Related adhesion focal tyrosine kinase (RAFTK), also known as proline-rich tyrosine kinase 2 and cellular adhesion kinase β, has been recently cloned and characterized as a member of the focal adhesion kinase (FAK) subfamily. RAFTK has an overall 48% amino acid homology to p125\textsuperscript{FAK} and contains a kinase domain but lacks a transmembrane region, myristylation sites, and Src homology region 2 and 3 domains. By Northern blot analysis, RAFTK is expressed in myeloid, lymphoid, and megakaryocytic hematopoietic cells. Like p125\textsuperscript{FAK}, we found that RAFTK interacts with the focal adhesion protein paxillin. In the lymphoid cell line BaF3 and the myeloid cell line 32Dcl3, RAFTK coprecipitates with paxillin. Using \textit{in vitro} binding assays, RAFTK and paxillin were shown to bind directly, through a segment of paxillin that required amino acids 100–227 and a domain in the C terminus of RAFTK. \textit{In vitro}, RAFTK could phosphorylate paxillin on tyrosine residues. These results suggest that RAFTK, as well as p125\textsuperscript{FAK}, may be important in phosphotyrosine-signaling events within the focal adhesion.

Related adhesion focal tyrosine kinase (RAFTK\textsuperscript{1}) is a protein tyrosine kinase that is a member of the focal adhesion kinase (FAK) subfamily (1–4). RAFTK was originally cloned from a cDNA library from CMK megakaryocytic cells (1). The cDNA encoding RAFTK is 3.6 kilobases with a translated product of 123 kDa. RAFTK has a “focal adhesion-targeting” domain, which is 52% homologous to the focal adhesion-targeting domain of p125\textsuperscript{FAK}, both of which are located in the C terminus of their respective proteins. RAFTK lacks a transmembrane region, myristylation sites, and SH2 and SH3 domains. These protein tyrosine kinase have highly related kinase domains (amino acids 419–680 in human RAFTK and amino acids 390–650 in human p125\textsuperscript{FAK}), but their N- and C-terminal domains differ (1).

Recently, RAFTK has been suggested to play a role in signal transduction of megakaryocytes and in hematopoietic cells. RAFTK has been shown to be expressed in platelets, CD34\textsuperscript{+} marrow cells, and primary bone marrow megakaryocytes, as well as in some nonhematopoietic cells, including brain cells (1). Although RAFTK and p125\textsuperscript{FAK} are structurally similar, it is not currently known whether their functions are also similar. p125\textsuperscript{FAK} is involved in integrin signaling, phosphorylates cytoskeletal proteins, and is known to associate with proteins at focal adhesions, the specialized structures in which the actin cytoskeleton is connected to transmembrane integrin molecules (4). One of the proteins that directly interacts with p125\textsuperscript{FAK} in the focal adhesions is paxillin (5). Paxillin is a substrate for p125\textsuperscript{FAK} and also serves as a binding site for vinculin, talin, CRK, CRKL, and c-Src (6–8). Paxillin is believed to be an important substrate and binding site for various oncogene products, such as BCR/ABL, v-Crk, and v-Src (9–11).

In preliminary studies, it was determined that, like p125\textsuperscript{FAK}, RAFTK localized in the focal adhesion. Also, RAFTK is expressed in most hematopoietic cells (myeloid, lymphoid, and megakaryocytic). In an effort to compare the function of RAFTK and p125\textsuperscript{FAK}, we have examined the interaction of RAFTK with paxillin in hematopoietic cells. We show that RAFTK can act as a kinase for paxillin.

\textbf{MATERIALS AND METHODS}

\textbf{Cells and Cell Culture—}The murine pre-B cell line BaF3, murine myeloid cell line 32Dcl3, and human DAMI and CMK megakaryocytic cells were maintained in RPMI 1640 medium (Mediatech, Washington, DC), 10% (v/v) WEHI conditioned medium as a source for interleukin 3, and 10% (v/v) fetal calf serum (PAA Laboratories Inc., Newport Beach, CA). Human megakaryocytic Mo7e cells were maintained in Dulbecco’s modified Eagle’s medium, 10 ng/ml granulocyte-macrophage colony-stimulating factor, and 15% (v/v) fetal calf serum. The cells were then incubated at 37 °C with 10% CO\textsubscript{2}. Monkey CV-1, human FS-2, and murine NIH3T3 fibroblast cell lines were maintained in Dulbecco’s modified Eagle’s medium, 10% (v/v) fetal calf serum. The BCR/ABL-expressing cell lines BaF3.p210BCR/ABL\textsuperscript{1} and 32D.p210BCR/ABL\textsuperscript{1} were generated by transfection of the vector containing the p210BCR/ABL\textsuperscript{1} DNA as described previously (12, 13). 32D.p210BCR/ABL\textsuperscript{1}, BaF3.p210BCR/ABL\textsuperscript{1}, and human erythroleukemic K562 cells were maintained in RPMI medium with 10% fetal calf serum; all cells were cultivated at 37 °C with 5% CO\textsubscript{2}.

\textbf{Northern Analysis—}RNA was isolated from various cells by extraction with guanidine isothiocyanate as described (14). Samples of 15 μg of total RNA were subjected to Northern analysis. cDNA for full-length paxillin (9), RAFTK C terminus (15), p125\textsuperscript{FAK} C terminus (16), and glyceraldehyde-3-phosphate dehydrogenase were used as specific probes.

\textbf{Preparation of Cell Lysates, Immunoprecipitations, and Immunoblotting—}Cells were lysed in lysis buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, and 0.42% NaF) containing inhibitors (10 μl of 100 μM phenylmethylsulfonyl fluoride, 10 μl of 100 μM Na\textsubscript{3}VO\textsubscript{4}, 5 μl of aprotinin (Sigma), and 2 μl of 10 mg/ml leupeptin (9). Immunoprecipitations were performed with the antipaxillin (clone SH11, as

\textsuperscript{1} The abbreviations used are: RAFTK, related adhesion focal tyrosine kinase; FAK, focal adhesion kinase; GST, glutathione S-transferase; IL, interleukin; PYK, proline-rich tyrosine kinase; SH2, Src homology region 2; SH3, Src homology region 3; PAGE, polyacrylamide gel electrophoresis.

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\textsuperscript{4} The related adhesion focal tyrosine kinase forms a complex with paxillin in hematopoietic cells.\textsuperscript{*}
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Described below were mouse monoclonal antibody and the anti-RAFTK (rabbit polyclonal antibody) (1). Immunoprecipitates were separated by gradient (6–12%) SDS-PAGE under reducing conditions, electrophoretically transferred to Immobilon™ polyvinylidene difluoride (Millipore, Bedford, MA), and processed for immunoblotting as per established methods using the enhanced chemiluminescence technique (Amersham Corp.) (17). Immunoblots with various antibodies are as shown under "Results." Antibodies used were rabbit polyclonal anti-RAFTK (R4250) antibody (1.5,000), mouse monoclonal antipaxillin (clone 5H11) antibody (1:10,000), mouse monoclonal antivinculin antibody (1:2000, clone VIN-11-c; Sigma), and mouse monoclonal anti-p125FAK antibody (1:1000, clone 77; Transduction Laboratories, Lexington, KY).

Production of Paxillin Monoclonal Antibodies—Mice were immunized by repeated injections of GST-full-length human paxillin fusion protein (amino acids 1–527) emulsified in Freund's complete adjuvant (Sigma) (18). Hybridomas were screened by enzyme-linked immunosorbent assay against the immunizing protein and further by immunoblotting. Positive hybridomas were cloned and isolated by enzyme-linked immunosorbent assay, and high titer ascites were produced by standard techniques (14). Monoclonal antipaxillin antibodies were purified on protein A-Sepharose beads (Pharmacia Biotech Inc.). Five separate antipaxillin antibodies were identified and characterized with respect to their ability to be used for immunoblotting, immunoprecipitations, and cell staining (data not shown). One hybridoma clone (5H11) was used in this study (IgG1 isotype) and was found to specifically identify paxillin in human and murine cells by immunoblot, immunoprecipitation, and cell staining (data not shown).

GST Fusion Protein Production and Precipitations—GST fusion proteins in the vector pGEX-2TK (Pharmacia) containing full-length human paxillin (amino acids 1–527, human sequence), the four tandem LIM domains of paxillin (LIM, amino acids 316–527), N-terminal paxillin (PE7) without the LIM domains (amino acids 1–315), paxillin internal deletions PE8 (amino acids 100–227 deletion mutant, the putative talin binding site), PE9 (amino acids 56–100 deletion mutant, adjacent to the proline-rich region), and PE10 (amino acids 43–60 deletion mutant, proline-rich region) were expressed and isolated as per previous protocols(8).

GST fusion proteins in the vector pGEX-2TK containing N-terminal human paxillin (amino acids 1–426) and C-terminal RAFTK (amino acids 680–1009) were expressed and isolated as per previous protocols (15).

RAFTK, p125FAK, and paxillin as probes. Total RNA was isolated, and Northern analysis was performed as described under "Materials and Methods." Cell line RNA is identified above each lane. Top panel, RAFTK as the probe; second panel, p125FAK as the probe; third panel, paxillin as the probe; bottom panel, glyceraldehyde-3-phosphate dehydrogenase (G3PDH) as a control probe.

RESULTS

Expression of RAFTK in Hematopoietic Cell Lines—To determine the expression of RAFTK in various cell lines, we performed Northern blot analysis. Several hematopoietic, megakaryocytic, and adherent fibroblast cell line RNAs were used to compare the levels of expression of p125FAK, RAFTK, and paxillin. The hematopoietic cell lines used were BaF3, 32Dcl3, and K562. The megakaryocytic cell lines used were CMK, DAMI, and Mo7e. The fibroblast cell lines used were CV-1, FS-2, and NIH3T3. The membrane was probed with RAFTK-, p125FAK-, paxillin-, and glyceraldehyde-3-phosphate dehydrogenase-specific probes sequentially.

Expression of RAFTK was found in megakaryocytic cell lines (Fig. 1). However, in nonhematopoietic cell lines, RAFTK expression was low. In myeloid and lymphoid cell lines 32Dcl3, BaF3, and K562, RAFTK was moderately expressed. Paxillin was expressed in both the hematopoietic and adherent fibroblast cell lines and only minimally in the megakaryocytic cell lines. In contrast, p125FAK was expressed at relatively consistent levels in the fibroblastic and megakaryocytic cell types tested, but at a somewhat lower level in myeloid and lymphoid cell lines.

RAFTK Coprecipitates with the Focal Adhesion Protein Paxillin—It has been previously shown that paxillin coimmunoprecipitates with p125FAK in hematopoietic cells (6). Using the BaF3 cell line, RAFTK was found to coimmunoprecipitate with paxillin in unstimulated, IL-3-stimulated, and p210BCR/ABL-transformed BaF3 cells (Fig. 2, A and B). Identical results were observed when cell lysates were immunoprecipitated with anti-RAFTK and immunoblotted with paxillin. These results indicate constitutive association of RAFTK and paxillin.

Previously, it has been shown that the C terminus of p125FAK binds directly to a region in the N terminus of paxillin (19). Since RAFTK is a member of the FAK family, and constitutive association of RAFTK and paxillin was observed, GST fusion protein precipitations were performed to determine which regions of paxillin would associate with RAFTK and vice versa. Lysates from BaF3 cells transformed by the oncogene BCR/ABL were precipitated with various GST fusion proteins of paxillin (full-length, PE7–PE10), RAFTK (C terminus), and p125FAK (C terminus). The samples were separated by gradient (6–12%) SDS-PAGE as described under "Materials and Meth-
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Fig. 2. RAFTK binds paxillin in hematopoietic cells. A, immunoprecipitates of cell lysates with control antibody against normal rabbit sera (NRS I.P., 20 × 10^6 cells) and immunoprecipitates of cell lysates with anti-RAFTK antibody (RAFTK I.P., 20 × 10^6 cells) were processed as described under “Materials and Methods,” applied to gradient SDS-PAGE (6–12%), and transferred to an Immobilon-P membrane. The membrane was immunoblotted with anti-RAFTK antibody, stripped, and reprobed with antipaxillin (5H11) antibody. B, immunoprecipitates of cell lysates with control antibody against interferon γ (3c11c8; Cntrl I.P., 20 × 10^6 cells) and immunoprecipitates of cell lysates with anti-paxillin (5H11) antibody (Paxillin I.P., 20 × 10^6 cells), were processed as described under “Materials and Methods,” applied to gradient SDS-PAGE (6–12%), and transferred to an Immobilon-P membrane. The membrane was immunoblotted with antipaxillin (5H11) antibody, stripped, and reprobed with anti-RAFTK antibody. The cells used are unstimulated BaF3 cells (BaF3(–)), BaF3-stimulated cells with IL3 (BaF3(+)), and BaF3.p210BCR/ABL cells. Molecular masses are shown in kDa. W.B., Western blot.

RAFTK Binds Directly to Paxillin—Previous groups have shown that the C terminus of p125FAK binds directly to paxillin (5, 7, 16). We determined whether the same would be true for RAFTK. To show direct binding between the C terminus of RAFTK and paxillin, we performed far Western blotting. Lysates of BaF3 cells were immunoprecipitated with the RAFTK rabbit polyclonal antibody (R4250). The samples were then separated on a 7.5% SDS-PAGE gel and subjected to far Western blotting as described under “Materials and Methods.” Fig. 3 shows that the GST protein alone does not bind to RAFTK, nor do the PE8-paxillin-GST fusion protein (putative talin binding region, deletion of amino acids 100–227) or the LIM domains. Furthermore, the results shown in Fig. 3 demonstrate that the full-length paxillin binds directly to RAFTK and supports the location of the binding region in the C terminus of RAFTK. The experiment was also performed in reverse, with paxillin immunoprecipitation and RAFTK far Western blotting, which reconfirmed the direct binding (data not shown).

RAFTK phosphorylates Paxillin in vitro—RAFTK contains a catalytic domain, which could potentially phosphorylate paxillin in a similar fashion as p125FAK. An in vitro phosphorylation experiment using lysates from the megakaryocytic cell line Mo7e was performed, because these cells contain very small amounts of endogenous paxillin. RAFTK immunoprecipitated with the R4250 antibody was incubated in a kinase reaction with various GST fusion proteins of paxillin and was tested for phosphorylation. Full-length paxillin, PE7 (N-terminal region of paxillin without the LIM domains), and the LIM domains were studied. After in vitro phosphorylation of the various paxillin fusion proteins, the samples were resolved on a 10% SDS-PAGE gel, and the results were visualized by autoradiography. The results in Fig. 4 demonstrate that RAFTK phosphorylates the N-terminal half of paxillin and not the LIM domains. In the N-terminal half of paxillin there are six tyrosine residues (at positions 31, 33, 40, 88, 118, and 181; human sequence), which could be potential substrate sites for the RAFTK kinase.
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Fig. 5. RAFTK binds directly to paxillin. Immunoprecipitates using lysates of unstimulated BaF3 cells (BaF3−) or BaF3 cells stimulated with IL-3 (BaF3+/−), or BaF3 cells p210BCR/ABL, cells with anti-RAFTK antibody (I.P., 20 × 10⁶ cells) were processed as described under “Materials and Methods.” GST-paxillin fusion proteins were added, and the kinase reaction was performed as described under “Materials and Methods.” The membranes were processed for a far Western blot as described under “Materials and Methods” with the various GST fusion proteins: GST, GST-paxillin (full-length), and GST-PE8. Molecular masses are shown in kDa.

Fig. 6. In vitro kinase assay with paxillin. Immunoprecipitates of cell lysates of Mo7e with anti-RAFTK antibody (20 × 10⁶ cells) were processed as described under “Materials and Methods.” GST-paxillin fusion proteins were added, and the kinase reaction was performed as described under “Materials and Methods” and applied to 10% SDS-PAGE. The fusion proteins used were GST-paxillin (full-length (F.L.), LIM, and PE7. Bands were visualized using autoradiography. Molecular masses are shown in kDa.

DISCUSSION

The cytoskeleton is essential for many cellular functions, including regulation of cell shape, flexibility, and adhesive properties (20). Part of the cytoskeleton and plasma membrane form a region known as the focal adhesion (21). Focal adhesions are structures that form adherent contacts with the extracellular matrix (21). Extracellular matrix proteins act through receptors known as integrins localized in focal adhesions. The intracellular domains of integrins interact directly with focal adhesion proteins such as tensin and p125FAK (22). Various events, such as exposure to cytokines and growth factors, can modulate the expression and function of adhesion receptors and the formation of focal adhesions (23). Phosphorylation of proteins within focal adhesions is closely associated with changes in the structure of the actin cytoskeleton. Some proteins contained in the focal adhesion include talin, tensin, vinculin, paxillin, p125FAK, and α-actinin (21). p125FAK is a unique tyrosine kinase localized to this region and has been shown to phosphorylate various proteins, on tyrosine, in focal adhesions by several extracellular stimuli (24).

p125FAK has been previously shown to be activated by integrin cross-linking, growth factor stimulation, and oncogenic signaling (25). After phosphorylation, p125FAK is known to associate with several proteins, including paxillin, tensin, Csk, and the Src family kinases (through tyrosine 397 on p125FAK) (26). p125FAK has also been shown to associate with GRB2 in macrophages via tyrosine residue 397 (27). This interaction links p125FAK to the Ras pathway (28). p125FAK may be important in the turnover of focal adhesion contacts during cell migration (29). The tyrosine phosphorylation of p125FAK precedes cell spreading and the formation of focal adhesion contacts in adherent fibroblasts. When cells are detached, p125FAK tyrosine kinase activity is rapidly reduced.

RAFTK is another protein tyrosine kinase recently cloned out of a megakaryocytic library similar to p125FAK. RAFTK was localized to chromosome 8 in humans (1) and, like p125FAK, was shown to become tyrosine-phosphorylated in response to thrombin stimulation (1). Tyrosine residue 402 of RAFTK is similar to that of tyrosine residue 397 of p125FAK (1). This site has the potential to bind the SH2 domains of Src family kinases.

To identify the role of RAFTK in hematopoietic cells, expression of RAFTK was examined in these cells. RNA from hematopoietic (32Dcl3 and BaF3), megakaryocytic (CMK, DAMI, and Mo7e), and adherent (CV-1, FS-2, and NIH3T3) fibroblasts were tested for expression of RAFTK, p125FAK, and paxillin. In the hematopoietic cells, the expression levels of p125FAK and paxillin were high, whereas those of RAFTK were only moderate. Interestingly, in the megakaryocytic cells, expression of RAFTK and p125FAK were high, but the levels of paxillin were extremely low. But in the adherent fibroblasts, the expression of p125FAK was high, levels of paxillin were moderate, and the expression levels of RAFTK were very low.

In hematopoietic cells, p125FAK is known to be expressed, and it associates with one of its major substrates, paxillin (6). Paxillin is a focal adhesion protein known to associate with several tyrosine kinases. Paxillin has also been shown to be important in signaling transduction pathways of hematopoietic cells (8). The results in this study indicate that in addition to p125FAK, RAFTK and paxillin associate with each other in hematopoietic cells. In megakaryocytes, it has been shown that both RAFTK and paxillin colocalize with each other (data not shown). Like p125FAK, the C terminus of RAFTK binds directly to the N terminus of paxillin. RAFTK binds in the region of amino acids 100–227 of paxillin. This is probably a different binding site from that of p125FAK, which binds in the region of amino acids 51–315 (19). p125FAK and RAFTK may bind to paxillin in different regions, because they may tyrosine phosphorylate different residues on paxillin. Their binding locations may give them greater access to the tyrosine residues they phosphorylate, and these data may show that p125FAK and RAFTK phosphorylate different tyrosines on paxillin.

RAFTK (1), cellular adhesion kinase β (3), and proline-rich tyrosine kinase 2 (PYK2) (2) are the same molecules and are cloned via different strategies with interesting findings. The results obtained from studies of RAFTK, cellular adhesion kinase β, and PYK2 may offer insight into the function of the FAK family. For example, the tyrosine phosphorylation state of cellular adhesion kinase β was not reduced on trypsinization or enhanced in response to plating 3Y1 cells onto fibronectin (3). PYK2 was isolated when libraries were screened with the GRB2 adaptor protein (2), and it has been shown that calcium influx in PC12 cells will cause rapid tyrosine phosphorylation of PYK2 (2). Also, carbachol, acting through the nicotinic acetylcholine receptor, and bradykinin stimulation will cause tyrosine phosphorylation of PYK2 in PC12 cells (2). These interactions are thought to play a role in the Ras signaling pathway, very similar to p125FAK (2). Another homologue to p125FAK was identified as fakB, a 120-kDa tyrosine kinase that is localized in focal adhesions of cells (30). Tyrosine phosphorylation of fakB is rapidly augmented in human B and T cells following antigen receptor cross-linking with antibody (30). fakB is a putative downstream component of antigen receptor signaling in both T and B lymphocytes.

The first substrate for RAFTK has been identified in this...
RAFTK and RAFTK, and additional comparison of these signalling pathways activated by phosphorylated (7, 8). These results suggest that there is overlap and possibly redundancy in the signaling pathway activated by p125FAK and RAFTK, and additional comparison of these signaling events is warranted.

Tyrosine phosphorylation of paxillin by a protein kinase can be important in interactions between paxillin and other proteins, especially with SH2 domains. Paxillin was originally identified in Rous sarcoma virus-transformed chick embryo fibroblasts through a monoclonal antibody produced by Glenney and Zokas (11). The paxillin cDNA, recently cloned, encodes for a protein with an interesting structure (9, 31). Paxillin has four tandem LIM domains C-terminal to various binding sites for SH2 and SH3 domains (9). The interactions between paxillin and SH2- and SH3-containing proteins are important in various signal transduction pathways. Several proteins other than p125FAK and RAFTK have been shown to tyrosine phosphorylate paxillin, such as v-Crk, v-Src, p5210BCR/ABL, and v-ABL (6, 7, 10). It has been shown that paxillin binds to the SH2 domains of CRK, CRKL, and Src (7, 8, 10). Thus, one can envision a role of RAFTK, to promote paxillin binding to other proteins, initially phosphorylating paxillin, and thereafter SH2-domain-containing proteins can bind paxillin.

In summary, we have shown that the tyrosine kinase RAFTK associates with and in vitro phosphorylates paxillin in hematopoietic cells. The effect of RAFTK on paxillin may be important in one of multiple signal transduction pathways. In hematopoietic cells, we show that there is constitutive binding between RAFTK and paxillin in response to IL-3 and the oncogene p5210BCR/ABL. This could be a mechanism through which the cell retains its normal homeostasis.

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