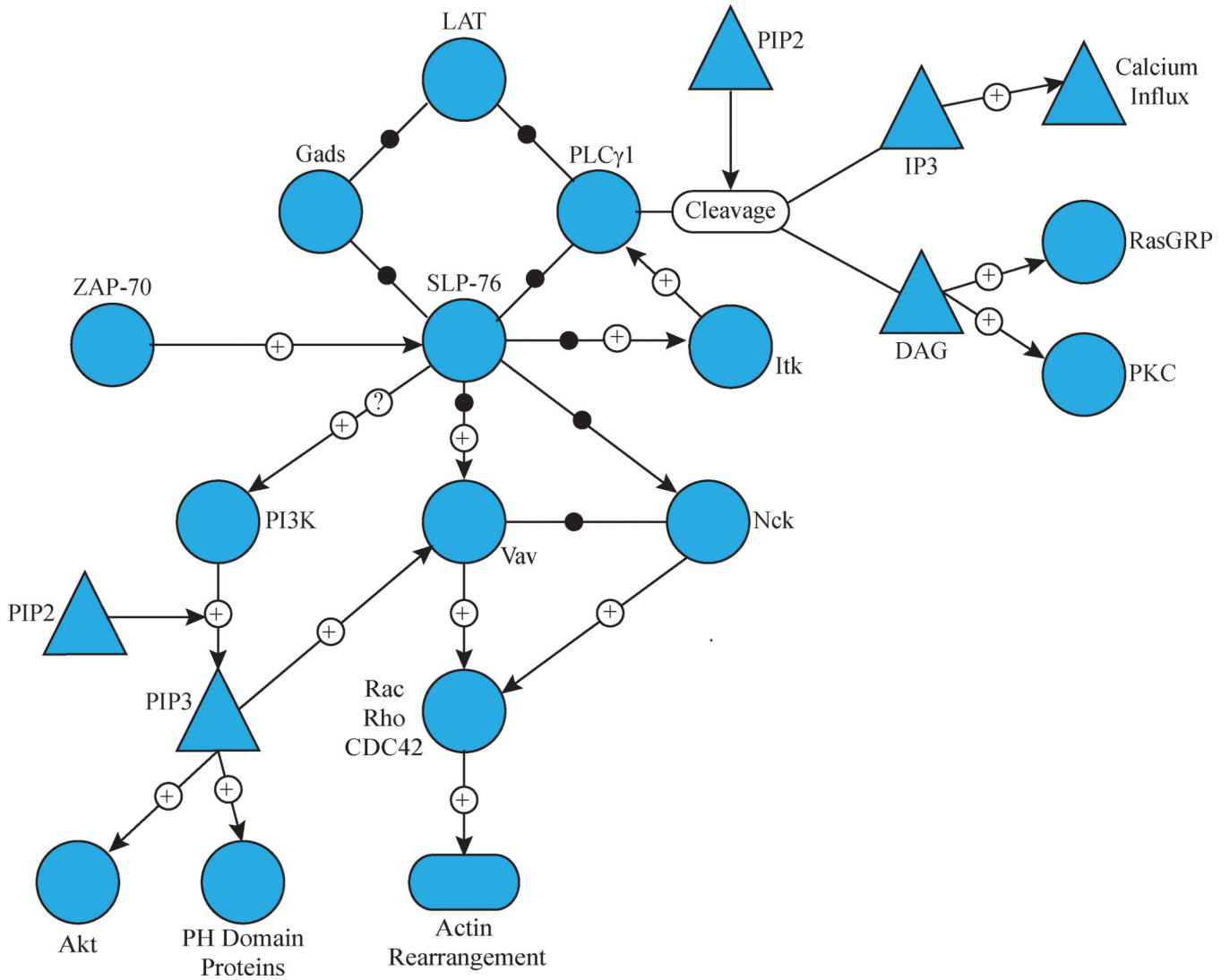inducing LAT phosphorylation by an uncharacterized mechanism.

Figure 2. Recruitment of Complexes Containing PLCγ1, Gads and SLP-76 to LAT
LAT phosphorylation results in the binding of PLCγ1 to LAT Y132 and Gads to LAT Y171
or Y191. Gads also binds to SLP-76, which stabilizes the interaction of PLCγ1 with LAT.
PLCγ1 cleaves PIP2 into IP3, which induces calcium influx, and DAG, which activates
RasGRP and PKC isoforms. SLP-76 is phosphorylated by ZAP-70 and subsequently binds
ITK, which phosphorylates and activates PLCγ1, and Vav and Nck, which are critical for
actin polymerization. SLP-76 is also required for the induction of PI3 kinase, resulting in the
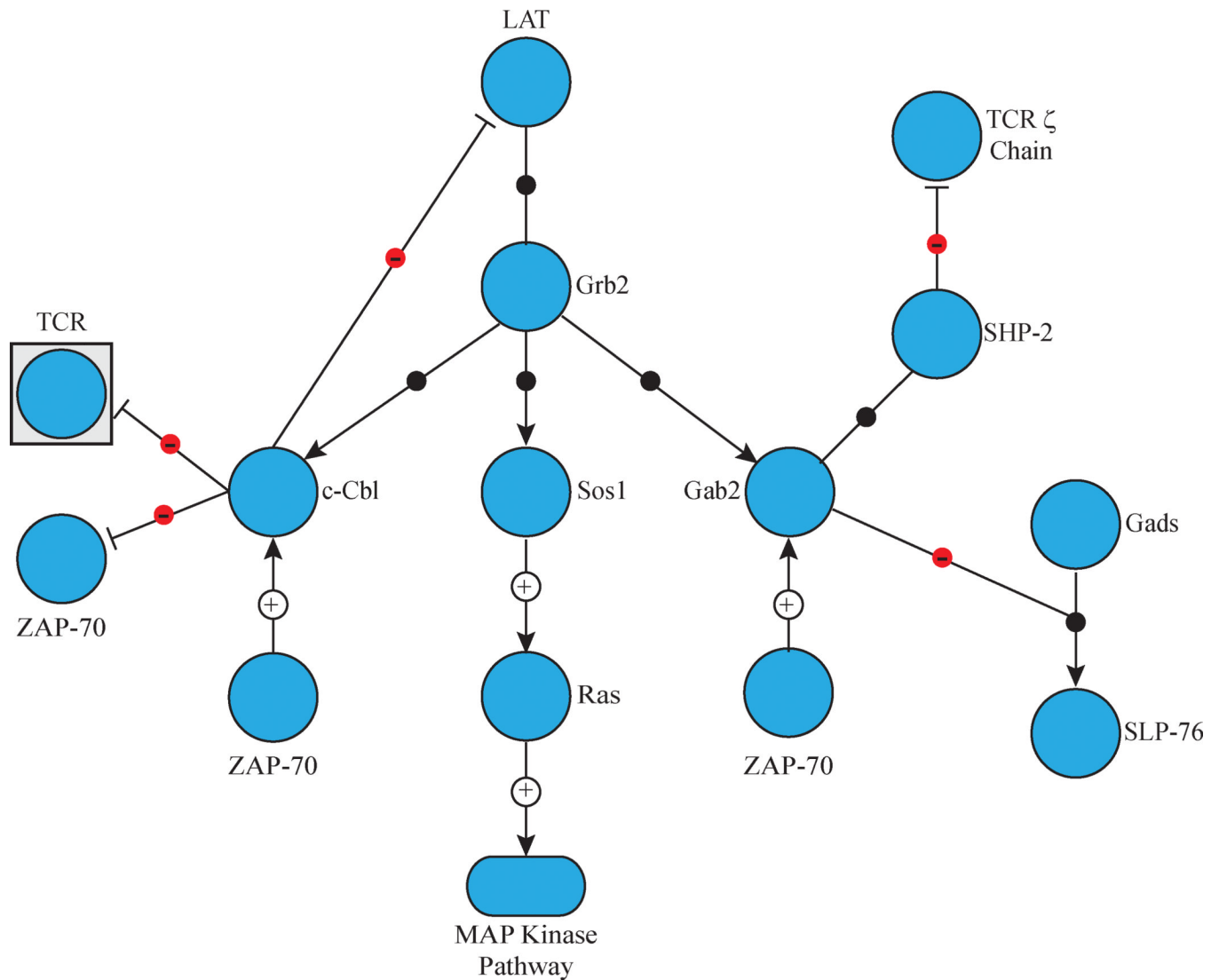induction of numerous downstream pathways.

Figure 3. Recruitment of Grb2-Mediated Complexes to LAT
LAT phosphorylation induces the binding of Grb2 to LAT Y171, Y191 and Y226. Grb2 simultaneously recruits several SH3 domain ligands to LAT. These include Sos1 and Sos2, which activated the MAP kinase pathway, c-Cbl, which facilitates the ubiquitination of multiple signalling proteins, and Gab2, which recruits and activates the phosphatase SHP-2 and inhibits the Gads/SLP-76 complex.
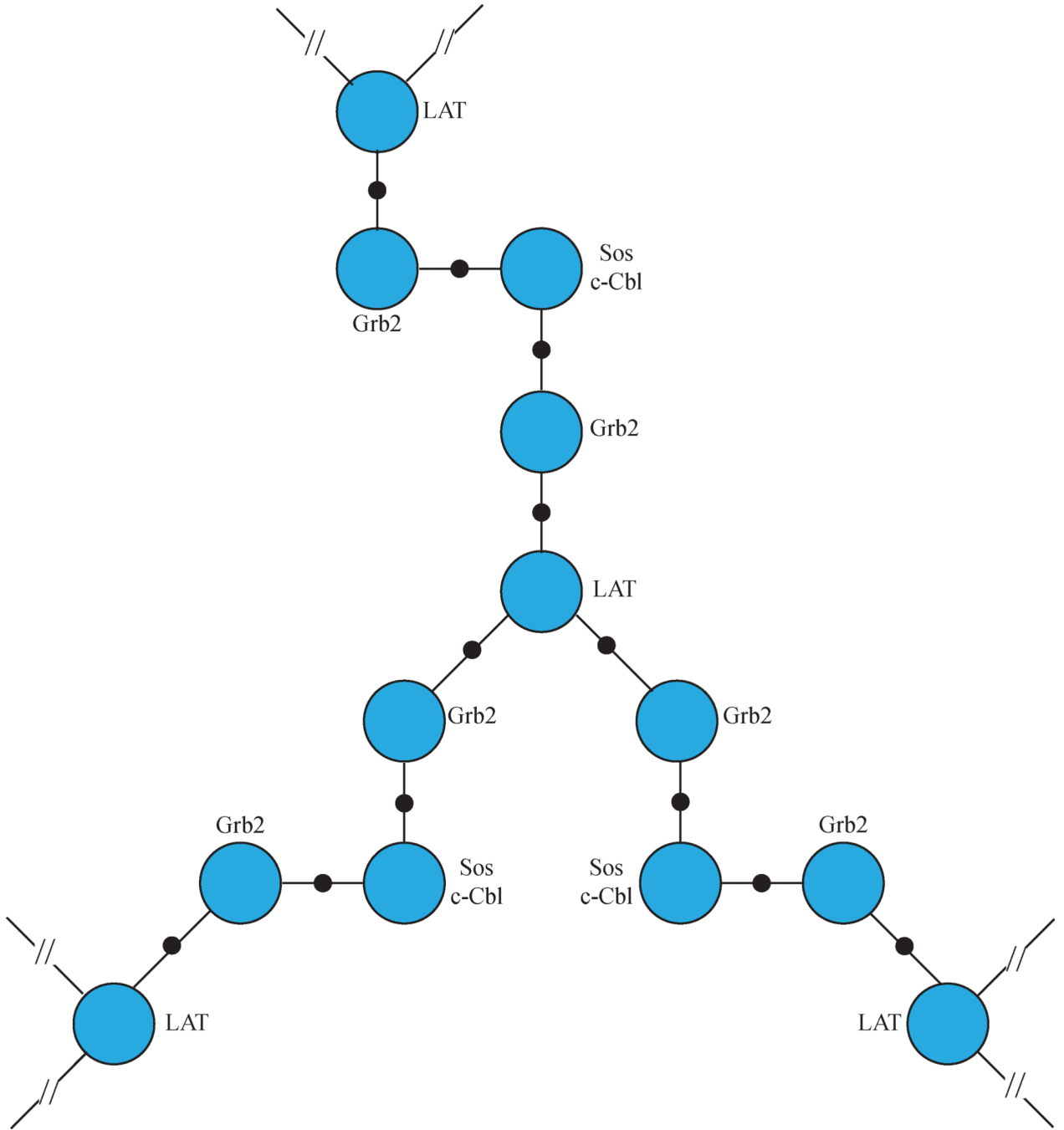
Figure 4. Grb2/SH3 Domain Ligand Complexes Facilitate LAT Clustering
Phosphorylated LAT is capable of simultaneously binding three Grb2 proteins. At the same
time, Grb2 is dimerized by its SH3 domain ligands Sos1 and c-Cbl. The ability to bind
multiple Grb2 molecules and the dimerization of Grb2 leads to the formation of a lattice of
LAT that drives LAT clustering.