The \( \beta_1 \) and \( \beta_3 \) Integrins Promote T Cell Receptor-mediated Cytotoxic T Lymphocyte Activation*

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Received for publication, March 17, 2003, and in revised form, April 9, 2003
Published, JBC Papers in Press, April 10, 2003, DOI 10.1074/jbc.M302709200

Recognition by CD8\(^+\) cytotoxic T lymphocytes (CTLs) of antigenic peptides bound to major histocompatibility class (MHC) I molecules on target cells leads to sustained calcium mobilization and CTL degranulation resulting in perforin-dependent killing. We report that \( \beta_1 \) and \( \beta_3 \) integrin-mediated adhesion to extracellular matrix proteins on target cells and/or surfaces dramatically promotes CTL degranulation. CTLs, when adhered to fibronectin but not CTL in suspension, efficiently degranulate upon exposure to soluble MHC-peptide complexes, even monomeric ones. This adhesion induces recruitment and activation of the focal adhesion kinase Pyk2, the cytoskeletal linker paxillin, and the Src kinases Lck and Fyn in the contact site. The T cell receptor, by association with Pyk2, becomes part of this adhesion-induced activation cluster, which greatly increases its signaling.

CD8\(^+\) cytotoxic T lymphocytes (CTLs) \(^{1}\) are activated upon engagement of their T cell receptor (TCR) by major histocompatibility (MHC)-peptide complexes on antigen-presenting cells (APCs) (1, 2). This interaction results in the formation of the immunological synapse, harboring in its center TCR, tyrosine kinases, CD8, and CD2 and in its periphery the immunological synapse, harboring in its center TCR, tyrosine kinase engagement of their T cell receptor (TCR) by major histocompatibility (MHC) molecule on other cells (13–17). Whereas LFA-1-mediated adhesion requires TCR triggering, \( \beta_1 \) and \( \beta_3 \) integrin-mediated adhesion of activated T cells, although enhanced upon TCR triggering, also takes place spontaneously (16, 18, 19). Therefore these integrins can sense changes in the extracellular environment, e.g. when T cells leave the vasculature and enter secondary lymphoid organs or inflamed tissues, where they become strongly exposed to ECM proteins (16). Integrin-mediated adhesion to ECM proteins results in activation and recruitment at the contact sites of the focal adhesion kinases FAK (20, 21), Itk (22), and Pyk2 (23–30), which promotes their association with the cytoskeleton linkers paxillin and talin (20, 23, 30, 31) and the Src kinases Fyn (29) and Lck (28, 30). Pyk2 is translocated to the T cell-target cell contact site after TCR triggering and plays an important role in degranulation of CTLs and natural killer cells (4, 32). Although the avidity and the redistribution of integrins is promoted by TCR signaling, the contribution of integrin-mediated signals to T cell activation is not well understood.

The availability of soluble recombinant MHC-peptide complexes triggered various studies aimed to elucidate the molecular basis of T cell activation (33–37), which often reached diverging conclusions. For example, monomeric MHC-peptide complexes have been reported to activate CD8\(^+\) T cells by cross-linking of TCR and CD8 (35) or by transfer of peptide from soluble to cell-associate MHC molecules (38, 39). By contrast, other studies concluded that activation of CD8\(^+\) T cells requires multimeric MHC-peptide complexes and co-engagement of CD8 (33, 34, 37). These discrepancies suggest that activation of CD8\(^+\) T cells involves additional factors.

To elucidate these divergences and to define the minimal molecular requirements for the activation of perforin-dependent cytotoxicity, we studied the MHC-peptide-driven activation of cloned T1 CTLs and CD8\(^+\) T cells from T1 TCR transgenic mice. The T1 TCR recognizes the Plasmodium berghei circumsporozoite (PbCS) peptide 252–260 (SYIPSAEKI) conjugated with photoreactive 4-azidobenzoic acid on Lys-259 (PbCS-
Role of β1/β2 Integrins in CTL Activation

EXPERIMENTAL PROCEDURES

Cells, Antibodies, and K⁺PbCS(ABA) Complexes—The T1 CTL clone was generated and propagated as described previously (41). P815 mastocytoma, A20 B cell lymphoma, and T-cells transfected with K⁺ or the mAb 32.2 (43) were cultured in DMEM containing 5% fetal calf serum. Rat γ/− T1 transgenic mice were obtained as described previously (42). Two days before taking their spleens, mice were injected intravenously with 50 nmol of PbCS(ABA) peptide. CD8⁺, tetramer−, and CD44 high splenocytes were isolated by Percoll separation and cultured for 4–6 days as T1 CTLs (41). Macrophages and B and T cells were isolated by Percoll gradient from spleen or bone marrow from BALB/c or Black six mice and immediately analyzed for surface expression of fibronectin by FACS.

The following antibodies were from Upstate Biotechnology (New York, NY): anti-Pyk2 (polyclonal), anti-paxillin (5H11), anti-Lck (3A5), anti-phosphotyrosine (4G10), and anti-ZAP-70 (polyclonal). Anti-Lck (2C10), anti-CD3 (2C11) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-LFA-1 (FD44.8), anti-CD3 (145-2C11-PE), and anti-CD61 (clone 2C9.G2) were from BD Pharmingen (San Diego, CA). Anti-fibronectin was from Molecular Probes (Eugene, OR) and washed twice with PBS, and sections of the cells parallel to the coverslip were analyzed on an LSM510 Zeiss confocal microscope (Zeiss, Germany). For analysis of conjugates, K⁺-transfected L cells were pulsed with 0.1 μM Dapi(Cy5)-YIPSAE(ABA)I, washed, adhered to Lab-Tek chambered coverglass (Nalge Nunc, Naperville, IL), incubated for 10 min with monomorphic K⁺PbCS(ABA), washed twice with pre-warmed DMEM, and fixed for 10 min at room temperature with 0.1% Brij 96, washed twice with PBS, and blocked for 20 min with PBS containing 1% BSA or 2% gelatin for detection of phosphotyrosine. Fixed cells were incubated with the different antibodies for 30 min at room temperature in the same buffer. Following three washes with PBS, the cells were incubated with anti-mouse Alexa 488 or anti-rabbit Cy5 (Molecular Probes, Eugene, OR) and washed twice with PBS, and sections of the cells parallel to the coverslip were analyzed on an LSM510 Zeiss confocal microscope (Zeiss, Germany). For analysis of conjugates, K⁺-transfected L cells were pulsed with 0.1 μM Dapi(Cy5)-YIPSAE(ABA)I, washed, adhered to Lab-Tek chambered coverglass, and conjugated for 15 min at 37°C with T1 CTLs and then fixed, permeabilized with 0.2% Triton X-100, and analyzed as described above. For co-localization imaging, all experiments were recorded in multitracking mode. Co-localization images were obtained by selecting the pixels having 30–100% intensities in each channel using IMARIS co-localization software (bitplane, Zurich, Switzerland).

RESULTS

Adhesion of CD8⁺ T Cells to Fibronectin Promotes Intracellular Calcium Mobilization and Degranulation—To assess the impact of cell adhesion on cell activation, we first assessed the intracellular calcium mobilization elicited by soluble K⁺PbCS(ABA) complexes on T1 CTLs that were adhered to immobilized fibronectin or kept in suspension. Cells in suspension exhibited transient calcium mobilization upon incubation with K⁺PbCS(ABA) tetramers that was lower as compared
with the strong and sustained calcium flux elicited by Pbc-S(ABA)-pulsed P815 cells (Fig. 1A). Tetramer-induced calcium mobilization was abolished by anti-CD8 mAb H35 (Fig. 1A). Importantly, soluble monomeric K^d/PbsCS(ABA) complexes had no effect on intracellular calcium, even at high concentration (1 μM) (Fig. 1A and data not shown). By striking contrast, T1 CTLs adhered to fibronectin exhibited sustained calcium mobilization upon incubation with soluble tetrameric and monomeric K^d/PbsCS(ABA) complexes (Fig. 1B). To generalize these observations, we examined the response of splenocytes from T1 TCR transgenic mice when challenged with soluble K^d/PbsCS(ABA) complexes. Essentially the same findings were obtained for splenocytes except that the calcium flux elicited by K^d/PbsCS(ABA) tetramer was more stable (Fig. 1, C and D). Again this response was abolished by anti-CD8 mAb or by using D227KK^d/PbsCS(ABA) complexes, which cannot co-engage CD8 (data not shown). These differences were not accounted for by absorption of K^d/PbsCS(ABA) complexes onto the plates, because no calcium mobilization was observed when T1 CTLs or T1 splenocytes were put on fibronectin-coated plates previously incubated for 20 min with soluble K^d/PbsCS(ABA) complexes and washed (data not shown).

We next examined T1 CTLs degranulation, which reflects perforin-mediated cytotoxicity. T1 CTLs in suspension exhibited no esterase release upon incubation with monomeric and tetrameric K^d/PbsCS(ABA) complexes. By contrast, T1 CTLs adhered to immobilized fibronectin efficiently degranulated in the presence of tetramer and, slightly less efficiently, monomeric complexes (Fig. 2A). This response was abolished by cytochalasin D, the ZAP-70/Syk-specific inhibitor piceatannol (46), and by anti-CD8 mAb (Fig. 2C). No detectable esterase release was observed in response to irrelevant monomeric K^d/cw3 complexes when CTLs were adhered to immobilized fibronectin. Furthermore, T1 CTL degranulation induced by sensitized P815 cells was substantially stronger on fibronectin-adhered CTLs as compared with CTLs in suspension (Fig. 2B). Essentially the same findings were obtained for the related S14 CTLs clone (data not shown). Taken together these findings indicate that adhesion of T1 CTLs and T1 splenocytes to immobilized fibronectin promotes calcium flux and degranulation in response to soluble monomeric K^d/PbsCS(ABA) complexes as well as recognition of sensitized target cells.

Cloned CTLs are propagated by periodic re-stimulation and hence are activated effector T cells, which express high levels of β1, β2, and β3 but not β6 integrins (Table I and data not shown) (14, 24). The high expression of β1 and β3 integrins enables T1 CTLs to spontaneously adhere to immobilized fibronectin (Fig. 2D). This adhesion was inhibited by the fibronectin-derived peptide GRGD and its cyclic variant, which selectively binds to β3 integrin (47), as well as by anti-β3 integrin antibody (Fig. 2D). Nonspecific adhesion of CTLs to immobilized BSA was 6-fold lower as compared with fibronectin (data not shown). This adhesion was also inhibited by PP2, cytochalasin D, and methyl-cyclodextrin, but not by piceatannol, indicating that it requires src kinases activity, functional cytoskeleton, and lipid rafts but not Zap-70/Syk or Syk kinase activity.

**CTLs Adhesion Induces Tyrosine Phosphorylation and Association of Pyk2 with Lck, Fyn, Paxillin, and TCR-CD3**—Upon adhesion of T1 CTLs to fibronectin, a dramatic increase in tyrosine phosphorylation of the focal adhesion kinase Pyk2, the cytoskeleton linker paxillin, and the src kinases Fyn and Lck was observed (Fig. 3, A and B). Because paxillin is a substrate for Pyk2 (23, 30, 48), its phosphorylation suggests that this CTL adhesion activates Pyk2. This adhesion also activates Fyn and Lck, which undergo autophosphorylation upon activation (49). By contrast, CTL adhesion caused no significant changes in tyrosine phosphorylation of ZAP-70, LAT, FAK, and CD3 (Fig. 3, A and B, and data not shown).

Tyrosine-phosphorylated paxillin, Fyn, and Lck, were co-immunoprecipitated with Pyk2 from the lysate of adherent but
not of non-adherent CTLs (Fig. 3C), indicating that, upon adhesion to fibronectin, these molecules associate with phosphorylated Pyk2. Similar findings have been reported for other systems (23, 28–30). The scant co-precipitation of paxillin is most likely explained by its association with the cytoskeleton, i.e. was lost in the detergent-insoluble fraction. Importantly, CTL adhesion also promoted association of the TCR/CD3 complex with Pyk2; remarkably, however, without increasing phosphorylation of CD3. LAT also co-precipitated with Pyk2, but this was not induced by cell adhesion (Fig. 3C).

**CTL Adhesion Induces Redistribution of TCR-CD3, CD8, Pyk2, and Paxillin**—Because T cell activation involves redistribution of signaling molecules to lipid rafts (10, 11, 50), we examined what impact adhesion of T1 CTLs to fibronectin has on the distribution of TCR-CD3, CD8, Pyk2, and paxillin. In accordance with previous reports on cells in suspension, GM1 and Thy-1 were located predominantly in the detergent-insoluble rafts and CD45 in the detergent-soluble fractions (Fig. 4A and data not shown) (50, 51). CTL adhesion did not alter this distribution and did not change the distribution of Fyn and Lck. By contrast, the fraction of raft-associated TCR increased substantially upon CTL adhesion. Moreover, Pyk2 and paxillin on cells in suspension were exclusively found in the detergent-soluble fraction but, upon adhesion, partitioned in rafts.

Confocal microscopy provided further information on adhesion-induced redistribution of signaling molecules. Although TCR-CD3 lined the cell surface of T1 CTLs in suspension, it was mainly found in clusters in and near the adhesion zone of...
fibronectin-adhered T1 CTLs (Fig. 4B). The same adhesion-induced redistribution was observed for CD8 and Thy-1, except that these molecules were in small aggregates on CTLs in suspension, which most likely reflect rafts that contain these molecules (Fig. 4, A and B) (11, 34, 52). Pyk2 on cells in suspension was mainly cytosolic (Fig. 4A) (27) but, upon adhesion, was concentrated in bright patches in and near the adhesion zone. Finally, tyrosine-phosphorylated proteins on cells in suspension were evenly distributed at the cell membrane but, upon adhesion, were found in bright patches mainly at and near the contact site. Upon CTL adhesion, the amount of protein-tyrosine phosphorylation increased by about 4-fold, mostly in rafts (data not shown). Taken collectively these results indicate that CTL adhesion to immobilized fibronectin induces translocation of Pyk2, paxillin, and TCR-CD3 in rafts, visible as large aggregates at the adhesion site, where tyrosine phosphorylation mainly occurred.

**Convergence of Adhesion- and TCR-mediated Signals**—On T1 CTLs in suspension monomeric K^d^-PbCS(ABA) complexes had no effect on tyrosine phosphorylation (Fig. 5A). However, on fibronectin-adhered T1 CTLs they increased the adhesion-induced phosphorylation of Lck/Fyn, paxillin, and Pyk2 and elicited phosphorylation of LAT, ZAP-70, and CD3\(\varepsilon\). This is in accordance with the finding that monomeric MHC peptide complexes induce intracellular calcium mobilization and esterase release on adherent, but not on T cells, in suspension (Figs. 1 and 2). On T1 CTLs in suspension tetrameric K^d^-PbCS(ABA) complexes induced tyrosine phosphorylation of Lck/Fyn, LAT, ZAP-70, and CD3\(\varepsilon\), and on adherent CTLs the same phosphorylation was induced as for the monomeric complexes, but
Fig. 5. CTL adhesion amplifies TCR signaling. To reach optimal activation, T1 CTLs in suspension (S) or bound to fibronectin (A) were incubated at 37 °C for 2.5 min (S) or 5 min (A) with 100 nM of monomeric (M) or 50 nM of tetrameric (T) Kd PbCS(ABA) complexes. A, CTLs were lysed and cell lysate analyzed by SDS-PAGE and Western blotting with anti-pY antibody. The bottom panel shows longer exposure for ZAP-70 and pY, following the same incubation with monomeric or tetrameric Kd PbCS(ABA) complexes, T1 CTLs in suspension (open bars) or adhered to fibronectin (closed bars) were lysed, and the kinase activities of immunoprecipitated Lck were assessed using [32P]ATP and immunoreceptor tyrosine-based activation motif (pY) peptides as substrates. Results shown are from one of three experiments. C, the distribution of pY proteins was analyzed by confocal microscopy after incubation with soluble Kd PbCS(ABA) complexes as in A. Each panel presents a section as indicated at the bottom. Scale bar, 5 μm.

much stronger, especially of paxillin and CD3ɛ.

Adhesion of T1 CTLs to immobilized fibronectin augmented the kinase activity of total Lck by about 2.3-fold (Fig. 5B). Upon incubation with monomeric Kd PbCS(ABA) complexes, the kinase activity increased by about 1.6-fold in adherent CTLs, but remained unchanged on CTLs in suspension. Tetrameric Kd PbCS(ABA) complexes caused a 2.7-fold increase in Lck kinase activity in adherent CTLs as compared with CTLs in suspension (Fig. 5B). Essentially the same changes in kinase activity were observed for CD8-associated Lck (data not shown).

As assessed by confocal microscopy, the tyrosine phosphorylation induced by Kd PbCS(ABA) complexes on T1 CTLs in suspension occurred primarily at the cell membrane (Fig. 5C). By contrast, on adhered cells a dramatic increase in phosphorylation elicited by MHC-peptide complexes was observed throughout the cell with a maximal intensity in and near the adhesion zone (Fig. 5C, panel 2'). Taken together these findings indicate that MHC-peptide complexes and adhesion elicit tyrosine phosphorylation of various molecules and that their combination results in strong signal amplification at the contact zone.

Co-engagement of CD8 and TCR by MHC-peptide Monomers Induces Co-aggregation of TCR, CD8, and Pyk2 in Adherent CTLs—Based on the observation that activation of T1 CTLs by soluble Kd PbCS(ABA) complexes requires that they co-engage CD8 and TCR (Figs. 1 and 2), we examined whether they induce proximity of CD8 and TCR. T1 CTLs stained in the cold with PE-labeled anti-CD3ε and Cy5-labeled anti-CD8 antibody exhibited substantial FRET data when incubated with soluble Kd PbCS(ABA) monomers but not in their absence or presence of irrelevant Kd-Cw3 170–179 complexes (Fig. 6A). Only background FRET was also observed in the presence of D227KKd PbCS(ABA) complexes, which are unable to co-engage CD8 yet, at the high concentrations used (1 μM), bind equally well to T1 CTLs (Ref. 40 and data not shown), confirming that MHC-peptide induces proximity of CD8 and TCR. Similar findings were obtained on CD8' lymph node cells from TCR transgenic mice but not on CD8' T cell hybridomas, where CD8 association with TCR is largely constitutive (42, 52).

Confocal microscopy showed that on fibronectin-adhered T1 CTLs, soluble Kd PbCS(ABA) but much less D227KKd PbCS(ABA) monomer induced co-localization of CD8 and TCR (Fig. 6B) similar as observed with non-adhered T1 CTLs (Fig. 6A). On adherent CTLs Kd PbCS(ABA) monomer induced extensive co-localization of CD8 with Pyk2 and Thy-1 in aggregates at the adhesion site (Fig. 6C). Significantly less co-localization was observed in the presence of D227KKd PbCS(ABA) complexes and almost none on adherent cells alone. Taken together these results demonstrate that Kd PbCS(ABA) complexes induce proximity of TCR and CD8. Moreover they promote co-localization of TCR, CD8, Pyk2, and the raft marker Thy-1 in large clusters at the adhesion site. Because D227KKd PbCS(ABA) complexes fail to do so, this implies a central role for CD8 in linking TCR- and adhesion-mediated activation events.
50% by anti-β₁ integrin and nearly by 90% by anti-β₂ integrin (LFA-1) antibody (Fig. 7A). Furthermore, the peptide GRGDS inhibited degranulation by 70%, whereas the control peptide GRGES had no effect. The cyclic GRGDS peptide, which selectively binds to β₃ integrins (47), caused only 15% inhibition. Essentially the same findings were obtained when cloned S14
cells were used as CTLs or A20 cells as targets (data not shown).
As assessed by FACS, the surfaces of P815 mastocytoma, A20 B lymphoma cells, L cells, macrophages, and splenic B cells, but not T cells, express fibronectin (Table I). This is consistent with the finding that most leukocytes secrete fibronectin and retain it at the cell surface for processing before depositing it at the extracellular matrix (53). Moreover, T1 CTLs express the fibronectin-binding integrins $\beta_1$ and $\beta_2$ but not $\beta_1$ and $\beta_3$ (Table I and data not shown) (14, 24). Taken together, this implies that binding of $\beta_1$ and $\beta_2$ integrins of the CTLs to cell-associated fibronectin on target cells greatly enhances antigen recognition. Indeed, T-cells, which express no ICAM (Table I), were well recognized by T1 CTLs, and this response was greatly impaired by the SH2GDS peptide and anti-$\beta_1$ integrin antibody but not affected by the GRGDS peptide (Fig. 7B).

Confocal microscopy of T1 CTLs conjugated with P815 cells sensitized with PbC(ABA) peptide showed in the contact site high enrichment of GM1, Pyk2, and paclixin (Fig. 7C). Co-localization of GM1 with Pyk2 and paclixin, respectively, were observed in bright clusters at contact site. Moreover, on T1 CTLs conjugated with K4-transfected L-cells previously sensitized with Cy5-labeled PbC(ABA), paclixin and the peptide were strongly enriched at the contact zone and co-localized with $\beta_1$ integrins (Fig. 7D). Taken together these results demonstrate that $\beta_1$ and $\beta_2$ integrins, by interacting with fibronectin on target cells, play an important role in target cell recognition and co-localize with Pyk2, paclixin, and the antigenic peptide in the CTLs-target cell contact site, similar to the role previously described for LFA1 and talin (3, 5, 49, 54).

**DISCUSSION**

A key finding of the present study is that MHC-peptide-driven CTL activation requires integrin-mediated adhesion. Activated T cells, such as cloned CTLs, express high levels of integrins like $\alpha_{\beta_1}$ (VLA4), $\alpha_{\beta_2}$ (VLA5), $\alpha_{\beta_1}$, $\alpha_{\mu}$, $\beta_3$, and $\alpha_{\beta_3}$ and adhere to ECM proteins (16, 18, 19). We show that this adhesion dramatically enhances perforin-dependent cytotoxicity of CD$^8^+$ effector T cells. Cells adhered to immobilized fibronectin stably flux calcium and efficiently degranulate upon incubation with soluble MHC-peptide complexes, even monomeric ones, whereas cells in suspension only exhibited transient calcium flux, given that the MHC-peptide complexes are multimeric and co-engage CD8 $^+$ T cells when they are adherent, but not when they are in suspension. Activated CD8 $^+$ T cells avidly adhere to immobilized fibronectin but also to other immobilized ECM proteins (e.g. collagen, gelatin, and vitronectin) and even to artificial surfaces like polystryrene (Fig. 2D).

It has been shown recently that soluble monomeric MHC-peptide complexes can activate CD8 $^+$ T cells by transfer of the peptide from soluble MHC to T cell-associated MHC molecules (38, 39). Although this mechanism does not account for our findings, because they were reproduced with covalent K4-IASA*-YIPSAE(K)ABA complexes (40, 55), these studies support the conclusion that activation of CD8 $^+$ T cells requires cell adhesion.

The role of $\beta_1$ and $\beta_2$ integrins in CTLs function is 2-fold. First, adhesion of CTLs to immobilized ECM proteins, such as fibronectin, provides co-stimulation for the recognition of sensitized target cells and soluble MHC/peptide complexes (Figs. 1 and 2) (13, 46). Second, $\beta_1$ and $\beta_2$ integrins are directly involved in target cell recognition by CTLs (Fig. 7). We demonstrate that most cells express fibronectin at their surface and that recognition of vastly different target cells (e.g. P815 mastocytoma, A20 B lymphoma, and L cells) by CTLs is greatly impaired in the presence of the RGPD peptide or anti-$\beta_1$ antibody (Fig. 7 and Table I). Similar observations were made for other ECM proteins binding to $\beta_1$ and $\beta_2$ integrins (17, 56). Although the $\beta_2$ integrin LFA-1 plays an important role in antigen recognition (3, 8), our findings indicate that $\beta_1$ and $\beta_2$ integrins play a hitherto unappreciated important role as well. In particular, the ECM binding integrins allow antigen recognition in the absence of LFA-1 or ICAMs (Fig. 7) (57, 58).

The use of soluble MHC-peptide complexes and spontaneous adhesion to immobilized fibronectin allowed us to conclusively investigate $\beta_1$/$\beta_2$ integrin- and TCR/CD8-mediated signals separately and how they elicit CTL degranulation when they are combined. The hallmark of $\beta_1$/$\beta_2$ integrin-mediated adhesion is the formation of tyrosine-phosphorylated molecular aggregates containing Pyk2, Lck, Fyn, and paclixin in and near the cell adhesion zone (Figs. 3 and 4). The strong tyrosine phosphorylation of Pyk2, Lck, and Fyn argues that adhesion activates these tyrosine kinases. For Lck the increase in kinase activity was directly assessed (Fig. 3), and the activation of Pyk2 and Fyn is deduced from the phosphorylation of their substrates, paclixin and Pyk2, respectively (Figs. 3A and 5A) (23, 28–30). Several studies indicate that these complexes are raft-associated as follows. 1) They contain LAT, Fyn, Lck, and CD8, which are palmitoylated and partition in rafts (Fig. 4) (10, 11, 34, 42, 52, 59). 2) CTL adhesion induced translocation of Pyk2 and paclixin to rafts (Fig. 4). 3) The strong tyrosine phosphorylation of paclixin, Pyk2, Lck, and Fyn is indicative for localization in raft, where phosphatases are excluded (Figs. 3 and 4) (1, 11, 60). Moreover, because $\beta_1$ integrins, as well as phosphorylated paclixin and Lck, associate with the cytoskeleton, these rafts are cytoskeleton-associated (20, 23, 31).

Conversely, on CTLs in suspension soluble MHC-peptide complexes by co-engaging TCR and CD8 promote their association (Fig. 5) (42, 61). Cross-linking of the resulting TCR-CD3- CD8/Lck adds resulted in Lck activation and phosphorylation of CD3 (Fig. 5A) (34). ZAP-70 is then recruited to phosphorylated CD3 and, upon activation by Lck, phosphorylates LAT (Fig. 5, A and B) (11, 62). Phosphorylated LAT in turn interacts with various adaptors and signaling molecules such as Vav, SLP-76, PLC$\gamma$, Grb2, and SOS, which are involved in various downstream signaling events, including actin polymerization and mobilization of intracellular calcium (12, 63–65). Because the ZAP-70/Syk-specific inhibitor piceatannol (46) had no effect on CTL adhesion (Fig. 2D), but blocked the activation of adherent CTLs by MHC-peptide complexes (Fig. 2C), the recruitment and phosphorylation of ZAP-70 and in turn of LAT is induced by MHC-peptide complexes and not by cell adhesion. Thus, although CTL adhesion elicited strong tyrosine phosphorylation of Pyk2, Lck, Fyn, and paclixin, phosphorylation of TCR-CD3, ZAP-70, and LAT was induced only by MHC-peptide complexes (Figs. 3 and 5). However, TCR-CD8 triggering by MHC-peptide and $\beta_1$/$\beta_2$ integrin-mediated adhesion elicited clearly different activation events, and, when combined, they provide the powerful signaling, resulting in sustained calcium flux and CTL degranulation (Figs. 1 and 2).

What is the molecular basis for this signal integration and amplification? Our finding that Pyk2 associates with TCR-CD3 and LAT (Fig. 3) probably explains the dramatic changes in TCR signaling observed upon $\beta_1$ and $\beta_2$ integrin-mediated CTL adhesion (Figs. 1, 2, and 5). For example, in view of the importance of Lck activation in CTL activation driven antigen-specifically, it is interesting to note that adhesion of CTLs to fibronectin more strongly activates Lck than does the

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MHC peptide on CTLs in suspension (Figs. 3 and 5) (34). However, even though in adherent CTLs TCR-CD3 is part of the adhesion-induced molecular aggregates, adhesion-activated Lck only becomes effective upon cross-linking of TCR and CD8 by MHC peptide (Figs. 5 and 6). Thus CD8 plays a central role in converging of adhesion and TCR-mediated signals, namely by bringing adhesion-activated Lck to TCR-CD3.

Furthermore, adhesion of CTLs also efficiently activates Fyn (Fig. 3). Because Fyn associates with Pyk2 (29), this probably explains the strong tyrosine phosphorylation of Pyk2 (Figs. 3 and 5). Pyk2 in cytotoxic cells is recruited to the contact site with target cells and, upon activation, plays a critical role in the re-orientation of the microtubule-organizing center (27, 66). Consequently, this is consistent with the finding that, in degranulating CTLs, Pyk2 mediates re-orientation of the microtubule-organizing center (27, 66). Because Fyn associates with Pyk2 (29), this probably explains the strong re-orientation of the microtubule-organizing center (27, 66). Consistent with this is the finding that, in degranulating CTLs, Pyk2 re-orientation of the microtubule-organizing center (27, 66). Consequent to this is the finding that, in degranulating CTLs, Pyk2 re-orientation of the microtubule-organizing center (27, 66). Consequently, this is consistent with the finding that, in degranulating CTLs, Pyk2 mediates re-orientation of the microtubule-organizing center (27, 66). Because Fyn associates with Pyk2 (29), this probably explains the strong re-orientation of the microtubule-organizing center (27, 66). Consistent with this is the finding that, in degranulating CTLs, Pyk2 mediates re-orientation of the microtubule-organizing center (27, 66). Consequently, this is consistent with the finding that, in degranulating CTLs, Pyk2 mediates re-orientation of the microtubule-organizing center (27, 66). Consequently, this is consistent with the finding that, in degranulating CTLs, Pyk2 mediates re-orientation of the microtubule-organizing center (27, 66). Consequently, this is consistent with the finding that, in degranulating CTLs, Pyk2 mediates re-orientation of the microtubule-organizing center (27, 66). Consequently, this is consistent with the finding that, in degranulating CTLs, Pyk2 mediates re-orientation of the microtubule-organizing center (27, 66). Consequently, this is consistent with the finding that, in degranulating CTLs, Pyk2 mediates re-orientation of the microtubule-organizing center (27, 66). Consequently, this is consistent with the finding that, in degranulating CTLs, Pyk2 mediates re-orientation of the microtubule-organizing center (27, 66). Consequently, this is consistent with the finding that, in degranulating CTLs, Pyk2 mediates re-orientation of the microtubule-organizing center (27, 66). Consequently, this is consistent with the finding that, in degranulating CTLs, Pyk2 mediates re-orientation of the microtubule-organizing center (27, 66).

In conclusion, the present study shows that β1 and β2 integrins play an important role in the function of CTLs, both in sensing changes in the extracellular environment and in target cell recognition. These ECM-binding integrins directly implicate Pyk2, which is important for CTL degranulation. This focal adhesion kinase is involved in re-localizing the microtubule-organizing center and, together with Pyk2 and Fyn, forms cytoskeleton and raft-associated molecular aggregates. Such aggregates, including TCR-CD3, CD8, and LAT, are capable of integrating and amplifying adhesion and MHC-peptide-mediated signals, thus eliciting CTL effector functions.

Acknowledgment—We thank Sandra Levraud for excellent technical assistance.

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