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Genetic analysis of integrin activation in T lymphocytes

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Summary: Among the myriad receptors expressed by T cells, the *sine qua non* is the CD3/T cell receptor (CD3/TCR) complex, because it is uniquely capable of translating the presence of a specific antigen into intracellular signals necessary to trigger an immune response against a pathogen or tumor. Much work over the past 2 decades has attempted to define the signaling pathways leading from the CD3/TCR complex that culminate ultimately in the functions necessary for effective T cell immune responses, such as cytokine production. Here, we summarize recent advances in our understanding of the mechanisms by which the CD3/TCR complex controls integrin-mediated T cell adhesion, and discuss new information that suggests that there may be unexpected facets to this pathway that distinguish it from those previously defined.

Introduction

The function of T cells is critically dependent on the coordinated and controlled activities of a number of adhesion molecules, including integrins, expressed on their cell surface. Integrins expressed on resting human T cells include the β2 integrin LFA-1 (αLβ2), the β1 integrins α4β1, α5β1 and α6β1, and the α4β7 integrin (1). This constellation of integrins allows human T cells to interact with other cells via interaction with cell surface counter-receptors such as ICAM-1, ICAM-2, ICAM-3 (all via LFA-1), VCAM-1 (via α4β1) and MAdCAM-1 (via α4β7), as well as with extracellular matrix (ECM) proteins, such as fibronectin (via α4β1 and α5β1) and laminin (via α6β1). These adhesive interactions are critical for T cell extravasation out of the bloodstream into secondary lymphoid organs and peripheral tissue sites (2), as well as for the formation of a highly organized structure, known as the immunological synapse, that forms when antigen-specific T cells interact with and are activated by antigen-presenting cells (APCs) expressing a relevant MHC/peptide antigen complex (3–5). β1 integrin-mediated interactions with ECM pro-

teins are also critical to T cell function, as T cell–ECM interactions regulate T cell migration into and through tissue sites (1,6,7). In addition to mediating adhesion, integrin receptors can transmit intracellular signals that modulate T cell activation and functional responses, including proliferation and cytokine production initiated by the antigen-specific CD3/T cell receptor (CD3/TCR) complex (8–10) as well as regulation of ECM-degrading enzymes (11).

Integrin expression is regulated in order to temporally and spatially regulate the efficiency of T cell activation and the specificity of lymphocyte trafficking patterns (12). The more vigorous response of memory T cells to antigen when compared to naïve T cells is due in part to stable changes in integrin expression that are a hallmark of the differentiation program that occurs during the naïve to memory T cell transition. Thus, in comparison to naïve T cells, memory T cells express higher levels of several integrins, including LFA-1, α4β1, α5β1 and α6β1 (13,14). In addition, the spectrum of integrins and other adhesion molecules expressed by memory T cells is dictated in part by where and when a T cell first encountered antigen (1,15). These stable changes in integrin expression ensure the appropriate trafficking of these memory T cells to anatomic sites where they are most likely to re-encounter antigen in a secondary response. Transient increases in expression of specific integrins on activated, effector T cells have also been demonstrated. In vitro activation of human T cells results in induced expression of collagen-binding integrins such as α1β1 and α2β1 (16), and recent studies demonstrate a critical function for these integrins in several inflammatory disease models in mice (7).

The focus of this review is on the acute, transient regulation of the functional activity of integrins expressed on the T cell surface by various activating stimuli. While T cells in circulation are relatively non-adherent, they must be able to stop and respond quickly and specifically to foreign or tumor antigens. This ability of T cells to switch rapidly between non-adherent and adherent states is based on their ability to modulate integrin activity, known as "inside-out" signaling. On T cells, stimulation of the CD3/TCR results within minutes in increased integrin-mediated adhesion to ICAM-1, VCAM-1 and ECM proteins such as fibronectin and laminin (14,17–19) and is one of the earliest detectable changes in T cell function following CD3/TCR activation. The ability of the CD3/TCR to rapidly enhance integrin function results not only in enhanced interaction of T cells to APCs (20,21), but also probably diminishes T cell migration in tissue by enhancing T cell adhesion to the surrounding ECM (22). Ligation of co-receptors on T cells, including CD28 (23), CD7

(23) and CD2 (14,18), independent of CD3/TCR stimulation can also result in integrin activation. This suggests that one critical function of T cell co-stimulation may be to enhance integrin activation signals provided by the CD3/TCR. Chemokine receptors initiate changes in integrin activity that can be detected experimentally within seconds of chemokine stimulation (24–28). This mode of integrin activation is critical to the initiation of integrin-mediated adhesion of lymphocytes to endothelial cells under conditions of vascular shear flow (2). In vitro studies have also demonstrated that experimental conditions can be manipulated so that integrins become active. Phorbol esters such as PMA have been known for many years to be effective in rapidly enhancing integrin functional activity (14,17,29). In addition, pharmacological agents that mobilize intracellular calcium in T cells can enhance integrin function (23,30). Some, but not all, integrin-specific monoclonal antibodies can also directly increase integrin functional activity (31). Furthermore, alterations in the divalent cation milieu, such as the addition of excess Mn^{2+}, can enhance integrin-mediated adhesion (32,33). These studies with pharmacological activators of integrin function have provided critical information on the mechanisms by which integrin function can be modulated.

The physiological significance of activation-dependent integrin regulation to T cell function is now clear. First, two rare variant forms of leukocyte adhesion deficiency (LAD) have been reported where integrin expression is normal but integrin activation is defective (34–37). Like LAD patients that lack β2 integrins, patients with these variant forms of LAD are severely immunocompromised (34,35). Second, several mouse models have recently been reported where genetic manipulation of specific genes results in changes in integrin function or activation-dependent integrin regulation (21,38–42). These mouse models exhibit a variety of changes in T cell functional responses, which are discussed later in this review.

These rapid increases in integrin activity following T cell stimulation do not require changes in levels of integrin expression on the T cell surface. Rather, integrin activation involves qualitative alterations in cell surface integrins. Activation can induce conformational changes in integrin receptors that can enhance their affinity for soluble ligand (43,44). In addition, integrin-activating antibodies and divalent cations such as Mn^{2+} enhance integrin affinity by inducing conformational changes in integrins (45,46) that can be detected by the expression of neo-epitopes recognized by unique integrin-specific antbodies (47,48). Many activation signals that enhance integrin functional activity also induce cytoskeletal-

dependent clustering of integrins on the cell surface (21,38,39,49). Although current evidence suggests that avidity modulation via effects on integrin clustering on the cell surface is a major mechanism by which the CD3/TCR regulates integrin functional activity (50), changes in integrin conformation are also likely to participate in activation-dependent changes in integrin function on T cells (51).

Inside-out signaling is a complex process. The initial appearance of nimiety among T cell integrin regulators (including CD3/TCR, CD2, CD7, CD28 and chemokine receptors) gives way, upon deeper analysis, to a complex picture of non-redundant and distinct pathways of adhesion regulation (52). Furthermore, the same integrin regulator may have different effects depending on the cell type in which it is expressed. For example, CD3/TCR ligation of resting, peripheral blood-derived CD4$^+$ T cells (14) results in increased adhesion to fibronectin via β1 integrins. On the other hand, TCR cross-linking on cytotoxic T cell clones also leads to upregulated adhesion to fibronectin, but this appears to be mediated by β3 integrins (i.e. αvβ3) and is not inhibited by antibodies that block β1 integrins also expressed on these cells (53). This study reveals a further level of complexity, namely, that integrin regulators can exert distinct effects on different integrins on the same cell. Tanaka and colleagues (54) noted that while CD31 and CD3 can both regulate β1 and β2 integrins, CD31 enhances β1 integrin-mediated adhesion to a greater extent than β2 integrin-mediated adhesion, whereas the opposite is observed for CD3. This led to the intriguing speculation that integrin regulators modulate predominantly the activity of integrins that are germane to the site where integrin regulator ligation is likely to occur (54). Finally, under physiological conditions it is probable that several integrin regulators, such as CD3/TCR and CD28, may be co-ligated, and the integration of multiple signals probably adds yet another level of complexity to integrin activation. Recently, Katagiri et al. (20) reported that simultaneously engaging CD3 and CD28 leads to lower T cell adhesion to ICAM-1 than ligating CD3 alone, or CD3 in combination with CD2. However, other studies in the mouse have not observed inhibitory effects on integrin activation of co-ligation of CD3 with CD28 (21).

CD3/TCR signaling to integrins

Understanding how the CD3/TCR complex regulates integrin function has posed a challenge, in particular because CD3/TCR ligation triggers a remarkably complex and varied set of intracellular signaling cascades. Some of these pathways, most

notably those necessary for the induction of cytokine synthesis, have been elucidated to a considerable extent (55). Yet, despite the fact that the CD3/TCR complex was first described as an integrin regulator over a decade ago (14,17), this particular function of CD3/TCR remains incompletely defined. It is perhaps instructive to begin with a review of the known early signaling events that occur following CD3/TCR stimulation. The CD3/TCR complex is a multimer comprised of the TCR and the non-covalently associated subunits CD3 γ, δ and ε as well as TCR-ζ, which is present as a disulfide-linked homodimer. The CD3/TCR complex lacks intrinsic kinase activity; within seconds after TCR ligation, the CD3 and TCR-ζ subunits become phosphorylated by src kinases such as Lck that specifically phosphorylate ITAMs (immunoreceptor tyrosine-based activation motifs) in the cytoplasmic tails of the CD3/TCR complex. There is one ITAM (consensus sequence, D/E xx YxxL x$_{6-8}$ YxxL/I) (56) in each CD3 subunit, whereas TCR-ζ contains three ITAMs. ITAM phosphorylation recruits the protein tyrosine kinase ZAP−70 via interaction of its tandem SH2 domains with the tandem phosphotyrosines present in the ITAMs (57,58). Upon its recruitment, ZAP-70 subsequently becomes activated via tyrosine phosphorylation by Lck or Fyn (58,59). ZAP-70 phosphorylates two adapter proteins, the microdomain-localized transmembrane protein linker for activated T cells (LAT) (60) and SH2-domain containing leukocyte-specific phosphoprotein (SLP-76 (61)). Like ZAP-70, mutations in either of these two proteins in mice have dramatic effects on T cell development and function (62−64). ZAP-70 activation, followed by tyrosine phosphorylation of LAT and SLP-76, leads to the rapid recruitment of a phalanx of signaling proteins that initiate downstream signaling pathways that lead to induction of transcription of the IL-2 gene following CD3/TCR stimulation (65,66).

Several lines of evidence argue that the src kinase Lck and ZAP-70 are both critically required for CD3/TCR signaling to integrins (*Table* 1). In Jurkat cells lacking either Lck (67) or ZAP-70 (68), CD3/TCR signaling fails to enhance β1 integrin-mediated adhesion to fibronectin (69,70). Re-expression of these molecules restores CD3/TCR-mediated increases in β1 integrin function (69,70). CD3/TCR signaling to integrins requires the kinase activity of ZAP-70, as a kinase-inactive form of ZAP-70 (K369R) does not restore CD3/TCR-mediated increases in β1 integrin function in the ZAP-70-deficient Jurkat T cell line P116. Furthermore, expression of kinase-inactive ZAP-70 inhibits CD3/TCR-mediated increases in adhesion of peripheral T cells to fibronectin (70). Although these studies confirm the central role that these TCR-

proximal tyrosine kinases play in various TCR effector re- sponses, further analysis indicates that CD3/TCR signaling to β1 integrin activation may bifurcate early from other pre- viously characterized CD3/TCR signaling pathways and have its own unique characteristics. For example, while mutating the tyrosine at position 319 in the interdomain B region of ZAP-70 to phenylalanine (Y319F) abrogates CD3/TCR acti- vation of NF-AT, LAT phosphorylation, calcium flux, CD69 upregulation and IL-2 production (71,72), this mutation does not significantly affect CD3/TCR signaling to β1 inte- grins (70). In addition, over-expression of wild-type SLP-76, which enhances CD3/TCR-mediated activation of NF-AT (73), has no effect on CD3/TCR-mediated activation of inte- grins (70). Thus, it will be critical to further define the mech- anism by which ZAP-70 and its substrates, LAT and SLP-76, couple the CD3/TCR to integrin activation.

Although CD3 mAb-induced activation of the TCR fails to enhance β1 integrin function in ZAP-70-deficient Jurkat T cells (70), LFA-1-dependent conjugate formation of these same cells with superantigen-pulsed B cells is not impaired when compared to wild-type Jurkat T cells (74). This finding suggests potential differences in the mechanisms by which T cells regulate β1 and β2 integrins. However, since LFA-1 activ- ity during T-B conjugate formation is probably regulated by signals provided by the CD3/TCR as well as other receptors, the requirement for ZAP-70 in regulating LFA-1 function in- duced solely by CD3/TCR ligation is not clear at present. Jur- kat T cells may not represent the best system for assessing this issue, as differences have been noted between laboratories in the ability of CD3/TCR signaling to activate LFA-1-dependent adhesion of Jurkat T cells to immobilized ICAM-1 (74–76).

The use of pharmacological inhibitors and dominant-nega- tive constructs has also implicated the lipid kinase phosphati- dylinositol 3-kinase (PI 3-K) in regulating integrin activation in many cell types (77). Studies in T cells suggest that inhi- bition of the activity of the class I PI 3-K isoforms (α, β, γ and δ) can block the activation of integrins induced by a variety of cell surface receptors, including the CD3/TCR (21,44), CD2 (78), CD7 (79), CD28 (80) and chemokine receptors (28). In addition, neutrophils and macrophages from mice lacking the PI 3-K γ isoform exhibit defects in migration in response to chemoattractants that activate G pro- tein-coupled receptors (81–83). Although studies with macrophages suggest a role for PI 3-K β and δ, but not PI 3- K α, in regulating macrophage motility (84), the contribution of the various class I PI 3-K isoforms to regulating integrin activation by the CD3/TCR remains unknown.

Despite a common dependence of many integrin regulators on PI 3-K, the mode whereby PI 3-K is recruited appears to be different in each case. For example, PI 3-K may bind to CD7 and CD28 directly via recognition of tyrosine phos- phorylated YxxM sequences by the SH2 domain of the p85 regulatory subunit of PI 3-K (79,80). Mutagenesis of this se- quence in a CD2/CD28 chimera abrogates p85 association as well as CD2/CD28-stimulated adhesion (80). On the other hand, the CD2 cytoplasmic domain contains no tyrosine resi- dues, yet associates constitutively with p85 (78). Although CD3/TCR stimulation clearly leads to increased PI 3-K activity (85), the mechanism by which the CD3/TCR is coupled to PI 3-K signaling is unclear. Direct association of the p85 sub- unit with ITAM sequences has been reported (86,87). In ad- dition, two transmembrane adapter proteins that are phos-

Table 1. Key molecules implicated in CD3/TCR signaling to integrins

Molecule	Type	Comments	Refs
PKC	Ser-thr kinase	PKC inhibitors block integrin activation by CD3/TCR	17,75,105
Lck	Tyr kinase	Integrin activation by CD3/TCR impaired in Lck-deficient Jurkat T cells	69,74
PI 3-K	Lipid kinase	PI 3-K inhibitors block integrin activation by CD3/TCR	21,44
ZAP-70	Tyr kinase	β1 integrin activation by CD3/TCR impaired in ZAP-70-deficient Jurkat T cells; kinase-inactive ZAP-70 blocks β1 integrin activation by CD3/TCR in primary human T cells	70
Itk	Tyr kinase	β1 integrin activation by CD3/TCR blocked by kinase-inactive Itk or PH domain of Itk	69
ADAP	Adapter	Integrin activation and clustering induced by CD3/TCR impaired in ADAP KO T cells	38,39
Vav	GEF	Integrin activation and clustering induced by CD3/TCR impaired in Vav KO T cells	21
Cytohesin-1	GEF	PH domain of cytohesin-1 blocks LFA-1 activation induced by CD3/TCR	121,123
Rap1	GTPase	Enhanced integrin function upon expression of active Rap1; putative dominant-negative blocks integrin activation induced by CD3/TCR	20,42,118,120,121
H-ras	GTPase	Dominant-negative Ras blocks LFA-1 activation induced by CD3/TCR	109

phorylated upon CD3/TCR stimulation, LAT and TRIM, associate with PI 3-K in a CD3/TCR-dependent manner (60,88). Activation of PI 3-K in B cells by the B cell receptor is regulated by the Syk tyrosine kinase (89,90), suggesting the possibility that ZAP-70 may be involved in regulating activation of PI 3-K by the CD3/TCR in T cells.

One major function of PI 3-K is the generation at the plasma membrane of phosphatidylinositol (3,4,5)-trisphosphate [PI(3,4,5)-P$_3$], which can mediate the membrane recruitment of proteins containing pleckstrin homology (PH) domains (91). The Itk tyrosine kinase, which is a member of the Tec family of non-receptor tyrosine kinases, contains a PH domain that mediates binding to PI(3,4,5)-P$_3$ (92). Itk becomes activated following CD3/TCR stimulation, and Itk has been implicated in regulating PLC-γ1 activity (92). Furthermore, the association of Itk with both SLP-76 and LAT has been reported (93–95), which would place Itk in proximity to PLC-γ1 in membrane microdomains. Analysis of CD3/TCR-mediated activation of integrin function suggests that Itk may also represent a critical downstream effector of src family kinases and PI 3-K (69). Itk function is regulated not only by PI 3-K, which mediates recruitment of Itk to membrane microdomains, but also by microdomain-localized src family kinases such as Lck, which phosphorylates and thus increases Itk kinase activity (96). The ability to block CD3/TCR signaling to integrins by inhibiting either PI 3-K activity or Lck activity suggests a potential function for Itk in regulating integrin activity. In addition, a role for ZAP-70 in regulating CD3/TCR-mediated activation of Itk has been reported (97). In functional studies, expression of an active form of PI 3-K, together with CD4-mediated activation of Lck, is sufficient to induce increases in integrin function in T cells independently of CD3/TCR ligation (69). Furthermore, this increase in adhesion can be blocked by co-expression of a kinase-inactive form of Itk. A membrane-targeted form of Itk can also replace active PI 3-K and induce increased adhesion when Lck is activated by CD4 cross-linking. Thus, these studies are consistent with a role for Itk downstream of both Lck and PI 3-K in regulating integrin function. More significantly, expression of a kinase-inactive form of Itk in either Jurkat T cells or primary human T cell blasts inhibits CD3/TCR-mediated activation of integrins (69). The PH domain of Itk plays a critical regulatory role, as expression of just the PH domain of Itk can also block CD3/TCR signaling to integrins. Together, this work suggests that CD3/TCR signaling to integrins involves PI 3-K-dependent recruitment of Itk to membrane microdomains, via interaction of the PH domain of Itk with PI(3,4,5)-P$_3$. This recruitment of Itk to the plasma membrane places Itk in proximity to Lck, which can then activate Itk via tyrosine phosphorylation.

It is not known whether other members of the Tec family of tyrosine kinases are also involved in regulating integrin function. Although the Etk tyrosine kinase is expressed in Jurkat T cells and Etk has recently been implicated in regulating the migration of tumor cells (98), kinase-inactive Etk constructs do not inhibit CD3/TCR signaling to integrins (69). In addition, expression of the Etk PH domain does not block CD3-induced activation of β1 integrins (69) and expression of the Btk PH domain in Jurkat T cells does not inhibit CD3-induced activation of LFA-1 (99). Future analysis of mice deficient in expression of specific Tec family kinases (100) is likely to be informative. It is also interesting to note that other co-receptors that activate integrins on T cells, including CD2 and CD28, also activate Itk (101,102) and thus Itk may be involved in regulating integrin function initiated by other receptors in addition to the CD3/TCR.

There are several possible mechanisms by which Itk might regulate integrin activation by the CD3/TCR. The function of Itk in regulating calcium signaling via PLC-γ1 (100,103,104) may be critical to Itk-mediated regulation of integrin function, because calcium ionophores are sufficient to induce increased integrin-mediated adhesion (23,30). This is further supported by the observation that T cells migrating on lipid bilayers containing ICAM-1 stop upon recognition of MHC-antigen complexes, and this arrest is accompanied by an increase in intracellular calcium that is not inhibited by depletion of intracellular calcium (22). In addition, activating PLC-γ1 generates diacylglycerol, which activates PKC. The ability of phorbol esters such as PMA to upregulate adhesion suggests that PKC may be an important component of integrin regulation, and indeed CD3-stimulated adhesion through LFA-1 as well as β1 integrins is partially sensitive to staurosporine (17,75). In addition, GF109203X, a bisindolylmaleide that is a more specific PKC inhibitor than staurosporine, inhibits anti-CD3 stimulated T cell:B cell conjugate formation mediated by LFA-1 (105), further supporting a role for PKC in integrin regulation by the CD3/TCR complex. It will be critical in the future to identify the PKC isoforms involved in regulating integrin activity by both PMA and the CD3/TCR, as both reported cases of defective integrin activation in LAD variants exhibit specific defects in PMA-induced increases in integrin function (34,35).

Itk may also regulate integrin activation by coupling the CD3/TCR to cytoskeletal modification, since expression of either kinase-inactive Itk or the PH domain of Itk partially inhibits CD3/TCR-mediated actin polymerization (69). In ad-

dition, Itk has been reported to associate with cytoskeletal regulatory proteins, such as Wiskott–Aldrich syndrome protein (WASP) (106). Furthermore, another Tec family kinase member, Btk, has been proposed to play a role in regulating Rho-family GTPase activity in B cells (107). This is particularly interesting in terms of our understanding of integrin activation, since CD3/TCR stimulation activates GTPases and these GTPases play a central role in cytoskeletal reorganization (108). It is now clear that modulating GTPase activity can alter integrin function as well. Using a thymocyte cell line, O'Rourke and colleagues showed that a dominant-negative form of p21*ras* blocks the CD3-stimulated increases in adhesion to ICAM-1, and that mitogen-activated protein kinase (MAPK) is involved (109). In addition, expression of a constitutively active form of H-ras enhances LFA-1-dependent adhesion of T cells to ICAM-1 (110). However, constitutively active H-ras does not enhance the adhesion of Jurkat T cells to fibronectin (111), and studies in non-lymphoid cells have suggested a potential negative regulatory role for H-ras in integrin function (112).

PI 3-K, itself a potential downstream effector of Ras (113), can affect cytoskeletal reorganization by regulating the GTPases Rac and Rho (114). These small GTPases, in addition to Cdc42, have been implicated in activation-induced polarization and integrin-mediated adhesion of leukocytes (115,116). Expression of a constitutively active form of Rac, but not constitutively active forms of Rho, Cdc42 or ARF6, enhances the spreading and adhesion of Jurkat T cells to fibronectin in a manner that does not involve changes in integrin affinity (111). These results are similar in some respects to studies demonstrating that constitutively active R-ras can enhance integrin function, although the effects of R-ras in this system may be more dependent on modulating integrin affinity (117). Since R-ras is expressed at very low levels in Jurkat T cells (118), the function of R-ras in CD3/TCR signaling is currently unclear.

Several *in vitro* studies have focused on the function of the small GTPase, Rap1, in regulating integrin function (119). These studies, which utilize constitutively active Rap1 and a putative dominant-negative form of Rap1, suggest that modulation of Rap1 activity can alter both basal integrin activity and increases in integrin activity induced by several stimuli, including CD3/TCR stimulation, CD31 stimulation and inflammatory mediators such as LPS (20,118,120,121). The mechanism whereby Rap1 acts to modulate integrin activity in T cells may not require PI 3-K, as the adhesion to ICAM-1 triggered by a constitutively active form of Rap1, expressed in the BAF pro-B cell line, is not inhibited by wortmannin

(120). More recent studies have found that dominant negative Rap1, but not dominant negative H-Ras, inhibits CD3/TCR-mediated adhesion of Jurkat T cells to ICAM-1 (20). In addition, dominant-negative Rap1 or SPA-1, a Rap1 GTPase activating protein, expressed in hen egg lysozyme (HEL)-specific 3A9 transgenic T cells inhibits T cell adhesion to HEL-pulsed APCs.

Although these studies clearly demonstrate that activation of GTPase activity is in many cases sufficient to enhance integrin-mediated adhesion, the precise role that these GTPases play in signaling from the CD3/TCR to integrins remains an area deserving of further exploration. Furthermore, the role that other signaling proteins implicated in integrin activation, such as PI 3-K and Itk, play in coupling the CD3/TCR to these GTPases remains unclear. These relationships are likely to be complex and specific to each GTPase, as studies in mast cells suggest that adhesion induced by active H-ras, but not active R-ras, is dependent on PI 3-K (122).

Another potential link between PI 3-K and regulation of integrin activity by the CD3/TCR is cytohesin-1, a protein containing a PH domain that associates with the β2 integrin cytoplasmic domain (123) and also has GEF activity for the ADP ribosylation factor GTPases (124). Over-expression of cytohesin-1 enhances basal adhesion of Jurkat T cells to ICAM-1, and expression of the PH domain of cytohesin-1 blocks CD3-induced increases in LFA-1 activity on Jurkat T cells (123). These effects of cytohesin-1 on integrin function appear to be specific to LFA-1, as over-expression does not alter β1 integrin function in Jurkat T cells (123). PI 3-K regulates the membrane recruitment of cytohesin-1 (99). The effects of cytohesin-1 on LFA-1 function are complex, and depend on both the ability of cytohesin-1 to interact with LFA-1 as well as on the ARF-GEF activity of cytohesin-1 (125).

Novel mouse models of integrin activation

In vitro studies of the biochemical pathways that couple the CD3/TCR to integrins have only recently been complemented with an analysis of integrin activation events in various mouse knockout models (*Table 1*). This approach has led to the identification of the adapter protein adhesion and degranulation promoting adapter protein (ADAP), known previously as SLP-76 associated phosphoprotein of 130 kDa (SLAP-130) or Fyn binding protein (Fyb), as a critical protein involved in CD3/TCR-mediated activation of both β2 and β1 integrins (38,39). ADAP contains an SH3-like domain, a proline-rich sequence and numerous tyrosine phosphorylation sites. CD3/TCR

stimulation results in tyrosine phosphorylation of ADAP, and ADAP was initially identified as associating with both SLP-76 (126) and Fyn (127) upon CD3/TCR stimulation. However, early studies failed to reach a consensus on whether tyrosine-phosphorylated ADAP relayed negative or positive signals for IL-2 transcription (128,129). In vitro studies suggested a potential role for ADAP in regulating integrin-dependent cell responses, as ADAP is found concentrated at contact sites between Jurkat T cells and anti-CD3 coated beads (130), and is postulated to link the CD3/TCR complex to the cytoskeleton via its interaction with Ena/VASP proteins and association with the Arp2/3 complex (130). Similar findings have been reported in macrophages, where a protein complex consisting of ADAP, SLP-76, Ena/VASP proteins, Nck and WASP forms during the initiation of Fc receptor-mediated signaling events that lead to phagocytosis (131). Over-expression of ADAP enhances integrin-dependent migration of T cells in vitro (132), as well as integrin-mediated adhesion of the RBL-2H3 mast cell line and the release of β-hexosaminidase by this cell line following FcεRI stimulation (133).

Two groups have now analyzed T cell function in mice lacking ADAP (38,39). Unlike mice deficient in SLP-76 (63,64), ADAP-deficient T cells develop normally in a non-TCR transgenic background. However, ADAP-deficient T cells exhibit dramatically reduced proliferation, cytokine production and induction of the activation markers CD25 and CD69 in response to anti-CD3 antibody stimulation (38,39). In addition, anti-CD3 stimulation fails to result in enhanced adhesion of ADAP-deficient T cells to integrin ligands such as ICAM-1 and fibronectin. This appears to be specific to activation-dependent integrin regulation, as there is comparable basal adhesion of wild-type and ADAP-deficient T cells to these integrin ligands. In addition, the ability of PMA to enhance the integrin-mediated adhesion of ADAP-deficient T cells to levels comparable to wild-type T cells suggests that ADAP deficiency does not globally impair the ability of integrins to respond to activation signals. These defects in integrin activation in ADAP-deficient mice are accompanied with a loss of integrin clustering in response to anti-CD3 stimulation (38,39). This deficit in integrin clustering upon anti-CD3 stimulation in ADAP-deficient T cells is fairly specific, as global actin polymerization and clustering of the TCR following anti-CD3 stimulation are not impaired in ADAP-deficient T cells.

Although ADAP was initially identified based on its interaction with both SLP-76 and Fyn, loss of ADAP does not appreciably alter proximal TCR signaling events (38,39). Thus, when compared to wild-type T cells, ADAP-deficient T cells exhibit comparable calcium mobilization and MAPK activation in response to anti-CD3 stimulation. In addition, anti-CD3 stimulation of ADAP-deficient T cells results in tyrosine phosphorylation of SLP-76, PLC-γ1, LAT, p120^{Cbl}, Vav1, Fyn and Lck at levels comparable to wild-type T cells. Thus, these early signaling events are not sufficient to couple the CD3/TCR to integrins in the absence of ADAP. It is interesting to note that mice lacking the LFA-1 integrin exhibit profound hypocellularity in secondary lymphoid organs, due to an inability of LFA-1-deficient T cells to migrate efficiently into these tissue sites (134). In contrast, ADAP-deficient mice do not exhibit such dramatic hypocellularity in lymph nodes (38,39). Because trafficking of T cells into lymph nodes from the blood involves chemokine receptor-mediated activation of integrins such as LFA-1, this suggests that loss of ADAP may not dramatically impair integrin activation induced by G protein-coupled receptors.

The loss of CD3/TCR-induced clustering of LFA-1 in ADAP-deficient T cells is consistent not only with the known interaction of integrins with the cytoskeleton but also with the interaction of ADAP with Ena/VASP proteins (130). Interestingly, in contrast to the positive regulatory role that ADAP plays in CD3/TCR signaling to integrins, platelets from mice lacking VASP exhibit enhanced integrin activation following thrombin stimulation (135). Furthermore, in vitro studies have demonstrated that sequestration of Ena/VASP proteins away from the plasma membrane enhances the speed of fibroblast movement (136). Since over-expression approaches have also implicated ADAP in regulating T cell motility (132), it will be critical to determine if T cell motility is altered in ADAP-deficient mice. Since β1 integrin stimulation on T cells also leads to ADAP tyrosine phosphorylation (132), this adapter protein may also participate in outside-in as well as inside-out signaling events.

Similar to what has been identified in ADAP-deficient mice, T cells from mice lacking the Vav1 guanine-nucleotide exchange factor (137) also exhibit a defect in CD3-induced increases in β2 and β1 integrin-mediated adhesion as well as CD3-induced integrin clustering (21). This report documented a critical function for Vav1 in mediating T cell adhesion to APCs expressing a relevant MHC/peptide complex (21). However, unlike ADAP-deficient T cells, Vav1-deficient T cells also exhibit defects in TCR clustering and actin reorganization (21,138,139). Vav1 has been proposed to regulate cytoskeletal reorganization via a signaling pathway that involves WASP (140), and in fact WASP-deficient T cells exhibit impaired TCR clustering (21). However, unlike both ADAP-deficient and Vav1-deficient T cells, CD3/TCR signaling to

integrins and CD3-induced clustering of LFA-1 is not impaired in the absence of WASP. Thus, these studies suggest that in addition to ADAP, CD3/TCR signaling to integrins and CD3-mediated clustering of integrins involves Vav1, but not WASP. Like ADAP, Vav1 may also regulate integrin-dependent signaling, as integrin-mediated tyrosine phosphorylation of the tyrosine kinase Pyk2 is impaired in Vav1-deficient T cells, but not in WASP-deficient T cells (21).

The biochemical mechanism by which Vav1 regulates CD3/TCR signaling to integrins is not known. However, it is interesting to note that unlike ADAP-deficient T cells, CD3/TCR-mediated calcium flux and activation of MAPK is impaired in Vav-deficient T cells (141). Vav1 is also known to regulate the exchange activity of Rho family GTPases such as Rac (137). Thus, the effects of loss of Vav1 on integrin activation may be related to changes in the coupling of the CD3/TCR to GTPases such as Rac. Consistent with some of the *in vitro* studies using constitutively active Rac (111), transgenic mice expressing constitutively active Rac in T cells exhibit enhanced basal adhesion to integrin ligands such as fibronectin (41). Similar effects on basal integrin activity have also been observed in mice expressing constitutively active forms of RhoA (40) and Rap1 (42). Together, these studies emphasize that activation of any of these GTPases is sufficient to induce changes in the basal activity of integrin receptors.

In mice lacking ADAP or Vav, loss of CD3/TCR signaling to integrins is associated with loss of CD3-mediated clustering of integrins (21,38,39). In addition, LFA-1 exhibits a more clustered distribution on T cells isolated from transgenic mice expressing constitutively active Rap1 when compared to wild-type controls (42). Expression of constitutively active Rap1 also does not enhance T cell binding of soluble mouse ICAM-1 (42). Together, these studies provide further evidence that alteration of integrin clustering that leads to increased avidity is a major mechanism by which integrin activity is regulated in T cells (47,51,142). However, it should be noted that the affinity of integrins expressed on T cells can clearly be regulated by T cell stimulation. Early reports showed that CD3/TCR ligation of the HUT-78 T cell line leads to increased binding of soluble fibronectin (43). CD3 stimulation of freshly isolated peripheral blood T cells also increases binding of soluble FN and the appearance of an activation epitope on β1 integrins. However, this is not observed with Jurkat T cells under similar activation conditions, and the addition of soluble fibronectin does not effectively inhibit T cell adhesion to immobilized fibronectin (44). Other studies have also documented that anti-CD3 stimulation elicits T cell adhesion to immobilized but not soluble β1 and β2 integrin ligands (50,143).

The TCR-ζ subunit and integrin activation

The structural requirements within the CD3/TCR that are necessary for integrin regulation remain poorly characterized. We have utilized chimeric receptors expressing the cytoplasmic domain of the TCR-ζ subunit to explore this issue. Previous studies using such chimeric receptors showed that the TCR-ζ cytoplasmic tail can couple to signal transduction pathways that result in many of the early and late events associated with CD3/TCR signaling, including an increase in intracellular free calcium, generation of inositol phosphates, protein tyrosine phosphorylation, expression of the activation marker CD69, induction of IL-2 production, and cytolysis (144–146). In addition, these studies indicated that the ITAMs present in the cytoplasmic tail are necessary and sufficient for intracellular signaling (146–148). Studies using TCR-ζ-deficient T cell lines noted that antigen-specific stimulation of IL-2 production was sensitive to truncations of the TCR-ζ cytoplasmic tail. A TCR-ζ mutant that was truncated midway through the third ITAM (retaining residues 1–129) led to reduced IL-2 production in a T cell hybridoma line, and a transfectant expressing a ζ chain containing only one ITAM (retaining residues 1–87) failed to produce any IL-2 in response to antigen stimulation (149). Interestingly, a consensus nucleotide binding sequence is found between the second and third ITAMs, and a single mutation within this sequence, substituting G^{114} for a valine, reproduces the phenotype observed in the 1–87 truncation (149). This suggests that sequences outside of the ITAM may be important for TCR-ζ function. To assess more directly the contribution of ITAMs to TCR-ζ signaling, Romeo and colleagues used a chimeric molecule in which the membrane-proximal ITAM was fused to the CD16 extracellular and CD7 transmembrane domain, and the effects of various point mutations on chimera-triggered calcium flux and cytolysis were tested (146). This assay revealed an absolute dependence on both tyrosines in the ITAM to carry out these functions, while mutations at other sites had only moderate effects. Furthermore, Wegener & Malissen showed that IL-2 production stimulated by a chimera containing the third TCR-ζ ITAM was completely impaired if any one of the tyrosines or leucines was mutated (148). The presence of three ITAM motifs in the cytoplasmic tail of TCR-ζ may provide a signal amplification mechanism (146,147,150,151); in addition, each ITAM may be coupled to discrete downstream signaling pathways (148,152–154). It may be significant that the non-consensus amino acids in the sequence YxxLx (6–8) YxxL/I differ among the three ITAMs, and mutational analyses have shown that alteration of

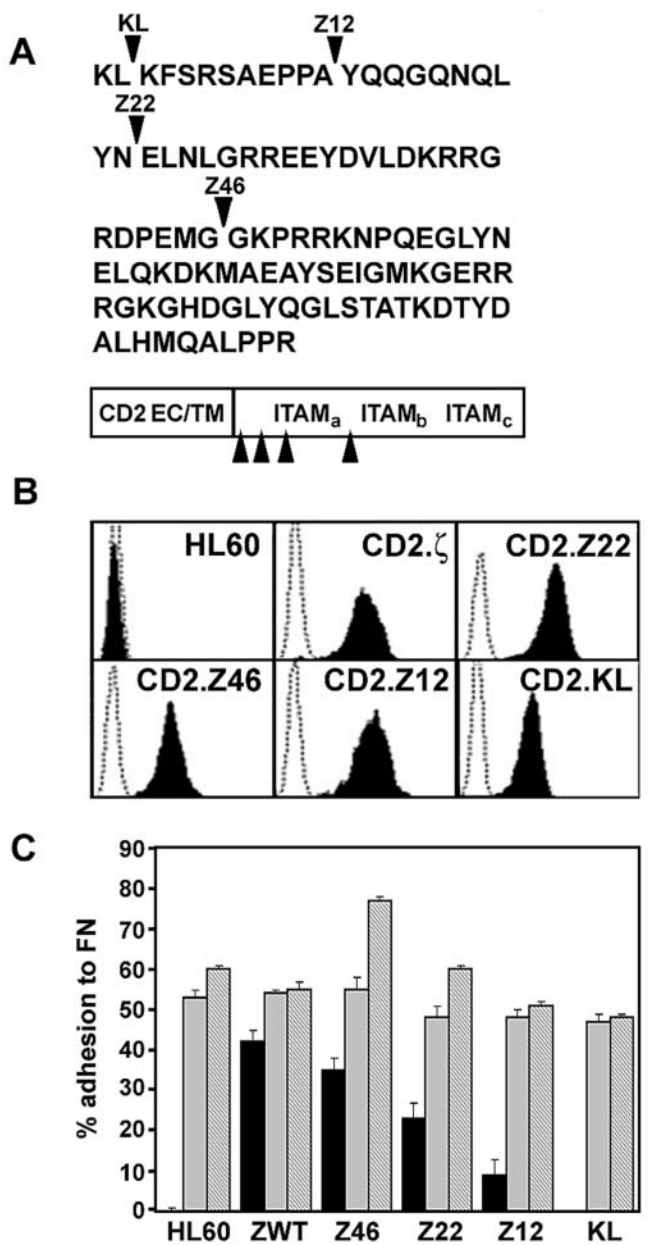residues within the ITAMs other than the two tyrosines and two leucines can affect ζ function (146,155).

To assess the ability of ζ to regulate $\beta1$ integrins, we constructed a chimeric protein containing the extracellular and transmembrane sequence of CD2 and the cytoplasmic sequence of TCR-ζ. This CD2.ζ chimera was expressed in HL60, a myelomonocytic cell line that lacks CD2 expression and has a $\beta1$ integrin profile and adhesion dynamics similar to T cells (78,80). Cross-linking CD2.ζ on these transfectants leads to a rapid and transient tyrosine phosphorylation of multiple proteins, indicating that the TCR-ζ cytoplasmic tail can couple to protein tyrosine kinases and trigger intracellular signal transduction pathways (Fig. 1a). Furthermore, CD2.ζ cross-linking dramatically enhances adhesion to fibronectin (Fig. 1b) that is inhibited by anti-$\alpha4$ and anti-$\alpha5$ integrin antbodies (Fig. 1c), sensitive to ligand density and the extent of cross-linking of the chimera (not shown). Adhesion increases to levels comparable to those obtained with PMA. In contrast to the sustained increase in adhesion mediated by direct activation of $\beta1$ integrins with the integrin-activating antibody TS2/16, the CD2.ζ-stimulated increase is transient, generally peaking at 10 min (Fig. 1b).

We next sought to determine the sequence requirements within the ζ cytoplasmic tail that are necessary for integrin regulation. Constructs encoding truncation mutants of CD2.ζ were generated; CD2.Z46, CD2.Z22, CD2.Z12 and CD2.KL retain 46, 22, 12 or 2 amino acids, respectively, of the recombinant ζ cytoplasmic sequence (Fig. 2a). These chimeric truncation mutants were expressed in HL60 (Fig. 2b). Cross-linking these molecules leads to increased protein tyrosine phosphorylation whose intensity correlates generally with the length of the cytoplasmic tail, with the exception of CD2.Z46, which elicits similar levels of tyrosine kinase activity as CD2.ζ (not shown). In addition, cross-linking CD2.Z46 enhances adhesion similar to that observed with CD2.ζ (Fig. 2c, and data not shown). Taken together, this suggests that truncating two of the three ITAMs from the ζ cytoplasmic tail does not impair its ability to couple to signaling pathways.

Surprisingly, CD2.Z22, which lacks ITAMs, revealed itself to be far from effete, as cross-linking this molecule leads to a significant increase in adhesion (Figs 2c and 3a). The adhesion stimulated by CD2.Z22 displays the same kinetics and fibronectin concentration dependence as CD2.ζ (not shown). Stimulation of adhesion is lost when the ζ cytoplasmic tail is further truncated to 12 amino acids in CD2.Z12 (Fig. 2c). Therefore, the first 22 residues of the ζ cytoplasmic tail are sufficient to upregulate adhesion. Integrin activation elicited by CD2.ζ and the mutant CD2.Z22 share a similar sensitivity
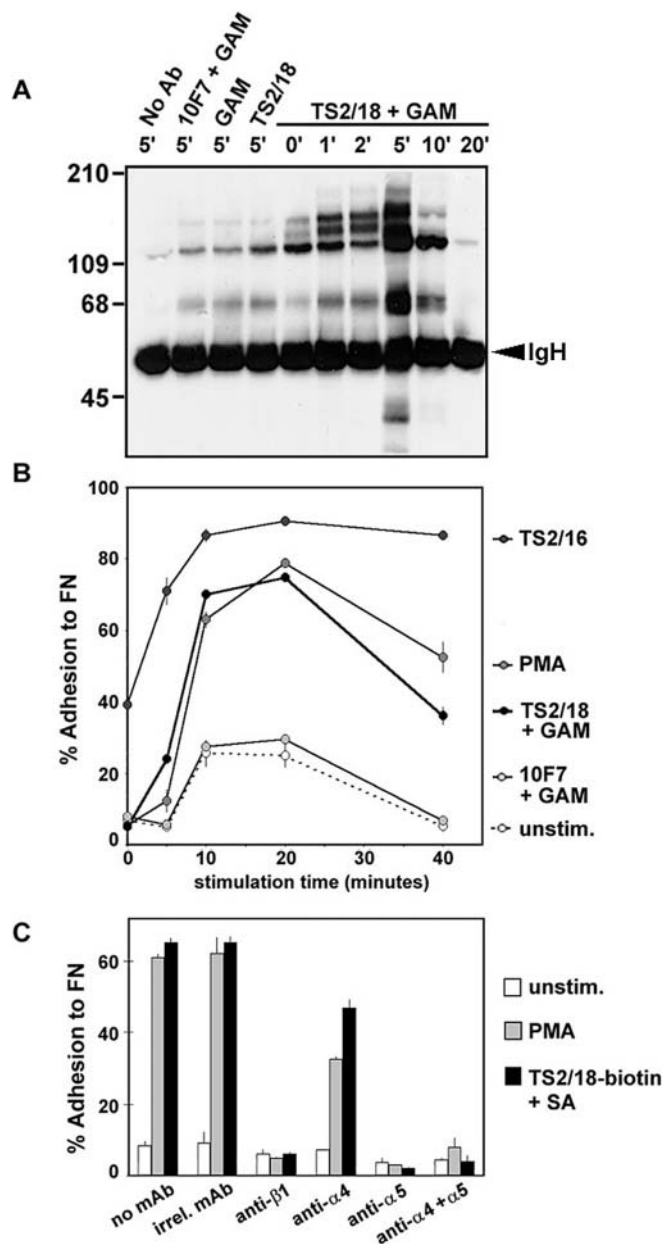
Fig. 1. CD2.ζ stimulation initiates intracellular signaling and upregulates integrin-mediated adhesion. (A) Stable CD2.ζ transfectants of HL60 cells were incubated with irrelevant antibody (10F7), anti-CD2 mAb TS2/18, or no antibody, and secondary goat antimouse antibody (GAM) was added as indicated. Cells were warmed for the time points shown and the lysates immunoprecipitated with antiphosphotyrosine antibody. Western blot analysis was carried out and tyrosine phosphorylated proteins were detected with the antiphosphotyrosine antibody 4G10. **(B)** Adhesion of CD2.ζ transfectants to fibronectin (FN) was assessed in the presence of TS2/18 or 10F7 plus secondary antibody, or PMA, or the $\beta1$ integrin-specific activating mAb TS2/16. **(C)** Integrin specificity was tested by the addition of $\beta1$-, $\alpha4$- and $\alpha5$-blocking antibodies to the assay and adhesion was measured after 10 min at 37 °C. In this assay, CD2.ζ was cross-linked using anti-CD2-biotin followed by streptavidin to avoid cross-linking the integrin-specific antibodies.

A

KL   Z12

KL KFSRSAEPPA YQQGQNQL

Z22

YN ELNLGRRREEYDVLDKRRG

Z46

RDPEMG GKPRRKNPQEGLYN
ELQKDKMAEAYSEIGMKGERR
RGKGHDGLYQGLSTATKDTYD
ALHMQALPPR

| CD2 EC/TM | ITAM_a | ITAM_b | ITAM_c |

B

C

Fig. 2. Sequence requirements for CD2.ζ-stimulated adhesion.
(A) Cytoplasmic sequence of CD2.ζ. The termination of each truncation mutant is indicated by an arrowhead. **(B)** Clonally derived HL60 transfectants were obtained and chimera expression assessed by flow cytometry. Open histograms represent staining with secondary antibody alone. Closed histograms represent staining with anti-CD2 mAb followed by secondary FITC-conjugated goat antimouse IgG. **(C)** Integrin regulation by each CD2.ζ truncation mutant was compared to untransfected HL60 cells and CD2.ζ cells. Cells were plated on fibronectin in the presence of TS2/18 plus GAM (black bars), PMA (light gray bars), or TS2/16 (hatched bars). Stimulation was for 10 min. Results are representative of at least three experiments for each transfectant, and are presented as adhesion above that obtained with an irrelevant antibody (10F7) plus GAM.

A.

B.
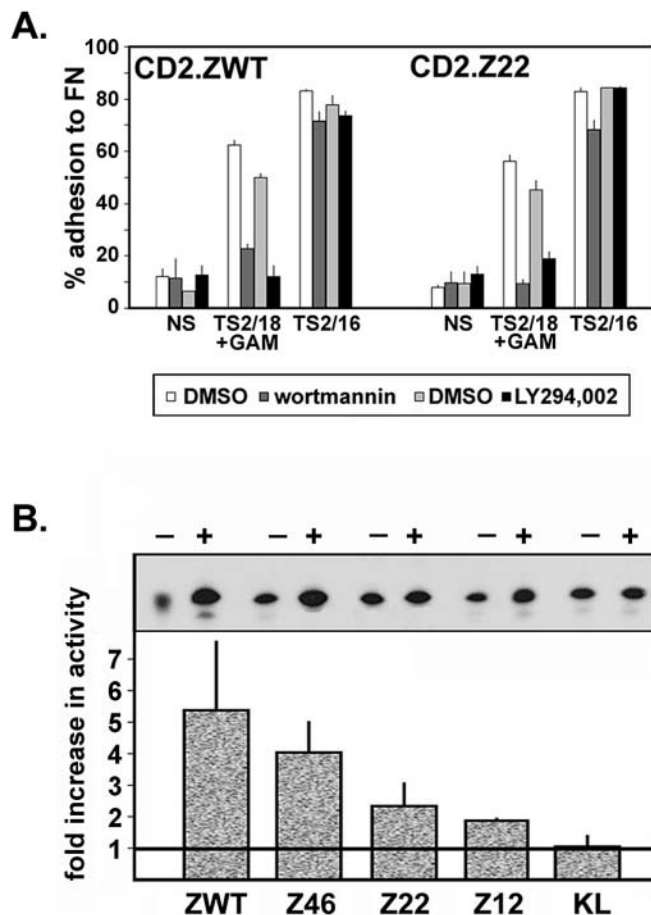
Fig. 3. PI 3-kinase plays a role in CD2.ζ-stimulated adhesion.
(A) CD2.ζ and CD2.Z22 transfectants were tested in parallel using the indicated stimulation conditions. Adhesion assays were carried out in the presence of 30 nM wortmannin (dark gray bars), 25 μM LY294 002 (black bars) or concentration-matched DMSO (white and light gray bars, respectively). Results are representative of at least three experiments for each transfectant. Optimal inhibitor concentrations were determined in preliminary titration assays (not shown). **(B)** In vitro lipid kinase assays were carried out using lysates prepared from transfectants stimulated for 5 min with TS2/18 (+) or 10F7 (−) followed by GAM. Equal amounts of lysate from each transfectant were immunoprecipitated with antiphosphotyrosine antibody and the immunoprecipitates mixed with phosphatidylinositol substrate in the presence of [γ-^{32}P]-ATP. Lipid was resolved by thin layer chromatography, and incorporation of phosphate determined by phosphorimaging and autoradiography. Representative results are shown in the top panel, and the average of at least two experiments for each transfectant are shown in the lower panel.

to wortmannin and LY294,002, two chemically distinct inhibitors of PI 3-K. Because integrin activation triggered by CD3/TCR cross-linking is also sensitive to these inhibitors, it suggests that β1 integrin regulation by the amino-terminal 22 residues of the TCR-ζ cytoplasmic tail utilizes the same signaling pathway(s) as the full length ζ cytoplasmic tail. Neither the p85 regulatory subunit of PI 3-K nor PI 3-K lipid

kinase activity could be co-precipitated with the CD2.ζ chimeras, consistent with other reports that failed to co-precipitate p85 with CD8/ζ or TCR-ζ from stimulated Jurkat T cells (156,157). However, p85 could be co-precipitated by anti-phosphotyrosine antibody, and a robust increase in lipid kinase activity is detected in antiphosphotyrosine precipitated lysates following CD2.ZWT or CD2.Z46 cross-linking (Fig. 3b). A modest increase in lipid kinase activity is observed upon activation of CD2.Z22 and CD2.Z12, while cross-linking CD2.KL had no effect. In contrast, rapamycin, PD098059, bisindolylmaleimide or U73122 which, respectively, inhibit the PI 3-K substrate p70S6 kinase, MEK, PKC and PLC-γ, did not impair the stimulation of β1-integrin mediated adhesion (not shown).

To further address the importance of intact ITAMs in TCR-ζ regulation of β1 integrins, we created an additional mutant (CD2.ZYF) in which all six tyrosine residues within the ITAMs were changed to phenylalanine (Fig. 4a). This mutant was expressed in HL60 cells at levels comparable to CD2.ζ (Fig. 4b). Cross-linking CD2.ZYF leads to a substantial and reproducible increase in cell adhesion to fibronectin (Fig. 4c). This increase is somewhat less than that elicited by CD2.ζ; consistent with this finding, activation of CD2.ZYF leads to relatively reduced protein tyrosine phosphorylation (not shown). The increased adhesion mediated by CD2.ZYF is completely blocked by both wortmannin (Fig. 4c) and LY294 002 (not shown). In light of the hypothesis that PI 3-K may be recruited to the CD3/TCR signaling complex through interactions of the p85 SH2 domain with ITAM YxxL sequences in the TCR-ζ subunit (86,87), or indirectly through other ITAM-dependent means, it is notable that the PI 3-K-dependent activation of β1 integrins is likely to be ITAM-independent in the case of CD2.Z22 or CD2.ZYF.

These unexpected observations suggest that ITAMs are not essential for ζ-mediated β1 integrin activation. This conclusion is based on evidence from two distinct CD2.ζ mutations that lack functional ITAMs, either because the ζ cytoplasmic tail has been truncated to 22 residues (CD2.Z22) or the ITAM tyrosine residues have been altered to phenylalanine (CD2.ZYF). It suggests that a sequence within cytoplasmic residues 1–22 has signaling capability. It is, however, noteworthy that neither mutant exhibits the full integrin activating capability of the wild-type chimera. Furthermore, the truncation mutant CD2.Z46 (whose cytoplasmic tail contains the first 20 membrane-proximal residues in addition to the first ITAM) is indistinguishable from wild-type CD2.ζ, suggesting that maximal integrin activation by TCR-ζ requires both the membrane-proximal domain and at least 1 ITAM.

An earlier report found that CD8.ζ22 (a chimera equivalent to CD2.Z22), when expressed in Jurkat T cells, is incapable of mobilizing intracellular calcium or eliciting protein tyrosine phosphorylation (147). However, an ITAM-independent signaling phenomenon similar to our own results was observed by Romeo and colleagues (146), who noted that a chimeric protein containing a ζ cytoplasmic tail truncated halfway through the first ITAM, although incapable of triggering cytolysis, still retains a significant capacity to elicit Ca++ mobilization. Furthermore, this was only the case when the "half
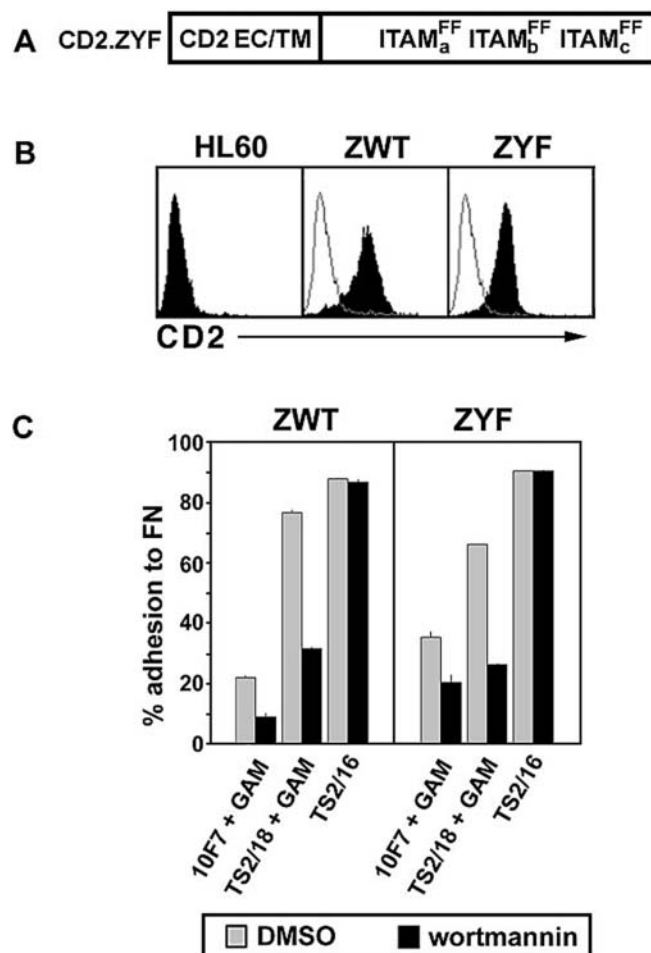
Fig. 4. ITAMs are not required for CD2.ζ-stimulated adhesion. (A) Schematic of CD2.ZYF, in which each ITAM tyrosine residue has been mutated to phenylalanine. (B) Stable HL60 transfectants were screened for chimera expression by flow cytometry. Open histograms, staining with FITC-conjugated goat antimouse IgG alone. Closed histograms, staining with anti-CD2 mAb followed by FITC-conjugated goat antimouse IgG. (C) CD2.ζ and CD2.ZYF were tested in adhesion assays in the presence of 50nM wortmannin (black bars) or carrier control (gray bars). Cells were left unstimulated (10F7 + GAM), or the chimera was cross-linked (TS2/18 + GAM), or β1 integrins were directly activated using TS2/16. Representative data from at least five experiments are shown.

ITAM'' was preceded by the membrane-proximal ζ sequence: if this sequence was substituted for that of the membrane-proximal 17 residues of CD7, the activity was lost. Together with our own observations, these data suggest that a TCR-ζ cytoplasmic tail may contain limited signaling capability within, or otherwise dependent upon, the first 17 membrane-proximal residues.

It is unclear how TCR-ζ, lacking intact ITAMs, can enhance β1 integrin activity. The first 22 residues of the ζ cytoplasmic tail do not contain any recognizable signaling motifs that would, for example, bind to SH2 or SH3 domains (158). The carboxy-terminal sequence of CD2.Z22 is similar to the protein tyrosine binding (PTB) motif NPxY recognized by Shc and IRS-1 (158). However, the tyrosine residue within this putative motif is, in full-length TCR-ζ, part of the first ITAM. This raises the possibility that the activity observed upon CD2.Z22 ligation may be due to the unmasking of a cryptic signaling motif not normally functional in the context of the full-length ζ cytoplasmic tail. Regardless, this NpxY motif is not present in the CD2.ZYF mutant that can still activate β1 integrins. Although the absence of ITAMs suggests that Syk (the ZAP-70 relative that is expressed in HL60 cells) is not conventionally recruited, CD2.Z22 ligation activates protein tyrosine kinases. Although the identity of these kinases remains to be elucidated, they appear to be the same as those activated upon CD2.ζ cross-linking, as the tyrosine phosphoprotein patterns in each transfectant have the same kinetics and suggest that identical substrates are being phosphorylated.

The ability of the TCR ζ cytoplasmic tail to upregulate β1 integrin function in the absence of functional ITAMs is surprising, considering the critical role that ZAP-70 plays in regulating CD3/TCR signaling to integrins (70). It is important to note that since we analyzed ζ mutations in myelomonocytic HL60 cells, the cellular context may have affected the signaling pathways available for integrin regulation. It is possible that HL60 cells and Jurkat T cells or T cell blasts represent the milieux within T cells at various stages of activation or maturation, and that for certain functions such as integrin activation, T cells can utilize different signaling routes to activate integrins. This idea is supported by evidence that, for example, memory CD4+ T cells have decreased expression of SLP-76 and apparently need to engage fewer signaling intermediates to activate MAPK compared to their effector T cell counterparts (159); in fact, these investigators argue that ZAP-70 does not become phosphorylated and may be bypassed altogether (159,160). Another example of different signaling pathways triggered by TCR ligation was revealed in

mice lacking PKC-θ, a PKC isoform that is rapidly recruited to the membrane upon CD3/TCR activation. PKC-θ is dispensible for thymocyte TCR-mediated NF-κB activation but essential for the same function in mature T cells (161). Consequently, T cell positive and negative selection are normal, but T cell responses to antigen are severely impaired (161). Similarly, mutations the SH2 domain of SLP-76 reveal a lesser dependence on this region in thymocytes compared to mature T cells (162,163).

It is also important to note that TCR-ζ complexed in a large oligomer with the TCR and CD3 subunits may not have the same freedom to move within the plane of the plasma membrane as when it is expressed as a chimera. This restriction could impact on its ability to form associations with other proteins and consequently other signaling pathways. It is possible that the integrin regulatory capability of the CD2.Z22 and CD2.ZYF mutants is conferred by their association with other proteins. One of the confounding factors in attempting to identify what signaling molecules may be interacting with TCR-ζ in the absence of functional ITAMs is that the membrane-proximal region of the ζ cytoplasmic domain lacks any recognizable protein–protein interaction motifs. Even if the CD2 ecto- and transmembrane interact with endogenous proteins, this activity does not upregulate integrin activity *per se*, considering the failure of CD2.Z12, or mutants of the CD2/CD28 chimera or CD2 itself to upregulate adhesion (80). Novel TCR-associated proteins have been described recently, such as TRIM (88) and CAST, which associate with a membrane-proximal cytoplasmic sequence of CD3ε (164). Possibly, these or other as yet unidentified proteins could assist in initiating the signaling observed upon CD2.Z22 and CD2.ZYF ligation and, by extension, may also play a role in TCR-ζ signaling to β1 integrins.

In addition to providing insights into potential alternate signaling mechanisms in TCR-ζ, the results of our structure–function analysis using CD2.ζ expressed in HL60 cells could bear on other ITAM-containing signaling molecules naturally expressed in myeloid cells, such as the high affinity IgE receptor γ chain and DAP-12, which in myeloid cells can couple to MDL-1, TREM-1, TREM-2 and SIRPβ1 (165). Might these proteins also have an alternate signaling pathway at their disposal? Our work suggests that TCR-ζ may have capabilities that are independent of their ITAM motifs. Future studies should explore the possibility that other proteins considered to signal through ITAMs contain alternative protein–protein interaction motifs. It will be necessary to identify the signaling machinery to which TCR-ζ can couple in the absence of functional ITAMs. Do proteins found to be relevant to

TCR/CD3-regulated integrin activation, such as ADAP and Itk, also play a role in this hypothetical alternate signaling pathway? Discerning such pathways may require examining functions, such as integrin regulation, that are not part of the typical assay repertoire utilized in mutational analyses.

TCR-dependent regulation of integrin activity, probably in combination with other T cell integrin regulators, is a key component in regulating T cell adhesion, which is central to T cell-mediated immunity. We have summarized our current knowledge of the molecules identified to be critical players in the control of integrins following CD3/TCR ligation. The pathways that the TCR utilizes to control integrin function are complex, but they clearly involve control of the T cell cytoskeleton by the TCR. Furthermore, these studies suggest that TCR control of integrin function potentially contains unexpected (and as yet unidentified) facets that differ from signaling pathways utilized to control other T cell effector responses.

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