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The β_1 and β_3 Integrins Promote T Cell Receptor-mediated Cytotoxic T Lymphocyte Activation*

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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 β_1 and β_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 cytoskeleton 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 β_2 integrin LFA-1 and the cytoskeletal linker talin (1, 3–7). Upon TCR triggering, LFA-1 dramatically increases its binding to ICAMs on APCs, which promotes conjugate formation and T cell activation (8). Antigen-specific T cell activation is initiated in the immunological synapse in detergent-insoluble glycolipid-enriched complexes, which, by including kinases (*e.g.* Lck and Fyn) and their substrates (*e.g.* LAT) and excluding phosphatases (*e.g.* CD45), are privileged sites for TCR signal induction (5, 6, 9–12).

Cloned CTLs are propagated by periodic re-stimulation, and hence are activated T cells, which express high levels of LFA-1 and β_1 and β_3 integrins. The latter interact with extracellular matrix (ECM) proteins, like fibronectin, vitronectin, and collagen, as well as with counter receptors (*e.g.* vasular cell adhesion molecule) on other cells (13–17). Whereas LFA-1-mediated adhesion requires TCR triggering, β_1 and β_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* circumsporozite (PbCS) peptide 252–260 (SYIPSAEKI) conjugated with photoreactive 4-azidobenzoic acid on Lys-259 (PbC-

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[1] The abbreviations used are: CTLs, cytotoxic T lymphocytes; TCR, T cell receptor; MHC, major histocompatibility complex; APCs, antigen-presenting cells; LFA-1, lymphocyte function associated antigen-1; ICAMs, intracellular adhesion molecule; ECM, extracellular matrix; PbCS, *P. berghei* circumsporozite; ABA, 4-azidobenzoic acid; DMEM, Dulbecco's modified Eagle's medium; FACS, fluorescence-activated cell sorting; mAb, monoclonal antibody; FRET, fluorescence resonance energy transfer; PBS, phosphate-buffered saline; BSA, bovine serum albumin; pY, phosphotyrosine; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; PE, phycoerythrin; GM1, Galβ1,3GalNacβ1,4NeuAcα2,3Galβ1,4Glc-ceramide.

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S(ABA)) in the context of K^d (40, 41). In the absence of cell adhesion, *i.e.* on CTLs in suspension, MHC·peptide complexes by co-engaging CD8 and TCR·CD3 promote the formation of raft-associated TCR·CD3-CD8/Lck adducts (34). Cross-linking of these adducts results in Lck activation, CD3 phosphorylation, recruitment and activation of ZAP-70, phosphorylation of LAT, and mobilization of intracellular calcium (34, 42).

Here we show that CTLs in suspension do not degranulate in response to MHC·peptide complexes and that for this cell adhesion is required. The β_1 and β_3 integrin-mediated adhesion of CTLs to ECM induces strong tyrosine phosphorylation and association of Pyk2 with the cytoskeleton linker paxillin and the Src kinases Lck and Fyn. These adhesion-induced, raft-associated molecular aggregates also contain TCR·CD3 and are able to integrate and to amplify adhesion- and TCR-mediated signals, thus promoting MHC·peptide-driven CTL degranulation.

EXPERIMENTAL PROCEDURES

Cells, Antibodies, and K^d·PbCS(ABA) Complexes—The T1 CTL clone was generated and propagated as described previously (41). P815 mastocytoma, A20 B cell lymphoma, and L-cells transfected with K^d or the mutant D227KK^d (43) were cultured in DMEM containing 5% fetal calf serum. Rag −/− T1 TCR 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 (2102), anti-CD3ϵ (M-20) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-LFA-1 (FD44.8), anti-β_2 integrin (2E6), anti-CD8β mAbs H35.17 and KT112 were from American Type Culture Collection (ATCC) (Manassas, VA). Anti-CD45 (clone 69), anti-CD29 (clone Ha 2/5), 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). Anti-Fyn (polyclonal) was from Dr. M. F. White (Harvard Medical School) linear GRGDS, GRGES, and cyclic GRGDS peptides were from Bachem (Bubendorf, Switzerland). Monomeric cholera toxin B subunit-peroxidase and -fluorescein isothiocyanate conjugates were from Sigma (Buchs, Switzerland). Western blotting, immunoprecipitations, and immunodetection were performed as described (34) using the ECL detection system (Amersham Biosciences, Little Chalfond, UK). Soluble monomeric and tetrameric K^d·PbCS(ABA) complexes were prepared, as described previously (34, 44). Cy5-labeled PbCS(ABA) was obtained by reacting Fmoc (*N*-(9-fluorenyl)methoxycarbonyl)-Dap-YIPSAEK(ABA)I in Me_2SO/dimethylformamide/diisopropylethylamine (5/4.5/0.5) with Cy5-*N*-hydroxysuccinimide ester (5% molar excess) at room temperature for 4 h. A 3-fold larger volume of piperidine (10% in dimethylformamide) was added, and after 9 min of incubation the peptide derivative was precipitated with diethylether purified by reversed-phase high performance liquid chromatography and analyzed by mass spectrometry as described previously (34).

FACS and FRET—For FACS and FRET analysis, CTLs clones were stained at 4 °C in PBS containing 1% BSA and 0.1% sodium azide. Cell-associated fluorescence was measured using a FACSCalibur (BD Biosciences). For FRET, T1 CTLs were stained at 4 °C for 40 min with Cy5-labeled anti-CD8α mAb KT112-Cy5 and PE-labeled anti-CD3ϵ mAb 145–2C11 in the absence or presence of 1 μM K^d·PbCS(ABA), and FRET was measured by the Cy5 fluorescence measured upon excitation of PE as described previously (42).

Calcium Mobilization, Esterase Release, and in Vitro Kinase Assays—CTLs in serum-free DMEM were adhered for 30 min at 37 °C on polystyrene tissue culture plates or glass coverslips previously coated overnight at 4 °C with 2 μg/ml of superfibronectin (Sigma) and incubated at 37 °C for 5 min with 100 nM monomeric or 50 nM tetrameric K^d·PbCS(ABA) complexes or P815 cells pulsed with 10 nM PbCS(ABA) peptide. For calcium mobilization T cells were loaded with 5 μM fura-2/AM (Sigma) and adhered onto glass coverslips coated with fibronectin. After washing off non-adhered cells, calcium-dependent fura-2 fluorescence was measured on a Zeiss Axiovert microscope equipped with a charge-coupled device camera and a light monochromator (Princeton Instruments). The camera output was recorded and analyzed using the Metafluor calcium-imaging software (Universal Imaging Corp.). Alternatively, T cells were loaded with 5 μM indo-1/AM (Sigma) for 45 min at 37 °C and calcium-dependent indo-1 fluorescence was measured on a FACStar (BD Biosciences, Erembodegen, Belgium) as described (34).

For esterase release experiments, T1 CTLs, untreated or preincubated for 30 min at room temperature with the indicated anti-integrin antibodies (10 μg/ml) or the peptides GRGDS or GRGES (1.5 mg/ml) or cyclic GRGDS (60 μg/ml), were washed and incubated in DMEM for 90 min at 37 °C with P815, A20, or L-cells (effector/target of 1/1) previously sensitized for 120 min at 37 °C with the indicated concentrations of PbCS(ABA) peptide. For esterase release in response to soluble K^d·PbCS(ABA) complexes, T1 CTLs in serum-free DMEM (10^6 cells/ml) were either previously adhered to fibronectin-coated plates or kept in suspension in rotating polycarbonate vials. Released esterases were measured in the supernatants as described (36); 100% of esterase release refers to the value measured upon lysis of the cells in 1% Triton X-100.

For measurements of the kinase activity of CD8-associated Lck, T1 CTLs, either adhered to fibronectin or in suspension, were incubated at 37 °C for 2.5 min with monomeric (100 nM) or tetrameric (50 nM) soluble K^d·PbCS(ABA) complexes. After lysis in *n*-octylglucoside (80 mM) CD8 was immunoprecipitated with anti-CD8α mAb 53.6.72. The immunoprecipitates were incubated at 37 °C for 5 min with [^{32}P]ATP and biotinylated peptide corresponding to the immunoreceptor tyrosine-based activation motif c of CD3ζ, and its phosphorylation was assessed as described previously (34).

Adhesion Assay—The adhesion of T1 CTLs to immobilized fibronectin was performed as described (45). In brief, CTLs pretreated or not for 30 min at 37 °C with 30 μM PP2 (Calbiochem, San Diego, CA), 100 μM cytochalasin D (Calbiochem), 100 μM piceatannol, or 10 mM methyl-β-cyclodextrin (Sigma), were incubated in 96-well plates (Polylabo, Illkirch, France) coated with fibronectin. The peptides GRGDS or GRGES (1.5 mg/ml) were added at the beginning of the incubations. After incubation for 30 min at 37 °C, adherent cells were stained with crystal violet, and the optical density of their lysates was measured at 570 nm.

Isolation of Rafts and Confocal Microscopy—Rafts were isolated as previously described (34), except that 0.2% Brij 96 (Fluka, Buchs, Switzerland) was used instead of 1% Triton X-100. Rafts were solubilized in octyl-β-D-glucoside (50 mM) containing EDTA (5 mM). The remaining insoluble material contained none of the molecules under study, as judged by SDS-PAGE and Western blotting. For confocal microscopy, T1 CTLs were adhered to fibronectin-coated Lab-Tek chambered coverglass (Nalge Nunc, Naperville, IL), incubated for 10 min with monomeric K^d·PbCS(ABA), washed twice with pre-warmed DMEM, and fixed for 10 min at room temperature with 3% paraformaldehyde in PBS. Alternatively, T1 CTLs were washed and fixed directly. CTLs were permeabilized 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 Cy3 (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^d-transfected L cells were pulsed with 0.1 μM Dap(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 0.02% Triton X-100, and analyzed as described above. For co-localization images 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^d·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^d·PbCS(ABA) tetramers that was lower as compared

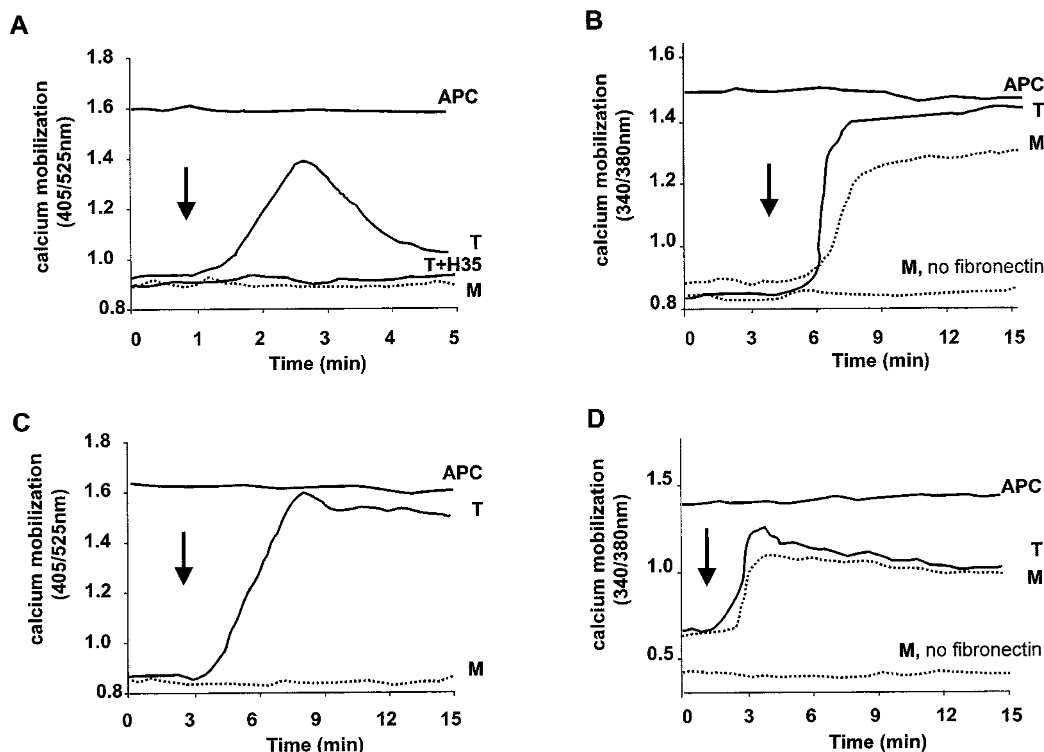

FIG. 1. **Adhesion is required for CTL activation by soluble MHC·peptide.** Intracellular calcium mobilization of T1 CTLs clone (*A* and *B*) or splenocytes from T1 TCR transgenic mice (*C* and *D*) in response to P815 cells (*APC*) pulsed with PbCS(ABA) or to 100 nM monomeric (*M*, *broken line*) or 50 nM of tetrameric (*T*) K^d·PbCS(ABA) complexes. T cells were kept in suspension (*A* and *C*) or adhered to fibronectin (*B* and *D*). The *arrows* indicate the addition of soluble K^d·PbCS(ABA) complexes. CTLs were treated with Fab′ fragments of anti-CD8β mAb H35 (*H35*).

with the strong and sustained calcium flux elicited by PbC-S(ABA)-pulsed P815 cells (Fig. 1*A*). Tetramer-induced calcium mobilization was abolished by anti-CD8 mAb H35 (Fig. 1*A*). Importantly, soluble monomeric K^d·PbCS(ABA) complexes had no effect on intracellular calcium, even at high concentration (1 μM) (Fig. 1*A* 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·PbCS(ABA) complexes (Fig. 1*B*). To generalize these observations, we examined the response of splenocytes from T1 TCR transgenic mice when challenged with soluble K^d·PbCS(ABA) complexes. Essentially the same findings were obtained for splenocytes except that the calcium flux elicited by K^d·PbCS(ABA) tetramer was more stable (Fig. 1, *C* and *D*). Again this response was abolished by anti-CD8 mAb or by using D227KK^d·PbCS(ABA) complexes, which cannot co-engage CD8 (data not shown). These differences were not accounted for by absorption of K^d·PbCS(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·PbCS(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·PbCS(ABA) complexes. By contrast, T1 CTLs adhered to immobilized fibronectin efficiently degranulated in the presence of tetrameric and, slightly less efficiently, monomeric complexes (Fig. 2*A*). This response was abolished by cytochalasin D, the ZAP-70/Syk-specific inhibitor piceatannol (46), and by anti-CD8 mAb (Fig. 2*C*). 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. 2*B*). 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·PbCS(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 β_1, β_2, and β_3 but not β_7 integrins (Table I and data not shown) (14, 24). The high expression of β_1 and β_3 integrins enables T1 CTLs to spontaneously adhere to immobilized fibronectin (Fig. 2*D*). This adhesion was inhibited by the fibronectin-derived peptide GRGDS and its cyclic variant, which selectively binds to β_3 integrin (47), as well as by anti-β_1 integrin antibody (Fig. 2*D*). 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

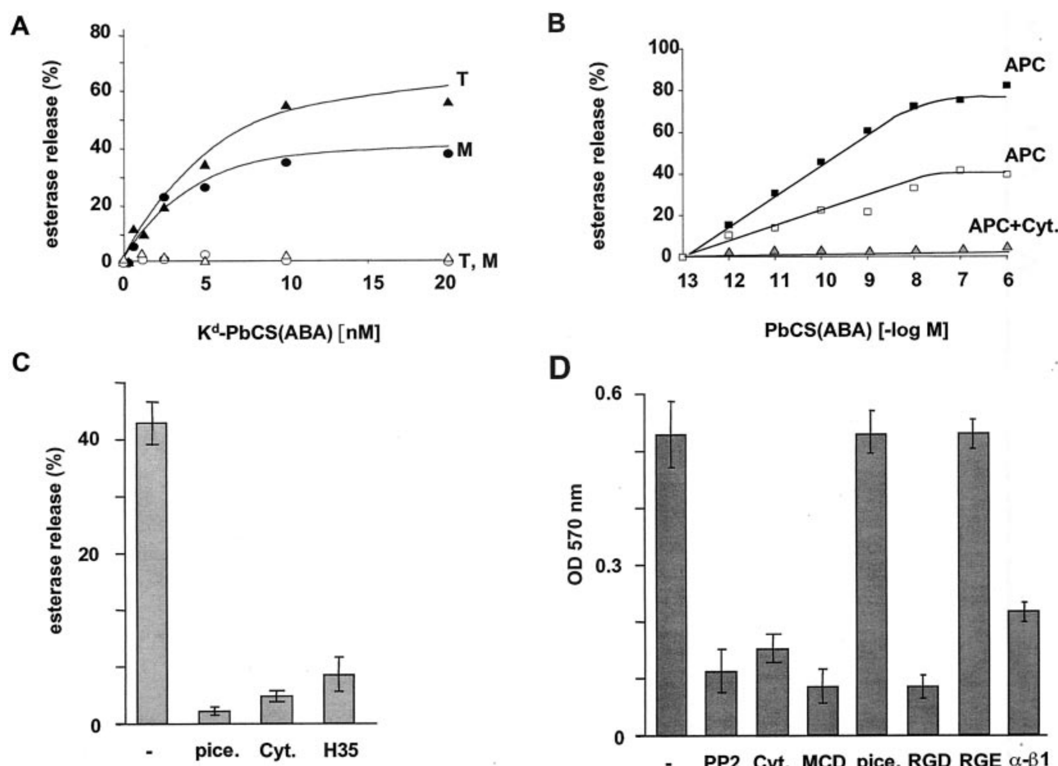

FIG. 2. **Monomeric K^d·PbCS(ABA) complexes induce degranulation of fibronectin-adhered T1 CTLs.** Esterase release of T1 CTLs clone in response to soluble K^d·PbCS(ABA) complexes (*A*) or P815 pulsed with PbCS(ABA) (*B*). Responses of CTLs in suspension are shown as *open symbols*, and those of CTLs bound to immobilized fibronectin are as *closed symbols*. 100% of esterase release refers to the total amount of esterases. One representative of four experiments is shown. *C*, esterase release of T1 CTLs clone in response to 15 nM soluble K^d·PbCS(ABA) monomeric complexes when cells were treated with piceatannol (*pice.*) or cytochalasin D (*Cyt.*) or blocking anti-CD8 mAb H35. *D*, T1 CTLs were incubated with GRGDS or GRGES peptides or with PP2, methyl-β-D-cyclodextrin (*MCD*), piceatannol (*pice.*), cytochalasin D (*Cyt.*), or blocking anti-β_1 antibody, added to fibronectin-coated plates, and cell adhesion was measured by means of staining with crystal violet. Results shown are from one of three experiments.

TABLE I
Cell surface expression of K^d, integrins, and adhesion molecules

The surface expressions were measured by FACS using specific antibodies (see "Experimental Procedures"). The *numbers* indicate the mean fluorescence intensities (MFI) corrected for control staining of three independent staining experiments performed in duplicate. Background represents cells not stained. Values for control staining, performed with the secondary antibody alone, were in the range of 1–15% of the specific staining.

	Background	Integrin			LFA-1	ICAM-1	Fibronectin	K^d
		β_1	β_2	β_3				
				MFI				
T1 CTL	4	480	498	140	210	135	4	1670
P815	4	500	185	15	102	100	140	1203
A20	4	85	250	9	8	58	55	—
K^d-transfected L-cells	3	ND[a]	ND	ND	ND	5	112	590
Macrophages, Mac-1^+ splenocytes	12	ND	ND	ND	ND	ND	72	ND
B-cells, $CD19^+$ splenocytes	13	ND	ND	ND	ND	ND	59	ND
T cells, $CD3^+$ splenocytes	10	ND	ND	ND	ND	ND	14	ND

[a] ND, not determined.

not of non-adherent CTLs (Fig. 3*C*), 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. 3*C*).

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. 4*A* 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·CD3 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

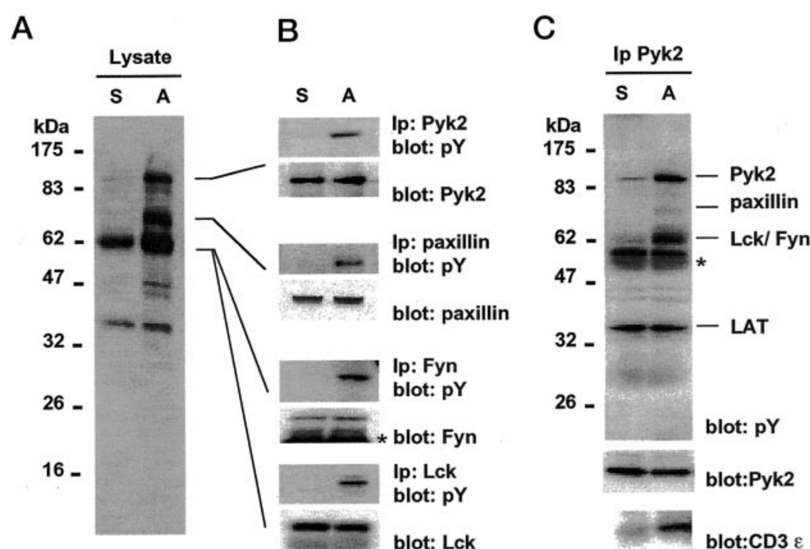

FIG. 3. **CTL adhesion induces phosphorylation of Pyk2, Lck, Fyn, and paxillin.** *A*, protein tyrosine phosphorylation of total lysate of T1 CTLs in suspension (*S*) or bound to fibronectin (*A*). Equal amounts of protein were loaded per lane, resolved by SDS-PAGE, and Western blotted with anti-phosphotyrosine (*pY*) antibody. *B*, cell lysates were immunoprecipitated (*Ip*) with the indicated antibodies and analyzed by Western blotting. The *asterisk* indicates IgG heavy chain. *C*, Pyk2 was immunoprecipitated from the same lysates, and the immunoprecipitates were analyzed by SDS-PAGE and Western blotting with pY mAb, anti-Pyk2, and anti-TCR·CD3 antibody.

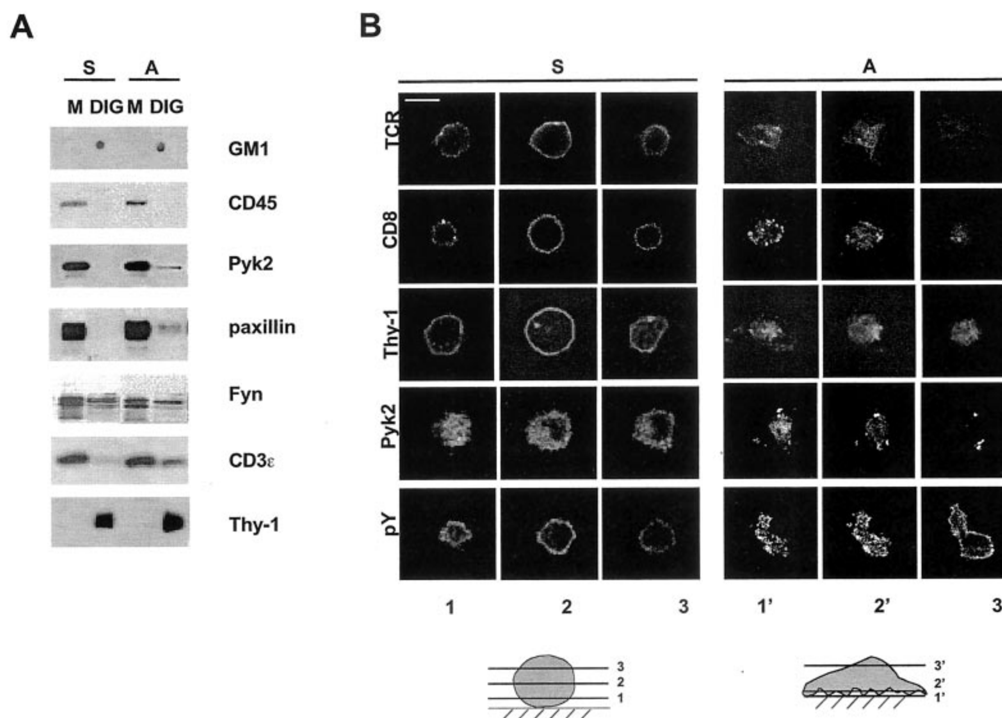

FIG. 4. **CTL adhesion results in redistribution of TCR·CD3, Pyk2, and paxillin to rafts.** T1 CTLs in suspension (*S*) or bound to fibronectin (*A*) were fractionated in detergent-soluble (*M*) and detergent-insoluble (*DIG*) fractions, and the distributions of GM1, CD45, Pyk2, TCR·CD3, paxillin, Fyn, and Thy-1 were analyzed by SDS-PAGE and Western blotting. 10% of each fraction was loaded per lane, and one representative of three experiments is shown. *B*, T1 CTLs were stained for TCR, CD8, Thy-1, Pyk2, or phosphotyrosine and examined by confocal microscopy. *Each panel* presents a section parallel to the coverslip, as indicated at the *bottom*. *Scale bar*, 5 μm.

fibronectin-adhered T1 CTLs (Fig. 4*B*). 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. 4*A*) (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. 5*A*). 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 CD3ζ. 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 CD3ζ, and on adherent CTLs the same phosphorylation was induced as for the monomeric complexes, but

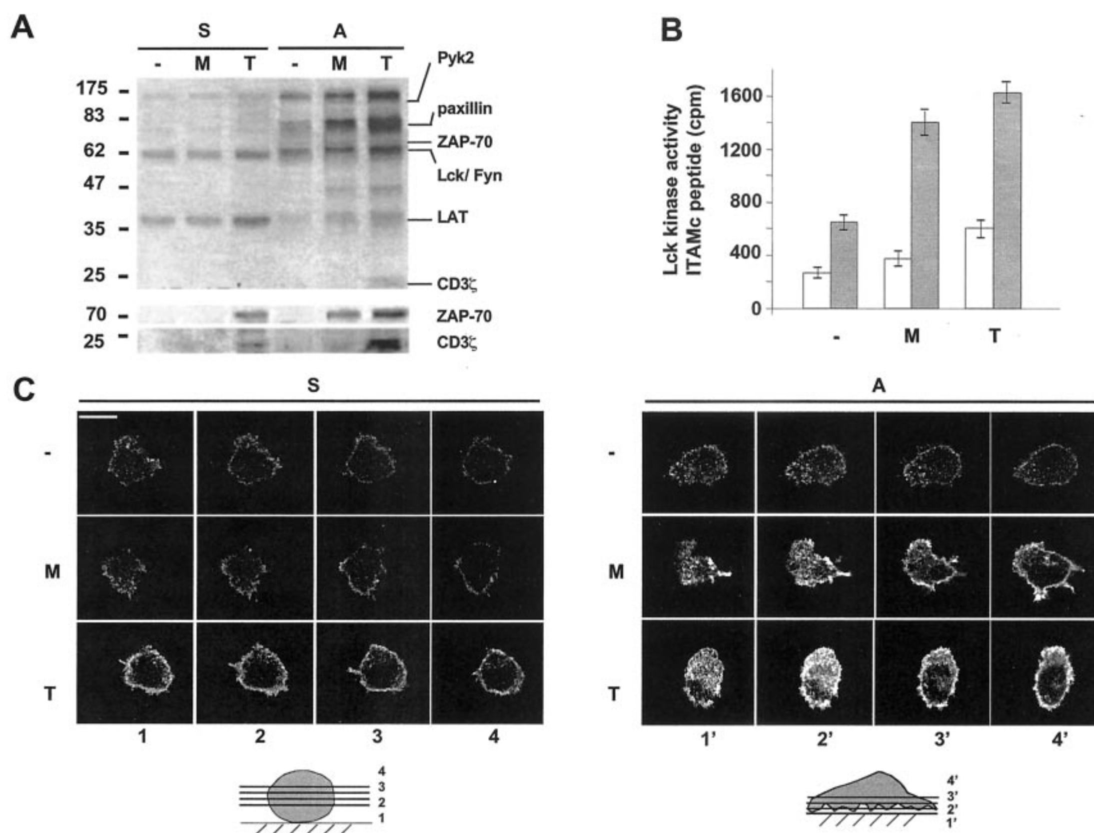

FIG. 5. **CTL adhesion amplifies TCR signaling.** To reach optimal activation, T1 CTLs in suspension (*S*) or bound to fibronectin (*A*) were incubated at 37 °C for 2.5 min (*S*) or 5 min (*A*) with 100 nM of monomeric (*M*) or 50 nM of tetrameric (*T*) K^d·PbCS(ABA) complexes. *A*, CTLs were lysed and cell lysate analyzed by SDS-PAGE and Western blotting with anti-pY antibody. The *bottom panel* shows longer exposures for ZAP-70 and CD3ζ. *B*, following the same incubation with monomeric or tetrameric K^d·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 [^{32}P]ATP and immunoreceptor tyrosine-based activation motif c peptide 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 K^d·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 CD3ζ.

Adhesion of T1 CTLs to immobilized fibronectin augmented the kinase activity of total Lck by about 2.3-fold (Fig. 5*B*). Upon incubation with monomeric K^d·PbCS(ABA) complexes, the kinase activity increased by about 1.6-fold in adherent CTLs, but remained unchanged on CTLs in suspension. Tetrameric K^d·PbCS(ABA) complexes caused a 2.7-fold increase in Lck kinase activity in adherent CTLs as compared with CTLs in suspension (Fig. 5*B*). 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 K^d·PbCS(ABA) complexes on T1 CTLs in suspension occurred primarily at the cell membrane (Fig. 5*C*). 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. 5*C*, *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 K^d·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 K^d·PbCS(ABA) monomers but not in their absence or presence of irrelevant K^d·Cw3 170–179 complexes (Fig. 6*A*). Only background FRET was also observed in the presence of D227KK^d·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-transfected T cell hybridomas, where CD8 association with TCR is largely constitutive (42, 52).

Confocal microscopy showed that on fibronectin-adhered T1 CTLs, soluble K^d·PbCS(ABA) but much less D227KK^d·PbCS(ABA) monomer induced co-localization of TCR and CD8 (Fig. 6*B*) similar as observed with non-adhered T1 CTLs (Fig. 6*A*). On adherent CTLs K^d·PbCS(ABA) monomer induced extensive co-localization of CD8 with Pyk2 and Thy-1 in aggregates at the adhesion site (Fig. 6*C*). Significantly less co-localization was observed in the presence of D227KK^d·PbCS(ABA) complexes and almost none on adherent cells alone. Taken together these results demonstrate that K^d·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 D227KK^d·PbCS(ABA) complexes fail to do so, this implies a central role for CD8 in linking TCR- and adhesion-mediated activation events.

β_1 Integrin, Pyk2, and Paxillin Function in Target Cell Recognition by CTLs—We next examined the role of $\beta_1$and β_3 integrins in the recognition of sensitized target cells by T1 CTLs. The degranulation of T1 CTLs observed upon incubation with PbCS(ABA) sensitized P815 cells was inhibited by about

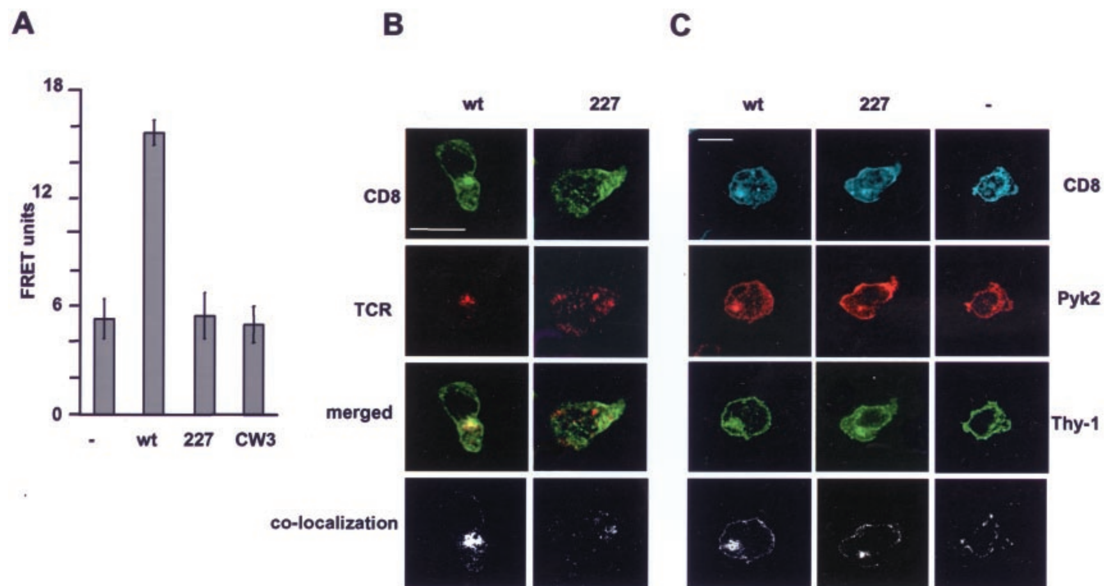

FIG. 6. **MHC·peptide complexes induce proximity and colocalization of TCR, CD8, and Pyk2 at the site of cell adhesion.** *A*, measure of TCR and CD8 proximity by FRET in the absence or presence of 1 μM monomeric K^d·PbCS(ABA)PbCS (wt or D227K) complexes or irrelevant K^d·Cw3 170–179 complexes. *B*, distribution of CD8 and TCR on fibronectin-adhered T1 CTLs after stimulation of the cells with wt or 227 mutant monomeric K^d·PbCS(ABA). Co-localization obtained by IMARIS co-localization software is shown in *white*. *C*, distribution of TCR, Pyk2, and Thy-1 at the site of cell contact to fibronectin examined by confocal microscopy following stimulation of the cells as described in *B*. Triple co-localization is shown in *white*. *Scale bar*, 5 μm.

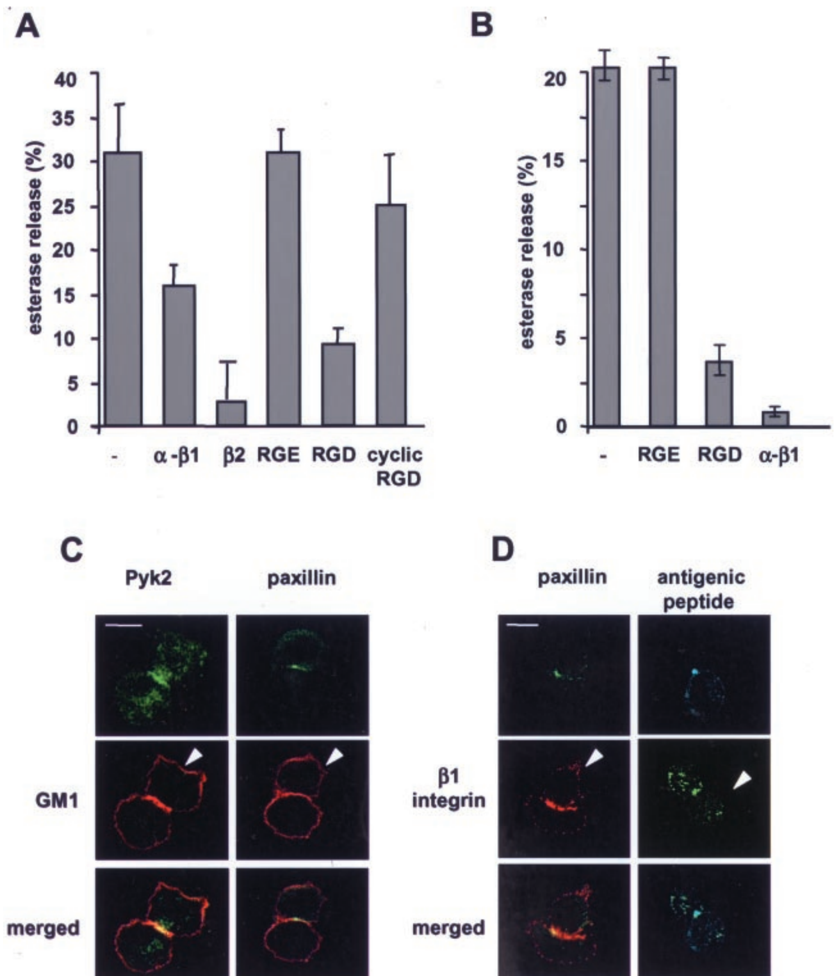

FIG. 7. **Adhesion molecules focus at the T cell-APC interface.** *A*, esterase release of T1 CTLs in response to P815 cells pulsed with 10 nM PbCS(ABA) in the absence or presence of blocking antibodies specific for β_1 and β_2 integrins or the peptides GRGDS (*RGD*), GRGES (*RGE*), or cyclic GRGDS (*RGD*) β_3 integrin-binding peptide. *B*, esterase release of T1 CTLs in response to L-cells transfected with K^d pulsed with 100 nM PbCS(ABA), as described in *A*. *C*, P815 cells pulsed with 10 nM PbCS(ABA) and conjugated with T1 CTLs, and the distributions of GM1, paxillin, and Pyk2 were analyzed by confocal microscopy. The *arrow* indicates the target cell. *D*, distribution of paxillin and β_1 integrin or Cy5-labeled antigenic peptide and β_1 integrin in T1 CTLs conjugated with K^d-expressing L-cells pulsed with 100 nM Cy5-labeled antigenic peptide. *Scale bar*, 5 μm.

50% by anti-β_1 integrin and nearly by 90% by anti-β_2 integrin (LFA-1) antibody (Fig. 7*A*). Furthermore, the peptide GRGDS inhibited degranulation by 70%, whereas the control peptide GRGES had no effect. The cyclic GRGDS peptide, which selectively binds to β_3 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 β_1 and β_3 but not β_5 and β_7 (Table I and data not shown) (14, 24). Taken together, this implies that binding of β_1 and β_3 integrins of the CTLs to cell-associated fibronectin on target cells greatly enhances antigen recognition. Indeed, L-cells, which express no ICAM (Table I), were well recognized by T1 CTLs, and this response was greatly impaired by the GRGDS peptide and anti-β_1 integrin antibody but not affected by the GRGES peptide (Fig. 7*B*).

Confocal microscopy of T1 CTLs conjugated with P815 cells sensitized with PbCS(ABA) peptide showed in the contact site high enrichment of GM1, Pyk2, and paxillin (Fig. 7*C*). Co-localization of GM1 with Pyk2 and paxillin, respectively, were observed in bright clusters at contact site. Moreover, on T1 CTLs conjugated with K^d-transfected L-cells previously sensitized with Cy5-labeled PbCS(ABA), paxillin and the peptide were strongly enriched at the contact zone and co-localized with β_1 integrins (Fig. 7*D*). Taken together these results demonstrate that β_1 and β_3 integrins, by interacting with fibronectin on target cells, play an important role in target cell recognition and co-localize with Pyk2, paxillin, 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 $\alpha_4\beta_1$ (VLA4), $\alpha_5\beta_1$ (VLA5), $\alpha_v\beta_1$, $\alpha_{IIb}\beta_1$, and $\alpha_v\beta_3$ and adhere to ECM proteins (16, 18, 19). We show that this adhesion dramatically enhances perforin-dependent cytotoxicity of $CD8^+$ 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 are multimeric and co-engage CD8 (Fig. 1) (34, 37). This difference explains the controversy about whether monomeric MHC·peptide complexes can activate T cells (34, 35, 37). Indeed they can activate $CD8^+$ 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 polystyrene (Fig. 2*D*).[2]

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 K^d·"IASA"-YIPSAEK(ABA) complexes (40, 55),[2] these studies support the conclusion that activation of $CD8^+$ T cells requires cell adhesion.

The role of β_1 and β_3 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, β_1 and β_3 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 RGD peptide or anti-β_1 antibody (Fig. 7 and Table I). Similar observations were made for other ECM proteins binding to β_1 and β_3 integrins (17, 56). Although the β_2 integrin LFA-1 plays an important role in antigen recognition (3, 8), our findings indicate that β_1 and β_3 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 β_1/β_3 integrin- and TCR/CD8-mediated signals separately and how they elicit CTL degranulation when they are combined. The hallmark of β_1/β_3 integrin-mediated adhesion is the formation of tyrosine-phosphorylated molecular aggregates containing Pyk2, Lck, Fyn, and paxillin 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, paxillin and Pyk2, respectively (Figs. 3*A* and 5*A*) (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 paxillin to rafts (Fig. 4). 3) The strong tyrosine phosphorylation of paxillin, Pyk2, Lck, and Fyn is indicative for localization in raft, where phosphatases are excluded (Figs. 3 and 4) (1, 11, 60). Moreover, because β_1 integrins, as well as phosphorylated paxillin 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 adducts resulted in Lck activation and phosphorylation of CD3 (Fig. 5*A*) (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, PLCγ, 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. 2*D*), but blocked the activation of adherent CTLs by MHC·peptide complexes (Fig. 2*C*), 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 paxillin, 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 β_1/β_3 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 β_1 and β_3 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

[2] M.-A. Doucey, D. F. Legler, M. Faroudi, N. Boucheron, P. Baumgaertner, D. Naeher, M. Cebecauer, D. Hudrisier, C. Rüegg, E. Palmer, S. Valitutti, C. Bron, and I. F. Luescher, unpublished results.

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). Consistent with this is the finding that, in degranulating CTLs, Pyk2 and its cytoskeleton linker paxillin (Figs. 4 and 6) are focused in the contact site with target cells, irrespective of whether these cells express ICAM or not (Fig. 7). In addition, Fyn associates with the Fyn-binding protein Fyb, also known as SLAP-130 (SLP-76-associated phosphoprotein) or ADAP (adhesion and degranulation adaptor protein), which regulates β_1/β_3 integrin-mediated adhesion and their cross-talk with the TCR (67–69).

In conclusion, the present study shows that β_1 and β_3 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 paxillin 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.

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