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The Cytoplasmic Tyrosine Kinase FER Is Associated with the Catenin-Like Substrate pp120 and Is Activated by Growth Factors

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The *FER* gene encodes a cytoplasmic tyrosine kinase with a single SH2 domain and an extensive amino terminus. In order to understand the cellular function of the FER kinase, we analyzed the effect of growth factor stimulation on the phosphorylation and activity of FER. Stimulation of A431 cells and 3T3 fibroblasts with epidermal growth factor or platelet-derived growth factor results in the phosphorylation of FER and two associated polypeptides. The associated polypeptides were shown to be the epidermal growth factor receptor or the platelet-derived growth factor receptor and a previously identified target, pp120. Since pp120 had previously been shown to interact with components of the cadherin-catenin complex, these results implicate FER in the regulation of cell-cell interactions. The physical association of FER with pp120 was found to be constitutive and was mediated by a 400-amino-acid sequence in the amino terminus of FER. Analyses of that sequence revealed that it has the ability to form coiled coils and that it oligomerizes in vitro. The identification of a coiled coil sequence in the FER kinase and the demonstration that the sequence mediates association with a potential substrate suggest a novel mechanism for signal transduction by cytoplasmic tyrosine kinases.

Many cytoplasmic tyrosine kinases are now recognized as integral components of the cellular machinery for relaying signals for cell growth and differentiation. For example, the *src* family kinases have been shown to mediate signaling in lymphoid cells (reviewed in reference 3). Extracellular signals that are mediated by cell surface receptors, such as the T-cell receptor, stimulate the phosphorylation of some of the *src* family kinases. The phosphorylated kinases bind the src homology 2 (SH2) domains of adaptor molecules and, in turn, phosphorylate these adaptors. These events are believed to trigger the intracellular cascade leading to lymphocyte activation. In addition, some *src* family kinases form complexes with growth factor receptors and are activated upon growth factor stimulation (8, 17, 35). These cytoplasmic kinases may therefore be involved in multiple signaling pathways. Their membrane localization positions them to function as points of convergence for signals generated by diverse extracellular agents. Another cytoplasmic kinase family of prominence are the Janus (JAK) kinases, which have been shown to play a major role in cytokine receptor signaling (reviewed in reference 29). By phosphorylating subunits of transcription factors that regulate cytokine-responsive genes, the JAK kinases provide a direct link between extracellular signals and gene regulation. Recently, it was demonstrated that the focal adhesion kinase (FAK) is activated upon the clustering of integrins or their binding to the extracellular matrix (4, 20, 24, 34, 37). The activation of FAK leads to other binding and activation events, including the activation of c-*src* kinase and the binding of FAK to the adaptor molecule GRB2 (55). Thus, FAK is likely to mediate the regulation of signal transduction by the cytoskeleton. It is therefore apparent from the analyses of cytoplasmic tyrosine kinases that these kinases are engaged in the early events in a diverse array of signaling pathways.

The *FER* and *FPS/FES* genes encode cytoplasmic tyrosine kinases that are structurally similar (25, 36). Both gene products contain a single SH2 domain that lies immediately upstream of the kinase domain. The remaining halves of the coding sequences encode the amino termini of the proteins, which do not share any similarity to other signal transduction components. Whereas FER expression is detected in all cell types that have been examined, expression of the *FPS/FES* gene is restricted to myeloid and endothelial cells (12, 18, 19, 40). The restricted expression of the *FPS/FES* gene has prompted investigations into the role of the kinase in hematopoietic cell signaling. These studies have led to the demonstration that FPS/FES interacts with the β subunit that is common to the interleukin-3 and granulocyte-macrophage colony-stimulating factor receptors in an erythroleukemic cell line (23). Stimulation of these cells with interleukin-3 or granulocyte-macrophage colony-stimulating factor results in an increase in the phosphorylation of the FPS/FES kinase. Similarly, the FPS/FES kinase was found to interact with the interleukin-4 receptor, and stimulation of T-cell lines with interleukin-4 resulted in an increase in the phosphorylation of the kinase (30). Other studies showed that overexpression of FPS/FES in a colony-stimulating factor 1-dependent cell line can at least partially abrogate the factor dependence for cell proliferation (1). These results suggest that FPS/FES may mediate signaling from a number of different receptors for cytokines and growth factors that are specific for hematopoietic cells. The requirement for FPS/FES in hematopoietic cell function is further implicated by experiments showing that antisense inhibition of FPS/FES expression led to apoptosis of differentiated granulocytes (41). More recently, the overexpression of FPS/FES in transgenic mice revealed an additional role of the kinase in angiogenesis (18). Together, these data have helped to solidify the role of FPS/FES in hematopoietic cell signaling. In addition to some cytokine receptors, the FPS/FES kinase also binds and phosphorylates BCR, which may serve as an effector of the kinase (42). Biochemical analyses of FPS/FES kinase showed that its SH2 domain and tyrosine 713 both have a positive regulatory effect on kinase activity (27). Deletion of the SH2

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sequence or mutation of tyrosine 713 to phenylalanine diminishes the activity of the kinase. These properties are consistent with the positive effects of the SH2 domain and the analogous tyrosine on the transforming activity of the viral homolog, v-*fps* (33, 59). Unlike those of the *src* family kinases, the amino acid sequences of FER and FPS/FES do not contain signals for myristoylation that will enable the kinases to become membrane associated. Overexpression of the *FPS/FES* gene in fibroblasts does not result in cell transformation (18). However, cells that overexpress a myristylated mutant are morphologically transformed, and the mutant kinase exhibits elevated kinase activity (18).

The ubiquitous expression of FER suggests that it may have a function that is fundamental to all cells. In contrast to the case with FPS/FES, relatively little is known about the cellular function of FER. We have been interested in identifying the role of FER in cell signaling. Given the ubiquitous expression of the FER kinase, we reason that it may be involved in a fundamental aspect of regulation, such as cell growth. We therefore set out to determine if FER may be regulated by mitogenic stimulation with growth factors. In this report, we show that FER is associated with a catenin-like molecule and that the kinase is activated upon growth factor stimulation.

MATERIALS AND METHODS

Cells and antibodies. NIH 3T3 fibroblasts, Swiss 3T3 fibroblasts, and A431 epidermal carcinoma cells were obtained from the American Type Culture Collection. NIH 3T3 and Swiss 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% donor calf serum and fetal bovine serum, respectively. A431 cells were grown in DMEM with 10% Fetal Clone II (HyClone). NIH 3T3 cells that express $\sim 10^5$ copies of the human epidermal growth factor (EGF) receptor per cell (EGFR-3T3; wt2) were provided by Graham Carpenter of Vanderbilt University and were grown in DMEM with 10% donor calf serum and G418 at 0.3 mg/ml (57). Polyclonal anti-FER1 antiserum is specific for residues 502 to 675 and was described before (13). The antiserum was used in all immunoprecipitations, except those that involved denaturing conditions. Two other anti-FER sera were also used for immunoblotting and denaturing immunoprecipitations. Anti-FER4 antiserum was generated with a TrpE fusion protein that consists of amino acids 149 to 332 of FER, and anti-FER5 antiserum was prepared by immunizing rabbits with a glutathione *S*-transferase (GST) fusion protein that contains residues 451 to 564 of the FER sequence. All three anti-FER antisera were effective in recognizing human as well as rodent FER kinases. Ascites fluids that contained monoclonal antibodies (MAbs) to FAK (MAb 2A7), pp120 (MAb 2B12), and AFAP110 (MAb F1) were provided by Thomas Parsons of the University of Virginia School of Medicine (32). MAbs to phosphotyrosine (PY20), to the EGF receptor, and to the carboxyl termini of pp120 isoforms were purchased from Transduction Laboratories. A polyclonal antipeptide antiserum to the platelet-derived growth factor (PDGF) receptor was from Upstate Biotechnology. Rabbit anti-mouse immunoglobulin G was from Pierce Chemicals.

Immunoprecipitation and immunoblotting experiments. Confluent cultures in 10-cm-diameter dishes were starved overnight in 10 ml of DMEM containing 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.2) and 0.2% serum. On the day of the experiment, the medium was changed to 4 ml of DMEM containing 10 mM HEPES (pH 7.2) and bovine serum albumin (BSA) at 1 mg/ml. After cultivation for 2 to 4 h in that medium, the cultures were stimulated by the addition of EGF or PDGF to 100 and 30 ng/ml, respectively. Cells were stimulated at 37°C for 5 min or longer, as specified below. They were rinsed twice with ice-cold phosphate-buffered saline and were lysed by being scraped into 0.8 ml of lysis buffer (1% Triton X-100, 5% glycerol, 20 mM Tris-HCl [pH 7.7], 1 mM EDTA, 0.15 M NaCl, 1 mM sodium orthovanadate, 40 μM ammonium molybdate) supplemented with leupeptin and pepstatin at 20 μg/ml. The cell lysates were clarified by centrifugation at 10,000 × *g* for 10 min and were assayed for protein concentration by using the Micro BCA reagents (Pierce). The cell lysates (0.5 to 1.5 mg of protein) were used for immunoprecipitation with 5 μl of anti-FER1 antiserum, 2 μl of ascites fluid, or 2 μg of purified MAbs. Immune complexes were collected by being bound to 25 μl of protein A-Sepharose CL4B directly or in the presence of 8 μg of rabbit anti-mouse immunoglobulin G. After a 2-h incubation at 4°C, the immune complexes were washed three times with lysis buffer and boiled in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. The immunoprecipitated polypeptides were fractionated by SDS-PAGE in 7.5% gels and were electrophoretically transferred to a piece of Immobilon P membrane (Millipore). After the transfer, the membrane was blocked by incubation in TBST (0.2% Tween 20, 10 mM Tris-HCl [pH 7.7], 0.15 M NaCl, 1 mM EDTA) that contained 5% dry milk. The membrane was then incubated with first antibody in TBST that contained 2% BSA. The antibody dilutions were 1 μg/ml for PY20, 0.5 μg/ml for other purified antibodies, and 1:5,000 for crude antisera or ascites fluids. The filter was washed with TBST and incubated with peroxidase-conjugated second antibody in TBST containing 2% BSA. This was followed by washing with TBST and antibody detection using the Enhanced Chemiluminescence kit from Amersham. The filter was then stripped of the antibodies by incubation in a buffer that contained 7 M guanidine hydrochloride, 0.1 M KCl, 50 mM glycine (pH 10.3), 0.05 mM EDTA, and 20 mM 2-mercaptoethanol for 10 min at room temperature (53). Following extensive washing with water, the filter was again blocked with dry milk in TBST before being reprobed with a different antibody.

Denaturing immunoprecipitation of FER was performed by first immunoprecipitating FER from Swiss 3T3 cell lysates with anti-FER1 serum as described above. After the immune complexes had been washed with lysis buffer, they were denatured by boiling in 100 μl of denaturing buffer (1% SDS, 50 mM Tris-HCl [pH 7.7]) for 5 min. The Sepharose beads were pelleted by centrifugation, and the supernatants were saved. To the supernatants were added 0.1 ml of 2× immunoprecipitation buffer (2% Triton X-100, 1% Nonidet P-40, 50 mM Tris-HCl [pH 7.7], 0.3 M NaCl, 2 mM EDTA, 1 mM sodium vanadate), 0.9 ml of water, and 5 μl of anti-FER5 serum. The immune complexes were immobilized by being bound to protein A-Sepharose, washed, and fractionated by SDS-PAGE as described above.

Immune complex kinase assays. Swiss 3T3 cell lysates were immunoprecipitated with anti-FER1 serum, and the immune complexes were washed three times with radioimmunoprecipitation assay (RIPA) buffer (1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 5% glycerol, 20 mM Tris-HCl [pH 7.7], 0.15 M NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 40 μM ammonium molybdate) and once with kinase buffer (50 mM Tris-HCl [pH 7.7], 10 mM $MnCl_2$). Each immunoprecipitate was divided into two aliquots, one of which was used directly for gel fractionation and immunoblotting. The other aliquots were used for kinase assays. To those beads were added 25 μl of kinase buffer that also contained 5 μM [γ-^{32}P]ATP. The reaction mixtures were incubated at 26°C for 10 min, and the reaction was terminated by the addition of SDS-PAGE sample buffer. The reaction products were fractionated by SDS-PAGE, and the gel was stained with Coomassie brilliant blue and destained. To enhance the detection of tyrosine phosphorylation, the gel was incubated in 1 M KOH at 55°C for 1.5 h. After the alkaline treatment, the gel was fixed again and dried. Phosphorylated polypeptides were detected by autoradiography.

Kinase activity was also measured by using [Val-5]angiotensin II as a substrate as described before (13). After the immune complexes had been washed with RIPA buffer and kinase buffer, the beads were incubated in 25 μl of kinase buffer that contained 50 mM Tris-HCl (pH 7.7), 10 mM $MnCl_2$, BSA at 1.25 mg/ml, 5 mM [Val-5]angiotensin II, and 10 μM [γ-^{32}P]ATP (specific activity, 50,000 to 100,000 cpm/pmol). The reaction mixtures were incubated for 10 min at 26°C, and the reaction was terminated by the addition of 45 μl of ice-cold stop buffer (5% trichloroacetic acid, 7 mM ATP). The mixtures were incubated on ice for 5 min, and insoluble materials were removed by centrifugation. The phosphorylated peptide was recovered by being bound to phosphocellulose paper, and phosphorylation was measured as described before (13).

In vitro binding assays. In previous work, we used degenerate oligonucleotide primers to isolate partial cDNAs of tyrosine kinases (48). Among those isolated was a partial cDNA fragment of human FER, which was used to isolate more overlapping FER cDNA clones from a fetal lung cDNA library. GST fusion proteins, GST-SH2 (residues 451 to 564) and GST-CCD (residues 9 to 434), were obtained by subcloning cDNA fragments into the vector pGEX-KG (21). Bacteria (*Escherichia coli* JM109) that expressed the fusion proteins were grown at room temperature to an optical density at 600 nm of 0.6. Protein expression was induced with 0.4 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and the culture was incubated for an additional 3 h. The bacteria were lysed as described previously, and the lysate was incubated with glutathione-Sepharose (Pharmacia) for 1 h at 4°C (21). The beads were washed three times with tissue culture cell lysis buffer as described above. Lysates of Swiss 3T3 cells (~0.5 mg of protein each) were then added to the immobilized GST fusion proteins (approximately 10 μg of protein each), and the mixtures were incubated at 4°C for 2 h. The beads were then washed three times with lysis buffer and boiled in SDS-PAGE sample buffer. Bound proteins were analyzed by Western blotting (immunoblotting) as described above.

Chemical cross-linking. The GST-CCD fusion protein was isolated from bacteria by being bound to glutathione-Sepharose as described above. The protein was digested in situ with thrombin (0.5 μg/ml) in phosphate-buffered saline at room temperature for 16 h. The liberated CCD peptide was recovered from the supernatant by centrifugation and was analyzed by SDS-PAGE and Coomassie blue staining. The isolated CCD peptide (10 μg) was incubated in the presence of 0.1 mM bifunctional cross-linking reagent [bis(sulfosuccinimidyl)suberate (Pierce)] in a total volume of 30 μl. After 5 min at room temperature, the reaction was quenched by the addition of 2 μl of 1 M Tris-HCl (pH 7.5). The reaction products were fractionated by SDS-PAGE and analyzed by Western blotting with anti-FER4 serum. Identical incubations were also carried out with ovalbumin, and the reaction products were analyzed by SDS-PAGE and staining with Coomassie blue.

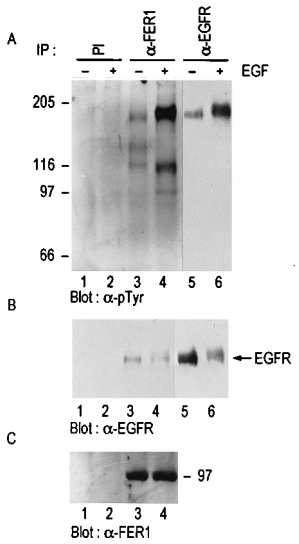

FIG. 1. EGF stimulates the phosphorylation of a complex that coimmunoprecipitates with FER kinase from A431 cells. (A) Lysates were prepared from untreated A431 cells (lanes 1, 3, and 5) or from cells that had been stimulated with EGF for 5 min (lanes 2, 4, and 6). Immunoprecipitation (IP) was carried out with preimmune serum (PI) (lanes 1 and 2), anti-FER1 (α-FER1) antiserum (lanes 3 and 4), or a monoclonal anti-EGF receptor (EGFR) antibody (lanes 5 and 6). The anti-EGF receptor immunoprecipitations were performed with 0.1 mg of cell lysates, whereas those for the preimmune and anti-FER sera used 1.5 mg of lysates. The immunoprecipitates were fractionated by SDS-PAGE, and phosphotyrosine-containing polypeptides were detected by blotting with an antiphosphotyrosine (α-pTyr) antibody. Lanes 1 to 4 were exposed to film for 15 s, and lanes 5 and 6 were exposed for 2 s. The numbers on the left side indicate the locations and sizes, in kilodaltons, of molecular mass markers. (B) The membrane was stripped and reprobed with a monoclonal anti-EGF receptor antibody. The arrow indicates the position at which the receptor migrated. Exposure times were 30 s for lanes 1 to 4 and 2 s for lanes 5 and 6. (C) The filter from panel B was stripped and probed with anti-FER1 antiserum. The migration of the 97-kDa FER kinase is indicated on the right.

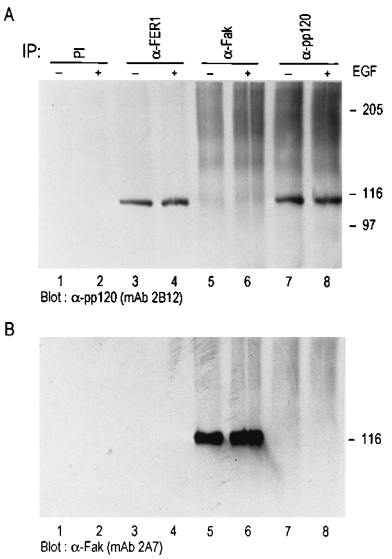

FIG. 2. Identification of pp120 as one of the polypeptides that are associated with FER in A431 cells. (A) Lysates were prepared from untreated A431 cells (lanes 1, 3, 5, and 7) or A431 cells that had been stimulated with EGF for 5 min (lanes 2, 4, 6, and 8). The lysates were immunoprecipitated (IP) with preimmune serum (PI) (lanes 1 and 2), anti-FER1 (α-FER1) antiserum (lanes 3 and 4), a monoclonal anti-FAK antibody (2A7) (lanes 5 and 6), or a monoclonal anti-pp120 antibody (2B12) (lanes 7 and 8). Polypeptides that were coimmunoprecipitated were analyzed by blotting with the MAb to pp120 (MAb 2B12). (B) The membrane was stripped of the antibodies and reprobed with the monoclonal anti-FAK antibody (MAb 2A7).

RESULTS

Effect of growth factor stimulation on the FER kinase. To determine if FER may be activated upon stimulation of cells with growth factors, we treated cultures of A431 cells with EGF and immunoprecipitated FER from the cell lysates. A complex of phosphorylated polypeptides was immunoprecipitated by the anti-FER serum from a lysate of EGF-treated cells, and they had molecular masses of 175, 115, and 97 kDa (Fig. 1A, lane 4). Polypeptides with similar molecular masses were also present in the immunoprecipitate prepared from a lysate of untreated cells, although with reduced reactivity to the antiphosphotyrosine antibodies (lane 3). An additional 145-kDa polypeptide was detected in the immune complex from untreated cells but not in that from EGF-stimulated cells. The electrophoretic mobility and extensive phosphorylation of the 175-kDa polypeptide in the anti-FER immunoprecipitates suggested that it may be the EGF receptor. Subsequent reprobing of the membrane with anti-EGF receptor antibodies confirmed that the anti-FER immunoprecipitate did contain the EGF receptor (Fig. 1B). The receptor was present in the anti-FER immunoprecipitates prepared from both untreated and EGF-stimulated cell lysates. The filter was also reprobed with antibodies to FER, and they detected a 97-kDa polypeptide that comigrated with the phosphorylated polypeptide that was immunoprecipitated from the lysate of EGF-stimulated cells. We were not able to detect FER in the anti-EGF-receptor immunoprecipitates (data not shown). These results indicate that in A431 cells, FER forms complexes with the EGF receptor and with two additional polypeptides that are phosphorylated on tyrosines.

To date, a number of targets of tyrosine kinases have been identified, and several of them have molecular masses in the range of 110 to 125 kDa (reviewed in reference 54). These include the *ras* GTPase-activating protein (GAP), FAK, pp120, and a 110-kDa actin filament-associated polypeptide (AFAP). We performed an analysis to determine if the 115-kDa polypeptide that coimmunoprecipitated with FER was one of these previously identified substrates. Immunoprecipitates were prepared from untreated and EGF-stimulated A431 cells with anti-FER, anti-FAK, and anti-pp120 antibodies. They were then analyzed by immunoblotting with monoclonal anti-FAK and anti-pp120 antibodies. As shown in Fig. 2A, the analysis readily revealed the presence of the substrate pp120 in the FER immune complex (lanes 3 and 4). In contrast, the monoclonal anti-FAK antibodies detected FAK only in the anti-FAK immunoprecipitate, and not in the anti-FER or anti-pp120 immunoprecipitates (Fig. 2B). These results show that in A431 cells FER forms a complex with the substrate pp120. The association between FER and pp120 is highly specific, because pp120 is not detectable in immune complexes prepared with preimmune serum or anti-FAK serum (Fig. 2A, lanes 1, 2, 5, and 6). Interestingly, the association of FER and pp120 was detectable in lysates of untreated cells, and EGF stimulation did not appear to result in any significant change in the extent of the interaction. In similar experiments, we found that the FER immune complex did not contain any *ras* GAP or AFAP-p110 (data not shown).

Phosphorylation of the FER-pp120 complex is stimulated by PDGF as well as EGF. We used A431 cells in the initial characterizations because EGF elicits robust biochemical changes by virtue of the extraordinary number of EGF receptors ($\sim 2 \times 10^6$ per cell) in these cells (11). However, the high density of

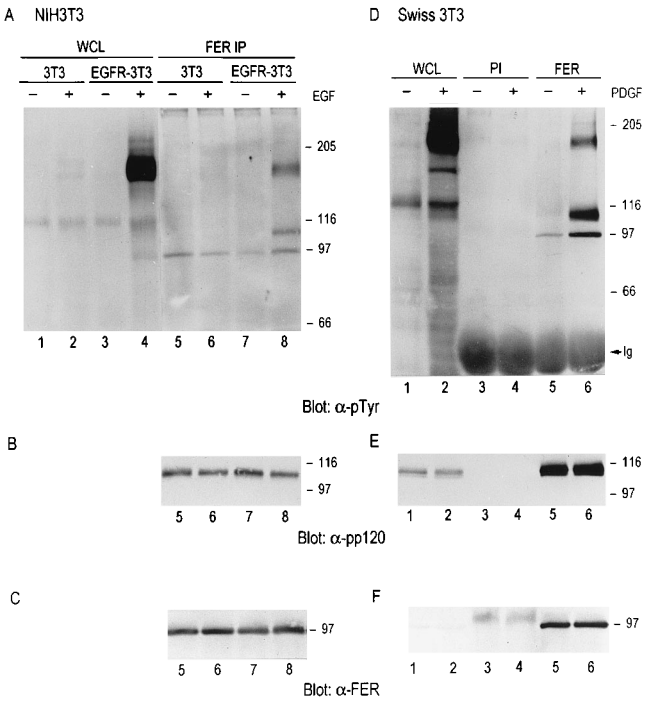

FIG. 3. EGF and PDGF stimulate the phosphorylation of the FER-pp120 complex in mouse fibroblasts. NIH3T3 cells (3T3), NIH 3T3 cells overexpressing the human EGF receptor (EGFR-3T3), and Swiss 3T3 cells were rendered quiescent by serum deprivation. Cells were stimulated with EGF or PDGF for 5 min and were lysed. Aliquots of whole-cell lysates (WCL; 20 μg of protein each) or anti-FER1 immunoprecipitates (FER IP) were analyzed by immunoblotting. (A) Lysates (lanes 1 to 4) and anti-FER1 immunoprecipitates (FER IP) (lanes 5 to 8) from NIH 3T3 cells and EGFR-3T3 cells were analyzed by blotting with an antiphosphotyrosine (α-pTyr) antibody. (B) Part of the membrane from panel A was reprobed with a monoclonal anti-pp120 antibody (MAb 2B12). (C) The membrane from panel B was stripped and probed with anti-FER5 antiserum. (D) Lysates (lanes 1 and 2), preimmune immunoprecipitates (PI) (lanes 3 and 4), and anti-FER1 immunoprecipitates (lanes 5 and 6) from Swiss 3T3 cells were analyzed by blotting with an antiphosphotyrosine antibody. The dark band at the bottom of lanes 3 to 6 was from cross-reactivity to the rabbit immunoglobulin (Ig) in the sera. Panels E and F show the results of reprobing the membrane from panel D with a monoclonal anti-pp120 antibody (MAb 2B12) and with anti-FER5 antiserum, respectively.

EGF receptors in A431 cells also results in a significant level of receptor phosphorylation in cells that have been deprived of growth factors (Fig. 1A, lane 5). To determine if the constitutive association of FER and pp120 in A431 cells was due to the high basal level of tyrosine phosphorylation of the EGF receptor, we analyzed the composition of the FER immune complex prepared from NIH 3T3 fibroblasts that had been engineered to express $\sim 10^5$ human EGF receptors per cell. When these cells were deprived of serum growth factors, they showed no significant extent of tyrosine phosphorylation (Fig. 3A, lane 3). When they were stimulated with EGF, extensive tyrosine phosphorylation of a number of polypeptides was observed. The most extensively phosphorylated component was a 175-kDa polypeptide, which presumably was the human EGF receptor that is overexpressed (lane 4). When anti-FER immune complexes were analyzed, a number of phosphorylated polypeptides were found to be coprecipitated from a lysate of EGF-stimulated cells (Fig. 3A, lane 8). These polypeptides had apparent molecular masses of 97, 115, and 175 kDa. This phosphorylated complex was detected only in the lysate of the EGF receptor-overexpressing cells that had been stimulated with EGF. From the untreated cells, the same antiserum immunoprecipitated a 97-kDa polypeptide that was less extensively phosphorylated (lane 7). When treated with EGF, untransfected NIH 3T3 cells showed only a minor increase in tyrosine phosphorylation of total cellular proteins, and the corresponding anti-FER immunoprecipitate contained only a 97-kDa polypeptide that was moderately phosphorylated (Fig. 3A, lanes 2 and 6). When the filter was reprobed with a MAb to pp120, pp120 was detected in the FER immunoprecipitates prepared from lysates of untreated and EGF-stimulated cells (Fig. 3B). The coprecipitation of FER and pp120 was detected for NIH 3T3 cells as well as for cells that overexpress the EGF receptor. Unlike the EGF receptors in the A431 cells, the EGF receptors in the EGFR-3T3 cells could be rendered inactive by deprivation of growth factors. These results therefore show that the constitutive association of FER and pp120 is not unique to A431 cells and that it is not dependent on a high basal level of tyrosine phosphorylation of the EGF receptor.

The analyses were extended to determine if the phosphorylation of the FER-pp120 complex is specific to EGF only. Swiss 3T3 fibroblasts were treated with type B PDGF (PDGF-BB), and cell lysates were immunoprecipitated with preimmune serum or anti-FER antiserum. The PDGF treatment resulted in the tyrosine phosphorylation of an extensive array of cellular polypeptides (Fig. 3D, lanes 1 and 2). From a lysate of PDGF-treated cells, the anti-FER serum immunoprecipitated a complex of polypeptides that are phosphorylated on tyrosines (lanes 5 and 6). These polypeptides had molecular masses of 180, 115, and 97 kDa. The 115- and 97-kDa polypeptides in the immunoprecipitates were shown by successive rounds of immunoblotting to be pp120 and FER, respectively (Fig. 3E and F). The 180-kDa polypeptide, which was presumably the PDGF receptor, comigrated with the most extensively phosphorylated cellular targets. It is therefore apparent that PDGF elicits a similar pattern of tyrosine phosphorylation of polypeptides that coimmunoprecipitate with FER. As in NIH 3T3 cells, the FER kinase is constitutively associated with pp120 in Swiss 3T3 fibroblasts (Fig. 3E, lanes 5 and 6).

The phosphorylation of the FER-pp120 complex in response to PDGF was analyzed in greater detail. The phosphorylation of the complex was stimulated by PDGF in a dose- and time-dependent manner (data not shown). We also analyzed the ligand-receptor specificity of the phosphorylation of the FER-pp120 complex, since it was shown previously that the two PDGF receptor isotypes may elicit different responses (10). It was also shown previously that Swiss 3T3 cells express comparable numbers of α- and β-PDGF receptors (56). Stimulation with PDGF-AA and stimulation with PDGF-BB resulted in comparable extents of tyrosine phosphorylation of cellular proteins (Fig. 4A, lanes 2 and 3). The slight difference in the molecular weights of the two receptor isoforms is evident in the most extensively phosphorylated polypeptides detected in the cell lysates. From the analyses of the anti-FER immunoprecipitates, it is evident that PDGF-AA and PDGF-BB were equally effective in stimulating the phosphorylation of the FER-pp120 complex (lanes 5 and 6). It is noteworthy that the 180-kDa phosphoproteins that were present in the anti-FER immunoprecipitates also showed the slight difference in electrophoretic mobilities that is attributed to the difference in the molecular weights of the receptor isotypes (lanes 5 and 6) (10).

The anti-FER immunoprecipitates prepared from EGF- or PDGF-stimulated cells contained a 97-kDa polypeptide, the phosphorylation of which is stimulated by growth factor treatment. The phosphoprotein comigrates with authentic FER kinase that can be detected by immunoblotting with different FER antibodies. To ascertain that the 97-kDa phosphoprotein was indeed the FER kinase, we subjected the components of

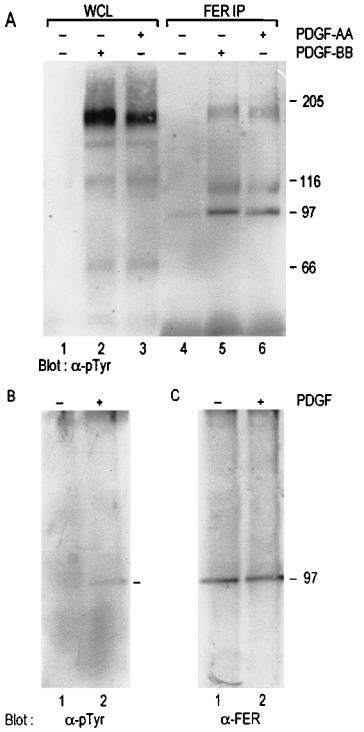

FIG. 4. PDGF-AA and PDGF-BB are equally effective in stimulating the phosphorylation of FER and pp120. (A) Swiss 3T3 cells were left untreated (lanes 1 and 4) or were stimulated with PDGF-BB (lanes 2 and 5) or PDGF-AA (lanes 3 and 6) at 30 ng/ml. Whole-cell lysates (WCL) (lanes 1 to 3) or anti-FER1 immunoprecipitates (FER IP) (lanes 4 to 6) were analyzed by blotting with an antiphosphotyrosine (α-pTyr) antibody. (B) Lysates from quiescent Swiss 3T3 cells (lane 1) or cells that had been stimulated with PDGF-BB (lane 2) were immunoprecipitated with anti-FER1 antiserum. The immunoprecipitates were washed with lysis buffer and denatured by boiling in 1% SDS as described in Materials and Methods. The eluted materials were diluted and immunoprecipitated with anti-FER5 antiserum. The immunoprecipitates were analyzed by Western blotting with an antiphosphotyrosine antibody. (C) The membrane from panel B was stripped and reprobed with anti-FER5 antiserum.

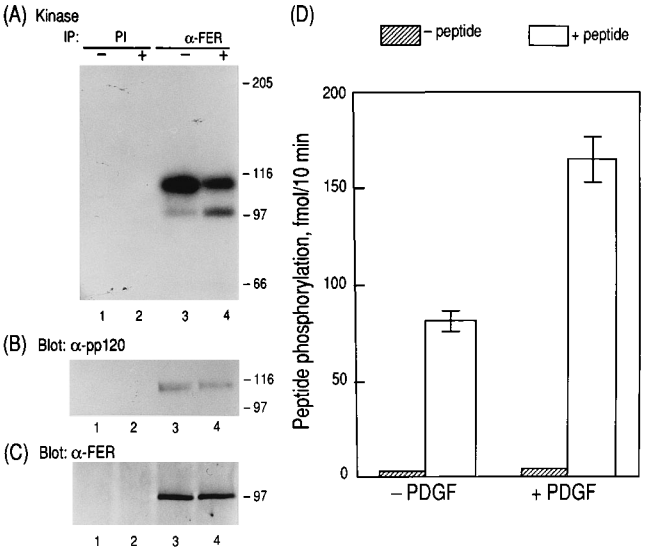

FIG. 5. PDGF treatment of Swiss 3T3 cells stimulates the FER kinase. (A) Quiescent Swiss 3T3 cells were left untreated (−) (lanes 1 and 3) or were treated with PDGF-BB (+) (lanes 2 and 4), and cell lysates were immunoprecipitated (IP) with preimmune serum (PI) (lanes 1 and 2) or with anti-FER1 (α-FER) antiserum (lanes 3 and 4). The immunoprecipitates were washed with RIPA buffer and were each divided into two aliquots, one of which was incubated in the presence of [γ-^{32}P]ATP. The reaction products were fractionated by SDS-PAGE and visualized by autoradiography. The other set of anti-FER immunoprecipitates were analyzed directly by sequential Western blotting with anti-pp120 (B) and anti-FER (C) antibodies to ensure that all samples contained equal amounts of FER immunoprecipitates. (D) Lysates of untreated Swiss 3T3 cells (−PDGF) or cells stimulated with PDGF (+PDGF) were analyzed in an immune complex kinase assay using [Val-5]angiotensin II as an exogenous substrate. Peptide phosphorylation was measured by quantitating the incorporation of ^{32}P-phosphate into acid-soluble materials that bind to phosphocellulose paper squares. The assays were carried out in the presence (unfilled bar) or absence (shaded bar) of [Val-5]angiotensin II to account for background incorporations that may have resulted from the immunoprecipitates alone. Results shown are averages of those from triplicate assays.

the anti-FER immunoprecipitates to a second round of immunoprecipitation under denaturing conditions. Following disruption of the complex, a single 97-kDa phosphoprotein was immunoprecipitated by the second anti-FER immunoprecipitation (Fig. 4B, lane 2). Reprobing of the same filter with anti-FER antiserum confirmed that the 97-kDa phosphoprotein was indeed the FER kinase (Fig. 4C). These results therefore further validated the specificity of the immunoprecipitation and also established the fact that FER undergoes an increase in tyrosine phosphorylation upon PDGF stimulation.

PDGF stimulates FER kinase activity. Since the enzymatic activities of many tyrosine kinases are stimulated by tyrosine phosphorylation, we performed an analysis to determine if PDGF treatment resulted in any change in FER kinase activity. In immune complex kinase assays, we detected the phosphorylation of polypeptides of 97 and 115 kDa (Fig. 5A, lanes 3 and 4). PDGF treatment resulted in an increase in the phosphorylation of the 97-kDa polypeptide but a reduction in the phosphorylation of the 115-kDa polypeptide. There was no detectable phosphorylation in immunoprecipitates prepared with preimmune serum (lanes 1 and 2). We also performed similar immune complex kinase assays but instead measured the kinase activity with [Val-5]angiotensin II as a soluble substrate. The results showed that the kinase activity in the anti-FER immune complex is stimulated 1.8- to 2-fold upon PDGF treatment (Fig. 5D).

FER associates with specific isoforms of pp120. We have shown that FER is associated with the substrate pp120 in A431 cells as well as in mouse fibroblasts. pp120 was originally identified as a substrate that undergoes increased phosphorylation in cells transformed with v-*src* or in cells stimulated with growth factors (31). Recently, it was found that pp120 is expressed as a number of distinct isoforms with molecular masses of between 90 and 115 kDa (50). We have shown that the forms of pp120 that coimmunoprecipitated with FER were primarily 115 kDa in size. To analyze the characteristics of these polypeptides in greater detail, we compared the composition of the pp120 isoforms in the anti-FER complex with those of the isoforms expressed in A431 cells and in Swiss 3T3 cells. For these analyses, we used a MAb that recognizes the carboxyl termini of the published pp120 sequence, which is common to all isoforms (50). As Reynolds et al. showed previously, epithelial cells such as A431 cells express a heterogeneous mixture of pp120 isoforms that are predominantly 95 to 100 kDa in size (Fig. 6B, lanes 1 and 2) (50). These isoforms show a significant basal level of tyrosine phosphorylation that is further stimulated by EGF treatment (Fig. 6A, lanes 1 and 2). However, only the high-molecular-weight isoforms of pp120 were detected in the FER immunoprecipitates (lanes 3 and 4). Unlike epithelial cells, Swiss 3T3 cells express predominantly the high-molecular-weight isoforms of pp120 and only

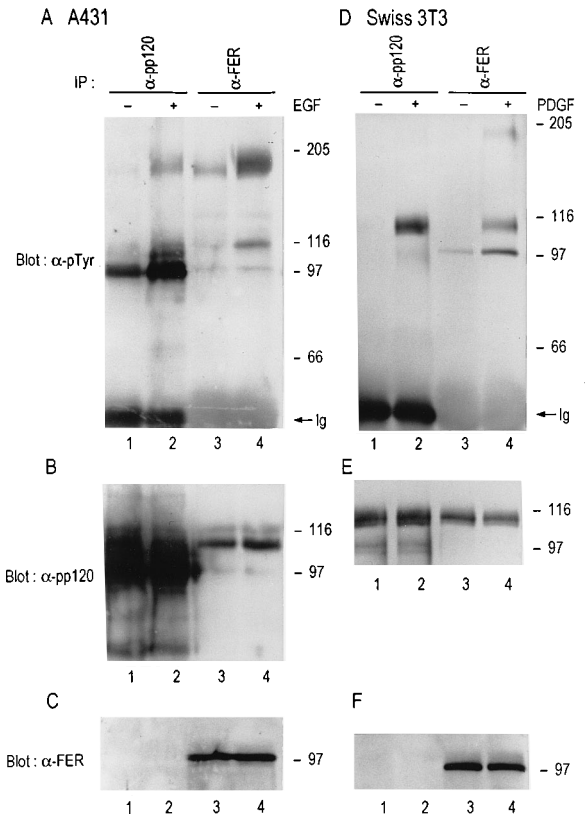

FIG. 6. Specificity of the FER-pp120 interactions in A431 and Swiss 3T3 cells. A431 and Swiss 3T3 cells were left untreated (lanes 1 and 3) or were treated with EGF or PDGF (lanes 2 and 4), and lysates were immunoprecipitated (IP) with a MAb to the C termini of pp120 isoforms (α-pp120) (lanes 1 and 2) or polyclonal anti-FER1 (α-FER) antiserum (lanes 3 and 4). The amounts of cell lysates used for the anti-pp120 immunoprecipitations were half that used for the anti-FER immunoprecipitations. Ig, immunoglobulin. (A) Immunoprecipitates from A431 cells were analyzed by blotting with an antiphosphotyrosine (α-pTyr) antibody. Panels B and C show the results of reprobing the membrane with the MAb to the C termini of pp120 isoforms and with anti-FER5 antiserum, respectively. (D) Immunoprecipitates from Swiss 3T3 cells were analyzed by blotting with an antiphosphotyrosine antibody. Panels E and F show results of reprobing the membrane from panel D with a monoclonal anti-pp120 antibody and with anti-FER5 antiserum, respectively.

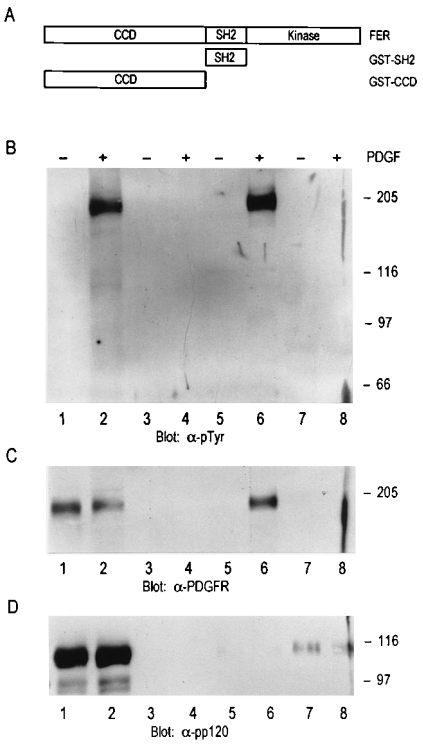

FIG. 7. Identification of the FER sequence that mediates complex formation with pp120. (A) Schematic illustration of the three major domains in the FER kinase and the two GST fusion proteins containing the SH2 domain (GST-SH2) and the amino terminus (GST-CCD). Lysates were prepared from untreated Swiss 3T3 cells (lanes 1, 3, 5, and 7) and cells that had been stimulated with PDGF-BB (lanes 2, 4, 6, and 8). The lysates were incubated with GST (lanes 3 and 4), GST-SH2 (lanes 5 and 6), or GST-CCD (lanes 7 and 8) proteins that had been purified by being bound to glutathione-Sepharose. (B) Proteins that bound to the beads as well as aliquots of whole-cell lysates (20 μg each) (lanes 1 and 2) were fractionated by SDS-PAGE and analyzed by blotting with antiphosphotyrosine (α-pTyr) antibodies. Panels C and D show results of reprobing the membrane from panel A with a polyclonal anti-PDGF receptor (PDGFR) antiserum and with a MAb to the C termini of pp120, respectively.

trace amounts of the smaller isoforms (Fig. 6E, lanes 1 and 2). PDGF stimulated the tyrosine phosphorylation of pp120, but no significant phosphorylation was detected in quiescent cells (Fig. 6D, lanes 1 and 2). Again, the smaller isoforms appeared to be excluded from the FER immunoprecipitates (Fig. 6E, lanes 3 and 4). The results of these experiments suggest that the association of FER and pp120 requires sequences that are present only in the high-molecular-weight isoforms of pp120. For both A431 and Swiss 3T3 cells, we were not able to detect FER in the anti-pp120 immunoprecipitates (Fig. 6C and F).

The amino terminus of FER mediates complex formation with pp120. The association of FER and pp120 is observed in quiescent Swiss 3T3 cells when no tyrosine phosphorylation of pp120 can be detected. The constitutive nature of the association suggests that it does not involve interaction between the SH2 domain of FER and phosphotyrosines. To identify the part of the FER sequence that mediates the complex formation, we analyzed the binding specificities of GST fusion proteins that contained different segments of the FER sequence (Fig. 7A). Lysates of untreated and PDGF-stimulated Swiss 3T3 cells were incubated with the GST fusion proteins, and the complexes that formed were analyzed by immunoblotting. The SH2 domain of FER forms a complex with a phosphorylated polypeptide of 180 kDa, which was shown to react with antibodies to the PDGF receptor (Fig. 7B and C, lanes 6). A fragment that contained 400 amino acids of the amino terminus of FER (CCD) was able to associate with pp120 (Fig. 7D, lanes 7 and 8). In a number of separate experiments, we consistently observed a reduction in the amount of pp120 that complexed with the CCD fragment in lysates of PDGF-treated cells. Also, the pp120 in these complexes showed no reactivity to antiphosphotyrosine antibodies (Fig. 7A). Although the SH2 domain of FER showed a slight ability to bind pp120, we found no additive or synergistic effects when the two sequences were expressed contiguously in one fragment (data not shown).

The amino terminus of FER is an alpha-helical coiled coil. The observation that the amino terminus, but not the SH2 domain, of FER mediates the association with pp120 is consistent with the constitutive nature of the association. The 400 residues in the amino terminus of FER do not bear any resemblance to other motifs found in other signal transduction molecules, such as the SH3 and pleckstrin homology motifs (46). Upon a close examination of the sequence, we found that it may possess features that are characteristic of alpha-helical coiled coils. The sequence was therefore analyzed by using an algorithm designed to detect coiled coil domains (39). The

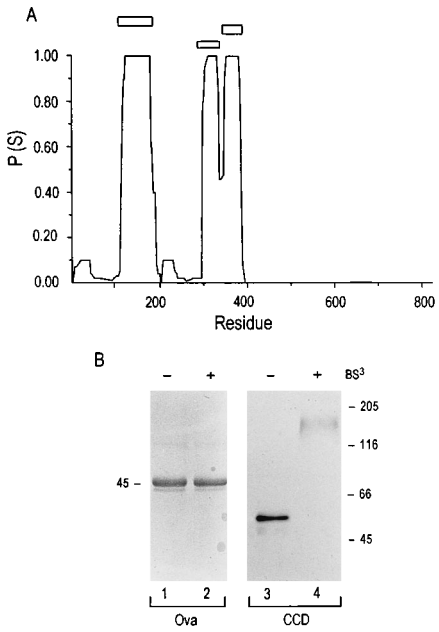

FIG. 8. The amino terminus of FER forms a coiled coil. (A) The amino acid sequence of FER was analyzed by using the PEPCOIL algorithm in the Genetics Computer Group package. The *P*(S) score, which measures the probability that any residue and its surrounding sequence will form coiled coils, is plotted against the number of amino acids in the sequence. A more detailed description of the analysis is presented in the text. (B) Chemical cross-linking of the purified amino-terminal fragment of FER. The CCD fragment, which was expressed as a bacterial GST fusion protein, was purified and excised with thrombin. Ovalbumin (Ova) (lanes 1 and 2) or the CCD peptide (lanes 3 and 4) was incubated in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of the cross-linking reagent bis(sulfosuccinimidyl)suberate (BS^3), and the reaction products were fractionated by SDS-PAGE. The reaction products in lanes 1 and 2 were stained with Coomassie blue, whereas those in lanes 3 and 4 were detected by blotting with anti-FER4 antiserum.

analysis revealed that a 57-amino-acid segment from residue 122 to 178 had a maximum score of 1.76, whereas a second extended segment from residue 301 to 390 had a maximum score of 1.60 (Fig. 8A). The latter segment had a discontinuity between residues 348 and 354. The probabilities of forming coiled coils for these sequences were between 0.96 and 1.0. In such analyses, sequences that had been found to be coiled coils all scored above 1.30, whereas those that scored between 1.1 and 1.3 were generally extended amphipathic alpha-helices in globular proteins (39). For comparison, the same algorithm previously calculated a maximum score of 1.91 for yeast GCN4, which was experimentally shown to form coiled coils (45). To verify that the amino terminus of FER does indeed have the ability to form coiled coils, we analyzed the ability of the sequence to oligomerize by chemical cross-linking. The CCD fragment was expressed in bacteria as a GST fusion protein and was recovered after in situ cleavage with thrombin. Following a brief incubation with the chemical cross-linker bis(sulfosuccinimidyl)suberate, the 52-kDa CCD fragment was quantitatively converted to higher-molecular-mass species (Fig. 8B, lanes 3 and 4). The molecular mass of the cross-linked materials was about 150 kDa, suggesting that the majority of the fragment was covalently linked as homotrimers. In contrast, no significant oligomerization was detected in parallel incubations that contained ovalbumin and bis(sulfosuccinimidyl)suberate (Fig. 8B, lanes 1 and 2).

DISCUSSION

In this report we have shown that the cytoplasmic kinase FER forms a complex that includes the substrate pp120 and a phosphorylated growth factor receptor. Upon stimulation of cells with EGF or PDGF, FER becomes associated with the phosphorylated growth factor receptor. In fibroblasts, the stoichiometry of receptor association with FER is quite low. Our estimate is that less than 1% of the PDGF receptor is complexed with FER in stimulated Swiss 3T3 cells. The low abundance of the receptor in the anti-FER immunoprecipitates precluded any determination of its presence or absence in the complex isolated from quiescent cells. For A431 cells, the physical association with the EGF receptor occurs even in the absence of EGF stimulation. However, the high receptor density in these cells results in a significant basal level of receptor phosphorylation. Results of the in vitro binding experiments suggest that interaction with the receptor may be mediated by the SH2 domain of the FER kinase and phosphorylated tyrosines of the receptor. Our data are therefore consistent with a model in which the activated receptor interacts with the FER-pp120 complex, possibly through interactions with the phosphotyrosines on the receptor. However, this aspect of the proposed mechanism in which FER becomes activated will have to be validated in the future when the composition of the FER-pp120 complex becomes better defined. At the moment, we cannot exclude the possibility that the complex contains additional components that are not phosphorylated and are therefore not yet identified. These additional components may contain SH2 domains that can mediate the interaction with the activated receptors. Stimulation of the PDGF receptor in Swiss 3T3 cells results in a twofold activation of the kinase activity associated with FER. The stimulation of the peptide kinase activity correlates with an increase in the in vitro phosphorylation of FER itself, thus suggesting that FER is activated. However, we cannot formally exclude the possibility that an associated kinase, distinct from FER, was stimulated by PDGF treatment. The extent of kinase activation is comparable to that observed for the activation of c-*src* kinase activity by PDGF or colony-stimulating factor 1 (8, 35, 49). As was noted previously for the activation of c-*src* kinase, the magnitude of the activation is most likely underestimated, because the immune complex kinase assays measure the total cellular enzyme activity but only a small fraction of that activity is affected by the growth factor receptor through direct physical association (8, 35). In the immune complex kinase assay, we observed a reduction in the in vitro phosphorylation of pp120 in the anti-FER immunoprecipitate prepared from PDGF-treated cells. This can be attributed to the fact that the pp120 in the FER immune complex was already extensively phosphorylated.

We have shown that FER and pp120 are parts of a stable complex. The tyrosine kinase substrate, pp120, was originally identified by using MAbs prepared against polypeptides that are phosphorylated in v-*src*-transformed cells (31, 32). Tyrosine phosphorylation of pp120 is stimulated also in cells treated with EGF or PDGF (9, 31). The predicted amino acid sequence of pp120 contains multiple copies of the so-called *armadillo* repeat, which are also present in a *Drosophila melanogaster* segment polarity gene product, β-catenin, plakoglobin (γ-catenin), and the adenomatous polyposis coli gene product (47, 51). β-Catenin and plakoglobin are cytoplasmic proteins that form complexes with α-catenin and cell adhesion molecules called cadherins, which mediate the formation of calcium-dependent cell-cell junctions (16). The cadherin-catenin complex has been shown to play a major role in morphogenesis. In addition, the complex has a tumor suppressive property

by virtue of its ability to regulate cell growth (6, 14). The catenins are present in multiple cellular pools and form multiple complexes with distinct compositions, possibly involving other additional polypeptides (26, 44). One such complex includes α- and β-catenins as well as the adenomatous polyposis coli gene product (52, 58). Recently, it was shown that pp120 is also associated with some components of the cadherin-catenin complex in MDCK cells (50). On the basis of the presence of the *armadillo* repeats in the sequence of pp120 and its association with the cadherin-catenin complex, it was suggested that pp120 may be a novel catenin (50). Prior to the discovery of the association of pp120 and the catenins, tyrosine phosphorylation had been implicated in the regulation of the cadherin-catenin complex. Transformation of epithelial cells and fibroblasts with v-*src* leads to the phosphorylation of the cadherin-catenin complex and the deterioration of intercellular junctions (2, 22, 43). Stimulation of some epithelial cells with EGF also results in the tyrosine phosphorylation of β-catenin and plakoglobin (28). However, it remains to be determined how tyrosine phosphorylation of the catenins regulates the function of the cadherin-catenin complex.

Although pp120 is expressed as several isoforms at ratios that are cell specific, those isoforms that are associated with FER are exclusively the high-molecular-weight isoforms in epithelial cells as well as in fibroblasts. As Reynolds et al. showed previously, the different isoforms differ in the sequences of their amino termini (50). This suggests that the amino termini of the longer isoforms contain the sequence that is required for association with FER. We have not been able to detect FER in Western blot analyses of anti-pp120 immunoprecipitates. It is possible that the subpopulation of pp120 that is in the FER-pp120 complex is not accessible to the antibodies for immunoprecipitation. This possibility is not unlikely, given that pp120 probably interacts with multiple partners. We have not determined if FER and pp120 interact directly or if the interaction is mediated by other components. That issue, as well as the possible involvement of other catenins in the FER-pp120 complex, is currently being analyzed further. We estimate that between 10 and 15% of the pp120 in the cell is associated with FER. We believe that the FER-pp120 association may be negatively regulated by the phosphorylation of pp120. This theory is based on the fact that the extent of phosphorylation of pp120 in the FER immunoprecipitates was much less than that of the total pp120 (Fig. 6D). Also, the pp120 precipitated by the GST-CCD fusion protein from PDGF-treated cell lysates was not detectably phosphorylated. Together, these data suggest that FER preferentially associates with unphosphorylated or underphosphorylated p120.

We showed that the 400 residues in the amino terminus of FER can mediate association with pp120 in vitro. The ability of the CCD fragment to associate with pp120 in vitro was reduced in lysates prepared from PDGF-stimulated cells. We also consistently observed a diminution in the phosphorylation of pp120 in immune complex kinase assays using lysates of PDGF-stimulated cells, whereas the phosphorylation of FER itself and that of an exogenous peptide substrate were stimulated. One possible explanation is that the association of FER and pp120 is modulated by the extent of phosphorylation of pp120. Although we have not excluded other explanations for the apparent difference, this question will have to be investigated further, as it may provide a clue to how the FER kinase may regulate the function of pp120. The immune complex kinase assay demonstrates that FER can phosphorylate pp120, at least in vitro. While this does not provide formal proof that FER phosphorylates pp120 in vivo, their physical association argues strongly for an enzyme-substrate relationship between FER and pp120. Although pp120 is commonly referred to as a substrate of v-*src*, there is as yet no evidence that the *src* kinase phosphorylates pp120 directly (50). There is a possibility that some of the pp120 isoforms, such as those that are specific to epithelial cells and do not associate with FER, may be targets of more than one tyrosine kinase. Although the cadherin-catenin complexes are believed to be essential to epithelial function, the components of the complexes are also expressed in other cell types, such as fibroblasts, and their function in nonepithelial tissues is not known. The identification of the FER-catenin complex should facilitate our efforts to understand the function of pp120 and other catenins in epithelial and nonepithelial cells.

The involvement of the amino terminus of FER in the association with pp120 is consistent with the constitutive nature of the association. Interestingly, the sequence is predicted to form coiled coils, which are known to mediate protein oligomerization. As predicted, the CCD fragment forms oligomers in vitro. It remains to be determined if the coiled coil sequences are required solely for oligomerization of FER or if they mediate interactions with other components, such as pp120. In recent years, coiled coil domains have been shown to be present not only in fibrous proteins, but also in regulatory components such as G-protein subunits (7, 15, 38). Moreover, coiled coil domains in some proteins may be quite dynamic and may play an active role in mediating structural changes in response to regulatory signals (5). Therefore, the amino terminus of FER may be a key element in the function of the kinase in relaying signals generated by growth factor stimulation. The association of FER and pp120 suggests that FER may be involved in a signal transduction pathway that regulates, or is regulated by, cell-cell interactions. The structural features in FER that we have identified are consistent with this proposed function of the kinase.

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