

T Cell Receptor- and β_1 Integrin-mediated Signals Synergize to Induce Tyrosine Phosphorylation of Focal Adhesion Kinase (pp125^{FAK}) in Human T Cells

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Summary

The β_1 subfamily of integrins is thought to play an important role in both the adhesion/migration and proliferation/differentiation of T cells. β_1 integrins can provide T cell costimulation through interaction of very late antigen (VLA) 4 (VLA-4) ($\alpha_4\beta_1$) and VLA-5 ($\alpha_5\beta_1$) with the extracellular matrix protein fibronectin (FN), or by VLA-4 binding to its cell surface ligand, vascular cell adhesion molecule (VCAM) 1. The mechanism by which β_1 integrin members transduce T cell-costimulatory signals is poorly understood. Studies in non-T cells have demonstrated regulation of the tyrosine focal adhesion kinase pp125^{FAK} by β_1 integrin engagement and, most recently, indicate a role for pp125^{FAK} in linking integrin-mediated signal transduction to the Ras pathway (Schaller, M. D., and J. T. Parsons. 1994. *Curr. Opin. Cell. Biol.* 6: 705–710; Schlaepfer, D. D., S. K. Hanks, T. Hunter, and P. Van der Geer. 1994. *Nature (Lond.)*. 372:786–790). Although pp125^{FAK} kinase occurs in T cells, there are no reports on its regulation in this cell type. The studies described in this article characterize novel regulation of pp125^{FAK} by the T cell receptor (TCR)-CD3 antigen complex and β_1 integrins, and provide the first account, in any cell type, of integrin $\alpha_4\beta_1$ -mediated pp125^{FAK} tyrosine phosphorylation. We demonstrate a rapid and sustained synergistic increase in tyrosine phosphorylation of human pp125^{FAK} in Jurkat T cells after simultaneous (a) triggering of the TCR-CD3 complex, and (b) $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrin-mediated binding of these cells to immobilized FN or $\alpha_4\beta_1$ integrin-mediated binding to immobilized VCAM-1. Studies with normal peripheral blood-derived CD4⁺ human T blasts confirm the synergistic action of a TCR-CD3 complex-mediated costimulus with a FN- or VCAM-1-dependent signal in the induction of T cell pp125^{FAK} tyrosine phosphorylation. In vitro kinase assays performed on pp125^{FAK} immunoprecipitates isolated from Jurkat cells and normal CD4⁺ T cells identified a coprecipitating 57-kD tyrosine-phosphorylated protein (pp57), distinct from pp59^{fyn} or pp56^{lck}. These results indicate, for the first time, the involvement of a specific kinase, pp125^{FAK}, in $\alpha_4\beta_1$ - and $\alpha_5\beta_1$ -mediated T cell-costimulatory signaling pathways. In addition, the data demonstrate novel regulation of pp125^{FAK} tyrosine phosphorylation by the TCR-CD3 complex.

The integrins are a family of heterodimeric transmembrane proteins, formed by the noncovalent association of an α subunit and a β subunit, that function as cell surface adhesion receptors for both extracellular matrix (ECM)¹ and cell surface protein ligands. There are presently 15 different, structurally related integrin α subunits and at least 8 different β subunits described (1–3). In adherent cells, integrins localize to focal adhesions, subcellular structures oc-

curing in regions of tight contact between the cell and underlying ECM. Focal adhesions serve as transmembrane junctions between the ECM and cytoskeleton and consist of three distinct components: (a) an extracellular component made up of ECM proteins; (b) a transmembrane region comprised of integrin proteins, particularly members of the β_1 family of integrins (very late antigen molecules); and (c) an array of intracellular cytoskeletal proteins (4). The work of Kupfer and co-workers indicates that focal adhesions occur in T cells at points of contact with APC and target cells (5–7).

Integrins not only serve as a physical link between the ECM and cytoskeleton but also appear to be involved in

¹ Abbreviations used in this paper: ECM, extracellular matrix; FAK, focal adhesion kinase; FN, fibronectin; PDGF, platelet-derived growth factor; pFN, plasma FN; RGD, Arg-Gly-Asp; SH2, Src homology 2; VCAM, vascular cell adhesion molecule.

the transduction of intracellular signals. Engagement of integrins by their extracellular ligands is reported to result in a number of intracellular signals, including increases in intracellular pH, intracellular Ca^{2+} concentration, and tyrosine phosphorylation of cellular proteins, as well as activation of gene transcription (for reviews see references 1, 2). A recently described protein tyrosine kinase associated with focal adhesions, pp125^{FAK}, was found to be involved in the signal transduction pathways mediated by β_1 and β_3 integrin family members (for reviews see references 8, 9). Cells interacting with surfaces coated with various ECM proteins were found to upregulate the enzyme activity and tyrosine phosphorylation of pp125^{FAK}, and this up-regulation could be inhibited by β_1 or β_3 integrin-specific mAbs. In addition to integrins, platelet-derived growth factor (PDGF) (10) and the neuropeptides bombesin, vasopressin, and endothelin (11, 12) were shown to induce tyrosine phosphorylation of pp125^{FAK} in 3T3 cells. Recently, pp125^{FAK} was reported to physically associate with the cytoplasmic domains of β_1 integrins in vitro (8).

Several lines of evidence suggest that focal adhesion kinase (FAK) signal transduction may be involved in transmitting signals to both the cytoskeleton and the nucleus. First, tyrosine phosphorylation of the focal adhesion-associated cytoskeletal proteins paxillin and tensin appears to be dependent upon pp125^{FAK} autophosphorylation on tyrosine residue (Tyr) 397 (8). Secondly, the Src family kinases pp60^{src}, pp59^{lck}, and pp51^{ck} can bind to pp125^{FAK} through their Src homology 2 (SH2) domains, and the SH2-dependent pp60^{src} binding is also dependent upon autophosphorylation of pp125^{FAK} Tyr397 (13–15). Lastly, recent studies show that phosphorylation of pp125^{FAK} Tyr925 promotes SH2 domain-mediated binding of the kinase to the Grb2 adaptor protein (16). Thus, current integrin-regulated signaling models link pp125^{FAK} autophosphorylation to changes in the cytoskeletal architecture and activation of the Ras pathway of signal transduction (8, 16–18).

Numerous investigations indicate that β_1 integrins play an important role in the adhesion/migration and proliferation/differentiation of T lymphocytes through interaction of the T cell integrins $\alpha_4\beta_1$ (CD49d/CD29) and $\alpha_5\beta_1$ (CD49e/CD29) with the CS-1 and Arg-Gly-Asp (RGD) sites, respectively, on the ECM protein fibronectin (FN) (for reviews see references 3, 19, 20). In addition, the integrin $\alpha_4\beta_1$ can also interact with the cell surface ligand vascular cell adhesion molecule (VCAM) 1 (CD106), which is expressed on inflamed endothelium, follicular dendritic cells, and tissue macrophages (21, 22). Binding of T cell β_1 integrins to FN and VCAM-1 provides signals that are co-stimulatory with TCR-CD3-mediated signals in inducing T cell proliferation and cytokine production (23–28). Morimoto and colleagues showed that the interaction of T cells with FN through β_1 integrins induces tyrosine phosphorylation of a 105-kD protein (29) and the up-regulation of the DNA-binding protein AP-1. However, beyond these findings, little is known about the mechanisms by which β_1 integrin-mediated signals are transduced in T cells.

Kanner and colleagues demonstrated that human pp125^{FAK} is expressed in T cells, that its basal level of tyrosine phosphorylation is generally increased in T cell leukemia/lymphoma cell lines, and suggested that it is not a component of the TCR-CD3 signaling pathway (31, 32). The studies reported here investigate the regulation of T cell pp125^{FAK} by β_1 integrin-mediated signaling. We used the purified β_1 integrin ligands FN and VCAM-1 to perform these studies, and examined Jurkat T cells as well as peripheral blood-derived CD4⁺ T cell blasts. Our results show a strong synergistic effect in signaling through the TCR-CD3 complex and the $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins, resulting in a rapid and sustained induction of pp125^{FAK} tyrosine phosphorylation. In addition, we describe an unknown tyrosine-phosphorylated 57-kD protein (pp57) identified in in vitro kinase assays of pp125^{FAK}-specific immunoprecipitates.

Materials and Methods

Cell Culture. A subclone from the Jurkat T cell line was kept in culture at low density (below 10^6 /ml) in RPMI 1640 supplemented with 10% FCS, L-glutamine, and antibiotics. The Jurkat line stains positive in flow cytometry for the integrins α_L (CD11a), α_4 (CD49d), α_5 (CD49e), β_1 (CD29), and β_2 (CD18), as well as for CD2 and CD3. The adherent murine fibroblast line 3T3 was cultured in DMEM containing 10% FCS, L-glutamine, and antibiotics. Isolation of purified resting CD4⁺ T cells was performed as previously described (33). Briefly, mononuclear cells were isolated from buffycoats from healthy donors first by density centrifugation on a Ficoll gradient, followed by an immunomagnetic negative selection with specific mAbs and magnetic particles. T cell blasts were obtained by stimulating resting CD4⁺ T cells with PHA-M, 1:200 final dilution (GIBCO BRL, Gaithersburg, MD), and 10 ng/ml PMA. On day 3, cells were placed at low cell density (2×10^5 /ml) in culture media supplemented with 100 U/ml rhIL-2 (Hoffman-La Roche, Nutley, NJ). rhIL-2 was again added on day 6 of culture, after which the cells were left to rest for another 7–10 d before assaying.

Antibodies and Ligands. The pp125^{FAK} mAb 2A7 (IgG₁) (34) and biotinylated antiphosphotyrosine mAb 4G10 were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). The anti-pp125^{FAK} polyclonal rabbit serum BC3 was kindly provided by Dr. T. Parsons (University of Virginia, Charlottesville, VA) (35). The pp125^{FAK} polyclonal rabbit serum hFAK1 was produced by immunizing rabbits with the KLH-conjugated synthetic peptide SDEAPPKPSRPGYSPRSSE (single-letter amino acid code) corresponding to amino acids 556–575 of the human pp125^{FAK} sequence (produced by Dr. G. Reddy, Oligopeptide Core Facility, University of Chicago, Chicago, IL), and was affinity-purified over a peptide column (31). Affinity-purified polyclonal anti-p59^{lck} rabbit antisera, produced by immunizing rabbits with a glutathione S-transferase fusion protein containing the NH₂-terminal 144 amino acids of murine Fyn, was the gift of Dr. M. Clark (University of Chicago). Rabbit polyclonal anti-p56^{lck} antisera 688 directed against the NH₂ terminus of p56^{lck} was a gift from Dr. L. E. Samelson (National Institutes of Health [NIH], Bethesda, MD) (36). pp56^{lck} mAb was purchased from Transduction Laboratories (Lexington, KY). The pp60^{src} polyclonal rabbit serum generated against the entire chicken pp60^{src} protein was a gift from Dr. B. Cobb (University of Chicago) (37). Murine-derived mAb mOKT3 (IgG_{2a}) was obtained from the American Type Culture Collection (Rockville, MD), and a humanized version

hOKT3 (CDR grafted on human IgG₁) was provided by Dr. J. A. Bluestone (University of Chicago) (38). The rat anti-human mAb13 (CD29) and mAb16 (CD49e) were gifts from Dr. K. Yamada (NIH) (39). The NIH49d-1 (CD49d) mAb (IgG₁) was generated by Drs. Y. Tanaka and S. Shaw (NIH). The HP1/2 (CD49d) mAb was a gift from Dr. R. Lobb (Biogen, Inc., Cambridge, MA) (40). The mAb 5H7 (human HLA class I) was a gift from Dr. S. Woodle (University of Chicago) (41). MOPC-21 (IgG₁) was purchased from Cappel (Organon Teknika, Durham, NC). The recombinant fusion protein LFA-3-Ig, a construct of the extracellular domains of human LFA-3 gene fused to the hinge, CH2, and CH3 domains of human IgG₁, was provided by Dr. P. S. Hochman (Biogen, Inc.) (42). The recombinant fusion protein VCAM-1-Ig, comprised of the two NH₂-terminal domains of the human VCAM-1 gene fused to the hinge, CH2, and CH3 domains of human IgG₁, was produced at Biogen, Inc., as previously described (43). Human plasma FN (pFN) was purchased from GIBCO BRL.

Cell Stimulation and Preparation of Cell Extracts. Cells were stimulated in six-well tissue culture-treated plates (Costar Corp., Cambridge, MA) precoated with indicated stimulating antibodies and/or ligands. Plates were prepared by incubating with indicated concentrations of antibody/ligand diluted in PBS overnight at 4°C, followed by washing three times with PBS. In experiments where hOKT3 was one of the stimulatory ligands, all plates were precoated with goat anti-human IgG (10 µg/ml). For activation, cells were washed in PBS and resuspended in serum-free medium (X-Vivo 20; Biowhittaker, Gaithersburg, MD), and either 2 million Jurkat cells or 5–6 million T cell blasts were added to each well in a final volume of 2 ml. Plates were centrifuged at 200 g for 1 min to allow for immediate contact between cells and antibodies/ligands, and then incubated at 37°C for indicated periods of time. For harvest, supernatants containing nonadherent cells were removed and pelleted, and the remaining adherent cells were lysed *in situ* in buffer containing 0.5–1% Triton, 50 mM Tris-HCl, pH 7.4, 1,500 mM NaCl, 10 µg/ml aprotinin, 5 µg/ml leupeptin, 1 mM Pefabloc SC (Boehringer Mannheim Corp., Indianapolis, IN), 2 mM NaVanadate, 2 mM EDTA, 10 mM NaF, and 20 mM iodoacetamide. The adherent cell homogenate was then added to Eppendorf tubes containing pelleted nonadherent cells from the same wells, and then incubated on ice for 10 min. Cell membranes were pelleted by microcentrifugation at 4°C for 15 min, and the lysate-containing supernatant was transferred to a fresh microcentrifuge tube. Conditions indicated as “resting” represent cells obtained directly out of suspension culture. These cells were pelleted, washed once in PBS, pelleted again, and lysed in Eppendorf tubes, after which membranes were removed by microcentrifugation.

Immunoprecipitation. Immunoprecipitations were performed as described previously (44). Briefly, cell lysates prepared from equivalent numbers of Jurkat cells (12×10^6 cells) or CD4⁺ T cell blasts ($\sim 100 \times 10^6$ cells) were precleared (except in blocking studies) with either protein A- or protein G-Sepharose (ImmunoSelect; GIBCO BRL) and immunoprecipitated by incubation at 4°C with antibody preadsorbed to appropriate affinity beads, followed by washing of immunoprecipitates with lysis buffer before further analysis.

Western Blotting. Eluates from cell-equivalent Jurkat cell immunoprecipitations or CD4⁺ T cell blast immunoprecipitations were separated by SDS-PAGE and electroblotted onto polyvinylidene difluoride membrane. Membranes were blocked in 10% BSA and blotted with the biotinylated antiphosphotyrosine mAb 4G10 (αpTyr blots) followed by incubation with appropriate

horseradish peroxidase-conjugated secondary reagent and chemiluminescent development according to the manufacturer's instructions (Lumiglow; Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD; or enhanced chemiluminescence; Amersham Corp., Arlington Heights, IL). Where indicated, antiphosphotyrosine blots were stripped by 30-min incubation at 56°C in 100 mM β-2-ME, 2% SDS, and 62.5 mM Tris-HCl, pH 6.8, and effective stripping was confirmed by incubating blots in developing reagents and reexposing to autoradiography film. Finally, membranes were washed, blocked in 5% milk, and reblotted with BC3 primary antibody or hFAK1 (αFAK blots). pp125^{FAK} tyrosine phosphorylation was determined by densitometric scanning of αpTyr blots using a computing densitometer (model 325E; Molecular Dynamics, Inc., Sunnyvale, CA). Values for pp125^{FAK} tyrosine phosphorylation are expressed relative to a designated condition in each experiment.

Adhesion Assays. 24-well tissue culture-treated plates (Costar Corp.) were precoated with stimulating antibodies and/or ligands. Plates were prepared similarly as for cell activation conditions (see above). Jurkat cells were washed in PBS, resuspended in serum-free media, added to tissue culture wells at a density of 0.5 million/well, and incubated at 37°C for 15 min. After incubation, plates were washed repeatedly with cold PBS, which was removed by flipping of the plates. Remaining attached cells were quantitated using a kit (CytoTox 96TM; Promega Corp., Madison, WI) according to the manufacturer's guidelines. The assay is based on an enzymatic reaction of cytosolic lactate dehydrogenase with a provided substrate, which results in formation of a red formazan product that can be read out at 490 nm in an ELISA reader. Percent binding was determined by comparison with a serial dilution of Jurkat cells as standard.

In Vitro Kinase Assay. Cells were lysed in 1% Triton X-100 and 0.025% deoxycholate containing lysis buffer and immunoprecipitated on beads without preclearing. Immunoprecipitates, representing either 12×10^6 Jurkat cells or 40×10^6 CD4⁺ T cell blasts, were washed three times with lysis buffer, once with TBS (100 mM NaCl, 10 mM Tris, 5 mM MnCl₂), and once with kinase buffer (10 mM Tris, pH 7.4, and 5 mM MnCl₂). Pellets were dried thoroughly and resuspended in 20 µl kinase buffer containing 10 µCi [³²P]ATP (3.3 µM, sp act 3,000 Ci/mM; New England Nuclear, Boston, MA). Samples were incubated for 20 min at 30°C, washed three times with lysis buffer, boiled 5 min after addition of sample buffer, resolved by SDS-PAGE, and detected by autoradiography.

V8 Protease Mapping. V8 protease mapping was performed using a modification of the method of Cleveland et al. (45). In vitro kinase substrates were resolved by SDS-PAGE and visualized by autoradiography. Bands of interest were gel excised, incubated in pH 6.7 digestion buffer (10% glycerol, 0.1% SDS, 0.125 M Tris, pH 6.8, 1 mM EDTA) for 30 min at room temperature, and then placed in wells of a 12% SDS-PAGE gel with digestion buffer containing 50 µg/ml *Staphylococcus aureus* V8 protease (ICN Nutritional Biochemicals, Cleveland, OH). Digestion buffer was overlaid with tracking buffer containing bromophenol blue and subjected to electrophoresis until the tracking dye was focused within the gel stack. Power was then turned off for 30–60 min before completion of electrophoresis and visualization of digested fragments by autoradiography.

Results

TCR-mediated and pFN-dependent Signals Can Individually and Synergistically Induce Tyrosine Phosphorylation of pp125^{FAK}

in Jurkat T Cells. Guan et al. described that, in 3T3 cells, FN could induce β_1 integrin-mediated tyrosine phosphorylation of a 116-kD protein, which was later identified as pp125^{FAK} (35, 46). Resting human T cells, unlike adherent cells, express surface β_1 integrin molecules that do not support adhesion to ligand-bearing substrate (47). T cell β_1 integrin-mediated adhesion can, however, be induced by various mechanisms, including triggering through the TCR-CD3 complex or PMA treatment (47). Consequently, to study the role of pp125^{FAK} in β_1 integrin-mediated human T cell costimulation, we initially examined the induction of pp125^{FAK} tyrosine phosphorylation by pFN immobilized alone or coimmobilized with the CD3-specific murine mAb mOKT3. Experiments were performed using either the anti-Fak mAb 2A7 or the anti-human Fak peptide antisera hFAK1 (34). The specificity of hFAK1 for pp125^{FAK} was confirmed in reciprocal preclearing experiments of Jurkat cell lysates performed with hFAK1 and 2A7 (data not shown). Antiphosphotyrosine (α pTyr) Western blots showed that both 2A7 and hFAK1 immunoprecipitates from Jurkat cells stimulated with either pFN or mOKT3 alone displayed modest tyrosine phosphorylation of a 116-kD protein that was absent in control immunoprecipitates (Fig. 1 A, lanes 2–5, 8, and 9), whereas combined pFN and mOKT3 stimulation resulted in significant synergistic tyrosine phosphorylation of this protein (Fig. 1 A, lanes 6 and 7). Western blotting with BC3 (α pp125^{FAK}) demonstrated that the 116-kD tyrosine-phosphorylated protein comigrated with pp125^{FAK} and, consequently, will be referred to as pp125^{FAK} in the remainder of the text (Fig. 1 B). hFAK1 immunoprecipitates from bulk, unstimulated Jurkat cells demonstrated three times less tyrosine-phosphorylated pp125^{FAK} than immunoprecipitates from cells stimulated with pFN for 10 min (Fig. 1 A, lanes 1 and 3), and six times less tyrosine phosphorylation than hFAK1 immunoprecipitates from cells stimulated with mOKT3 (Fig. 1 A, lanes 1 and 5). Synergistic β_1 integrin- and TCR-CD3 complex-mediated pp125^{FAK} tyrosine phosphorylation occurred within 10 min of initiation of receptor cross-linking: the amount of pp125^{FAK} tyrosine phosphorylation for the combined stimulus of mOKT3 and pFN at 10 min was 1.4- and 2.5-fold higher than the additive phosphorylation of pFN alone plus OKT3 alone for 2A7 and hFAK1 immunoprecipitates, respectively (Fig. 1 A, lanes 2, 4, and 6 and lanes 3, 5, and 7). As hFAK1 was significantly more efficient than 2A7 in immunoprecipitating human pp125^{FAK}, and Western blotting of immunoprecipitates of either antibody demonstrated similar results, subsequent experiments were performed with hFAK1 immunoprecipitates.

Kinetics of Synergistically Induced pp125^{FAK} Tyrosine Phosphorylation in Jurkat T Cells. Time course studies were performed to characterize the kinetics of T cell pp125^{FAK} tyrosine phosphorylation in detail (Fig. 2). A very rapid and synergistic increase in pp125^{FAK} tyrosine phosphorylation can readily be observed after 1 min of stimulation with combined mOKT3 and pFN (Fig. 2, top, lane 4), with near maximum levels occurring by 5 min (Fig. 2, top, lane 7). After achieving maximal induction, the level of synergistic

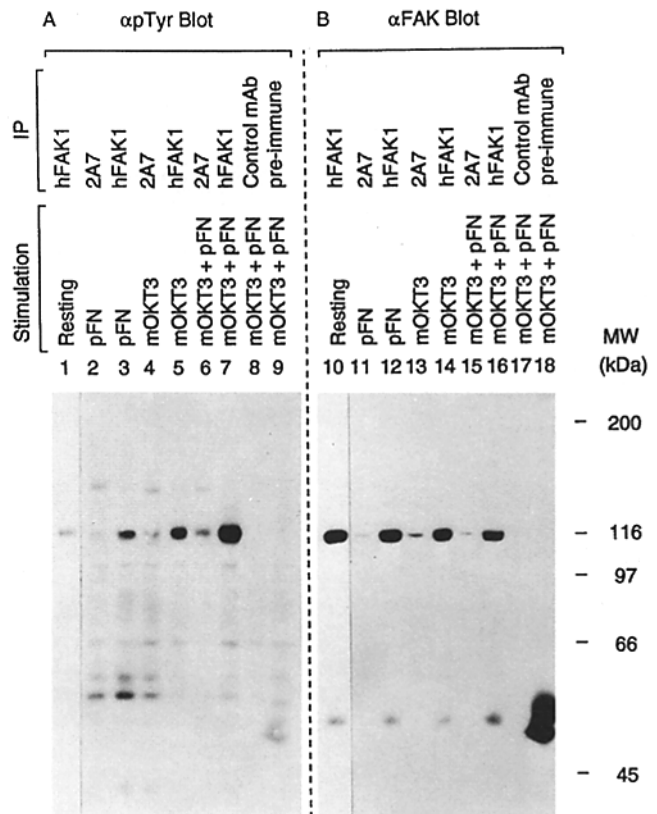


Figure 1. TCR-mediated and pFN-dependent signals synergize in tyrosine phosphorylation of pp125^{FAK} in Jurkat T cells. Jurkat T cells were either unstimulated (lane 1) or stimulated for 10 min (lanes 2–9) with either pFN (1 μ g/ml; lanes 2 and 3), mOKT3 (1 μ g/ml; lanes 4 and 5), or a combination of the two (lanes 6–9). Lysates prepared from 12×10^6 cells were immunoprecipitated with either the pp125^{FAK}-specific mAb 2A7 (lanes 2, 4, and 6) or the human pp125^{FAK}-specific polyclonal serum hFAK1 (lanes 1, 3, 5, and 7). Isotype-matched mAb (MOPCI-21) (lane 8) and preimmune sera (lane 9) immunoprecipitates were used as controls. Samples were first analyzed by Western blotting with (A) the antiphosphotyrosine mAb 4G10 (α pTyr blot), followed by stripping and reblotting with (B) the anti-pp125^{FAK} BC3 antiserum (α FAK blot). Relative FAK tyrosine phosphorylation values (determined separately for hFAK1 immunoprecipitations and 2A7 immunoprecipitations) were as follows: hFAK1 immunoprecipitations, bulk 0.04, pFN 0.14, mOKT3 0.25, mOKT3 + pFN 1; 2A7 immunoprecipitations, pFN 0.21, mOKT3 0.52, mOKT3 + pFN 1.

pp125^{FAK} tyrosine phosphorylation was maintained for at least 2 h (Fig. 2, top, lanes 10, 13, 16, and 19).

Both $\alpha_4\beta_1$ - and $\alpha_5\beta_1$ Integrin-mediated Signals Induce Tyrosine Phosphorylation of T Cell pp125^{FAK}. For further specificity analysis, we tested other purified ligands capable of interacting with the Jurkat T cells for their ability to induce pp125^{FAK} phosphorylation. In addition to pFN, which contains per molecule two RGD sites for $\alpha_5\beta_1$ binding and one CS-1 site for $\alpha_4\beta_1$ binding, we tested the $\alpha_4\beta_1$ integrin ligand VCAM-1 (CD106) and the CD2 ligand LFA-3 (CD58) (Fig. 3 A). VCAM-1 and LFA-3 were used as recombinant human Ig fusion proteins and immobilized indirectly through goat anti-human IgG. The hOKT3 mAb that was used is a humanized (IgG₁) version of the murine

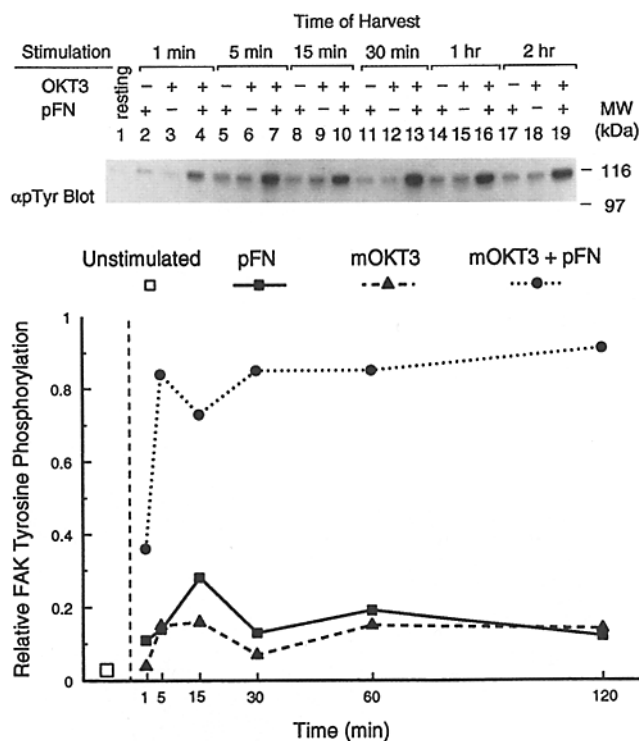


Figure 2. Tyrosine phosphorylation of pp125^{FAK} in T cells is induced rapidly and maintained for at least 2 h. (Top) Jurkat T cells were either unstimulated (lane 1) or stimulated for indicated periods with either pFN (1 μg/ml; lanes 2, 5, 8, 11, 14, and 17), mOKT3 (1 μg/ml; lanes 3, 6, 9, 12, 15, and 18), or a combination of the two (lanes 4, 7, 10, 13, 16, and 19). Lysates prepared from 12 × 10⁶ cells were immunoprecipitated with hFAK1 antisera and subjected to Western blotting with the antiphosphotyrosine mAb 4G10 (αpTyr blot). (Bottom) Mean relative pp125^{FAK} tyrosine phosphorylation as determined from two independent experiments. Values for the mean and standard deviation at each time point were as follows: pFN, 0.11 ± 0.04 (1 min), 0.14 ± 0.04 (5 min), 0.28 ± 0.18 (15 min), 0.13 ± 0.07 (30 min), 0.19 ± 0.04 (1 h), and 0.12 ± 0.05 (2 h); for mOKT3, 0.04 ± 0.02 (1 min), 0.15 ± 0.16 (5 min), 0.16 ± 0.14 (15 min), 0.07 ± 0.02 (30 min), 0.15 ± 0.1 (1 h), and 0.14 ± 0.01 (2 h); for mOKT3 + pFN, 0.36 ± 0.01 (1 min), 0.84 ± 0.15 (5 min), 0.73 ± 0.09 (15 min), 0.85 ± 0.21 (30 min), 0.85 ± 0.09 (1 h), and 0.91 ± 0.12 (2 h). Measurements were determined relative to the highest level of Fak tyrosine phosphorylation observed in each experiment.

mAb mOKT3. Together, pFN and hOKT3 demonstrated the same synergistic effect on pp125^{FAK} tyrosine phosphorylation as was shown previously (Fig. 3 A, top, lanes 1–3). VCAM-1 alone was capable of inducing tyrosine phosphorylation of pp125^{FAK} to levels similar to those achieved by the combination of pFN and hOKT3 (Fig. 3 A, top, lanes 3 and 4). A threefold synergistic increase in these levels occurred when VCAM-1 was costimulated with hOKT3 (Fig. 3 A, top, lanes 1, 4, and 5). The combination of hOKT3 and LFA-3 did not induce pp125^{FAK} tyrosine phosphorylation over the level obtained with hOKT3 alone (Fig. 3 A, top, lanes 1 and 7), even though the CD2-LFA-3 pathway is functional in this particular Jurkat subclone as indicated by the induction of IL-2 secretion (data not shown).

To identify the T cell pFN receptors involved in the pp125^{FAK} tyrosine phosphorylation, we performed mAb

blocking studies (Fig. 3 B). The greatest inhibition (~90% inhibition) of combined mOKT3-pFN-induced pp125^{FAK} tyrosine phosphorylation was observed after blocking with either a β₁-specific mAb (Fig. 3 B, top, lanes 3 and 8) or the combination of α₄- and α₅-specific mAbs (Fig. 3 B, top, lanes 3 and 7). No blocking was seen with control mAb against a nonpolymorphic determinant of HLA class I (Fig. 3 B, top, lanes 3 and 4). The α₄-specific mAb alone partially blocked pp125^{FAK} tyrosine phosphorylation (Fig. 3 B, top, lanes 3 and 5), as did the α₅-specific mAb (Fig. 3 B, top, lanes 3 and 6). The pp125^{FAK} tyrosine phosphorylation remaining after β₁-specific mAb blocking approximated that found with mOKT3 alone (Fig. 3 B, top, lanes 2 and 8). Together, these results indicate that the synergistic effect of combined mOKT3 and pFN stimulation on the induction of pp125^{FAK} tyrosine phosphorylation in Jurkat cells is completely mediated by β₁ integrins and, more specifically, is the result of both α₄β₁- and α₅β₁-mediated interactions.

The VCAM-1-dependent increase in tyrosine phosphorylation of pp125^{FAK} was also examined to determine the integrin receptors involved. The data shown in Fig. 3 C indicate that the dominant integrin receptor involved in combined hOKT3-VCAM-1-induced tyrosine phosphorylation of pp125^{FAK} is α₄β₁, as both the α₄-specific mAb (Fig. 3 C, top, lane 5) and the β₁ integrin-specific mAb (Fig. 3 C, top, lane 7) could block induction down to levels achieved with hOKT3 alone (Fig. 3 C, top, lane 1). As expected, mAbs against the α₅ integrin (Fig. 3 C, top, lane 6) or HLA class I (Fig. 3 C, top, lane 4) did not alter the pp125^{FAK} tyrosine phosphorylation induced by coimmobilized hOKT3 and VCAM-1 (Fig. 3 C, top, lane 3).

FACScan[®] cytofluorometric analysis of the Jurkat T cells showed that they expressed high levels of integrins α₄ (CD49d) and β₁ (CD29) and significantly lower levels of integrin α₅ (CD49e), and were negative for the mAb ACT-1 (data not shown), which recognizes a conformational epitope on α₄β₇ (48). Although the α₄β₇ integrin is reported to mediate adhesion to FN and VCAM-1 (49, 50), these findings exclude a potential role for α₄β₇-mediated induction of pp125^{FAK} tyrosine phosphorylation in these Jurkat cells.

PMA-induced Increased Adhesion of Jurkat Cells to FN Does Not Lead to Increased Tyrosine Phosphorylation of T Cell pp125^{FAK}. We postulated that the synergistic effect of TCR-CD3 complex- and pFN-mediated signals on the regulation of T cell pp125^{FAK} tyrosine phosphorylation was due to augmentation of β₁ integrin signaling as a result of TCR-CD3-mediated enhancement of β₁ integrin adhesion to pFN. To investigate this hypothesis, we examined whether the phorbol ester PMA, another reported enhancer of β₁ integrin-mediated T cell adhesion to pFN, would augment both binding of the Jurkat cells to pFN and pFN-induced tyrosine phosphorylation of pp125^{FAK} (Table 1) (47). As depicted in Table 1, although PMA significantly enhanced adhesion of Jurkat cells to pFN, it did not affect pFN-mediated tyrosine phosphorylation of FAK. These results indicate that increased binding of Jurkat cells to pFN will not necessarily result in enhanced pp125^{FAK} tyrosine phosphorylation.

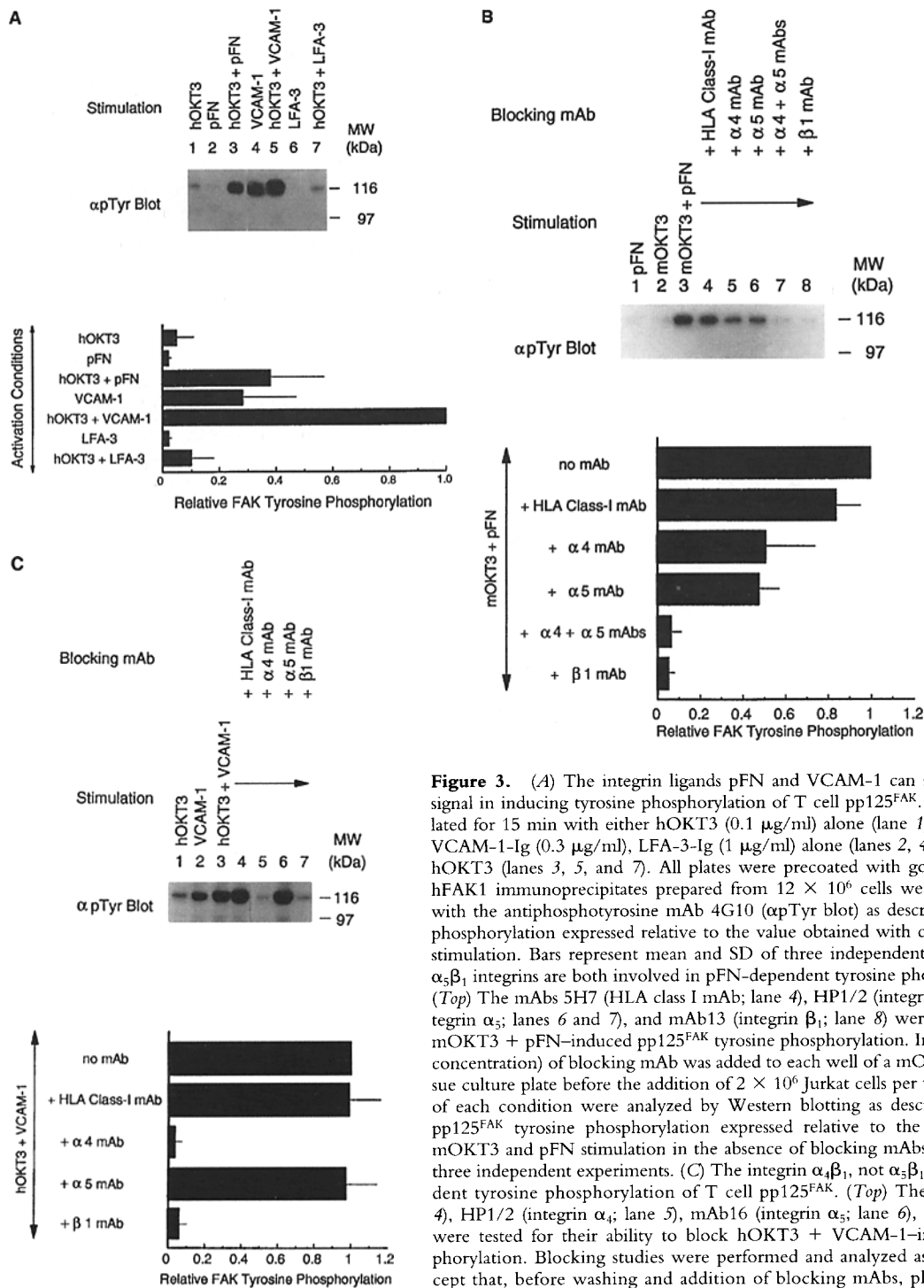


Figure 3. (A) The integrin ligands pFN and VCAM-1 can synergize with a TCR-mediated signal in inducing tyrosine phosphorylation of T cell pp125^{FAK}. (Top) Jurkat T cells were stimulated for 15 min with either hOKT3 (0.1 μ g/ml) alone (lane 1), or the ligands pFN (1 μ g/ml), VCAM-1-Ig (0.3 μ g/ml), LFA-3-Ig (1 μ g/ml) alone (lanes 2, 4, and 6), or in combination with hOKT3 (lanes 3, 5, and 7). All plates were precoated with goat anti-human IgG (10 μ g/ml). hFAK1 immunoprecipitates prepared from 12×10^6 cells were analyzed by Western blotting with the antiphosphotyrosine mAb 4G10 (α Tyr blot) as described. (Bottom) pp125^{FAK} tyrosine phosphorylation expressed relative to the value obtained with combined hOKT3 and VCAM-1 stimulation. Bars represent mean and SD of three independent experiments. (B) The $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins are both involved in pFN-dependent tyrosine phosphorylation of T cell pp125^{FAK}. (Top) The mAbs 5H7 (HLA class I mAb; lane 4), HP1/2 (integrin α_4 ; lanes 5 and 7), mAb16 (integrin α_5 ; lanes 6 and 7), and mAb13 (integrin β_1 ; lane 8) were tested for their ability to block mOKT3 + pFN-induced pp125^{FAK} tyrosine phosphorylation. In each condition, 10 μ g/ml (final concentration) of blocking mAb was added to each well of a mOKT3 + pFN-coated six-well tissue culture plate before the addition of 2×10^6 Jurkat cells per well. hFAK1 immunoprecipitates of each condition were analyzed by Western blotting as described above (Fig. 3 A). (Bottom) pp125^{FAK} tyrosine phosphorylation expressed relative to the value obtained with combined mOKT3 and pFN stimulation in the absence of blocking mAbs. Bars represent mean and SD of three independent experiments. (C) The integrin $\alpha_4\beta_1$, not $\alpha_5\beta_1$, is involved in VCAM-1-dependent tyrosine phosphorylation of T cell pp125^{FAK}. (Top) The mAbs (HLA class-I mAb; lane 4), HP1/2 (integrin α_4 ; lane 5), mAb16 (integrin α_5 ; lane 6), and mAb13 (integrin β_1 ; lane 7) were tested for their ability to block hOKT3 + VCAM-1-induced pp125^{FAK} tyrosine phosphorylation. Blocking studies were performed and analyzed as described above (Fig. 3 A) except that, before washing and addition of blocking mAbs, plates were incubated for 30 min with 10 μ g/ml of human immunoglobulin to bind unbound goat anti-human Ig Fc. (Bottom)

pp125^{FAK} tyrosine phosphorylation expressed relative to the value obtained with combined hOKT3 and VCAM-1 stimulation in the absence of blocking mAbs. Bars represent the mean and SD of three independent experiments.

pp125^{FAK} Immunoprecipitate-associated pp57. The role of pp125^{FAK} in T cell signaling is not yet clear. Consequently, *in vitro* kinase assays were performed to identify pp125^{FAK}-associated kinase substrates that may be members of known signaling pathways. Two specific bands, ~ 116 and 57 kDa, were identified as kinase substrates in both 2A7 and hFAK1 immunoprecipitates from Jurkat cells incubated on plates

coated with mOKT3 and pFN and 2A7 immunoprecipitates from tissue culture flask-adherent 3T3 cells (Fig. 4 A, lanes 1, 2, and 5, and data not shown). Presumably, the 116-kDa band represents phosphorylated pp125^{FAK}. As pp125^{FAK} has been reported to associate with both pp60^{src} and pp59^{lyn}, a principal tyrosine kinase in T cells, experiments were performed to determine if pp59^{lyn} associates with pp125^{FAK} in

Table 1. Effects of PMA on Jurkat Cell Adhesion to pFN and Tyrosine Phosphorylation of pp125^{FAK}

	Relative FAK tyrosine phosphorylation*	Percentage cells adherent [‡]
pFN	0.15 (±0.1)	1 (±1.4)
pFN + PMA	0.11 (±0.06)	43 (±9.8)

In all experiments, PMA was added at 10 ng/ml final concentration to the medium, mOKT3 and pFN were both coated at 1 µg/ml, and Jurkat cells were stimulated for 15 min at 37°C.

*Tyrosine phosphorylation of pp125^{FAK} was determined by Western blots of hFAK1 immunoprecipitates as described. The mean and SD of three independent experiments are shown and expressed relative to FAK tyrosine phosphorylation in the condition pFN + hOKT3 = 1.

‡Adhesion results are expressed as the mean and SD of two independent experiments.

Jurkat cells and represents the lower pp57 band observed in *in vitro* kinase studies (13). Jurkat cells activated with mOKT3 and pFN and immunoprecipitated with one of three different pp59^{Fyn} antibodies and one pp56^{Lck} antibody revealed no

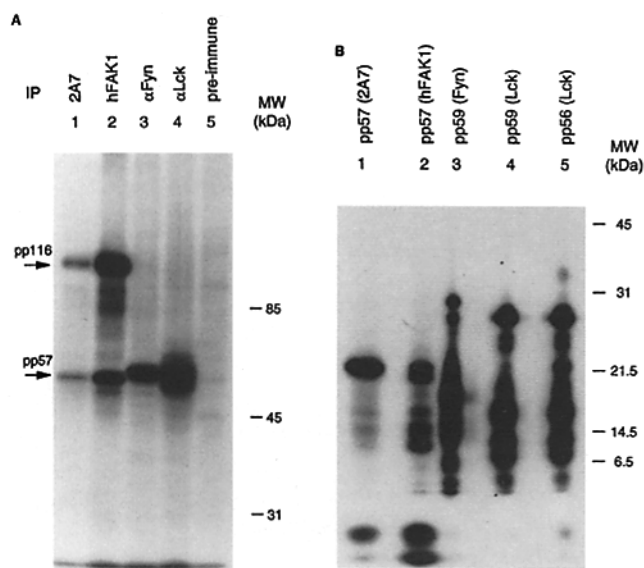


Figure 4. A phosphorylated 57-kD protein (pp57), distinct from pp59^{Fyn} and pp56^{Lck}, is detected in human T cell pp125^{FAK}-specific immunoprecipitates. (A) *In vitro* kinase assay of lysates from Jurkat T cells stimulated for 15 min with a combination of pFN (1 µg/ml) and mOKT3 (1 µg/ml) and immunoprecipitated with either the pp125^{FAK}-specific mAb 2A7 (lane 1), the human pp125^{FAK}-specific polyclonal serum hFAK1 (lane 2), a Fyn-specific antiserum (lane 3), a Lck-specific antiserum (lane 4), or control preimmune serum (lane 5). The specific immunoprecipitates were subjected to *in vitro* kinasing with [³²P]ATP and resolved by SDS-PAGE, followed by autoradiography. Arrows indicate the anti-pp125^{FAK}-specific pp116 and pp57 bands. (B) V8 protease analysis of phosphoproteins identified from *in vitro* kinase reactions depicted in Fig. 4 A. The specific bands analyzed were the 57-kD-specific band from 2A7 (pp57[2A7]) and hFAK1 (pp57 [hFAK1]) immunoprecipitates (lane 1 and 2, respectively), the 59-kD-specific band from anti-Fyn immunoprecipitates (pp59 [Fyn]) (lane 3) and the 59- and 56-kD-specific bands from Lck immunoprecipitates (pp59 [Lck] and pp56 [Lck]) (lanes 4 and 5, respectively). Specific bands were cut out and subjected to V8 protease digestion, followed by resolution by SDS-PAGE and autoradiography.

coprecipitating pp125^{FAK} protein by Western blot analysis with the anti-pp125^{FAK} antibody BC3 (data not shown). This failure of Western blot analysis to reveal an association between pp125^{FAK} and pp59^{Fyn} does not preclude the possibility of the pp57 band representing pp59^{Fyn}, because of the high specific activity of labeling in the *in vitro* kinase assays. Therefore, V8 protease digests of *in vitro* kinase-labeled pp59^{Fyn} and pp56^{Lck} proteins were compared with similar digests of pp57 to look for similarities (Fig. 4, A and B). Jurkat T cells were stimulated for 10 min with the combination of mOKT3 and pFN, lysed, and immunoprecipitated with 2A7, hFAK1, anti-pp59^{Fyn} antisera, anti-pp56^{Lck} antisera, anti-pp60^{src} antisera, and anti-pp56^{Lck} mAb. The specific immunoprecipitates were used as substrates in *in vitro* kinase reactions and run out on SDS-PAGE. The pp60^{src} and pp56^{Lck} antibodies failed to precipitate kinase activity from the Jurkat lysate (data not shown). In contrast, strongly labeled specific bands, which approximated but were not identical in molecular mass to the pp125^{FAK}-specific pp57 band, were obtained from the kinase-labeled pp59^{Fyn} and pp56^{Lck} immunoprecipitates (Fig. 4 A, lanes 3 and 4). The pp57 band in the 2A7 and hFAK1 immunoprecipitates and the phosphorylated band of 59 kD (pp59) in the pp59^{Fyn} immunoprecipitate were cut out of the gel and used for V8 protease digest analysis. The pp56^{Lck} immunoprecipitate showed two major phosphorylated bands at 59 (pp59) and 56 kD (pp56), which were both cut out and subjected to V8 protease digestion. The V8 protease digest analysis revealed that, whereas the phosphopeptide map of pp57 from the 2A7 immunoprecipitates was almost identical to that of hFAK1-derived pp57 (Fig. 4 B, lanes 1 and 2), it was clearly distinct from the pp59 (Fyn), pp59 (Lck), and pp56 (Lck) maps (Fig. 4 B, lanes 3–5). These results indicate that T cell pp57 represents neither pp59^{Fyn} nor pp56^{Lck} protein tyrosine kinase.

In vitro kinase-labeled pp116 (pp125^{FAK}) and pp57 derived from both 2A7 and hFAK1 immunoprecipitates were analyzed to determine the specific phosphoamino acid content. Phosphoamino acid analysis indicated that pp116 (pp125^{FAK}) and pp57 were, in both cases, almost exclusively tyrosine phosphorylated (data not shown).

pp125^{FAK} Tyrosine Phosphorylation and pp57 in Normal Human Peripheral Blood-derived CD4⁺ T Cells. To ascertain whether the findings on pp125^{FAK} regulation derived from studies with the Jurkat cell line could be extended to normal CD4⁺ T cells, we performed a set of experiments on resting human peripheral blood-derived CD4⁺ T cell blasts. Fig. 5, A and B, shows the results of the effect of various stimulatory conditions on pp125^{FAK} tyrosine phosphorylation in normal resting CD4⁺ T cell blasts. The data confirm the results found in the Jurkat T cell line, namely, optimal β₁ integrin ligand (pFN and VCAM-1)-induced pp125^{FAK} tyrosine phosphorylation requires a TCR-CD3-mediated costimulus.

pp57 was also found in normal peripheral blood-derived CD4⁺ T cell blasts. *In vitro* kinase assays on hFAK1 immunoprecipitates from hOKT3 plus pFN-stimulated CD4⁺ T cell blasts show the pp116 and pp57 bands observed in similarly stimulated Jurkat T cells (data not shown).

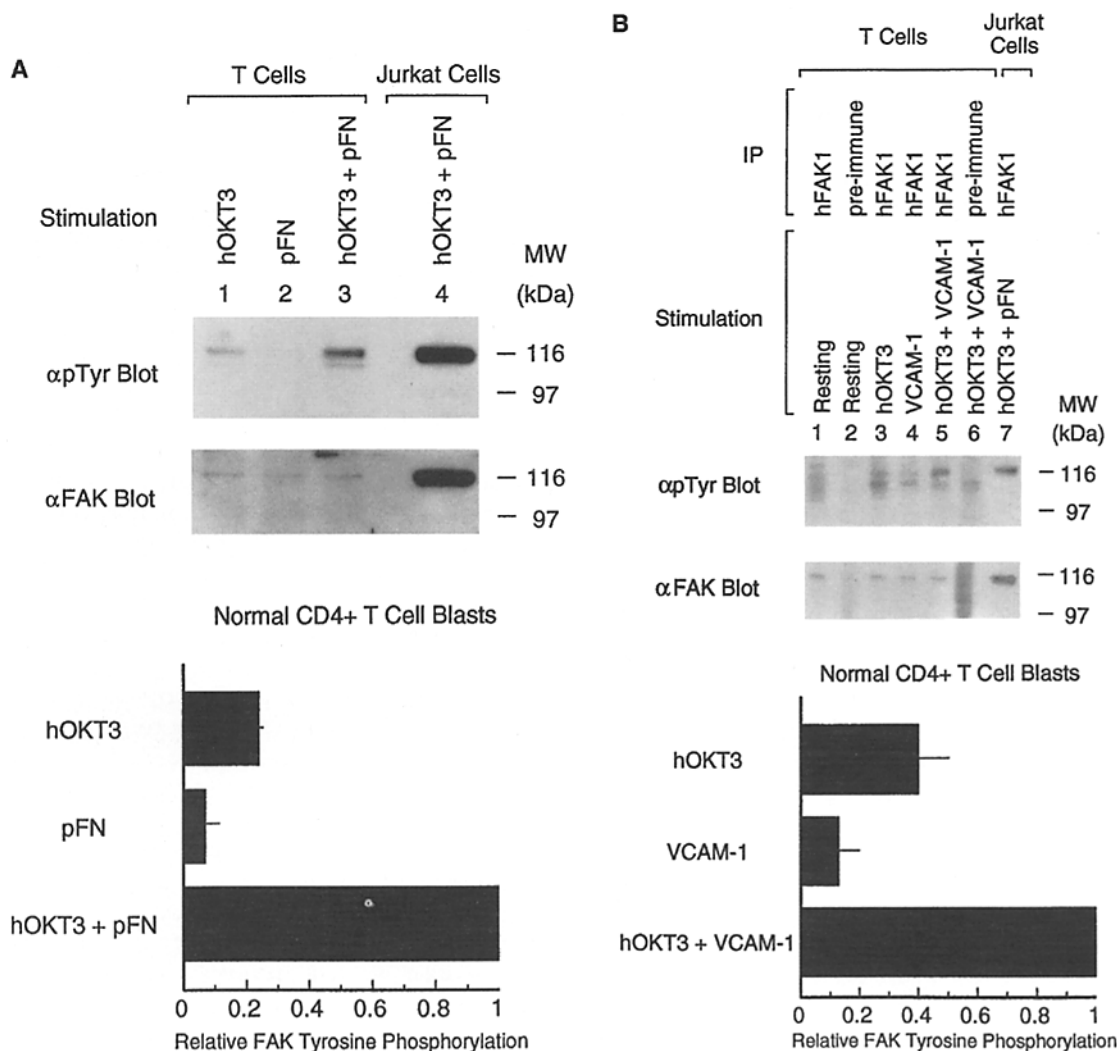


Figure 5. A TCR-mediated signal synergizes with both pFN- and VCAM-1-dependent signals in the induction of tyrosine phosphorylation of pp125^{FAK} in human peripheral blood-derived CD4⁺ T cell blasts. (A) (Top) Western blot analysis of hFAK1-immunoprecipitated lysates derived from 90×10^6 CD4⁺ T cell blasts (lanes 1–3) and 12×10^6 Jurkat T cells (control; lane 4) stimulated for 15 min with either hOKT3 (0.1 μ g/ml; lane 1), pFN (1 μ g/ml; lane 2), or a combination of the two (lanes 3 and 4). (Bottom) pp125^{FAK} tyrosine phosphorylation expressed relative to the value obtained with combined hOKT3 and pFN stimulation. Bars represent the mean and SD of two independent experiments. (B) (Top) 115×10^6 CD4⁺ T cell blasts (lanes 1–6) were unstimulated (lanes 1 and 2) or stimulated for 15 min with either hOKT3 (0.1 μ g/ml; lane 3), VCAM-1 (0.3 μ g/ml; lane 4), or a combination of the two (lanes 5 and 6), immunoprecipitated with hFAK1 (lanes 1, 3, 4, 5, and 7) or preimmune serum (lanes 2 and 6), and subjected to Western blot analysis. Jurkat T cells (control; lane 7) were stimulated with hOKT3 + pFN. (Bottom) pp125^{FAK} tyrosine phosphorylation expressed relative to the value obtained with combined hOKT3 and VCAM-1 stimulation. Bars represent the mean and SD of three independent experiments. For both A and B, immunoprecipitated lysates were subjected to Western blot analysis with the antiphosphotyrosine mAb 4G10 (α pTyr blot) followed by the anti-pp125^{FAK} antisera BC3 (α FAK blot) as described in Materials and Methods.

Discussion

Work with purified FN and VCAM ligands has demonstrated that β_1 integrins can provide costimulatory signals for human T cells (for review see reference 19). However, the mechanism by which these β_1 integrin-mediated signals are transduced in T cells is poorly understood. In non-lymphoid cells, signaling through β_1 integrins was recently shown to induce tyrosine phosphorylation and increased enzyme activity of the tyrosine kinase pp125^{FAK} (51, 52). Tyrosine-phosphorylated pp125^{FAK} is reported to associate with a number of established signal-transducing molecules, including the Src family tyrosine kinases pp60^{src} and pp59^{lck},

phosphatidylinositol 3-kinase, and the adaptor protein Grb2 (3, 15, 16, 53).

We present here, for the first time, data demonstrating the involvement of pp125^{FAK} in costimulatory signal transduction through β_1 integrins in human T cells. We also provide the first description of integrin $\alpha_4\beta_1$ -mediated regulation of pp125^{FAK} tyrosine phosphorylation. Our results show that engagement of T cell β_1 integrin by natural ligand (pFN or VCAM-1) results in pp125^{FAK} tyrosine phosphorylation. In addition, we demonstrate that, in contrast to naturally adherent cells, a synergistic effect on pp125^{FAK} tyrosine phos-

phorylation can be achieved when a costimulus, specifically TCR-CD3 cross-linking, is provided concomitantly with β_1 integrin engagement. Lastly, we describe a tyrosine-phosphorylated 57-kD protein (pp57) present in pp125^{FAK}-specific immunoprecipitates.

Resting primary T cells express $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrin receptors that do not effectively bind their ligands, as determined by the inability of these integrins to promote adhesion of T cells to appropriate ligand-bearing substrates (26, 47). β_1 integrin-mediated adhesion can, however, be achieved in T cells after CD3-TCR cross-linking, PMA treatment, or stimulation with certain chemokines (47, 54, 55). Consequently, although we found that incubation of T cells with either immobilized pFN or VCAM-1 could induce tyrosine phosphorylation of pp125^{FAK}, we reasoned that the magnitude of this induction would significantly increase if the β_1 integrin ligands were coimmobilized with OKT3 mAb. Our results demonstrate that, in both Jurkat cells and human CD4⁺ T cell blasts simultaneous β_1 integrin-mediated binding to pFN or VCAM-1 and triggering of the TCR-CD3 complex does indeed result in synergistic tyrosine phosphorylation of pp125^{FAK}. However, we also found that incubation of T cells with immobilized OKT3 alone induces low but significant levels of pp125^{FAK} tyrosine phosphorylation. This finding suggests the possibility that the costimulatory effect of OKT3 on pFN- or VCAM-1-induced pp125^{FAK} tyrosine phosphorylation may not be due solely to TCR-mediated induction of β_1 integrin binding. In this regard, it is interesting to note that tyrosine phosphorylation of pp125^{FAK} in 3T3 cells is reported to occur not only as a result of integrin engagement, but also after treatment with growth hormone (PDGF) (10) and neuropeptides (bombesin, vasopressin, and endothelin) (11, 12). In addition, we observed that, although treatment of Jurkat cells with PMA results in increased cell adhesion to pFN, it does not lead to increased pFN-induced tyrosine phosphorylation of pp125^{FAK} (Table 1). Faull et al. recently reported that, although both PMA treatment and TCR-CD3 cross-linking induced increased adhesion of T cell lines to immobilized FN through $\alpha_5\beta_1$, only TCR-CD3-mediated activation induced high-affinity integrin receptors as defined by the ability of these receptors to bind soluble FN (56). Taken together, this report and our data suggest the possibility that, in T cells, only high-affinity β_1 integrin binding is capable of inducing tyrosine phosphorylation of pp125^{FAK}. An alternative (or additional) explanation for the synergistic action of TCR-CD3 complex- and β_1 integrin-mediated signals on pp125^{FAK} tyrosine phosphorylation may derive from the data of Cobb et al. characterizing the association between tyrosine-phosphorylated pp125^{FAK} and the tyrosine kinases pp60^{src} and pp59^{lyn} in chick embryo cells (13, 57). A model consistent with these data is that TCR-CD3 complex triggering results in activation and colocalization of pp59^{lyn} (or another Src-family kinase member), with pp125^{FAK} leading to association of the two kinases and either (a) direct pp59^{lyn}-mediated tyrosine phosphorylation of pp125^{FAK} and/or (b) maintenance of pp125^{FAK} tyrosine phosphorylation as a result of pp59^{lyn} SH2 domain binding,

rendering FAK resistant to the action of endogenous tyrosine phosphatases. Although we have not been able to demonstrate an association between pp125^{FAK} and pp59^{lyn} in Jurkat cells, it is possible that such an association does exist but is below our level of detection. Further detailed studies on this complex and important issue are in progress.

Brugge and co-workers showed that $\alpha_{IIb}\beta_3$ -mediated tyrosine phosphorylation of pp125^{FAK} in platelets requires not only binding to fibrinogen but also ADP as cofactor (58). Interestingly, $\alpha_2\beta_1$ -mediated tyrosine phosphorylation of pp125^{FAK} in platelets binding to collagen did not require such a costimulus. Synergistic pp125^{FAK} tyrosine phosphorylation has also been reported after FN adherence and Fc ϵ RI aggregation of a rat basophilic leukemia cell line (59). As discussed above, our results show that, in T cells, β_1 integrin-mediated coupling to the pp125^{FAK} pathway requires costimuli for optimal tyrosine phosphorylation of pp125^{FAK}. Together, these data and our findings suggest the existence of critical differences in requirements for integrin-mediated pp125^{FAK} tyrosine phosphorylation, dependent on both the specific integrin subfamily member and the cell type involved.

pp125^{FAK} tyrosine phosphorylation occurs rapidly and is sustained in Jurkat cells stimulated with coimmobilized OKT3 and pFN. Maximal tyrosine phosphorylation is achieved ~5-15 min after initiation of stimulation and is maintained at high levels for at least 2 h. The rapid induction of T cell pp125^{FAK} tyrosine phosphorylation suggests the possibility that pp125^{FAK}-mediated signaling might play a role in short-lived T cell interactions, such as those occurring when cytotoxic T cells engage their targets or when migrating T cells adhere to endothelium and extravasate into tissue. In contrast, the sustained kinetics of tyrosine phosphorylation might be indicative of a T cell-costimulatory function of pp125^{FAK}, as it appears that prolonged interaction between T cells and costimulatory ligand-bearing APC is required for downstream proliferation to occur.

Experiments with blocking antibodies and specific recombinant ligands demonstrated the direct involvement of β_1 integrins in tyrosine phosphorylation of pp125^{FAK}. Moreover, they confirmed a role for the integrin $\alpha_4\beta_1$ and indicated a previously undescribed participation of $\alpha_4\beta_1$ integrin in pp125^{FAK} regulation. The finding that combined inhibition by the α_4 - and α_5 -specific mAbs was similar to that achieved by the β_1 -specific mAb alone indicates that the $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins can account for all the pFN-induced β_1 integrin-mediated tyrosine phosphorylation of pp125^{FAK} in Jurkat cells. Blocking experiments with specific mAbs in Jurkat cells stimulated with coimmobilized VCAM-1 and OKT3 mAb indicated an exclusive role for the integrin $\alpha_4\beta_1$ in the increased VCAM-1-mediated tyrosine phosphorylation of pp125^{FAK}.

In vitro kinase experiments and phosphoamino acid analyses identified a single, specific 57-kD tyrosine phosphoprotein (pp57) associated with pp125^{FAK} immunoprecipitates from both Jurkat cells and CD4⁺ T blasts. In adherent cells, pp125^{FAK} has been reported to associate with several proteins that may be tyrosine phosphorylated in

vitro, including members of the Src family of protein tyrosine kinases (pp60^{src}, pp59^{lyn}, and pp50^{csk}) and the cytoskeletal protein paxillin (8, 13, 15, 16). Obvious candidates for pp57, based on their molecular mass, ability to be phosphorylated or autophosphorylate on tyrosine, and their prominent roles in T cell signaling, were the Src family members pp59^{lyn} and pp56^{lck}. However, V8 protease digest analyses indicated that both pp59^{lyn} and pp56^{lck} could be eliminated as likely pp57 candidates. We were unable to precipitate kinase activity from Jurkat lysates with either pp60^{src}- or pp56^{lyn}-specific antibodies. Based on its molecular mass, pp57 is not likely to represent pp50^{csk} or paxillin. A phosphorylated species of similar molecular mass to pp57 has been identified in pp125^{FAK} immunoprecipitates from thrombin-treated platelets incubated with [γ -³²P]ATP (51). The presence of pp57 in T cells as well as non-T cells suggests that pp57 may be a component of the Fak signaling pathway common to many cell types.

We describe findings on the synergistic involvement of

the TCR-CD3 complex and the integrins $\alpha_4\beta_1$ and $\alpha_5\beta_1$ in regulation of human T cell pp125^{FAK} tyrosine phosphorylation. Recently, we have observed a direct correlation between synergistic pp125^{FAK} tyrosine phosphorylation and synergistic mitogen-activated protein tyrosine kinase activation and IL-2 production in Jurkat cells costimulated with OKT3 and either FN or VCAM-1 (data not shown). These results complement earlier findings in Jurkat cells demonstrating that $\alpha_2\beta_1$ integrin stimulation promotes accumulation of GTP-bound Ras (18). Together, these data suggest that adherent cell models linking β_1 integrin-dependent pp125^{FAK} tyrosine phosphorylation to activation of the Ras signaling pathway might also apply to T cells. In conclusion, the results presented in this article indicate a potential role for the pp125^{FAK} signaling pathway in transducing $\alpha_4\beta_1$ - and $\alpha_5\beta_1$ -derived T cell-costimulatory signals and expand the list of regulators of pp125^{FAK} to include the TCR-CD3-signaling complex.

We thank Drs. P. Hochman, K. Yamada, Y. Tanaka, S. Shaw, and J. Bluestone for generously contributing reagents. Drs. M. Clark and B. Cobb were very helpful in providing expert advice. Special thanks to Dr. Reidy of the Oligopeptide Core Facility of the University of Chicago for supplying synthetic peptides.

This work was supported by National Institutes of Health grant A-34541-01 to G. A. van Seventer. Additional support was provided by grants from the Louis Block Fund and University of Chicago Cancer Research Center grant CA-14599-20.

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Received for publication 9 February 1995 and in revised form 22 June 1995.

Note added in proof: After this paper was received for publication, it was reported that $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins mediate induction of pp125^{FAK} tyrosine phosphorylation in the human H9 T cell line (Nojima, Y., K. Tachibana, T. Sato, S. F. Schlossman, and C. Morimoto. 1995. *Cell. Immunol.* 161:8-13).

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