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Lymphocyte antigen receptor activation of a focal adhesion kinase-related tyrosine kinase substrate

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ABSTRACT **One of the earliest responses of T and B lymphocytes to stimulation through their antigen receptors is the activation of protein tyrosine kinases and the tyrosine phosphorylation of multiple cellular substrates. Here we describe a tyrosine kinase substrate, fakB, a putative homologue of the focal adhesion kinase pp125^{FAK}. Tyrosine phosphorylation of fakB was rapidly augmented in human T and B cells following antigen receptor cross-linking with antibody, while pp125^{FAK} was nonresponsive. Costimulation of the T-cell antigen receptor (TCR/CD3) with either the CD2 or CD4 costimulatory receptors induced synergistic fakB tyrosine phosphorylation in normal human T cells. Engagement of TCR/CD3 induced the stable association of fakB with ZAP-70, the TCR/CD3 ζ-chain-associated tyrosine kinase involved in antigen receptor-induced T-cell activation. In addition, preformed complexes of fakB and ZAP-70 were observed in T-cell leukemia lines. Phosphorylation of fakB on serine, threonine, and tyrosine residues was observed both *in vivo* and *in vitro*, where a functional increase of *in vitro* kinase activity was observed following TCR/CD3 stimulation. fakB is thus a focal adhesion kinase-related tyrosine kinase substrate that is differentially regulated from that of pp125^{FAK} and likely plays a role in antigen-induced lymphocyte signaling.**

The identification of the focal adhesion kinase (pp125^{FAK}) was facilitated by the generation of the monoclonal antibody (mAb) 2A7, one of several isolated following immunization of mice with immunoaffinity-purified phosphotyrosine-containing proteins from *src*-transformed chicken embryo fibroblasts (1). cDNAs isolated from a chicken λgt11 library with 2A7 encoded a unique tyrosine kinase structure (2), subsequently identified in mouse (3) and human (4, 5). Activation of pp125^{FAK} has been observed via increased tyrosine phosphorylation and *in vitro* kinase activity (1–3, 6–12). Such activation occurs following stimulation of β_1-integrins (3, 6–8), gpIIbIIIa integrin (9), IgE receptors (10), G-protein-coupled receptors (11), neuropeptide receptors (12), and oncogenic transformation (1, 7, 13) in adherent cells.

In T and B lymphocytes, activation of their antigen receptors by cross-linking with antibody results in the activation of tyrosine kinases and the subsequent tyrosine phosphorylation of intracellular substrates including phospholipase C-γ1, ras-GTPase activating protein (GAP), Vav, and the T-cell antigen receptor (TCR)/CD3 ζ-chain. Tyrosine phosphorylation of intracellular signaling components leads to downstream events such as cytokine expression, clonal expansion, and effector function. In the present study, we have identified a FAK-related protein that is a substrate of tyrosine kinases in T and B lymphocytes following stimulation of their antigen receptors. This protein forms stable complexes with the TCR/CD3-linked tyrosine kinase ZAP-70 in T cells, and it appears to be stimulated differentially from pp125^{FAK}.

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MATERIALS AND METHODS

Cells. T-cell lines (Jurkat, CEM, HPB-ALL, and HUT-78) and B-cell lines (Ramos, Daudi, Raji, and Nalm-6) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics. T-cell blasts were prepared by phytohemagglutinin P expansion of human peripheral blood mononuclear cells for 6 days in culture as described (14).

Peptides, Antibodies, and Reagents. Peptides were synthesized on an Applied Biosystems model 430A synthesizer using Boc/benzyl-based chemistry with the thiol of an added cysteine protected with the ethylcarbamoyl group. After purification by reverse-phase HPLC, this protecting group was removed with base and the peptides were conjugated to maleimido-derivatized ovalbumin. Antiserum to ZAP-70 was prepared using ovalbumin conjugated to a synthetic 19-amino acid peptide (residues 289–307) derived from the human ZAP-70 sequence (15). The corresponding peptide blocked both immunoprecipitation and immunoblotting activity of the antisera to ZAP-70. Unconjugated and biotinylated anti-CD3 mAb (G19-4), biotinylated anti-CD4 mAb (G17-2), and biotinylated anti-CD2 mAb (9.6) (14, 16) were used for receptor cross-linking. For biotinylated reagents, cells were incubated with mAbs for 10 min, followed by ligation with avidin (5 μg/μg of mAb) for 1 min prior to cell lysis. mAbs 2A7 (anti-pp125^{FAK}) and 2B12 (anti-p120) have been described (1). Antiserum to TCR/CD3 ζ-chain was kindly provided by J. Sancho and C. Terhorst (Harvard University), and affinity-purified anti-phosphotyrosine (anti-pTyr) was prepared as described (17). Rabbit F(ab')$_2$ anti-IgM was purchased from Jackson ImmunoResearch.

Immunoprecipitation and Western Immunoblotting. Cells were resuspended to 2×10^7 per ml in growth medium and lysed before or after receptor cross-linking in 0.5 ml of radioimmune precipitation assay (RIPA) detergent buffer (17). Immunoprecipitates were prepared by the addition of 10 μg of mAb or 10 μl of antiserum to lysates for 2 hr at 0°C followed by 50 μl of protein A-Sepharose beads (Pharmacia) for 1 hr. Immune complexes were subjected to SDS/8% PAGE and immunoblotted with antiserum to FAK (714) or affinity-purified rabbit anti-phosphotyrosine (17).

Phosphoamino Acid Analysis. T cells were labeled with [^{32}P]orthophosphate for 3 hr at 1 mCi/ml (1 Ci = 37 GBq), and gel-purified immunoprecipitated proteins were subjected to complete hydrolysis in 5.7 M HCl at 110°C for 1.5 hr followed by two-dimensional phosphoamino acid analysis (17).

***In Vitro* Kinase Assay.** Jurkat T cells (2×10^7) were lysed or stimulated with anti-CD3 mAb G19-4 at 10 μg/ml for 3 min and then lysed as described above. Immunoprecipitates of fakB and ZAP-70 were prepared, washed, and incubated for 5 min in 20 mM Pipes, pH 7.2/5 mM $MgCl_2$/5 mM $MnCl_2$/10

Abbreviations: FAK, focal adhesion kinase; GAP, GTPase activating protein; TCR, T-cell antigen receptor; BCR, B-cell antigen receptor; mAb, monoclonal antibody; SH, *src* homology.

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μCi of [γ-^{32}P]ATP. Immunoprecipitates were subjected to SDS/8% PAGE and analyzed by autoradiography. Individual bands were identified, isolated from the gel, and quantitated by scintillation spectroscopy; data are expressed as total cpm.

RESULTS AND DISCUSSION

By immunization of rabbits with peptide–ovalbumin conjugates using sequences derived from the unique carboxyl-terminal region of pp125^{FAK}, antisera were generated against both pp125^{FAK} and a slightly smaller phosphoprotein we denote fakB (Fig. 1*A*). Detection of fakB by immunoprecipitation with antiserum 693 (peptide 2) or antiserum 714 (peptide 1) was observed by immunoblotting with anti-pTyr (Fig. 1*A*, *Upper*) or by immunoblotting with antiserum 714, which apparently detects both pp125^{FAK} and fakB (Fig. 1 *A Lower* and *B*). Antiserum to the carboxyl-terminal 17 amino acids (peptide 3) of pp125^{FAK} (antiserum 713) detected only the slower migrating pp125^{FAK} isoform by immunoprecipitation followed by immunoblotting with anti-pTyr, antiserum 714 (Fig. 1*A*), or antiserum 713 (unpublished observations). Neither FAK isoform was detected with preimmune serum or with immune serum incubated with the immunizing peptides. Clearly, the fakB protein shares homology with pp125^{FAK} in two unique epitope sequences derived from pp125^{FAK} via antiserum cross-reactivity, and both phosphoproteins were constitutively phosphorylated on serine, threonine, and tyrosine residues in T cells (Fig. 1*B*).

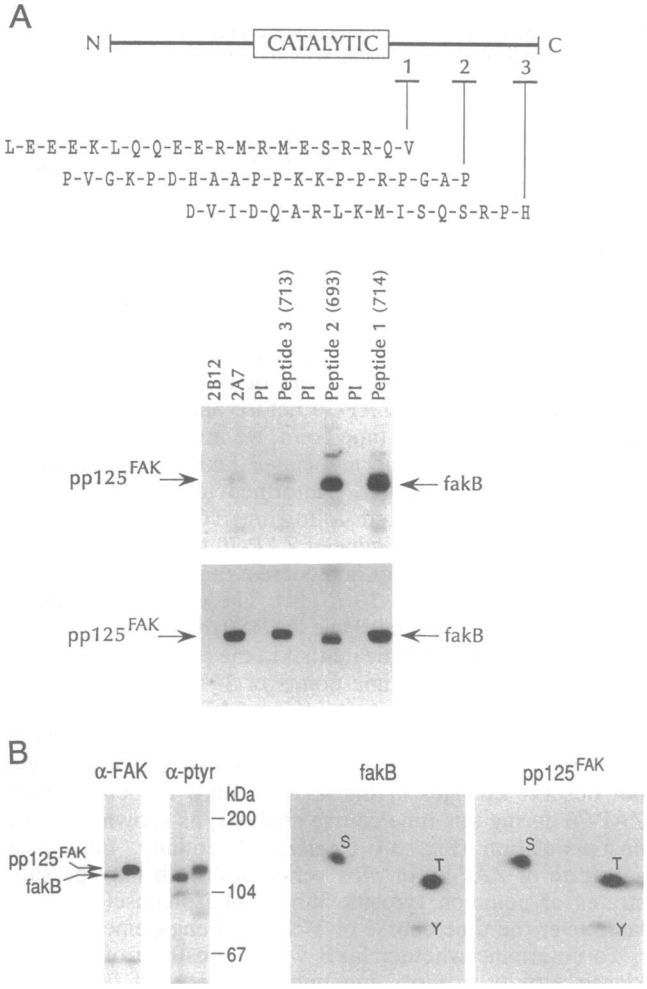

FIG. 1. fakB expression and basal tyrosine phosphorylation in T cells. (*A*) Synthetic peptides from the FAK sequence (2) used for conjugation to ovalbumin for the generation of rabbit antiserum. Immunoprecipitates of pp125^{FAK} [mAb 2A7 and antiserum 713 (peptide 3)] and fakB [antiserum 693 (peptide 2) and 714 (peptide 1)] from Jurkat T cells were analyzed by Western blot with anti-phosphotyrosine (*Upper*). 2B12, mAb to p120, a pp60^{src} substrate (1). PI, preimmune serum. An identical immunoblot was immunoblotted with anti-peptide serum 714 (*Lower*). (*B*) Immunoprecipitates of fakB (antiserum 693) and pp125^{FAK} (mAb 2A7) were analyzed by Western blot with antiserum 714 (*Left*) or anti-phosphotyrosine (*Right*). Phosphoamino acid analysis of fakB and pp125^{FAK} immunoprecipitated from [^{32}P]orthophosphate-labeled Jurkat T cells. Localization of ninhydrin-stained phosphoamino acid standards is indicated as S (phosphoserine), T (phosphothreonine), and Y (phosphotyrosine).

To address whether tyrosine phosphorylation of pp125^{FAK} or fakB was augmented by activation of the TCR/CD3, we stimulated the Jurkat T-cell line with antibody to TCR/CD3, immunoprecipitated pp125^{FAK} and fakB, and immunoblotted the proteins with anti-pTyr. Augmentation of fakB tyrosine phosphorylation was observed following TCR/CD3 cross-linking, which peaked within 2 min and returned to baseline within 60 min (Fig. 2*A*). In contrast, stimulation of pp125^{FAK} phosphorylation above basal levels was not observed. Analysis of fakB activation in the Ramos B-cell line following B-cell antigen receptor (sIgM, BCR) ligation revealed kinetics identical to those observed in T cells (Fig. 2*A*), without an effect on basal B-cell pp125^{FAK} tyrosine phosphorylation (unpublished observations). No changes in fakB expression levels were observed during antigen receptor cross-linking (Fig. 2*A*). In addition, TCR and BCR stimulation in four TCR/CD3$^+$ T-cell lines and four sIgM$^+$ B-cell lines, respectively, showed potent increases in fakB phosphotyrosine levels (Fig. 2*B*). Longer exposure of the immunoblot filters to autoradiographic film revealed that fakB was constitutively phosphorylated on tyrosine at low levels prior to activation in all T and B cells examined and normal T cells (unpublished observations). The T- and B-cell lines expressed virtually identical levels of fakB (unpublished observations), in contrast to the higher but variable levels of pp125^{FAK} observed in the same cells (5). To investigate whether the costimulatory receptors CD2 and CD4 on T cells could synergize with TCR/CD3 to stimulate fakB in normal human T cells, peripheral blood lymphocytes were expanded in phytohemagglutinin P to generate sufficient numbers of TCR/CD3$^+$CD2$^+$CD4$^+$ T cells, and fakB was immunoprecipitated from cell lysates following TCR/CD3, CD2, and CD4 ligation alone and in combination (Fig. 2*B Right*). Clearly, neither CD2 nor CD4 accessory molecules stimulated fakB activation alone; however, both receptors could synergistically costimulate the TCR/CD3-driven fakB tyrosine phosphorylation signal. As previously observed for TCR/CD3 receptor stimulation, no changes in fakB expression were observed following coligation of multiple receptors on normal T cells (unpublished observations).

We investigated the possibility that fakB was part of the T-cell receptor complex and searched for phosphotyrosine-containing proteins that coimmunoprecipitated with fakB. A 70-kDa protein was identified in fakB immunoprecipitates following TCR/CD3 engagement by pTyr immunoblot analysis (Fig. 3*A*). Recent studies have identified the TCR/CD3 ζ-chain-associated tyrosine kinase ZAP-70 (15), a 70-kDa *src* homology 2 (SH2) domain-containing kinase of the SYK family (18). Analysis of ZAP-70 immunoprecipitates on an anti-pTyr immunoblot revealed a band of 120 kDa that comigrated with fakB (Fig. 3*A Left*). To confirm that the 120-kDa band observed in immunoprecipitates of ZAP-70 was fakB, an identical immunoblot was probed with anti-FAK serum 714 (Fig. 3*A Center*). Conversely, a similar immunoblot was probed with anti-ZAP-70 to confirm the identity of ZAP-70 in fakB immunoprecipitates (Fig. 3*A Right*). ZAP-70 clearly forms a complex with fakB prior to

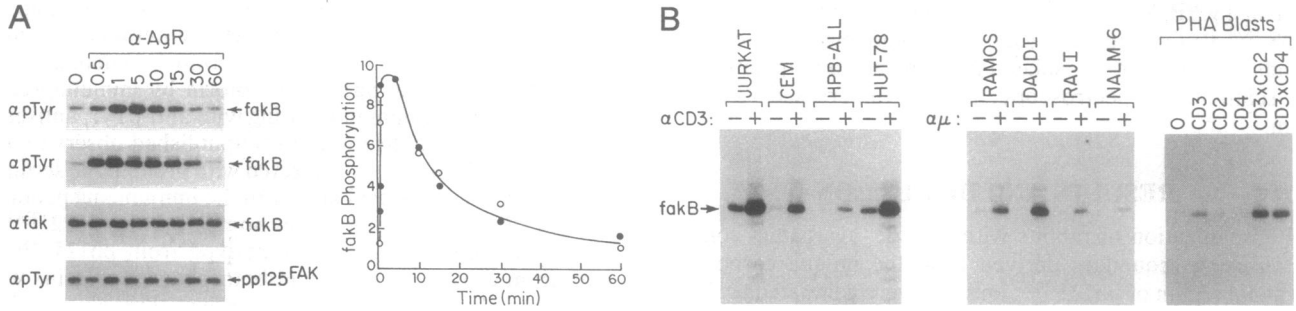

FIG. 2. Activation of fakB through the T- and B-cell antigen receptors (AgR). (*A*) Immunoprecipitates of fakB and pp125^{FAK} were prepared from Jurkat T cells following stimulation with mAb G19-4 (αCD3) or from Ramos B cells stimulated with affinity-purified anti-IgM (αμ) for the time indicated. fakB immunoprecipitates using antiserum 693 were prepared from Jurkat T cells (top panel) or Ramos B cells (second panel) and were immunoblotted with anti-pTyr. fakB immunoprecipitates from Jurkat T cells (third panel) were immunoblotted with antiserum 714. Immunoprecipitates of pp125^{FAK} (mAb 2A7) from Jurkat T cells (bottom panel) were immunoblotted with anti-pTyr. Immunoblot filters were analyzed by PhosphorImager, and the respective volumes of the bands were plotted according to the relative amount of fakB in the immunoprecipitates (●, T cells; ○, B cells). (*B*) Immunoprecipitates of fakB (antiserum 693) from the indicated T- and B-cell lines were prepared before (−) and after (+) stimulation with antigen receptor-specific antibody as described above. T-cell blasts were prepared by phytohemagglutinin (PHA) P expansion of human peripheral blood mononuclear cells. Receptors indicated were ligated with biotinylated mAbs and avidin for 1 min. Immunoprecipitates of fakB (antiserum 693) were immunoblotted with anti-pTyr.

T-cell activation, and complexes of fakB/ZAP-70 were also observed following TCR/CD3 ligation, as observed by the induction of ZAP-70 association with fakB following receptor ligation in immunoblots probed with anti-ZAP-70 (Fig. 3*A Right*). Although fakB and ZAP-70 were constitutively in stable complexes prior to receptor stimulation, as shown by anti-FAK immunoblotting, additional complexes were observed only after receptor ligation, as detected by immunoblotting with ZAP-70 specific antisera. Perhaps the antiserum

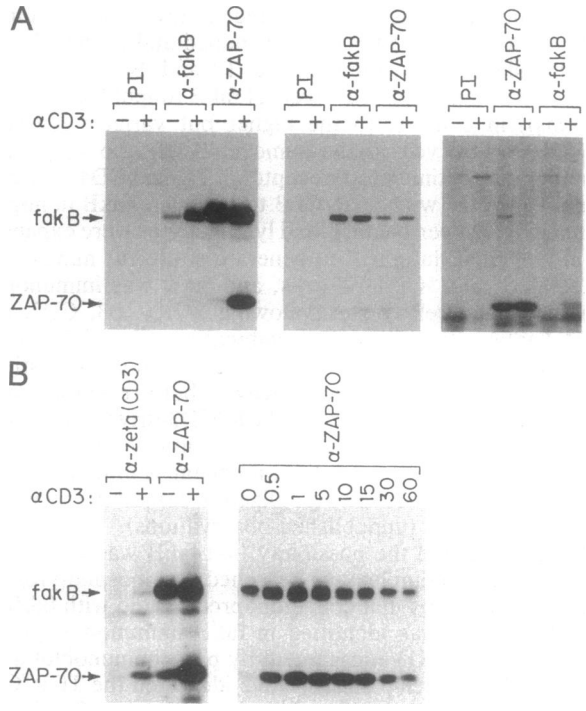

FIG. 3. Association of fakB with ZAP-70. (*A*) Immunoprecipitates of fakB and ZAP-70 from Jurkat T cells before (−) and after (+) stimulation with soluble anti-CD3 mAb G19-4 for 5 min were immunoblotted with anti-pTyr (*Left*), anti-FAK antiserum 714 (*Center*), or antiserum to ZAP-70 (*Right*). PI, preimmune serum. (*B*) Immunoprecipitates of ZAP-70 from unstimulated (−) or anti-CD3-stimulated (+) T cells were compared with immunoprecipitates of TCR/CD3 ζ-chain and analyzed by pTyr immunoblotting (*Left*). Immunoprecipitates of ZAP-70 from T cells following anti-CD3 activation for the indicated time period (minutes) were analyzed by pTyr immunoblotting (*Right*).

to fakB detects a conformationally distinct complex of fakB/ZAP-70 as compared with the complexes detected by antiserum to ZAP-70. Nonetheless, it appears that preformed complexes exist, and inducible complexes were detectable following antigen receptor cross-linking. In addition, complexes of fakB/ZAP-70 bind to the TCR/CD3 ζ-chain only after cross-linking TCR/CD3 (Fig. 3*B Left*), where only a minor fraction of the total pool of complexes was observed associated with the ζ-chain. Although we observed a high level of tyrosine phosphorylation of fakB associated with immunoprecipitates of ZAP-70 before and after TCR/CD3 stimulation at the 5-min time point, we investigated whether increases or decreases in associated fakB phosphorylation status changed kinetically. Preformed fakB/ZAP-70 complexes were coregulated following TCR/CD3 ligation, wherein the kinetics of induced ZAP-70 tyrosine phosphorylation followed increases and decreases of phosphorylation of the bound fakB (Fig. 3*B Right*). These changes in phosphotyrosine content of fakB and ZAP-70 over the time course of TCR/CD3 ligation indicate that in the experiments in Fig. 3 with only individual time points of 3–10 min, apparently similar levels of phosphotyrosine on fakB may be observed before and after TCR/CD3 cross-linking while changes in ZAP-70 phosphorylation levels are observed. Further, we did not observe changes in the level of fakB associated with ZAP-70 during this time course analysis, as shown at 5 min poststimulation (Fig. 3*A Center*). Potentially, fakB is a substrate of ZAP-70 or vice versa, and both may be substrates of common tyrosine kinase/phosphatase(s) stimulated over the time course of TCR/CD3 engagement.

To determine whether fakB was a substrate of tyrosine kinase activity *in vitro*, immunoprecipitates of fakB were assayed in the presence of divalent cations and [γ-^{32}P]ATP and subjected to SDS/PAGE. Detection of a band corresponding to fakB that increased with TCR/CD3 activation was observed in immunoprecipitates from T cells, where a coprecipitated band of 70 kDa corresponding to ZAP-70 increased concomitantly. Quantitation of the incorporation of [γ-^{32}P]ATP into fakB and ZAP-70 is shown in Fig. 4. Both bands contained phosphotyrosine, in addition to phosphoserine and phosphothreonine *in vitro* (unpublished observations). In immunoprecipitates of ZAP-70, ZAP-70 and the associated fakB were augmented in their incorporation of phosphate following T-cell stimulation with anti-CD3 (Fig. 4). In addition, immunoprecipitates of fakB or ZAP-70 coincubated with the exogenous substrate [Val^5]angiotensin II during the *in vitro* kinase reaction did not show additional

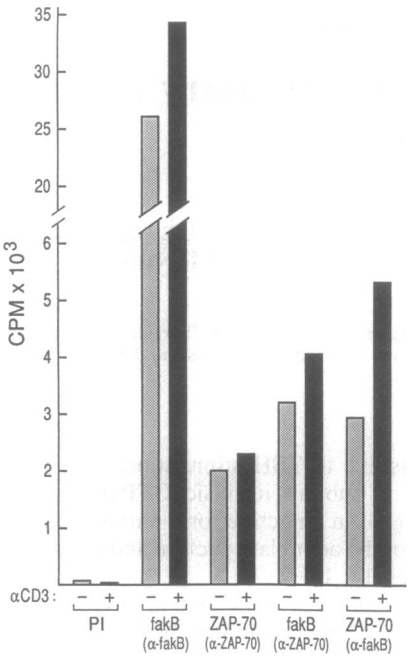

FIG. 4. Stimulation of *in vitro* kinase activity associated with fakB/ZAP-70 complexes. Immunoprecipitates of fakB and ZAP-70 were analyzed separately for kinase activity *in vitro* before (−, stippled bars) and after (+, shaded bars) stimulation of Jurkat T cells with anti-CD3. Antisera used for specific immunoprecipitations are indicated in parentheses below the name of the protein analyzed for incorporation of [γ-^{32}P]ATP. Data are expressed as cpm of the band isolated from an SDS/polyacrylamide gel. fakB coprecipitating with antiserum to ZAP-70 and ZAP-70 coprecipitating with anti-fakB were analyzed in parallel and are indicated as proteins immunoprecipitated with the antiserum denoted in parentheses to the other component in the fakB/ZAP-70 complex. PI, preimmune serum.

differences from those observed for autophosphorylation (unpublished observations). Although only limited increases in phosphate incorporation were observed in these *in vitro* reactions following TCR/CD3 ligation, fakB immunoprecipitates apparently contain tyrosine kinase activity, wherein fakB and ZAP-70 were in preformed complexes. Whether fakB and/or ZAP-70 promoted the phosphorylation activity in trans or in cis remains unknown. The augmented tyrosine phosphorylation of fakB and ZAP-70 following TCR/CD3 engagement observed in anti-pTyr immunoblots compared with the less potent but correlative increases in *in vitro* phosphate incorporation could be due to additional tyrosine kinases not physically associated with fakB/ZAP-70 that induce their hyperphosphorylation *in vivo*.

The FAK-related protein fakB is a putative downstream component of antigen receptor signaling in T and B lymphocytes. Clearly, pp125^{FAK} does not appear to be involved in this signaling pathway but is expressed in T and B cells (5) and may be regulated through alternative receptors. Expression of fakB in fibroblasts (unpublished observations) suggests that it may also be responsive to growth factor receptors, β_1-integrins, and oncogenes. However, the stable association of fakB with the ZAP-70 tyrosine kinase suggests that it likely plays a specialized role in T-cell signaling pathways following antigen receptor stimulation.

The indication that a potentially novel isoform of FAK has a role in antigen receptor-driven signaling in lymphocytes suggests that this family of kinases is linked to a broad spectrum of receptor-induced cascades. Clearly, integrin-linked activation of pp125^{FAK} (3, 6–9, 19–21) suggests that this kinase may phosphorylate cytoskeletal components such as paxillin (8) and potentially alter cellular morphology. Likewise, fakB may play a critical FAK-related role in lymphocytes during cell–cell contact, where reorganization of cytoskeletal elements is observed during antigen presentation to T cells by antigen-presenting cells (22). Alternatively, fakB may differ in its activity from that of pp125^{FAK}, converging with other key elements of the tyrosine kinase cascade such as phospholipase Cγ1, Vav, and ras-GAP. If activation of ZAP-70 is proximal to TCR/CD3 stimulation by antigen, then fakB may be functionally responsive to ZAP-70 tyrosine phosphorylation facilitating its role in the signaling cascade. The unique structure of pp125^{FAK} does not contain SH2 or SH3 domains (2–5), suggesting that these sequences are likely not present in fakB but are clearly important structural features of ZAP-70 (15, 18, 23). Although no interaction of soluble fusion proteins containing ZAP-70 SH2 domains with a 120-kDa phosphoprotein *in vitro* has been observed (23), other elements such as regulatory signaling components or the TCR/CD3 chains (24, 25) may facilitate their interaction. The differential regulation of fakB and pp125^{FAK} during TCR or BCR-driven signaling indicates that in hematopoietic cells, fakB may perform a specific FAK-related function.

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