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T-cell receptor signaling to integrins

Summary: Integrin adhesion receptors are critical for antigen recognition by T cells and for regulated recirculation and trafficking into and through various tissues in the body. T-cell receptor (TCR) signaling induces rapid increases in integrin function that facilitate T-cell activation by promoting stable contact with antigen-presenting cells and extracellular proteins in the environment. In this review, we outline the molecular mechanisms by which the TCR signals to integrins and present a model that highlights four key events: (i) initiation of proximal TCR signals nucleated by the linker for activated T cells (LAT) adapter protein and involving Itk, phospholipase C- γ 1, Vav1, and Src homology 2 domain-containing leukocyte-specific phosphoprotein of 76 kDa; (ii) transmission of integrin activation signals from the LAT signalosome to integrins by protein kinase (PK) C and the adapter protein, adhesion and degranulation-promoting adapter protein; (iii) assembly of integrin-associated signaling complexes that include PKD, the guanosine triphosphatase Rap1 and its effectors, and talin; and (iv) reorganization of the actin cytoskeleton by WAVE2 and other actin-remodeling proteins. These events coordinate changes in integrin conformation and clustering that result in enhanced integrin functional activity following TCR stimulation.

Keywords: integrin, signal transduction, adapter protein, T-cell receptor, T-cell activation, adhesion

Introduction

An efficient adaptive immune response requires T-cell recirculation through lymphoid tissue and a highly sensitive and specific ability of T cells to respond to low amounts of peptide antigen following interaction with antigen-presenting cells (APCs). Adhesive interactions that are critical for T-cell trafficking and antigen recognition are facilitated by integrins, a large family of $\alpha\beta$ heterodimeric cell surface proteins that promote the interaction of cells with other cells and extracellular matrix proteins (1). Integrins such as the β 2-integrin leukocyte function-associated antigen-1 (LFA-1) (α L β 2) and the β 1-integrin α 4 β 1 promote T-cell interactions with endothelial cells that are critical for T-cell recirculation and recruitment into inflammatory sites. In addition, LFA-1 facilitates antigen-dependent interactions of T cells with APCs and target cells. Other integrins, such as α E β 7, α 1 β 1, and α 2 β 1,

have been proposed to have unique and specific functions in the retention of T cells in specific anatomic locations (2, 3). Thus, integrins participate in T-cell activation by promoting T-cell contact with APCs during antigen recognition and facilitating the migration and retention of T cells in different micro-environments in the body.

Prior to antigen stimulation, T lymphocytes exhibit a high rate of motility that facilitates rapid movement into and through secondary lymphoid organs (4, 5). Consequently, integrin receptors expressed on unactivated T cells exhibit a low basal state of functional activity that does not promote strong adhesion between T cells and relevant counterreceptors and extracellular matrix ligands. However, the functional activity of integrins is rapidly enhanced following stimulation of the antigen-specific CD3/T-cell receptor (TCR) complex. Thus, TCR stimulation results in robust increases in integrin-mediated adhesion of T cells to purified integrin ligands and to antigen-laden APCs *in vitro* (6–8). The interface between a T cell and an APC is characterized by the immunological synapse, where an outer ring defined by LFA-1 and the integrin-associated protein talin surrounds clustered TCRs (9, 10). Furthermore, the motility of T cells is dramatically reduced following TCR signaling *in vitro* (11), and T cells exhibit a period of enhanced interaction with APCs in lymphoid organs during antigen stimulation *in vivo* (4, 5). Thus, integrin activation provides a mechanism by which T cells can enhance their interactions with the extracellular environment in order to receive and integrate extracellular signals critical for optimal antigen-dependent T-cell activation and differentiation (1).

Integrin activation initiated by TCR signaling occurs within minutes following T-cell stimulation and is one of the earliest detectable changes in the behavior of T cells following activation. Increased integrin-mediated adhesion following TCR stimulation does not require changes in levels of integrins on the T-cell surface. Consequently, alterations in integrin function following TCR signaling involve either qualitative alterations in integrin conformation or enhanced clustering of integrins on the T-cell surface. Changes in the redistribution of integrins in response to TCR signaling have been well documented, the most notable examples being the movement of the LFA-1 and $\alpha 4 \beta 1$ integrins to the periphery of the contact site between an activated T cell and an APC (9, 10, 12). However, changes in integrin conformation in the context of antigen stimulation of T cells, particularly *in vivo*, are incompletely understood. Analysis of integrin activation in platelets and analysis of integrin structure and function *in vitro* have clearly shown that integrins can adopt multiple con-

formations of differing affinities and that activation can rapidly induce changes in integrin conformation (8, 13–15). In the case of T cells, analysis of LFA-1 function using fluorescence photobleaching recovery and single-particle tracking has shown that LFA-1 mobility (regulated by cytoskeletal attachment) and conformation are clearly linked (16). These experiments indicate that multiple conformations of LFA-1 exist on the T-cell surface, each with a unique diffusion profile. On resting T cells, both inactive and active conformations of LFA-1 are mobile, while intermediate conformations of LFA-1 are tethered to the cytoskeleton and thus are immobile. Stimulation of T cells with the phorbol ester phorbol-12-myristate-13-acetate (PMA), which activates protein kinase (PK) C, releases cytoskeletal constraints on the intermediate LFA-1 receptors, thereby promoting receptor–ligand interactions. In contrast, LFA-1 in the active conformation becomes immobile following stimulation to stabilize adhesion, and this cytoskeletal attachment is further enhanced following ligand engagement. Thus, changes in the distribution of these various conformations of LFA-1 between mobile and cytoskeleton-attached immobile pools provides a dynamic mechanism by which T cells can regulate their adhesive potential.

The ability to rapidly modulate integrin function in response to external signals is common to many hematopoietic cells. In addition to antigen recognition by T cells, integrin activation is also central to the aggregation of platelets during wound healing, homeostatic recirculation of lymphocytes, and the migration of leukocytes and lymphocytes into inflammatory sites (8, 13). Thus, there is considerable interest in defining the mechanism by which cells rapidly modulate the functional activity of integrins in response to activation signals. Recent studies in T cells have elucidated intracellular signaling cascades that directly connect initial signaling events following TCR stimulation to changes in the association of signaling intermediates with integrins that mediate changes in integrin function. In this review, we discuss this new information on TCR signaling to integrins in the context of a model that highlights four key signaling events: (i) initiation of integrin activation signaling cascades by TCR-proximal signaling events nucleated by the linker for activation of T cells (LAT) adapter protein; (ii) transmission of signaling cascades to integrins by signaling effectors such as adhesion and degranulation-promoting adapter protein (ADAP) and PKC; (iii) assembly of signaling complexes containing the serine–threonine kinase PKD, the guanosine triphosphatase (GTPase) Rap1, and talin by integrin cytoplasmic domains; and (iv) reorganization of the actin cytoskeleton by WAVE2 and other signaling intermediates triggered by TCR engagement.

Initiation of integrin activation by the TCR: the LAT signalosome

TCR stimulation promotes rapid activation of the Src family tyrosine kinases p56^{lck} (Lck) and p59^{fyn-T} (Fyn), leading to phosphorylation of immunoreceptor tyrosine-based activation motifs on the TCR ζ and CD3 chains. These sites are then recognized by Src homology (SH) 2 domains of the related kinases Syk (spleen tyrosine kinase) and ZAP-70 (ζ -associated protein of 70 kDa), which in turn are activated by Lck-mediated phosphorylation. These recruited and activated Syk family kinases then phosphorylate a number of downstream target sites, including tyrosines on the transmembrane adapter protein LAT (Fig. 1).

LAT is sequestered to the inner leaflet of the plasma membrane by a glycosylphosphatidylinositol anchor, and cytoplasmic proteins nucleating with LAT following TCR stimulation coordinate various outcomes of TCR signaling (17). Genetic deletion of LAT in mice severely impairs TCR signaling, resulting in arrested T-cell development (18). Thus, the absence of mature T lymphocytes from LAT-deficient mice has impeded analysis of the role of LAT in regulating integrin

function. However, analysis of LAT-deficient Jurkat T cells indicates that LAT is specifically required for TCR-mediated increases in $\beta 1$ -integrin function (19). This study also showed that ZAP-70-dependent phosphorylation of tyrosine residues 171 and 191 in the LAT cytoplasmic domain is critical for the activation of $\beta 1$ integrins on Jurkat T cells. LAT tyrosines 171 and 191 can bind or recruit (directly or indirectly) a number of key signaling proteins, including phosphatidylinositol-3-kinase (PI3K), the tyrosine kinase Itk, and phospholipase C- $\gamma 1$ (PLC- $\gamma 1$) (17, 20). All three of these LAT-associated proteins also play critical roles in TCR signaling to integrins.

Itk

The Tec family tyrosine kinase Itk is an important link between LAT and integrin activation (21, 22) (Fig. 1). Lck-mediated activation and PI3K-dependent membrane recruitment downstream of TCR stimulation coordinately regulates Itk function (23). Itk couples PI3K and Lck to downstream activation of integrins, as integrin activation triggered by TCR-independent activation of PI3K and Lck can be blocked by mutant Itk constructs (24). In addition, disruption of Itk signaling blocks TCR signaling to $\beta 1$ integrins (24). Murine T cells deficient in Itk also show impaired antigen-dependent T-cell conjugate formation with APCs (25) and impaired adhesion to purified integrin ligands (26). Thus, TCR signaling to both $\beta 1$ and $\beta 2$ integrins requires Itk. Reorganization of the actin cytoskeleton following TCR stimulation is also impaired in Itk-deficient T cells (25). This is consistent with a function for Itk in promoting the interaction of LAT with the guanine nucleotide exchange factor (GEF) Vav1 (27), which facilitates activation of GTPases that trigger downstream polymerization of actin (28).

Phospholipase C- $\gamma 1$

Activated PLC- $\gamma 1$ hydrolyzes phosphatidylinositol-4,5-bisphosphate into inositol-1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ then promotes the release of intracellular calcium stores, while DAG activates PKC. Importantly, inducible phosphorylation of PLC- $\gamma 1$ by TCR-dependent tyrosine kinases is critical for PLC- $\gamma 1$ activation, and optimal PLC- $\gamma 1$ activation requires SH2 domain-mediated interaction of PLC- $\gamma 1$ with phosphorylated LAT. Itk in particular is important for optimal PLC- $\gamma 1$ phosphorylation and activation, and Itk-deficient T cells show impaired TCR-dependent calcium signaling (29, 30). The link between PLC- $\gamma 1$ and integrin function has now been established using PLC- $\gamma 1$ -deficient Jurkat cells (31). In the absence of PLC- $\gamma 1$, TCR-triggered LFA-1-mediated adhesion to intercellular adhesion molecule-1 (ICAM-1) and activation of the Rap1 GTPase (see below) are impaired. These signals can be rescued

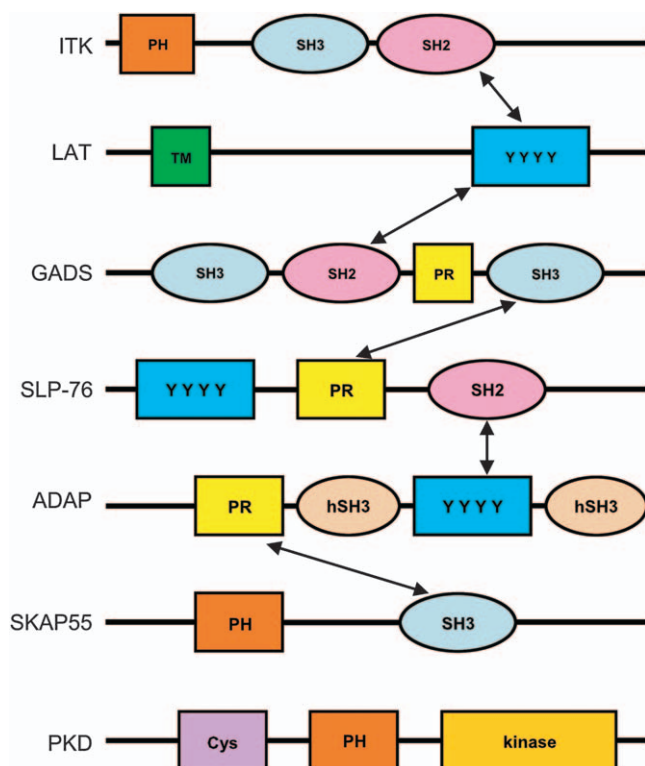


Fig. 1. Schematic diagram depicting several key enzymes and adapter proteins that regulate integrin activity downstream of the TCR. Domain abbreviations: YY (phosphotyrosine domain); PR (proline rich); SH3 (Src homology 3); hSH3 (helical SH3), SH2 (Src homology 2); TM (transmembrane); Cys (cysteine-rich domain).

following re-expression of PLC- γ 1 (32). However, stromal cell-derived factor 1/CXCL12 signaling to Rap1 occurs independently of PLC- γ 1 expression. This finding indicates that PLC- γ 1-dependent activation of Rap1 and integrin function is mechanistically distinct from chemokine-dependent activation of Rap1.

SH2 domain-containing leukocyte-specific phosphoprotein of 76 kDa

LAT is not alone in its indispensable role for TCR signaling. Mice deficient in the LAT-associated adapter protein SH2 domain-containing leukocyte-specific phosphoprotein of 76 kDa (SLP-76) also fail to produce mature peripheral T cells (33, 34) as a result of a block in T-cell maturation at the double-negative-3 stage of T-cell development in the thymus. It is perhaps surprising that a related signaling complex downstream of the TCR, a subset of the LAT signaling complex, also plays such an obligate role in T-cell activation. A number of other proteins can nucleate with SLP-76 in the LAT complex, in particular the adapter protein GADS, which binds and recruits SLP-76 through its proline-rich domain (35) (Fig. 1). Mechanistically, the role of LAT/SLP-76 has been investigated by the creation of LAT/SLP-76 chimeras consisting of the LAT membrane anchor fused to SLP-76 (36). This study highlights the ability of the chimera to rescue TCR signaling, including PLC- γ 1 activation, in LAT-deficient T cells, although it is difficult to interpret how forced interaction of LAT/SLP-76 bypasses the specific LAT tyrosine phosphorylation events normally occurring during physiologically regulated TCR signaling (37). Thus, it is likely that SLP-76 diversifies signals downstream of LAT phosphorylation rather than controlling a single linear pathway. This idea is consistent with the observation that T cells from mice conditionally expressing SLP-76 through the double-negative-3 stage (to permit production of CD4/CD8 double-positive thymocytes) still manifest TCR signaling defects (38). In this case, calcium signaling through the $\alpha\beta$ TCR is impaired, and positive selection to the CD4 or CD8 single-positive stage is dramatically blocked.

Given the critical role that the LAT/SLP-76 signaling module plays in T-cell function, an important question is the sub-cellular/supramolecular organization of the TCR/LAT/SLP-76 signaling module during T-cell activation. Several key studies have made use of advances in live cell time-lapse imaging, interference reflection microscopy, multicolor fluorescent protein tagging, and photobleaching recovery methodology. Following TCR stimulation, macroscopically detectable signaling 'clusters' form (39). These structures contain the TCR and related kinases, LAT, SLP-76, and several other proteins, but they are insufficient to promote full T-cell activation, as they still form in the absence of PLC- γ 1 (40). Some proteins, such as

ZAP-70, cycle into and out of these clusters. However, SLP-76 initially nucleates within the TCR/LAT cluster but then gradually dissociates in a microtubule-dependent manner (39, 41). The fate of this 'postclustered' SLP-76 pool appears to be distinct from conventional endosomal and Golgi compartments. However, recent analyses indicate that cholesterol/raft-dependent endocytosis of LAT/SLP-76-containing clusters may provide a mechanism for the downregulation of TCR signaling (41).

These studies raise the question of whether a pool of 'preactivated' SLP-76 might specifically regulate integrin function. One clue comes from the diversity of SLP-76-associated proteins, which include Vav1, Nck, Grb2, ADAP, and WAVE2. The balance of these interactions may coordinate downstream responses, including those that influence cytoskeletal regulation or integrin function. Indeed, recent work has indicated that TCR and ZAP-70-dependent T-cell activation promotes the assembly of a complex consisting of LAT, SLP-76, Nck, and Wiskott–Aldrich syndrome protein (WASp). This complex regulates the formation of actin 'rings' following TCR activation (42). However, while these TCR-induced cytoskeletal structures appear to trace directly to TCR signaling, the precise contribution of integrins to the formation of these actin rings is unclear. Finally, it is important to note that the specific role of SLP-76 in TCR signaling to integrins has not as yet been directly tested in a knockout or knockdown system.

Transmission of signaling cascades to integrins: ADAP

In the search for proteins that associate with LAT and/or SLP-76 following TCR activation, ADAP (also previously known as SLAP-130 or Fyb) has emerged as a more selective regulator of TCR signaling to integrins. ADAP was originally cloned following a screen from Jurkat T cells for proteins able to inducibly bind SLP-76 or the Src family tyrosine kinase Fyn following TCR stimulation (43, 44). ADAP has several protein interaction motifs, including an N-terminal proline-rich domain, central and C-terminal non-canonical helical SH3 (hSH3) domains, and more than a dozen putative tyrosine phosphorylation sites (Fig. 1). ADAP tyrosine phosphorylation is coordinately enhanced following TCR stimulation, and several ADAP tyrosine residues have been specifically implicated in mediating the ADAP–SLP-76 interaction by the SH2 domain of SLP-76 (43, 45–47) (Fig. 1). Co-immunoprecipitation studies identifying proteins constitutively associated with Fyn (48) also identified ADAP and a protein named Src kinase-associated protein of 55 kDa (SKAP 55 kDa) (49). Subsequent analysis identified a direct interaction between the proline-rich

domain in the N-terminus of ADAP with the SH3 domain in SKAP55 (49) (Fig. 1). A second interaction site between ADAP and SKAP55 has also been reported, involving the C-terminal non-canonical hSH3 domain of ADAP and tyrosine phosphorylated SKAP55 (50, 51). ADAP contains consensus sites for interaction with Ena/VASP homology proteins and, similar to SLP-76, has been identified in complexes with the Nck adapter protein and the actin regulatory protein WASp (52, 53). The extreme N-terminal domain of ADAP has been reported to bind to the actin-binding protein HIP-55 (54). Finally, the non-canonical hSH3 domains of ADAP, which are unable to bind standard proline-rich motifs typically recognized by SH3 domains, have recently been characterized as novel binding sites for cellular phosphoinositides and lipids (55–57).

ADAP and TCR signaling

What is known about how ADAP governs T-cell activation? Initial overexpression studies with ADAP failed to provide a consensus on whether ADAP plays a positive or negative regulatory role during T-cell activation (43, 45–47). However, the development of ADAP-deficient mice has clearly showed that ADAP is a positive regulator of TCR signaling and plays a particularly critical role in integrin activation (58, 59). Proximal TCR signaling, such as calcium mobilization and extracellular signal-regulated kinase (ERK) phosphorylation, occurs normally in ADAP knockout T cells. Thus, assembly of the LAT signalosome is intact in the absence of ADAP. This conclusion is also supported by the work using SLP-76-deficient mice reconstituted with SLP-76 bearing an R448K mutation in the SLP-76 SH2 domain (60), which uncouples ADAP from SLP-76. Consistent with data obtained with the ADAP^{-/-} mice, the SLP-76^{-/-} mice reconstituted with SLP-76-R448K show normal proximal TCR signaling responses, such as PLC- γ 1 activation, ERK activation, and calcium mobilization. However, in the absence of ADAP, TCR-dependent responses consistent with T-cell activation, including CD25 expression, interleukin-2 (IL-2) and interferon- γ production, and anti-TCR-mediated T-cell proliferation, are defective (58, 59). This impairment of mature T-cell activation is also seen in the SLP-76-R448K reconstituted mutant (60).

In addition to defects in T-cell activation responses, ADAP-deficient T cells exhibit impaired adhesion to β 1- and β 2-integrin ligands and impaired LFA-1 clustering following TCR stimulation (58, 59). However, treatment with PMA is able to rescue these adhesion defects, suggesting that ADAP operates in a pathway upstream or distinct from PKC. While these initial studies showed a role for ADAP in regulating integrin clustering, a more recent work with platelets has also indicated

that ADAP regulates affinity modulation of the α IIB β 3 integrin. Activated ADAP-deficient platelets exhibit reduced α IIB β 3 integrin-dependent adhesion to fibrinogen and the POW-2 Fab fragment ligand mimetic (61). This defect in α IIB β 3 function is physiologically relevant, as ADAP knockout mice also exhibit increased rebleeding from tail wounds (61). Although this observation does not directly address the role of ADAP in regulating integrin conformation on T cells, it does suggest that ADAP regulation of both the affinity and clustering of T-cell integrins is operative downstream of the TCR.

Consistent with a positive role for ADAP in peripheral T-cell activation, ADAP^{-/-} mice bearing the ovalbumin-specific DO11.10 transgenic TCR show impaired antigen-specific T-cell clonal expansion *in vitro* and *in vivo* when challenged with low antigen concentrations (Mueller et al., submitted). ADAP is absolutely required for clonal expansion at low clonal precursor frequencies, suggesting that ADAP is an important positive regulator of T-cell expansion under conditions that more closely approximate an immune response in a polyclonal animal. In addition to impaired clonal expansion, ADAP^{-/-} DO11.10 T cells also exhibit defective IL-2 production at low antigen doses, both *in vitro* and *in vivo*. Furthermore, ADAP^{-/-} T cells decrease expression of CD62L to a lesser extent than control animals. This finding suggests that CD4⁺ effector T-cell differentiation may be defective in the absence of ADAP. Thus, ADAP may control the strength of accumulated TCR signaling by limiting the length of T cell–APC interaction times. ADAP^{-/-} DO11.10 T cells also display reduced induction of the prosurvival gene Bcl-xL and increased labeling with the apoptosis marker Annexin V, following stimulation with antigen concentrations that result in impaired T-cell proliferation (Mueller et al., submitted). Thus, the deficient clonal expansion exhibited by ADAP^{-/-} T cells may also be in part because of impaired T-cell survival following antigen stimulation. These results suggest that ADAP may function in other signaling pathways downstream of the TCR that promote T-cell activation.

ADAP regulation of integrin function

How do the molecular interactions between ADAP and its subcellular binding partners regulate integrin function downstream of TCR ligation? At least one mechanism appears to involve the interaction between ADAP and SLP-76. Overexpression of ADAP enhances the ability of T-cell hybridoma cells and DO11.10 TCR transgenic T cells to conjugate with antigen-laden APCs and also enhances formation of the supramolecular activation cluster at the immunological synapse (62). In contrast, expression of a mutant ADAP with tyrosine to

phenylalanine substitutions at two tyrosine residues critical for ADAP association with SLP-76 inhibits immunological synapse formation and IL-2 production (62).

Overexpression of the ADAP-associated adapter protein SKAP55 can also enhance TCR-dependent T-cell adhesion to integrin ligands, LFA-1 clustering, and the formation of T cell–APC conjugates (63). Deletion of the SH3 domain of SKAP55, which interacts with the proline-rich domain of ADAP, abrogates this positive effect on integrin function. Knockdown of SKAP55 expression by RNA interference (RNAi) in a T-cell hybridoma line also abrogates LFA-1 clustering, while knockdown of the related protein SKAP55R or SKAP55 homologue (SKAP-HOM) has no effect (64). Although information about murine T cells lacking SKAP55 has not been published, B cells lacking SKAP-HOM exhibit defects in integrin-dependent adhesion to fibronectin (65). Thus, it appears that ADAP and SKAP55 compose a functional complex that couples TCR signaling to integrins. Interestingly, additional recent studies have highlighted a role for ADAP in the coordinate stabilization of SKAP55 protein expression (66). ADAP-deficient Jurkat T cells are also deficient in SKAP55 protein but not SKAP55 mRNA. Re-expression of ADAP dramatically increases the half-life of SKAP55 protein expression, a function that requires the proline-rich domain of ADAP and the SH3 domain of SKAP55.

Overexpression approaches utilizing mutant ADAP and SKAP55 constructs have recently been used to probe the mechanism by which the ADAP/SKAP55 complex controls TCR signaling to integrins (67). The ability of SKAP55 to enhance Jurkat T-cell adhesion to fibronectin or ICAM-1 following TCR stimulation requires a key tryptophan residue (W333) in the ADAP-interacting SH3 domain of SKAP55. A small segment of the proline-rich domain of ADAP is also critical for enhancement of integrin function following ADAP overexpression. In addition, the C-terminal domain of ADAP is required for this enhanced adhesion, suggesting that an additional ADAP interaction (presumably with SLP-76) is required for integrin activation. These studies also showed that TCR-dependent membrane targeting, but not activation, of the GTPase Rap1 is impaired in ADAP-deficient T cells (67). Thus, a constitutively preformed complex of ADAP/SKAP55 appears to regulate the intracellular localization of Rap1 in T cells, possibly by controlling access of the GTPase to sites of nascent integrin adhesion (see below).

The non-canonical hSH3 domains of ADAP may also be involved in ADAP-dependent regulation of integrin function. The ADAP hSH3 domains have a unique N-terminal helical region (55) and bind to a variety of phosphoinositides *in vitro*,

although there are differences between the two hSH3 domains in their lipid-binding affinities (56, 57). Removal of the critical hSH3 domain N-terminal helices involved in lipid binding inhibits the ability of ADAP to enhance integrin function when overexpressed in Jurkat T cells (57). This effect required removal of the helices from both hSH3 domains, and thus it is unclear if they act in tandem or if only one helical region is sufficient. The ability of these hSH3 domains to bind to phospholipids found at membranes in cells suggests a potential role for these domains in regulating the localization of ADAP to discrete regions of the T cell, such as the T cell–APC contact site during conjugate formation.

The ADAP hSH3 domains have also been reported to serve as a secondary binding site for a non-canonical RKXXYXXY phosphorylation motif in SKAP55 (50, 51). This secondary ADAP–SKAP55 interaction is not sufficient for the association of ADAP and SKAP55. However, Fyn-mediated phosphorylation of SKAP55 at this binding interface inhibits the secondary contact with ADAP and simultaneously promotes LFA-1-mediated adhesion of cells to ICAM-1. Thus, conformational changes in the ADAP/SKAP55 complex induced by changes in the binding interface between ADAP and SKAP55 may alter integrin function. However, the relationship between ADAP hSH3 domain binding to SKAP55 versus phospholipids and the impact of these interactions on Rap1 relocalization are not known.

Transmission of signaling cascades to integrins: PKC

PKC is a well-established intracellular effector of integrin activation as treatment of T cells with PMA, a potent PKC activator, is sufficient to enhance integrin-mediated adhesion (6, 7, 68). Inhibition of PKC activity also blocks TCR signaling to integrins (69). Thus, PKC appears to be both sufficient and necessary for integrin activation in T cells. The ability of phorbol ester treatment to bypass integrin activation defects in T cells lacking ZAP-70 (70), LAT (19), Itk (26), or PLC- γ 1 (32) indicates that PKC lies downstream of the TCR proximal signaling events nucleated by LAT that trigger integrin activation. This makes sense, given the known role that PLC- γ 1 plays in activating PKC following TCR stimulation. PMA can also enhance integrin function in T cells lacking ADAP (58, 59).

Which of the many isoforms of PKC is critical for TCR-mediated activation of integrins remains unresolved. It is possible that multiple PKC isoforms are each capable of promoting integrin-mediated adhesion following TCR stimulation. Alternatively, TCR signaling to integrins may

uniquely require specific PKC isoforms. Studies in other systems suggest that there may be integrin-dependent responses mediated by specific PKC isoforms. For example, PKC α overexpression induces α IIb β 3 integrin activation on platelets (13, 71), and the association of PKC α and ϵ with β 1 integrins has been reported (72–74). The PKC θ isoform plays a particularly important role in T-cell function. PKC θ localizes to the immunological synapse following TCR stimulation (75), and PKC θ -deficient mice exhibit defects in T-cell activation responses (76, 77). However, the specific role of PKC θ and other PKC isoforms in promoting integrin function following TCR stimulation remains incompletely characterized.

Assembly of integrin-associated signaling complexes: PKD and Rap1

The proximal signaling events initiated by TCR stimulation that lead to an enhanced integrin function must eventually act directly on integrins. Binding of intracellular proteins to integrin cytoplasmic domains has been proposed to alter the spatial segregation of the α and β subunit tails, resulting in changes in integrin conformation (78). In addition, dynamic changes in the linkage of integrins to the cytoskeleton regulates integrin mobility and clustering (16). Recent studies have highlighted several effector molecules that participate in integrin activation by promoting the assembly of integrin-associated signaling complexes. One such effector is the serine–threonine kinase PKD, which is a direct substrate of PKC and an additional target of DAG (79–81). The PKD family contains three members, PKD1 (also named PKC μ), PKD2, and PKD3, which share three structural domains (Fig. 1): an N-terminal cysteine-rich region, which is critical for PKD membrane localization, a central pleckstrin homology (PH) domain, and a C-terminal catalytic domain (80). A number of functions have been ascribed to PKD in non-lymphoid cells, including receptor recycling, motility, stress-induced NF- κ B activation, glucose transport, and apoptosis (79, 82–86). In lymphocytes, PKD has been proposed to regulate thymocyte development (87) and to modulate class-IIa histone deacetylase phosphorylation (88–90).

As a direct downstream effector of PKC, PKD represents a potential mechanism by which PKC activation leads to enhanced integrin function. Several lines of evidence support this hypothesis. First, similar to β 2 and β 1 integrins, PKD polarizes to the immunological synapse following TCR stimulation (91). Analysis of Jurkat T-cell interactions with superantigen-pulsed B cells shows that PKD translocates to the contact site within 1 min of T cell–APC contact and that

a majority of the PKD relocates to the cytosol after 15 min (91). These studies are consistent with earlier work showing a rapid, but transient, translocation of PKD to the plasma membrane following antigen receptor stimulation (92). Furthermore, the kinetics of PKD membrane localization and changes in integrin function following TCR stimulation are similar. Second, inhibition of PKD expression by RNAi leads to an inhibition of both TCR- and PMA-induced increases in Jurkat T-cell adhesion to fibronectin and clustering of β 1 integrins on the cell surface (93). Similar inhibitory effects are observed with overexpression of a mutant PKD construct lacking the PKD PH domain (93). Unlike TCR proximal signaling effectors described earlier (such as LAT, PLC- γ 1, and Itk), modulation of PKD expression and/or function inhibits integrin activation induced by PMA stimulation as well as TCR stimulation. Third, direct interactions between PKD and integrins have been reported. In T cells, PKD can be co-immunoprecipitated with β 1 integrins, particularly following stimulation (93). Similar results have been reported for PKD and the α v β 3 integrin in fibroblasts (83).

PKD mediates its effects on integrin activation by regulating the membrane localization of Rap1, a member of the Ras family of small GTPases that plays a particularly critical role in integrin activation (94). Rap1 was first identified as a protein that could reverse the morphological phenotype of Ras-transformed fibroblasts (95). However, a major Ras-independent function of Rap1 is the regulation of integrin function. Expression of a constitutively active form of Rap1 augments basal adhesion of T cells to purified integrin ligands (96–98). In contrast, inhibition of Rap1 activity with mutant Rap1 constructs or overexpression of Rap1 GTPase-activating proteins, such as SPA-1 or Rap1GAP1, blocks TCR signaling to integrins (99–101). Basal Rap1 activity also seems to be required for the maintenance of cell adhesion, as conditional overexpression of SPA-1 abrogates basal levels of active Rap1, resulting in loss of cell–matrix adhesion (100). Defects in Rap1 activity are also associated with a variant form of a human disease called leukocyte adhesion deficiency, where integrin activation is impaired on leukocytes and platelets (102). Like other Ras family GTPases, Rap1 contains a C-terminal CAAX sequence critical for localization of Rap1 to membranes. TCR stimulation enhances the localization of Rap1 to the plasma membrane (103) and to the T cell–APC contact site (99). In addition, activation of Rap1 has been shown to occur at the T-cell plasma membrane (103). Thus, the control of Rap1 membrane localization is likely critical to Rap1-dependent regulation of integrin function. PKD represents one mechanism by which Rap1 membrane localization and activation is controlled as

Rap1 directly interacts with the PH domain of PKD (93). Furthermore, expression of PKD constructs lacking the PKD PH domain inhibits TCR- and PMA-induced activation of Rap1, as well as membrane translocation of Rap1. Because these mutant PKD constructs translocate normally to the membrane, this finding suggests that these constructs block Rap1 activation and membrane translocation by inhibiting the normal activation-dependent movement of Rap1 mediated by endogenous PKD. Surprisingly, the kinase activity of PKD is not critical for PKD-dependent regulation of Rap1 function, suggesting that PKD functions primarily as an adapter protein that regulates the intracellular localization of Rap1 (93).

Both PKD and Rap1 can be co-immunoprecipitated with $\beta 1$ integrins on T cells, particularly after TCR stimulation or PMA stimulation (93). Thus, PKD provides a mechanism by which PKC-dependent signals link Rap1 to $\beta 1$ integrins. Furthermore, the localization of the PKD–Rap1 complex at the membrane is dependent on the expression of a functionally active $\beta 1$ integrin. In $\beta 1$ -integrin-deficient Jurkat T cells, TCR stimulation or PMA stimulation fails to result in Rap1 activation (93). Furthermore, activation-dependent association of PKD and Rap1 with $\beta 1$ integrins is dependent on the last five amino acids of the $\beta 1$ -integrin cytoplasmic domain, a region of the $\beta 1$ -integrin tail previously shown to be critical to integrin function in T cells (104).

Integrin activation and integrin recycling

Integrin heterodimers are continually internalized from the plasma membrane into endosomal compartments and then recycled back to the cell surface (105). The identification of an interaction between PKD and Rap1 that is important for integrin activation raises the potential role of integrin recycling in TCR signaling to integrins as both PKD and Rap1 have been proposed to be involved in receptor recycling. In epithelial cells, PKD localizes to the trans-Golgi network and regulates vesicular transport by phosphorylation-mediated activation of phosphatidylinositol-4-kinase III β (85, 106, 107). In addition, PKD knockdown or expression of kinase-inactive PKD in fibroblasts disrupts platelet-derived growth factor-dependent recycling of the $\alpha \nu \beta 3$ integrin to the plasma membrane from early endosomes (83). However, PKD was not clearly localized to endosomes in this study. PKC ϵ has also been reported to regulate the recycling of $\beta 1$ integrins through an intracellular vesicular compartment, and this recycling is critical to directional migration of mouse embryonic fibroblasts (74). An inhibitor of endosome recycling blocks stimulated adhesion of Jurkat T cells to fibronectin and ICAM-1 and also blocks Rap1 activation at the plasma membrane (103). In addition, active

Rap1 has been proposed to regulate trafficking of LFA-1 by association with the Rap1 effector RAPL (regulator of adhesion and cell polarization enriched in lymphoid tissues) (108) and the RAPL effector Mst1 (109)(also see below). Together, these studies suggest a potential role for PKD and Rap1 in controlling integrin activation by the association with integrins in endosomes (110, 111). Additional studies in this area, particularly using T cells, are clearly warranted. For example, the role of GTPases, such as Rab4 and Rab11, that regulate integrin recycling in TCR-mediated integrin activation remains unknown (105, 112, 113).

Mechanisms of Rap1 activation

Activation of Rap1 following TCR stimulation is facilitated by Rap1 GEFs. One key Rap1 GEF is C3G, which constitutively associates with the CrkL adapter protein. TCR stimulation results in tyrosine phosphorylation-dependent association of the C3G/CrkL complex with the adapter protein Cbl (114). Although C3G-deficient fibroblasts exhibit defects in Rap1 activation and cell adhesion (115), defects in T-cell function, including integrin activation, have not been reported in CrkL-deficient T cells (116). It is possible that other members of the Crk family of adapters compensate for the loss of CrkL in this model. The Cbl-b adapter protein, which also interacts with CrkL in a TCR-inducible manner, negatively regulates Rap1 signaling. Consequently, Rap1 activity and integrin function are elevated in Cbl-b-deficient T cells (117).

TCR-induced activation of the Fyn tyrosine kinase has been proposed to promote formation of the Cbl/CrkL/C3G complex and increase interactions between C3G and Rap1 (118). This proposal is consistent with studies showing that enhanced membrane localization of Rap1 following TCR stimulation or PMA stimulation results in an increased association of Rap1 with C3G in T cells (93). Thus, the PKD-dependent membrane localization of Rap1 to $\beta 1$ integrins likely facilitates the interaction of Rap1 with Rap1 GEFs such as C3G. It is also interesting to note that activation-dependent membrane localization of Rap1 is impaired in T cells lacking ADAP (67), which is believed to be a downstream substrate of Fyn.

Another family of Rap1 GEFs, the calcium and diacylglycerol-GEFs (CalDAG-GEFs), is regulated by calcium and DAG (94). Thus, TCR-induced activation of PLC- $\gamma 1$ likely results in activation of these GEFs. The GEF CalDAG-GEF1 (RasGRP2) has been proposed to be critical to Rap1 activation in mature T cells downstream of PLC- $\gamma 1$ (32). This idea is consistent with studies showing defects in TCR signaling to integrins in PLC- $\gamma 1$ -deficient T cells (32).

Although studies in Jurkat T cells indicate that Rap1 activation occurs predominantly at the plasma membrane (103), other

studies have suggested that Rap1 activation also occurs in intracellular compartments (119, 120). Recent findings that ADAP regulates the intracellular localization of Rap1 but not its activation suggest that Rap1-dependent regulation of integrin function can be disrupted by altered intracellular localization of active Rap1 in T cells (67). It will be important in the future to define the precise intracellular compartments where Rap1 is activated in ADAP-deficient T cells.

In addition to the TCR, other coreceptors that modulate T-cell activation can activate Rap1. Recent studies have shown that stimulation of the cytotoxic T-lymphocyte antigen-4 (CTLA-4) coreceptor, which negatively regulates T-cell activation, nevertheless results in robust activation of Rap1, increases in LFA-1-mediated adhesion to ICAM-1, and LFA-1 clustering (101, 121). Surprisingly, CTLA-4 ligation also enhances T-cell motility and reduces contact time between T cells and APCs (122).

Rap1 effectors

The identification of Rap1 effector proteins has provided important insights into mechanisms by which Rap1 regulates TCR signaling to integrins. A constitutively active Rap1 construct, Rap1G12V, was used as a bait in a yeast two-hybrid screen to identify RAPL as a Rap1-binding protein (108). Overexpression of RAPL enhances LFA-1 clustering and adhesion (108), and RAPL-deficient lymphocytes and dendritic cells exhibit impaired adhesion and migration (123). RAPL is also an integrin-associated protein as RAPL polarizes to the immunological synapse following antigen stimulation of T cells, colocalizes with LFA-1 following TCR or chemokine stimulation, and co-immunoprecipitates with LFA-1 in a Rap1-dependent manner (108). This interaction between RAPL and LFA-1 is dependent on lysine residues at positions 1097 and 1099 in the juxtamembrane region of the α L-subunit cytoplasmic domain. This is a functionally significant region of the α L cytoplasmic domain as deletion of the adjacent GFFKR motif results in a constitutively active LFA-1 integrin (124, 125). While lysines 1097 and 1099 are critical for Rap1-dependent activation of LFA-1, the β 2-subunit cytoplasmic domain appears to be dispensable for activation of LFA-1 by Rap1 (126). Mutation of these lysine residues to alanine impairs the ability of LFA-1 to redistribute to the leading edge induced by Rap1 activation or overexpression of RAPL (108). Because RAPL localizes to the leading edge properly in cells expressing this mutant LFA-1, this finding suggests that RAPL may play a critical role in localizing LFA-1 to discrete regions of the plasma membrane.

The serine–threonine kinase Mst1, a member of a family of kinases homologous to the Ste20 kinase in yeast (127), has

recently been identified as a RAPL effector (109). TCR-mediated activation of Mst1 is dependent on RAPL, and TCR-mediated adhesion to ICAM-1 and antigen-dependent conjugate formation are impaired following RNAi-mediated knockdown of Mst1 expression (109). Although Rap1 and RAPL have been shown to regulate both LFA-1 affinity and clustering (96, 108), overexpression of Mst1 only enhances LFA-1 clustering (109). This finding suggests that LFA-1 clustering is critical for TCR signaling to integrins that is mediated by Rap1. It also implies the existence of Mst1-independent mechanisms by which Rap1 regulates LFA-1 affinity.

Like Rap1, Mst1 is localized in intracellular vesicles (109). Subcellular fractionation experiments indicate that both Mst1 and RAPL are located in fractions that contain proteins found in early and late endosomes. This includes the GTPase Rab11, which has recently been shown to be critical for recycling of LFA-1 in neutrophils (128). RAPL appears to be critical for intracellular localization of Mst1, as Mst1 is not localized in perinuclear regions in RAPL-deficient cells (109). Thus, these studies suggest that Mst1 may regulate LFA-1 function by mediating the polarized vesicle transport critical for the movement of LFA-1 to the leading edge during the process of T-cell migration. Similar regulatory mechanisms may also promote the polarization of LFA-1 to the immunological synapse during antigen recognition.

A striking feature of Rap1 and the Rap1-associated signaling proteins PKD, RAPL, and Mst1 is their localization to membranes where integrins are found. This provides a mechanism by which Rap1 can act directly on integrins and modulate integrin affinity and/or clustering. PKD, RAPL, and Mst1 have also all been proposed to play a role in movement of receptors to the plasma membrane. PKD-dependent regulation of vesicular transport requires PKD kinase activity (85, 106, 107), while PKD-dependent regulation of TCR signaling to integrins does not appear to require PKD kinase activity (93). Thus, PKD may play a distinct role in regulating Rap1-dependent integrin regulation. For example, the PKD-dependent association of Rap1 with C3G suggests that PKD may be critical for localizing Rap1 not only with integrins but also with Rap1 GEFs. The PKD–Rap1 interaction may thus be central to the subsequent activation of Rap1 and triggering of downstream effectors such as RAPL and Mst1.

An additional Rap1 effector provides a link between Rap1 and the actin cytoskeleton. RIAM (Rap1–GTP-interacting adapter molecule) is a broadly expressed adapter protein that contains an RA (Ras association)-like domain, a PH domain, and several proline-rich sequences (129). Like RAPL, RIAM interacts preferentially with active Rap1, and overexpression of RIAM

enhances integrin-mediated adhesion. In addition, knockdown of RIAM inhibits adhesion induced by active Rap1 (71, 129) and inhibits the localization of active Rap1 at the plasma membrane (129). The ability of RIAM to associate with profilin, Ena/VASP proteins, and talin suggests that RIAM promotes Rap1-dependent integrin activation through effects on the actin cytoskeleton, particularly the interaction of talin with integrin cytoplasmic tails (71, 129). Given the known role of talin in regulating integrin affinity (71, 130, 131), RIAM may provide an Mst1-independent mechanism by which Rap1 regulates integrin affinity.

Assembly of integrin-associated signaling complexes: talin

The actin-binding protein talin was the first integrin-associated protein to be identified (132), and it provides a link between integrins and the actin cytoskeleton (133). Talin contains an N-terminal globular head region that contains a predicted FERM (four-point-one, ezrin, radixin, moesin) domain that mediates association with multiple integrin- β subunits and a C-terminal rod domain that contains vinculin- and actin-binding sites (133). Talin has been proposed to be a 'final common step' in integrin activation, as knockdown of talin expression in fibroblasts inhibits integrin activation, and this inhibition cannot be overcome by the expression of other integrin-activating proteins (130). The ability of Rap1 to activate the α IIb β 3 integrin requires talin as an active Rap1 construct is unable to activate α IIb β 3 in Chinese hamster ovary cells expressing a talin mutant that cannot bind to integrins (71). Rap1 has also been shown to regulate the membrane localization and association of talin with α IIb β 3 and promote the association of RIAM with the talin- α IIb β 3 integrin complex (71). Thus, Rap1-dependent integrin activation converges at the level of talin association with integrins. Recent studies have also shown that talin suppression in T cells leads to inhibition of TCR signaling to LFA-1 (131). However, it remains to be determined if Rap1-dependent integrin activation in T cells also requires talin and the formation of a talin-RIAM complex.

The ability of the isolated talin head domain to activate integrins and induce spatial separation of the α L and β 2 cytoplasmic domains indicates that direct association of talin with integrin- β subunit cytoplasmic domains is central to the process of integrin activation (78, 134). A conserved membrane-proximal NPXY motif in integrin β subunit cytoplasmic domains is critical for talin interaction with integrins (135, 136). Recent genetic studies in mice have confirmed that mutation of the tyrosine residues in the two NPXY motifs in the β 1 integrin cytoplasmic domain abolishes

β 1 integrin function on keratinocytes *in vivo* (137). Although phosphorylation of the tyrosine residues in the NPXY motifs has been proposed to regulate the interaction of talin with integrins (133), substitution of the tyrosine residues with phenylalanine does not lead to defects in β 1-integrin function *in vivo* (137).

The C-terminal NPXY motif of the β 1-integrin cytoplasmic domain is functionally important in TCR signaling to β 1 integrins (104) and lies in a region of the β 1-integrin cytoplasmic domain that is critical for Rap1 activation following PMA or TCR stimulation and for activation-dependent association of PKD and Rap1 with β 1 integrins (93). A similar region in the β 3 cytoplasmic domain regulates the interaction of the α v β 3 integrin with PKD (83). This region of the β 1-integrin tail has also been shown to be critical in adhesion, invasion, and metastasis of ESb lymphoma cells (138). While deletion of the C-terminal five amino acids of the β 1-integrin tail renders β 1 integrins unresponsive to TCR or PMA stimulation (93, 104), this region of the β 1-integrin cytoplasmic domain is not required for talin interaction with β 1 integrins (139). The ability of the C-terminal end of the β 1 tail to promote integrin association with the PKD-Rap1 complex suggests the possibility that this region of the β 1-integrin tail serves to integrate Rap1-dependent integrin activation with talin.

The isolated talin head domain has sixfold higher affinity for the β 3-integrin tail when compared with intact talin (140). This finding suggests that the integrin-binding site in intact talin is masked and that integrin activation involves regulated unmasking of this site in talin. The calcium-dependent protease calpain may represent one mechanism by which this occurs as calpain cleaves talin and separates the head domain from the actin-binding domain (140). The ability of calcium mobilizers to activate LFA-1 on T cells in a calpain-dependent manner is consistent with this model (141). Thus, TCR-mediated increases in intracellular calcium downstream of PLC- γ 1 likely participate in integrin activation by two distinct mechanisms: calpain-dependent cleavage of talin, which promotes the interaction of talin with integrins, and activation of calcium-dependent Rap1 GEFs, which facilitate activation of Rap1.

Reorganization of the actin cytoskeleton and integrin activation

TCR signaling induces rapid reorganization of the actin cytoskeleton at the interface between the T cell and an APC (28). The association of integrins with talin and the ability of the actin-destabilizing agent cytochalasin B to inhibit integrin activation clearly indicate that actin reorganization is central to TCR-mediated activation of integrins. In addition, RNAi-mediated

suppression of Arp2/3 actin regulatory proteins in Jurkat T cells has recently been shown to block TCR-mediated activation of integrins in Jurkat T cells (Gomez et al., submitted). These findings suggest that Arp2/3-dependent mechanisms of actin reorganization are critical for TCR signaling to integrins.

A key protein that binds to and activates the Arp2/3 complex is WASp (28). Although WASp deficiency leads to defects in T-cell activation and actin polymerization (142, 143), there are conflicting reports in the literature regarding the requirement for WASp in T-cell conjugate formation with APCs (144–148). However, activation-dependent adhesion of T cells to purified integrin ligands is normal in the absence of WASp (146), suggesting that WASp-independent mechanisms of regulating actin by Arp2/3 are critical for TCR signaling to integrins. One possible effector is the actin regulatory protein WAVE2, which is regulated by the GTPase Rac1 (28, 148). Suppression of WAVE2 in Jurkat T cells does in fact dramatically impair antigen-dependent conjugate formation and TCR-mediated activation of T-cell adhesion to fibronectin (148). Furthermore, overexpression of the WAVE2-associated protein PIR121 augments Jurkat T-cell adhesion to fibronectin and inhibition of PIR121 inhibits T-cell adhesion to fibronectin (149). Interestingly, increased integrin function is associated with overexpression of PIR121 in CD4⁺ T cells isolated from multiple sclerosis patients (149).

Upstream regulators of WAVE2 function have also been shown to be involved in integrin activation mediated by the TCR. Overexpression of Rac1 or expression of a constitutively active form of Rac1 enhances T-cell adhesion to fibronectin (149, 150). Furthermore, inhibition of Rac1 expression in T cells inhibits TCR signaling to integrins (149). In contrast, expression of a constitutively active form of the related GTPase cdc42, which regulates WASp, does not enhance T-cell adhesion to fibronectin (150). Finally, T cells deficient in expression of the GEF Vav1, which can activate both Rac1 and cdc42, exhibit impaired conjugate formation with APCs and adhesion to purified integrin ligands (146). Together, these results provide further evidence that TCR signaling to integrins is specifically dependent on WAVE2-dependent actin reorganization events.

General disruption of TCR-mediated actin polymerization is not sufficient to ablate integrin activation as suppression of expression of other actin regulatory proteins, such as dynamin 2 and HS1, disrupts actin accumulation at the T cell–APC contact but does not perturb antigen-dependent conjugate formation (151, 152). This finding suggests unique and specific roles for actin regulatory proteins in modulating actin-dependent integrin function.

ADAP may also participate in the modulation of the actin cytoskeleton as ADAP has been reported to bind to the EVH1

domain of Ena/VASP proteins, which interact with profilin at sites where actin polymerization takes place (52, 153). This role of ADAP in the regulation of the actin cytoskeleton was also highlighted in macrophages, where ADAP can be found in complexes with Nck, WASp, and VASP during phagocytosis (53). However, it should be noted that TCR-mediated actin polymerization appears grossly normal in ADAP knockout T cells (58, 59). Furthermore, VASP-deficient platelets exhibit enhanced thrombin-induced binding to fibrinogen, suggesting a negative role for VASP in integrin activation in platelets (154). Additional studies directly testing a role for Ena/VASP proteins in TCR signaling to integrins have not yet been reported. Recent work in macrophages indicates that ADAP might also be linked to the actin cytoskeleton through its interaction with HIP-55 (also known as mAbp1 or SH3P7) (54). Interestingly, HIP-55 binds to hematopoietic kinase-1 (HPK1), which regulates c-Jun N-terminal kinase (JNK) activation, localizes to the immunological synapse (155), and is regulated by PKD (156). However, the existence or putative role of an ADAP–HIP-55 interaction in T-cell function is unknown.

ADAP may also play a role in the polarized recruitment of the microtubule-organizing center (MTOC) to the T cell–APC contact site, a process that is mediated by the formin family of actin-nucleating proteins in an Arp2/3-independent manner (Gomez et al., submitted). Superantigen-induced MTOC polarization is impaired in Jurkat T cells with morpholino-mediated knockdown of ADAP (157). This function appears to trace the ability of synaptically organized ADAP to recruit the microtubule motor protein dynein and perhaps stabilize nascent microtubules extending from the MTOC. The possibility is thus raised that ADAP-deficient effector T cells may exhibit defects in the polarized secretion of cytokines or cytotoxic granules upon contact with target cells. However, the physiological relevance of this activity remains to be established, and differences have been reported between human and mouse T cells (157).

Signaling by integrins

While the majority of this review has focused on TCR signaling to integrins, it is important to reiterate that integrin-mediated signaling following ligand engagement also occurs. These integrin signaling events may stabilize and enhance integrin connections with the cytoskeleton and facilitate T-cell activation by providing coreceptor signals. Recent studies have suggested that ADAP itself may be involved in altering actin cytoskeletal events following LFA-1 engagement (158). Direct stimulation of LFA-1 with anti-LFA-1 antibodies promotes the generation of a ring-shaped ‘cloud’ of actin that is independent of TCR

activation and proximal signaling. Formation of this actin cloud is impaired following LFA-1 stimulation of ADAP-deficient T cells, and engagement of a CD8/ADAP chimera can induce actin cloud formation. Interestingly, phosphorylated ADAP and activated JNK are found in a complex with clustered LFA-1, and ADAP in fact can be co-immunoprecipitated with LFA-1 (158). Similar to results obtained with LFA-1 triggering, coengagement of the CD8/ADAP chimera with the TCR enhances IL-2 production, suggesting that the formation of this actin cloud may facilitate T-cell activation. These recent studies are consistent with earlier work showing that engagement of the $\alpha 4 \beta 1$ integrin induces tyrosine phosphorylation of ADAP (159).

TCR signaling to integrins: a model and key questions

We propose the following model by which the TCR induces increases in integrin function (Fig. 2). TCR stimulation results in the initiation of integrin activation signaling cascades that are nucleated by tyrosine phosphorylation of the LAT adapter protein and result in the assembly of the LAT signalosome. Critical signaling events for integrin activation at this stage include Itk-dependent activation of PLC- $\gamma 1$, tyrosine phosphorylation of the Rac1 GEF Vav1, and the inducible association of SLP-76 with LAT. Because TCR signaling is spatially segregated from integrins such as LFA-1 (9, 10), mechanisms

must exist by which these initial signaling events are transmitted from the LAT complex to integrins. We propose that the transmission of signaling cascades to integrins occurs by specific signaling effectors that are activated by discrete components of the LAT signalosome. Thus, DAG generation and increases in intracellular calcium by activated PLC- $\gamma 1$ result in the activation of PKC and calpain, respectively. Calcium may also activate calcium-dependent GEFs for Rap1. The inducible association of SLP-76 with the ADAP/SKAP55 complex initiates another transmission cascade. The mechanism by which signals are transmitted to a spatially segregated receptor remains unknown, although it is interesting to note that SLP-76 translocates to a perinuclear region following TCR clustering (39). Thus, intracellular vesicles may play a prominent role in signal transmission from the TCR to integrins.

There are two interdependent outcomes to these signaling cascades. One is the assembly of integrin-associated signaling complexes that control both the conformation and membrane distribution of integrins. We propose that PKC mediates the association of PKD with Rap1 and the ADAP/SKAP55 complex participates in this process by facilitating the TCR-dependent membrane targeting and association of the Rap1/PKD complex with integrins. This interaction is dependent on the C-terminal end of integrin β -subunit cytoplasmic domains and places Rap1 not only in proximity to integrins, but also to Rap1 GEFs such as

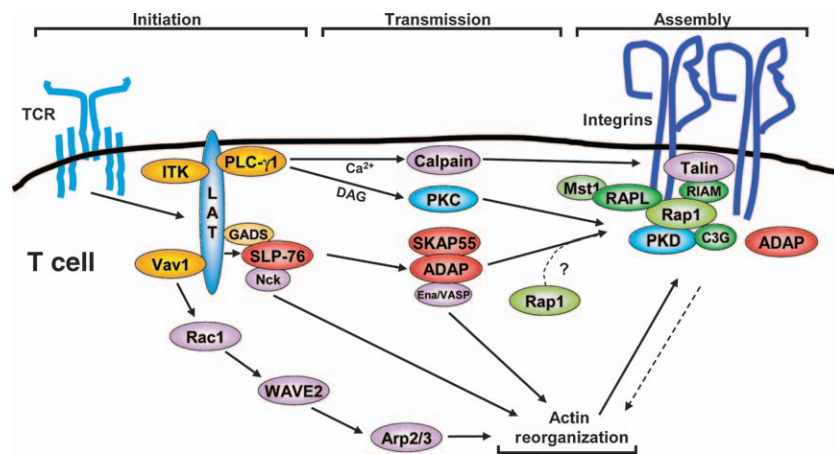


Fig. 2. Model summarizing key events in TCR signaling to integrins.

TCR engagement constitutes signal initiation and includes activation of proximal tyrosine kinases, tyrosine phosphorylation of LAT, and assembly of the LAT signalosome. Key components of the LAT signalosome for integrin activation are the docking of ITK (which promotes PLC- $\gamma 1$ activation), the docking of Vav1 (which promotes Rac1 activation and WAVE2 function), and the docking of SLP-76. Transmission of integrin-activating signals includes the PLC- $\gamma 1$ -dependent generation of calcium and DAG (which activates PKC), as well as the recruitment of the ADAP/SKAP55 module. These signals converge on integrins and promote the assembly of integrin-associated signaling complexes. Key components of these complexes include

recruitment of Rap1 to integrins by PKD and the recruitment of the Rap1 effectors RAPL (which recruits Mst1) and RIAM. Talin binding to integrins is promoted by calpain-mediated cleavage of talin, and RIAM may provide a link between Rap1-dependent integrin activation and talin. Reorganization of the actin cytoskeleton is also required for integrin activation, and integrin function is critically dependent on Rac1-dependent activation of WAVE2 and subsequent Arp2/3-dependent actin reorganization. Other components of this signaling cascade, such as SLP-76 and ADAP, may also facilitate actin reorganization events that occur following TCR stimulation. Signaling by integrins following ligand engagement may also enhance integrin interactions with the cytoskeleton. See text for further details.

C3G. Rap1 also nucleates the association of several Rap1 effectors with integrins. RAPL interacts with the α L-integrin subunit cytoplasmic domain, and RAPL-dependent activation of Mst1 is critical for regulating the membrane distribution of LFA-1. The membrane-proximal NPXY motif in integrin β -subunit cytoplasmic domains mediates the association of integrins with talin, which is 'activated' by calpain cleavage. The Rap1 effector RIAM provides a functional link between talin and Rap1, and interaction of both Rap1 and talin is required for optimal integrin activation (72). Talin likely regulates integrin conformation in a Rap1-dependent manner, while Mst1 may specifically control integrin clustering. The second outcome is that these events at the integrin are also dependent on efficient and specific reorganization of the actin cytoskeleton, which is initiated by Vav1-dependent activation of Rac1, which leads to activation of WAVE2 and Arp2/3-dependent actin polymerization. Actin polymerization may also be facilitated by SLP-76 (by Nck) (42) and ADAP/SKAP55 (by Ena/VASP proteins) (52). Thus, talin likely participates in both integrin affinity modulation and clustering. Recent studies in T cells highlight this dual role for talin in integrin activation (131). Following ligand engagement, the linkage of integrins with the actin cytoskeleton is likely to be stabilized and enhanced by integrin-specific signaling events that may involve intermediates such as ADAP itself (158).

Although recent work has identified several key signaling intermediates that are critical for integrin activation in T cells, important questions remain unresolved. Recent studies clearly suggest that integrin recycling is important in regulating integrin functional activity, particularly in the context of cell migration (110). It will be important to determine if intracellular vesicles are critical in the transmission of signaling from the TCR to integrins or in the assembly of integrin-associated complexes that regulate integrin conformation and/or clustering. The specifics of the assembly of integrin-associated complexes need further clarification. For example, PKD has been reported to associate specifically with the β 1- and β 3-integrin subunits (83, 93). Does PKD also associate with the

β 2-integrin subunit? Similarly, RAPL has been reported to associate specifically with the α L subunit (108). If these interactions are specific to certain integrin subunits and not others, there may be unique features of integrin activation pathways that selectively regulate different integrins. The α 4 β 1 integrin, like LFA-1, polarizes to the outer ring of the immunological synapse, even when T cells interact with APCs that do not express α 4 β 1 integrin ligands such as vascular cell adhesion molecule-1 (VCAM-1) (12). Thus, there may be cross talk between β 1 and β 2 integrins that participate in integrin activation pathways. It is also now clear that integrins expressed on any given T cell are heterogeneous in terms of their conformation and mobility (16). The intracellular mechanisms by which these different integrin states are maintained and altered upon activation are incompletely characterized. Do these different integrin affinity and mobility states interact with a different spectrum of intracellular regulators? How are these interactions dynamically regulated during the course of T-cell activation? The issue of how integrins are deactivated is also poorly understood. Finally, the significance of these integrin activation pathways in the context of an antigen-specific T-cell activation response *in vivo* remains an important area for future investigation. These studies will be challenging as effectors such as Rap1 have both positive and negative effects on T-cell activation (94). Analysis of the functional significance of key amino acids in the β 1-integrin cytoplasmic domain *in vivo* has already produced results that would not have been predicted based on previous *in vitro* studies (137). Thus, it will be critical in the future to combine our increasing knowledge of these signaling pathways with advances in characterizing T-cell immune responses *in vivo* to fully elucidate the role of TCR signaling to integrins in adaptive immunity.

Note: Menashe et al. have very recently reported that SKAP55 interacts constitutively with RIAM, thus providing a direct link between the ADAP/SKAP55 complex and activated Rap1 in T cells (160).

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