Tyrosine Phosphorylation of the Related Adhesion Focal Tyrosine Kinase in Megakaryocytes upon Stem Cell Factor and Phorbol Myristate Acetate Stimulation and Its Association with Paxillin*

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We have characterized signaling pathways involving the related adhesion focal tyrosine kinase (RAFTK, also known as PYK2 or CAK-β) in CMK human megakaryocytic cells. Stem cell factor, which potentiates the growth of megakaryocytes and their progenitors, and phorbol myristate acetate, which causes differentiation of megakaryocytic cell lines, induced the tyrosine phosphorylation of RAFTK but not of focal adhesion kinase. Stimulation of CMK cells with stem cell factor resulted in an increase in the autophosphorylation and kinase activity of RAFTK. Phosphorylation of RAFTK under these conditions was mediated by a protein kinase C-dependent pathway. Cytochalasin D, which disrupts the cytoskeleton, abolished the phosphorylation of RAFTK upon phorbol myristate acetate and stem cell factor stimulation, indicating that RAFTK association with the actin cytoskeleton appears to be critical for its phosphorylation. In addition, we observed an association of RAFTK with paxillin, a 68-kDa cytoskeleton protein. Using in vitro binding assays, RAFTK and paxillin were shown to bind directly through the C-terminal proline-rich domain. Transient overexpression of a dominant-negative mutant of RAFTK inhibited significantly the tyrosine phosphorylation of paxillin upon phorbol myristate acetate stimulation. These observations indicate that RAFTK might play an important role in the phosphorylation of signaling pathways within the focal adhesions and that RAFTK participates in signaling events that link signals from the cell surface to the cytoskeleton. Furthermore, this study suggests that RAFTK might be involved in megakaryocyte proliferation and differentiation.

Protein tyrosine kinases regulate various signal transduction pathways, including those controlling cell growth, differentiation, and activation (1–5). Integrins, cell surface receptors for proteins of the extracellular matrix, are also transducers of cytoplasmic signals (6–8), and activation of these pathways is linked to one or more protein tyrosine kinases (9, 10).

A cytoplasmic tyrosine kinase, focal adhesion kinase (FAK)†, has been identified as one of the cellular proteins that becomes phosphorylated in response to β1 or β2 integrin-mediated cell adhesion (8, 12–17). Supportive in vivo data from FAK-deficient mice revealed reduced cell motility and enhanced focal adhesion contact formation in FAK null cells (18).

Recently, we and others have isolated a cDNA encoding a novel human cytoplasmic tyrosine kinase termed RAFTK (for a related adhesion focal tyrosine kinase) (19), also known as PYK2 or CAK-β (20, 21). The RAFTK cDNA, encoding a polypeptide of 1009 amino acids, has the closest homology (48% identity, 65% similarity) to FAK. Analysis of their deduced amino acid sequences also indicates that RAFTK, like FAK, lacks a transmembrane region, myristylation sites, and SH2 and SH3 domains. In addition, like FAK, the RAFTK C-terminal domain contains a predicted proline-rich stretch of residues. RAFTK was reported to be highly expressed in the central nervous system (19) and is involved in Ca2+-induced regulation of ion channel and mitogen-activated protein kinase functions in PC-12 cells (20). Activation of PYK2 by the inflammatory cytokine tumor necrosis factor α and by stress signals such as UV light and osmotic shock was shown to couple with the c-Jun N-terminal kinase signaling pathway (22). Recently, a role for PYK2 and Src in linking G-protein-coupled receptors with mitogen-activated protein kinase activation was reported (23).

RAFTK expression is abundant in primary bone marrow megakaryocytes and their progeny, blood platelets (19). To address the role of RAFTK in signal transduction pathways in megakaryocytes, experiments were performed using the model CMK megakaryocytic cell line (24). The c-kit receptor and its cognate ligand stem cell factor (SCF) were investigated since they play a critical role in the adhesion, migration, motility, proliferation, and maturation of a number of hematopoietic cells, including megakaryocytes and platelets (25–29). Since SCF effects appear to be modulated by protein kinase C (PKC) (30), we focused on determining whether PKC might mediate the effects of SCF and Ca2+ on RAFTK phosphorylation. In this study, we report that SCF and phorbol myristate acetate (PMA) induced the tyrosine phosphorylation of RAFTK in a PKC-dependent pathway. The integrity of the actin cytoskeleton appears to be essential for RAFTK phosphorylation. Association of RAFTK with the cytoskeletal protein paxillin was observed through its proline rich-domain, and a dominant-negative mutant of RAFTK interfered with the PMA-induced phosphorylation of paxillin.
**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant SCF/KIT ligand and polyclonal anti-KIT antibodies were generously provided by Keith E. Langley and L. Bennett (Amgen Inc., Thousand Oaks, CA). Monoclonal anti-phosphotyrosine antibody (PY-20) and monoclonal anti-paxillin were obtained from ICN (Costa Mesa, CA); monoclonal antibodies anti-p56, anti-Shc, anti-Grb2, anti-FAK, anti-PCR-a, anti-PCR-b, and anti-PCR-c were obtained from Transduction Laboratories (Lexington, KY), Calbiochem (Berkly, CA), staurosporine, BAPTA-AM, calcium ionophore A23187, EGTA, and phorbol 12-myristate 13-acetate were obtained from Calbiochem. Electrophoresis reagents were obtained from Bio-Rad. All other reagents were purchased from Sigma.

**CMK Cells**—The CMK cell line, provided by T. Sato (Chiba University, Japan), was maintained in RPMI 1640 medium with 10% fetal calf serum as described previously (31). The CMK cell line has properties of cells of the megakaryocytic lineage because it can proliferate in response to cytokines and differentiate upon induction with PMA (24, 32). PMA was dissolved in dimethyl sulfoxide and stored at −20 °C until use, when it was diluted in RPMI 1640 medium.

**Antibodies**—Anti-RAFTK antisera was obtained from New Zealand White rabbits immunized with a bacterially expressed fusion protein consisting of glutathione S-transferase (GST) and the C terminus (amino acids 681−1009) of human RAFTK cDNA subcloned into the pGEX-2T expression vector as described (19). The serum (R-4250) inhibiting high titer (1:256,000) was used in subsequent experiments. Preimmune normal rabbit serum was used as a control antibody. No signal was observed when normal rabbit serum was used to immunoprecipitate lysates from unstimulated or SCF- or PMA-stimulated CMK cells (data not shown), indicating that the R-4250 antibody reacted specifically with RAFTK.

**Cell Stimulation, Immunoprecipitation, and Immunoblotting**—The CMK cells were starved overnight in RPMI 1640 medium with 0.5% fetal calf serum. Cells (10^6/ml) were stimulated for 0 to 30 min at room temperature with either SCF (100–500 ng/ml) or PMA (10–100 nM). For BAPTA treatment, CMK cells were pretreated for 30 min at room temperature with 100 μM BAPTA-AM (20 μM) before stimulation with other reagents. The stimulation was terminated by adding ice-cold RPMI 1640 medium containing sodium vanadate followed by centrifugation. The cells were lysed in modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM phenylmethylsulfonil fluoride, 2 μg/ml aprotinin, leupeptin, and pepstatin, and 1 mM Na_3VO_4). Total cell lysates were clarified by centrifugation at 10,000 × g for 10 min. Protein concentrations were determined using a protein assay (Bio-Rad). Identical amounts of protein from each sample were precleared by incubation with protein G-Sepharose CL-4B (Sigma) for 1 h at 4 °C. After the removal of protein G-Sepharose by brief centrifugation, the solution was incubated with different primary antibodies as described below for each experiment for 4 h or overnight at 4 °C. Immunoprecipitation of the antigen-antibody complex was accomplished by incubation for 1 h at 4 °C with protein G-Sepharose. Bound proteins were solubilized in 20 μl of 2 × Laemmli buffer. Samples were separated and analyzed by 7.5% or 10% SDS-PAGE and then transferred to nitrocellulose membranes. The membranes were blocked with 5% bovine serum albumin (Boehringer Mannheim) and probed with primary antibody for 1 h at room temperature. Immunoreactive bands were visualized using horseshad peroxiase-conjugated secondary antibody and the ECL reagents (Amer sham Corp.).

**GST Fusion Protein Production and Binding Studies**—The RAFTK C-terminal proline-rich domain (amino acids 681−1009), the RAFTK N-terminal domain (amino acids 26−286), and the shorter RAFTK C-terminal proline-rich domain (amino acids 700−972) GST fusion protein was expressed and purified by the polymerase chain reaction technique and cloned into the pGEX-2T expression vector (Pharmacia Biotech Inc.) and produced as described previously (19, 33). The GST fusion proteins were purified on a glutathione-Sepharose column by affinity chromatography according to the manufacturer’s recommendations (Pharmacia). For the binding experiments, 1 mg of cell lysate was mixed with 15 μg of GST fusion protein and incubated for 1 h at 4 °C on a rotary shaker. Pulling on glutathione-Sepharose 4B beads (Pharmacia) were added to preabsorb the complex. Following incubation for 3 h at 4 °C on a rotary shaker, the beads were centrifuged and washed three times with modified RIPA buffer. The bound proteins were eluted by boiling in Laemmli sample buffer and subjected to 7.5% SDS-PAGE and Western blot analysis.

**Western Blot Binding Assay (Far-Western)**—Immunoprecipitations were performed using anti-paxillin mouse monoclonal antibody and separated on 7.5% SDS-PAGE gels. The gels were then transferred, and the blots were incubated at 4 °C overnight in 5% dry milk and phosphate-buffered saline plus 0.1% Tween 20. The blots were incubated in GST fusion proteins for 2 h and then washed. Anti-GST mouse monoclonal antibody was added (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. After washing, anti-mouse Ig and horseshad peroxidase (Amersham Corp.) were added for 1 h. The membranes were processed using the ECL technique.

**In Vitro Kinase and Autophosphorylation Assays**—The immunoprecipitated complexes obtained by immunoprecipitating cell lysates with RAFTK antisera were washed twice with RIPA buffer and once in kinase buffer. The immune complex was then incubated in kinase buffer containing 25 μg of poly(GluTyr) (4:1, 20–50 kDa, Sigma) and 5 μCi of [γ-32P]ATP at room temperature for 30 min as described (33). For the autophosphorylation assay, the immune complexes were incubated in a kinase buffer containing 5 μCi of [γ-32P]ATP at room temperature for 30 min as described (33). The reaction was stopped by adding 4 × SDS sample buffer and boiling the sample for 5 min. Proteins were then separated on SDS-PAGE and detected by autoradiography.

**Transient Transfection of Cos Cells with a Dominant-negative Mutant of RAFTK**—A kinase-negative mutant of RAFTK (KM) was constructed by replacing Lys-475 with an Ala residue using a site-directed mutagenesis kit (Clontech, Palo Alto, CA) as described (20). The RAFTK cDNA in the pCDNA3-neo vector was constructed as described in our previous studies (19, 33), and the paxillin cDNA in the pRcCMV vector was kindly provided by R. Salgia and J. D. Griffin (Dana Farber Cancer Institute, Harvard Medical School, Boston, MA). Cos cells were transiently transfected with either the wild-type RAFTK construct, RAFTK-KM construct, or paxillin construct or were co-transfected with RAFTK and paxillin constructs or RAFTK-KM and paxillin constructs as described (33). After 72 h of transfection, the cells were starved for 4 h in serum-free medium and then either stimulated with PMA (50 ng/ml, 5 min at 37 °C) or left unstimulated. Cell lysates were prepared and immunoprecipitated with anti-RAFTK antibodies, anti-paxillin antibodies, or preimmune antibodies. The immune precipitates were washed and analyzed for tyrosine phosphorylation as described above.

**RESULTS**

**SCF Induces the Tyrosine Phosphorylation of RAFTK in CMK Megakaryocytic Cells**—To investigate whether RAFTK participated in growth-related signal transduction pathways in megakaryocytes, we studied model CMK megakaryocytic cells and focused on the c-KIT/SCF pathway, which is known to be important in the proliferation of this line (28, 29). The CMK cells were starved in RPMI 1640 medium followed by stimulation with SCF and then harvested at different time intervals as indicated. Cells were lysed and immunoprecipitated with polyclonal RAFTK R-4250 antibodies or FAK antibody. The precipitates were then immunoblotted with monoclonal anti-phosphotyrosine (PY-20) antibodies, FAK monoclonal antibodies, or RAFTK-specific antibodies as indicated. The tyrosine phosphorylation of RAFTK peaked at 5 min at an SCF concentration of 500 ng/ml (Figs. 1A and 2). Interestingly, no phosphorylation of FAK was observed under these conditions (Figs. 1B and 2). The maximum stimulation of RAFTK in CMK cells was observed within 1 min and peaked at 5 min (Fig. 2).

SCF treatment is known to increase cytoplasmic calcium levels (34) and to stimulate the phosphorylation of the components of c-KIT-associated signaling pathways (35, 36). Since SCF effects are modulated by PKC (30, 37), the role of PKC in RAFTK stimulation was investigated. SCF treatment of CMK cells induced (within 1 min) the rapid phosphorylation of RAFTK, which was inhibited by the PKC inhibitors calphostin C (1 μg/ml) and staurosporine (1 μg/ml) as shown (Fig. 3).

The autophosphorylation and kinase activity of RAFTK upon SCF stimulation in CMK cells were analyzed. SCF stimulation resulted in an increase in the autophosphorylation as well as the kinase activity of RAFTK (data not shown), which is essential for its phosphorylating role in CMK cell signaling.

**PMA Induces the Tyrosine Phosphorylation of RAFTK in CMK Megakaryocytic Cells**—To study whether RAFTK might participate in the pathways of megakaryocyte differentiation,
we examined whether PMA, which induces differentiation and maturation of these cells (24, 32, 37), was able to stimulate RAFTK phosphorylation (Figs. 1A and 2). Under these conditions, the phosphorylation of RAFTK peaked at 5 min at a PMA concentration of 50 nM.

Since PMA activates PKC, we sought to further characterize the relationship between the PMA-induced tyrosine phosphorylation of RAFTK in CMK cells and PKC activation. The addition of the PKC inhibitors calphostin C (1 mM) (Fig. 1D) or staurosporine (75 nM) (data not shown) inhibited RAFTK phosphorylation following the PMA treatment of CMK cells. In additional correlative experiments, PMA-sensitive isozymes were first down-regulated in CMK cells by prolonged treatment with PMA (1 h at 37 °C at 200 nM), after which these CMK cells were treated with SCF or PMA. This prolonged treatment with PMA completely abolished the subsequent effects of both the PMA and SCF treatments (data not shown), suggesting that the tyrosine phosphorylation of RAFTK by either SCF or PMA is mediated by a PKC-dependent mechanism. Interestingly, PMA stimulation did not result in an increase in the tyrosine phosphorylation of FAK (Fig. 1B), suggesting that these related molecules may have distinct roles in the different signaling pathways in megakaryocytes.

**RAFTK Phosphorylation in Megakaryocytes**

**Fig. 1.** Tyrosine phosphorylation of RAFTK in response to SCF and PMA treatment or stimulation in CMK megakaryocytic cells. A and B, immunoprecipitates of RAFTK (A) or FAK (B) from CMK cells after the following treatments: PMA (50 nM) for 10 or 20 min or SCF for 5 min at 100 or 500 ng/ml as indicated. The immunocomplexes were resolved by 7.5% SDS-PAGE, transferred to Immobilon-polyvinylidene difluoride membranes, and probed with either anti-phosphotyrosine antibodies PY-20, anti-RAFTK antibodies (1:1000), or antibodies for FAK-2A7 (1:1000) as indicated. Immunoprecipitates of lysates of CMK cells stimulated with SCF (500 ng/ml) for 5 min using normal rabbit serum (NRS) or irrelevant monoclonal antibody (Control mAb) were used as controls. Bands were visualized using the ECL system; unstimulated (UN). C and D, immunoprecipitates of RAFTK from CMK cells treated with SCF (500 ng/ml) (C) or PMA (50 nM) (D) for the indicated periods of time in the absence or presence of calphostin C (1 μM) were prepared. The immunocomplexes were washed, resolved by 7.5% SDS-PAGE, transferred to Immobilon-polyvinylidene difluoride membranes, and probed with either anti-phosphotyrosine PY-20 antibodies (1:1000) or anti-RAFTK antibodies (1:1000). Bands were visualized using the ECL system. Normal rabbit serum (NRS) was used as a negative control.

**Fig. 2.** Time course of tyrosine phosphorylation of RAFTK and FAK in response to SCF or PMA stimulation. Immunoprecipitates of RAFTK or FAK from CMK cells treated with SCF (500 ng/ml) or PMA (50 nM) for the indicated periods of time were prepared. RAFTK and FAK were immunoprecipitated from untreated or treated cells using RAFTK antibodies or FAK antibodies as indicated. The immunocomplexes were washed, resolved by SDS-PAGE, transferred to nitrocellulose, and probed with either anti-phosphotyrosine, anti-RAFTK antibodies (1:1000), or FAK antibodies (1:1000) as indicated.
RAFTK Phosphorylation in Megakaryocytes

**Effects of the Dominant-negative RAFTK (KM) on Paxillin Phosphorylation**—To analyze further the interaction between RAFTK and paxillin to test whether RAFTK is required as an upstream regulatory molecule of the paxillin signaling pathway, we used a catalytically inactive mutant of RAFTK (KM) that acts as a dominant-negative of the wild-type RAFTK in Cos cells. Cos cells were transiently transfected with KM, wild-type RAFTK (19, 33), or paxillin expression vectors. After 72 h, cells were starved for 4 h in serum-free medium and then stimulated with PMA, lysed, and analyzed for the phosphorylation of RAFTK. As expected, no tyrosine phosphorylation of RAFTK was observed in the presence of KM upon PMA stimulation (data not shown). Taken together, these results suggest that RAFTK forms a complex with paxillin in unstimulated and stimulated CMK cells and that the phosphorylation of RAFTK and paxillin is mediated by a PKC-dependent pathway.

**RAFTK Forms a Complex with Paxillin**—To address the role of RAFTK in the formation of focal adhesions, we characterized its association with the known focal adhesion protein paxillin. Cell lysates of CMK cells treated with PMA were immunoprecipitated with either RAFTK-specific antibodies or anti-paxillin antibodies. The immunoprecipitates were resolved and immunoblotted with antibodies PY-20 (1:2000) and vinculin at focal adhesion sites in CMK cells (33). Interestingly, upon calcium ionophore A23187 treatment, RAFTK tyrosine phosphorylation was inhibited in the presence of cytochalasin D, indicating that the integrity of the cytoskeleton is required for RAFTK phosphorylation following changes in intracellular calcium.

Similarly, SCF treatment of CMK cells induced rapid and transient tyrosine phosphorylation of RAFTK, which was inhibited in the presence of BAPTA (Fig. 3B), suggesting that SCF may induce RAFTK phosphorylation by elevating intracellular calcium levels. However, in the presence of cytochalasin D, SCF induction of RAFTK phosphorylation was completely inhibited (Fig. 3B), indicating again that the integrity of the cytoskeleton is required for RAFTK phosphorylation. Since SCF stimulation of RAFTK in the presence of calphostin C was also completely inhibited, this mechanism of RAFTK tyrosine phosphorylation appears to be mediated by a PKC-dependent pathway.

PMA phosphorylation of RAFTK was also blocked by calphostin C (Fig. 3C) or by BAPTA, further indicating that calcium regulation of PKC isoforms is involved in RAFTK stimulation. Cytochalasin D treatment inhibited the PMA stimulation of RAFTK, suggesting that the integrity of the megakaryocytic cytoskeleton is critical for RAFTK phosphorylation.

**FIG. 3. Tyrosine phosphorylation of RAFTK in response to treatment with calcium ionophore A23187, SCF, and PMA.** Immunoprecipitates of RAFTK from CMK cells were subjected to the following treatments: calcium ionophore A23187 (10 μM) (A), SCF (500 ng) (B), or PMA (50 nM) (C) in the absence or presence of BAPTA (20 μM) for 30 min, cytochalasin D (2 μM), or calphostin C (1 μM) for 5 min or for the period of time indicated. Cells were lysed in modified RIPA buffer. 800 μg of total cell lysate was immunoprecipitated with normal rabbit serum or anti-RAFTK antibodies. Immunoprecipitated complexes were resolved on 7.5% SDS-PAGE gel, transferred to nitrocellulose membranes, immunoblotted with antibodies R-4250 for RAFTK (1:1000) or with antibody to phosphorytrosine PY-20 (1:2000), and visualized by ECL.
sine phosphorylation of paxillin by competition with other tyrosine kinases (Fig. 6). However, when wild-type RAFTK and paxillin constructs (33) were co-transfected, there was an increase in paxillin phosphorylation. Interestingly, in Cos cells transfected with the paxillin construct alone, the phosphorylation of paxillin was observed. However, in Cos cells co-transfected with paxillin and RAFTK-KM, the expression of RAFTK-KM resulted in the inhibition of paxillin phosphorylation, indicating that intact RAFTK kinase activity is important for paxillin phosphorylation. These results demonstrate that RAFTK is required for paxillin phosphorylation and suggest that RAFTK can act as a mediator of the paxillin signaling pathway.

**DISCUSSION**

In this report, we have characterized new signaling pathways involving RAFTK in CMK human megakaryocytic cells.

**FIG. 4.** Association of RAFTK with paxillin. CMK cells untreated or treated with PMA (50 nM) in the absence or presence of calphostin C (1 μM) for the indicated periods of time were lysed in RIPA buffer. 500 μg of total cell lysate protein from each sample was immunoprecipitated with paxillin antibodies (A). Immunoprecipitated complexes were resolved on 7.5% SDS-PAGE, transferred to Immobilon (polyvinylidene difluoride) membranes, immunoblotted with either anti-phosphotyrosine PY-20 antibodies (1:2000), polyclonal antibodies R-4250 for RAFTK (1:1000), or monoclonal antibodies for paxillin (ICN) (1:1000) as indicated, and visualized by the ECL system. Control monoclonal antibody (mAb) was used as a negative control. B, untreated CMK cells or cells treated with SCF (500 ng/ml) for the indicated periods of time in the absence or presence of calphostin C (1 μM) were lysed in RIPA buffer. 800 μg of total cell lysate protein from each sample was immunoprecipitated with RAFTK antibodies. Immunoprecipitated complexes were resolved on 7.5% SDS-PAGE, transferred to Immobilon-polyvinylidene difluoride membranes, immunoblotted with either anti-PY-20 antibodies, RAFTK antibodies, or paxillin antibodies, and visualized by the ECL system. C, the GST-RAFTK C-terminal protein or GST protein alone as a control was added to unstimulated CMK cell lysates and immunoprecipitated with glutathione-Sepharose beads followed by immunoblotting with anti-paxillin antibody. Lanes 1, 2, and 4, unstimulated CMK cells; lanes 3 and 5, CMK cells stimulated with SCF or PMA as described in Fig. 1.

**FIG. 5.** RAFTK binds directly to paxillin. Lysates of unstimulated CMK cells (lanes 1 and 2) or CMK cells stimulated with SCF (lane 3) or PMA (lane 4) for 5 min were immunoprecipitated with anti-paxillin antibody and processed as described under “Experimental Procedures.” Samples were applied to a 7.5% SDS-PAGE and transferred to Immobilon-P membranes. The membranes were processed for Far-Western blotting as described under “Experimental Procedures” with the GST and GST C-terminal RAFTK.

**FIG. 6.** Effects of co-expression of wild-type RAFTK and paxillin or the RAFTK dominant-negative (KM) and paxillin on paxillin tyrosine phosphorylation. Cos cells were transiently co-transfected with dominant-negative RAFTK mutant plus paxillin constructs, wild-type RAFTK plus paxillin constructs, or the paxillin construct alone. Total cell lysates were analyzed by immunoprecipitating with paxillin antibody and immunoblotting with anti-phosphotyrosine antibody (PY-20) or anti-paxillin antibodies.

SCF and PMA induced the tyrosine phosphorylation of RAFTK but not of FAK. The effect of SCF, PMA, and Ca²⁺ on the phosphorylation of RAFTK appeared to be mediated by a PKC-dependent pathway. Association of RAFTK with paxillin was demonstrated, and cytochalasin D abolished the phosphorylation of RAFTK upon SCF and PMA stimulation.

Upon stimulation of CMK cells by SCF, RAFTK was phosphorylated and there was an enhanced autophosphorylation of RAFTK as well as an increase in its in vitro kinase activity as determined using a poly(GluTyr) (1:4) substrate. These results are consistent with our previous work (33), which indicated that RAFTK had intrinsic tyrosine kinase and autokinase activities.
PKC plays an important role in the cellular responses to various hormones, growth factors, neurotransmitters, and cytokines and transduces signals promoting lipid hydrolysis (39, 40). PKC regulates the action of a variety of ion channels, G-protein-coupled receptors, tyrosine kinase receptors, and nonreceptor tyrosine kinases (41, 42). Since the proliferative effects of SCF appeared to be modulated in part by PKC (30–37, 39–43), we sought to identify a role for PKC in RAFTK phosphorylation. Indeed, SCF induced the rapid phosphorylation of RAFTK, which was completely blocked by the PKC inhibitors calphostin C (Fig. 1) or staurosporine (data not shown). Furthermore, PMA, which induces the differentiation of CMK megakaryocytic cells (24, 32, 37), also stimulated the tyrosine phosphorylation of RAFTK, and this phosphorylation was abolished by the PKC inhibitors (Fig. 1) or by prolonged treatment with PMA. Rat brain PKC was able to phosphorylate the GST C-terminal RAFTK in a lipid-activator, Ca\textsuperscript{2+}-dependent manner.\footnote{D. Price and S. Avraham, unpublished data.} Taken together, these results indicate that RAFTK phosphorylation is mediated by a PKC-dependent pathway.

SCF induction of the tyrosine phosphorylation of RAFTK could be mediated by elevated intracellular calcium levels and/or by the activation of PKC through phospholipase C-\gamma (44). Indeed, RAFTK phosphorylation was inhibited following treatment by the PKC inhibitors calphostin C or staurosporine as well as by BAPTA (an intracellular Ca\textsuperscript{2+} chelator) (Fig. 3), indicating that PKC is involved in RAFTK phosphorylation. BAPTA blocked PMA- or SCF-induced RAFTK phosphorylation, suggesting that calcium was essential for PKC-mediated RAFTK phosphorylation (Fig. 3). Moreover, the calcium ionophore (A23187) also stimulated RAFTK phosphorylation and was inhibited by the PKC inhibitors calphostin C and staurosporine or by BAPTA (Fig. 3), indicating a role for PKC as a mediator of several signaling pathways including Ca\textsuperscript{2+} in RAFTK phosphorylation. The finding that FAK is not phosphorylated under these conditions is consistent with prior studies of FAK activation in Mo\textit{7e} megakaryocytic cells (45) and suggests important differences in the roles of FAK and RAFTK in cells of this lineage.

The cytoskeleton is essential for many cellular functions including the regulation of cell shape, flexibility, and adhesive properties (8, 9). Part of the cytoskeleton and plasma membrane form a region known as the focal adhesion (46). Focal adhesions are structures that form adhesive contacts with the extracellular matrix. Proteins contained in the focal adhesion include talin, \(\alpha\)-actinin, vinculin, paxillin, and other proteins (47–52). The signal transduction pathways initiated by the integrins involve the cytoskeleton-dependent activation of tyrosine kinases and the phosphorylation of a number of substrates including the FAK protein (9, 10, 13). Moreover, we have demonstrated that the C-terminal proline-rich region of RAFTK binds directly to paxillin (Fig. 5) and that overexpression of a dominant-negative mutant of RAFTK co-transfected with paxillin into Cos cells abolished the tyrosine phosphorylation of paxillin upon PMA stimulation (Fig. 6). The observation that in Cos cells transfected with the paxillin construct alone paxillin was phosphorylated indicates the involvement of other kinases in the phosphorylation of paxillin (52, 53). However, overexpression of the dominant-negative mutant of RAFTK prevented the tyrosine phosphorylation of paxillin upon PMA stimulation by other kinases. This association suggests a role for RAFTK in the linking and cross-talk between various signaling proteins localized in the cytosol and focal adhesion contacts. Interestingly, the RhoA-dependent assembly of focal adhesions in Swiss 3T3 cells was associated with an increased tyrosine phosphorylation and the recruitment of both p125\textsubscript{FAK} and PKC-\(\delta\) to focal adhesions (38).

We have recently shown that the stimulation of megakaryocytes with thrombin leads to the tyrosine phosphorylation of RAFTK (19). In this study, we observed that RAFTK is involved in PKC-mediated c-KIT growth factor and Ca\textsuperscript{2+}-signaling pathways in megakaryocytic cells. Taken together, these studies indicate the potentially important function of RAFTK phosphorylation in these cells.

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