The Related Adhesion Focal Tyrosine Kinase Is Tyrosine-phosphorylated after β1-Integrin Stimulation in B Cells and Binds to p130cas*

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Integrin ligation initiates intracellular signaling events, among which are the activation of protein tyrosine kinases. The related adhesion focal tyrosine kinase (RAFTK), also known as PYK2 and CAKβ, is a tyrosine kinase that is homologous to the focal adhesion kinase (FAK) p125FAK. The structure of RAFTK is similar to p125FAK in that it lacks a transmembrane region, does not contain Src homology 2 or 3 domains, and has a proline-rich region in its C terminus. Here we report that RAFTK is a target for β1-integrin-mediated tyrosine phosphorylation in both transformed and normal human B cells. Ligation of the B cell antigen receptor also induced tyrosine phosphorylation of RAFTK. Phosphorylation of RAFTK following integrin- or B cell antigen receptor-mediated stimulation was decreased by pretreatment of cells with cytochalasin B, indicating that this process was at least partially cytoskeleton-dependent. One of the tyrosine-phosphorylated substrates after integrin stimulation in fibroblasts is p130cas, which can associate with p125FAK. RAFTK also interacted constitutively with p130cas in B cells, since p130cas was detected in RAFTK immunoprecipitates. Although the function of RAFTK remains unknown, these data suggest that RAFTK may have a significant function in integrin-mediated signaling pathways in B cells.

Integrins are α/β heterodimeric receptors that are involved in cell-cell and cell-matrix interactions. Integrins play a role both in adhesion and in transducing signals involved in a variety of cellular functions. One of the intracellular signaling events initiated by integrins is the activation of tyrosine kinases (1). In many cell types, including fibroblasts, epithelial cells, and hematopoietic cells, there is prominent tyrosine phosphorylation of proteins of 105–130 kDa following integrin cross-linking (2–7). One of these proteins has been identified as the focal adhesion kinase (FAK)1 p125FAK in a large number of different cell types (8–14). It has been proposed that this kinase, which localizes to focal adhesion contacts (9, 10), is involved in linking adhesive events at the cell surface with intracellular pathways required for normal cellular function.

Recently, we and others have reported the identification and cloning of another related focal adhesion tyrosine kinase, called RAFTK (also known as PYK2 and CAKβ) (15–17). This kinase has 65% homology with p125FAK but is clearly distinct and could be distinguished using specific antibodies (18). Similar to p125FAK, RAFTK lacks a transmembrane region and does not contain any SH2 or SH3 domains but does have a proline-rich region in its C terminus. RAFTK is highly expressed in hematopoietic cells and coexpressed with p125FAK in megakaryocytes and B lymphocytes. It has also been reported that stimulation of platelets and megakaryocytes with thrombin induces the tyrosine phosphorylation of RAFTK, suggesting that it plays an important role in platelet signal transduction (15).

We have previously reported that integrin-mediated tyrosine phosphorylation of the 105–130-kDa substrates in B cells can occur in the absence of detectable p125FAK in certain B cell lines (SB cells) (14). In this report we demonstrate that RAFTK was tyrosine-phosphorylated after β1-integrin stimulation in normal and transformed human B cells. RAFTK was also tyrosine-phosphorylated in normal B cells following B cell antigen receptor (BCR) cross-linking. Furthermore, p130cas, one of the tyrosine-phosphorylated substrates following integrin ligation (19, 20), bound constitutively to RAFTK. These results suggest a potential role for RAFTK in integrin and BCR signaling pathways in B cells.

**EXPERIMENTAL PROCEDURES**

Antibodies—Monoclonal antibodies directed against CD29/β1-integrin (K20, IgG2a), and CD18 (10F12) were obtained from ascites. Antibodies directed against CD29/β1-integrin (K20, IgG2a) was provided by Dr. Brian Druker (Oregon Health Sciences Center). Affinity-purified Rabbit anti-mouse (RaM) was obtained from Jackson Laboratories (West Grove, PA). Anti-human IgG and IgM was obtained from Jackson Laboratories. Polyclonal antibodies directed against RAFTK were prepared as described previously (15). Anti-p130cas monoclonal antibody was obtained from Transduction Laboratories (Lexington, KY).

Normal B Cells and B Cell Lines—The human B cell lines ARH-77, SB, and Nalm-6 were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate, 50 units/ml penicillin, and 50 μg/ml streptomycin. Normal tonsillar B cells were enriched from single cell suspensions of tonsil by immunomagnetic bead depletion of T cells, monocytes, and natural killer cells as described previously (5). Tonsils were obtained according to appropriate Human Protection Committee validation and informed consent. Following immunomagnetic bead treatment, these cells were greater than 95% CD20+, and less than 5% CD3+, CD56+, and CD11b+ when analyzed by indirect immunofluorescence and flow cytometry.

Stimulation of Cells—Cells were washed two times and resuspended in Iscove's modified Dulbecco's media (Life Technologies, Inc.) at 5 × 10^6 cells/ml, starved at 37 °C for 30 min, and then cooled on ice for 15
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RESULTS

\( \beta_1 \) Stimulation of Human B Cells Induces the Tyrosine Phosphorylation of RAFTK—Stimulation of human B cells and B cell lines with mAbs directed against \( \beta_1 \)-integrin induces tyrosine phosphorylation of phosphoproteins ranging from 105 to 130 kDa. Since in certain cell lines such as SB, these substrates could be phosphorylated in the absence of any detectable p125\(^{\text{FAK}} \), we investigated whether RAFTK could be involved in the \( \beta_1 \)-integrin signaling pathway in human B cells. Normal tonsillar B cells or B cell lines (SB and ARH-77) were stimulated with anti-\( \beta_1 \)-integrin or anti-\( \beta_2 \)-integrin mAb followed by RoM for 30 min. Cellular lysates were subjected to immunoprecipitation with anti-RAFTK antibody followed by antiphosphotyrosine immunoblotting. Neither phosphotyrosine-containing proteins nor RAFTK were detected in immunoprecipitates formed using preimmune serum (Fig. 1A, left panel).

As seen in Fig. 1A, an increase in the tyrosine phosphorylation of RAFTK could be specifically observed in the B cell lines following \( \beta_1 \)-integrin stimulation. Stimulation by anti-\( \beta_2 \) integrin antibodies did not induce any significant increase in tyrosine phosphorylation. Stimulation of tonsillar B cells with anti-\( \beta_1 \) mAb as well as anti-IgM and -IgG antibodies induced an increase in the tyrosine phosphorylation of RAFTK (Fig. 1A, right panel). The increase in tyrosine phosphorylation of RAFTK in tonsillar B cells was less than that seen in cell lines, possibly due to an activation of the cells during their purification. The band migrating at approximately 50 kDa in Fig. 1A represents an Ig heavy chain. The membrane was then stripped and reprobed with anti-RAFTK antibody to confirm that equivalent amounts of RAFTK were loaded in each lane (Fig. 1A, lower panels). Depending on the resolution of the gels, RAFTK was seen to migrate either as a single band or as a doublet. To examine this further, we immunoprecipitated RAFTK from three B cell lines (ARH-77, SB, and Nalm-6) and normal tonsillar B cells. As seen in Fig. 1B, left panel, RAFTK was seen to migrate as two distinct bands in different ratios. Furthermore, following \( \beta_1 \) stimulation of Nalm-6 cells, both bands were tyrosine-phosphorylated (Fig. 1B, right panel).
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To determine the time course of the tyrosine phosphorylation of RAFTK, ARH-77 and SB cells were cultured with anti-β1-integrin mAb followed by RaM for increasing periods; RAFTK was then immunoprecipitated and analyzed by antiphosphotyrosine immunoblotting. As shown in Fig. 2A, there was an increase in the tyrosine phosphorylation of RAFTK in a time-dependent manner following specific β1 stimulation, which began after 5 min, increased until 60 min, and declined thereafter. A similar time course of tyrosine phosphorylation was seen following BCR cross-linking in normal tonsillar B cells (Fig. 2B).

Tyrosine Phosphorylation of RAFTK in Normal Tonsillar B Cells Requires an Intact Cytoskeleton following β1-Integrin and BCR Ligation—In B cells, the β1-integrin-induced increase in tyrosine phosphorylation of substrates from 105 to 130 kDa was inhibited by cytochalasin B (14). We thus investigated the cytoskeletal dependence of tyrosine phosphorylation of RAFTK. ARH-77 cells and normal tonsillar B cells were preincubated for 30 min at 37 °C with media alone (–CB) or cytochalasin B (+CB) prior to β1 or BCR stimulation, respectively. As shown in Fig. 3, the increase in RAFTK phosphorylation induced by stimulating cells with anti-β1 or anti-Ig antibodies was reduced by cytochalasin B pretreatment (+CB). These results suggest that the phosphorylation of RAFTK induced by ligation of β1 integrin or BCR was partially dependent on the integrity of the actin cytoskeleton.

RAFTK Associates In Vivo with the crk-associated Protein p130cas—One of the tyrosine-phosphorylated substrates after integrin stimulation is p130cas (19, 20). Since p130cas has been reported to bind to p125FAK (21), we investigated whether p130cas could also associate with RAFTK. Membranes containing immunoprecipitated RAFTK from the B cell line SB, which were either unstimulated or anti-β1-integrin- or anti-β2-integrin-stimulated, were probed with antiphosphotyrosine antibodies (4G10; Fig. 4A), anti-RAFTK antibodies (Fig. 4B), or anti-Cas antibodies (Fig. 4C). RAFTK was tyrosine-phosphorylated after anti-β1 stimulation, as detected by antiphosphotyrosine blotting (Fig. 4A). p130cas could be detected in RAFTK immunoprecipitates (Fig. 4C); however, the association appeared to be constitutive, since no increase was observed after β1 stimulation, despite an increase in the tyrosine phosphorylation of RAFTK after such stimulation. Conversely, membranes containing immunoprecipitated p130cas from SB cells were probed with anti-RAFTK antibodies (Fig. 4B) or with anti-Cas antibodies (Fig. 4C) and demonstrated the presence of RAFTK in the Cas immunoprecipitate. No Cas was detected in the preimmune serum immunoprecipitation of RAFTK. The band migrating at approximately 50 kDa in Fig. 4A represents the Ig heavy chain. A similar in vivo association could also be observed in the B cell lines Nalm-6 and ARH-77 (data not shown).
DISCUSSION

The signal transduction pathways initiated by integrin ligation involve cytoskeletal-dependent activation of tyrosine kinases and phosphorylation of a number of substrates (2–4). One of the kinases involved in integrin signaling is the focal adhesion kinase p125FAK. Tyrosine phosphorylation of p125FAK has been observed in a variety of cell types (8–13, 22), which suggests that this kinase is part of a general signaling pathway for integrin signaling. In previous studies we have observed that certain B cell lines remain responsive to integrin ligation, as determined by tyrosine phosphorylations of various substrates, in the absence of detectable p125FAK (14). This suggests that in these cells other kinases may provide functions similar to p125FAK. In this study we provide evidence in human B cells that a tyrosine kinase that is closely homologous to p125FAK, known as RAFTK (15), also participates in integrin signaling.

Since β1-integrin-induced tyrosine phosphorylation of 105–130-kDa substrates is observed in certain p125FAK-negative B cell lines, we investigated whether RAFTK was involved in this pathway. RAFTK was present in normal mature B cells and all B cell lines examined and was tyrosine-phosphorylated after β1- but not β2-integrin stimulation. However, we did not detect a significant increase in the autophosphorylation kinase activity in RAFTK following β1-integrin or BCR stimulation (not shown). This is somewhat analogous to p125FAK (in which phosphorylation of Tyr-397 is not necessary for kinase activity (23, 24)). Moreover, RAFTK phosphorylation also occurred in the SB and RPMI 8866 (not shown) B cell lines, which do not express detectable p125FAK, indicating that tyrosine phosphorylation of RAFTK may occur independently of p125FAK. We also investigated whether FAK could become tyrosine-phosphorylated in these studies, and no consistent phosphorylation of p125FAK was observed (data not shown). Further support of the nongeneralized involvement of p125FAK is the finding that ligation of integrins on human monocytes and T cell clones can lead to tyrosine phosphorylation of various substrates in the absence of detectable expression of p125FAK or tyrosine phosphorylation of p125FAK respectively (25, 26). Although homologous to p125FAK, stimulation of fibroblasts through β1-integrins did not induce tyrosine phosphorylation of CAKβ (identical to RAFTK; Ref. 17), suggesting that in different cell lineages the function of RAFTK may differ.

Ligation of integrins in association with stimulation of the T cell antigen receptor (TCR) provides a costimulatory signal to T lymphocytes (27). This suggests that there may be common links between these pathways. Following cross-linking of the TCR, p125FAK is tyrosine-phosphorylated. We have observed that stimulation of B cells through cross-linking the BCR induces tyrosine phosphorylation of RAFTK. Furthermore, both integrin- and BCR-induced RAFTK phosphorylation were partially decreased by pretreatment of cells with cytochalasin B, suggesting that RAFTK tyrosine phosphorylation may require the formation of a cytoskeletal complex, which provides a foundation for the compartmentalization and interactions of kinases and substrates. The functional effects of simultaneous ligation of integrin and antigen receptors on T and B cells suggest that there are shared pathways between TCR (or BCR) and β1-integrin signaling and that one of the common components may be the focal adhesion kinases.

The presence of potential SH2 binding motifs and a proline-rich region mediates the association of p125FAK with a number of other proteins. These include tyrosine kinases, including Fyn (28), Src (29), Csk (30), and the p85 subunit of phosphatidylinositol-3 kinase (31), through their SH2 domains. It has also been reported that the SH3 domain of p130Cas binds to the proline-rich region of p125FAK (21). p130Cas is one of the tyrosine-phosphorylated substrates following integrin ligation in a variety of cell types (19, 20). Furthermore, the SH3 binding motifs in p125FAK (APPKPSPR) and RAFTK (PPPKPSR) appear almost identical (15).

We observed an in vivo association between RAFTK and p130Cas. This association was constitutive, since there was no increase in the amount of p130Cas coinmunoprecipitated with RAFTK after β1 stimulation. This association was seen in B cell lines that both expressed or lacked p125FAK, indicating that in these B cells, p130Cas bound to RAFTK independently of p125FAK. The interaction p130Cas and RAFTK is also likely to be mediated through the SH3 domain of p130Cas with the C-terminal proline-rich region of RAFTK, and further studies are currently directed toward identifying the binding site. The p130Cas observed to be associated with RAFTK did not appear to be tyrosine-phosphorylated. p130Cas is tyrosine-phosphorylated in B cells following β1 stimulation, and this suggests that the pool of tyrosine-phosphorylated p130Cas may be distinct from that associated with RAFTK. The precise role, if any, of RAFTK in p130Cas phosphorylation is presently under investigation. The structure of p130Cas suggests that it may act as a docking protein and could function to bring various potential substrates into proximity of RAFTK. The tyrosine phosphorylation of RAFTK may induce such associations, since RAFTK contains a consensus high affinity binding site for the SH2 domains of Src kinases (17). Further studies of the various kinases and proteins associated with RAFTK should provide new insights into integrin signaling events.

The tyrosine kinase PYK2, which is identical to RAFTK, is involved in calcium release and mitogen-activated protein kinase function in neuronal cells (16). In addition, stimulation of megakaryocytes with thrombin leads to tyrosine phosphorylation of RAFTK (15). The evidence that RAFTK is involved in both integrin and BCR stimulation in B cells further supports the potentially broad function of this kinase in signaling pathways. However, RAFTK may serve as a link between these two distinct stimuli. Evidence for this is from studies of ligation of both integrins and BCR, in which there appears to be a functional cross-talk, leading to modulation of normal B cell proliferation and differentiation (32). Future studies will be directed toward understanding the function of RAFTK in integrin and BCR signaling pathways and gaining insight into the association of adhesion with antigen-induced activation of B cells.

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