Src Family Kinases Are Required for Integrin-mediated but Not for G Protein-coupled Receptor Stimulation of Focal Adhesion Kinase Autophosphorylation at Tyr-397*

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Plating suspended Swiss 3T3 cells onto fibronectin-coated dishes promoted phosphorylation of endogenous focal adhesion kinase (FAK) at Tyr-397, the major autophosphorylation site, and at Tyr-577, located in the activation loop, as revealed by site-specific antibodies that recognize the phosphorylated form of these residues. Treatment with the selective Src family kinase inhibitor pyrazolopyrimidine 2 (PP-2) markedly reduced the phosphorylation of both Tyr-397 and Tyr-577 induced by fibronectin. Furthermore, fibronectin-mediated FAK phosphorylation at Tyr-397 was dramatically reduced in SYF cells (deficient in Src, Yes, and Fyn expression). Stimulation of Swiss 3T3 cells with bombesin also induced a rapid increase in the phosphorylation of endogenous FAK at Tyr-397. In contrast to the results obtained with fibronectin, PP-2 did not prevent FAK Tyr-397 phosphorylation stimulated by bombesin at a concentration (10 μM) that suppressed bombesin-induced FAK Tyr-577 phosphorylation. Similarly, PP-2 did not prevent Tyr-397 phosphorylation in Swiss 3T3 cells stimulated with other G protein-coupled receptor agonists including vasopressin, bradykinin, endothelin, and lysophosphatidic acid. Lysophosphatidic acid also induced FAK phosphorylation at Tyr-397 in SYF cells. Our results identify, for first time, the existence of Src-dependent and Src-independent pathways leading to FAK autophosphorylation at Tyr-397 stimulated by adhesion-dependent signals and G protein-coupled receptor agonists in the same cell. A rapid increase in the tyrosine phosphorylation of the non-receptor tyrosine kinase FAK1 (1, 2), which localizes to focal adhesion plaques, has been identified as a prominent early event in cells stimulated by diverse signaling molecules that regulate cell proliferation, migration, and apoptosis (3–5). In particular, FAK is activated and tyrosine-phosphorylated in response to integrin clustering induced by cell adhesion or antibody cross-linking (1, 2, 6–9). In addition, FAK is rapidly tyrosine-phosphorylated in cells stimulated by mitogenic neuropeptide agonists including bombesin (10–16) and bioactive lipids including LPA (17–19) that act via heptahelical GPCRs, polypeptide growth factors (20–24), bacterial toxins (25, 26), and activated variants of pp60Src (27, 28). The importance of FAK-mediated signal transduction is underscored by experiments implicating this tyrosine kinase in embryonic development (29) and in the control of cell migration (30–33), proliferation (30, 34), and apoptosis (35, 36). In addition, there is increasing evidence linking overexpression of FAK to the invasive properties of cancer cells (37, 38). It is increasingly recognized that the tyrosine phosphorylation and activation of FAK is an important point of convergence in the action of integrins, GPCR agonists, growth factors, and oncogenes (5, 39, 40). However, the molecular pathways leading to FAK tyrosine phosphorylation in response to multiple extracellular factors remain incompletely understood.

Plating suspended cells onto fibronectin-coated dishes, a paradigm of integrin receptor activation (41), induces adhesion-dependent phosphorylation of FAK at multiple sites including tyrosines 397, 576, 577, 861, and 925 (32, 42–45). Tyr-397, the only apparent autophosphorylation site (46–51), has emerged as a critical residue in FAK-mediated signaling (5). The autophosphorylation of FAK at Tyr-397 creates a high affinity binding site for the SH2 domain of Src family kinases including Src, Yes, and Fyn and leads to the formation of a signaling complex between FAK and Src family kinases (46, 47, 49–52). A model has recently been proposed that envisages reciprocal catalytic activation of FAK and Src family kinases in response to adhesion-dependent signals. Src family kinases associated with FAK are thought to phosphorylate FAK at additional sites including Tyr-576 and Tyr-577 that are located in the activation loop of the kinase catalytic domain of FAK (32, 42, 53), thereby promoting maximal FAK catalytic activation. Because phenylalanine mutation of Tyr-576 and Tyr-577 reduced adhesion-mediated FAK autophosphorylation (at Tyr-397), it has been proposed that activation loop phosphorylation of FAK by Src stimulates intermolecular phosphorylation at Tyr-397, thereby leading to signal amplification at sites of integrin-mediated cell adhesion (32). Therefore, in this model Src family kinases are thought to play a major role leading to autophosphorylation of FAK at Tyr-397 as part of an adhesion-dependent signaling response. Recently, we demonstrated that GPCR agonists including bombesin and LPA also induce rapid activation of Src family kinases (54) and promote the formation of a FAK/Src signaling complex (55). However, it is not known whether Src family kinases are also required for promoting FAK phosphopho-
rylation at Tyr-397 in cells stimulated with GPCR agonists, as would be predicted by the signal amplification model.

In the present study, we report that stimulation with the mitogenic GPCR agonists bombesin, bradykinin, endothelin, vasopressin, and LPA induced rapid phosphorylation of endogenous FAK at Tyr-397 in intact Swiss 3T3 cells, an effect comparable to that stimulated by integrin-mediated cell adhesion. Treatment with the selective Src family kinase inhibitor PP-2 inhibited the phosphorylation of Tyr-397 induced by fibronectin. Furthermore, integrin-mediated FAK phosphorylation at Tyr-397 was dramatically reduced in SYF cells (deficient in Src, Yes, and Fyn expression). In striking contrast, PP-2, at a concentration that abolished activation loop phosphorylation, did not prevent FAK Tyr-397 phosphorylation in SYF cells stimulated by bombesin, LPA, or other GPCR agonists. In addition, LPA also induced FAK phosphorylation at Tyr-397 in SYF cells. Our results demonstrate, for the first time, that the signaling events leading to the phosphorylation of FAK at Tyr-397 induced by GPCR agonists are Src-independent and thus can be distinguished from those stimulated by integrin receptors that require Src family kinase activity.

EXPERIMENTAL PROCEDURES

Cell Culture—Stock cultures of Swiss 3T3 cells were maintained in DMEM, supplemented with 10% fetal bovine serum in a humidified atmosphere containing 10% CO2 and 90% air at 37 °C. For experimental purposes, Swiss 3T3 cells were plated in 100-mm dishes at 7 × 10^5 cells/dish in DMEM containing 10% fetal bovine serum and used after 5 days when the cells were confluent.

Cell Stimulation with Bombesin or Other Agonists—Confluent and quiescent Swiss 3T3 cells were washed twice with DMEM, equilibrated in the same medium at 37 °C for at least 30 min, and then treated with bombesin or other agonists for the times indicated. The stimulation was terminated by aspirating the medium and solubilizing the cells in 1 ml of ice-cold RIPA buffer containing 50 mM HEPES, pH 7.4, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 10 mM NaF, and 1 mM phenylmethylsulfonyl fluoride.

Immunoprecipitation—Lysates were clarified by centrifugation at 4 °C, rinsed with PBS, and then treated with 0.05% trypsin, 2 mM EDTA in PBS. The trypsin was inactivated by soybean trypsin inhibitor (0.5 mg/ml) with 0.25% bovine serum albumin in DMEM. Cells were collected by centrifugation, resuspended in DMEM containing 0.1% bovine serum albumin, and held in suspension for 1 h at 37 °C. Cell culture dishes (10 cm) were pre-coated with fibronectin purified from bovine plasma (100 μg/ml) or poly-L-lysine (100 μg/ml) in PBS overnight at 4 °C, rinsed with PBS, and warmed to 37 °C for 1 h prior to plating. Suspended cells were distributed onto ligand-coated dishes and incubated at 37 °C, and at various times following plating, protein extracts were made in RIPA buffer as described previously. Confluent cultures of SYF and YF cells were incubated for 40 h in DMEM containing 0.1% fetal bovine serum and then treated as described above for Swiss 3T3 cells.

Cell Stimulation with Fibronectin or Adherence to Poly-L-lysine—Confluent and quiescent Swiss 3T3 cells were harvested by limited trypsin/EDTA treatment (0.05% trypsin, 2 mM EDTA in PBS). The trypsin was inactivated by soybean trypsin inhibitor (0.5 mg/ml) with 0.25% bovine serum albumin in DMEM. Cells were collected by centrifugation, resuspended in DMEM containing 0.1% bovine serum albumin, and held in suspension for 1 h at 37 °C. Cell culture dishes (10 cm) were pre-coated with fibronectin purified from bovine plasma (100 μg/ml) or poly-L-lysine (100 μg/ml) in PBS overnight at 4 °C, rinsed with PBS, and warmed to 37 °C for 1 h prior to plating. Suspended cells were distributed onto ligand-coated dishes and incubated at 37 °C, and at various times following plating, protein extracts were made in RIPA buffer as described previously. Confluent cultures of SYF and YF cells were incubated for 40 h in DMEM containing 0.1% fetal bovine serum and then treated as described above for Swiss 3T3 cells.

 Autoradiograms were scanned by densitometric scanning of the bands. Values shown are the mean ± S.E. of at least three independent experiments and are expressed as the percentage of the maximal increase in FAK tyrosine phosphorylation value above control (unstimulated) values.

Fig. 1. Role of Src activity in FAK phosphorylation at Tyr-397 in response to fibronectin. A, Swiss 3T3 cells held in suspension were treated for 15 min in the absence (0) or in the presence of increasing concentrations of PP-2, as indicated, and then plated on either poly-L-lysine-coated dishes (P-LYS) or fibronectin-coated dishes (FIB) for 30 min and subsequently lysed. FAK Tyr-397 phosphorylation was analyzed by immunoprecipitation (IP) using anti-FAK Ab C-20 followed by Western blotting with anti-FAK-Tyr(P)-397. B, Swiss 3T3 cells held in suspension were treated for 15 min in the absence (0) or in the presence (+) of 10 μM PP-2 or 10 μM PP3, as indicated. Cells were then plated on poly-L-lysine-coated dishes (P-LYS) or fibronectin-coated dishes (FIB) for 30 min, as indicated. The cells were then lysed, and the extracts were incubated with anti-FAK antibody C-20. The immunoprecipitates were analyzed by Western blotting with anti-FAK-Tyr(P)-397 Ab. Inset, Swiss 3T3 cells held in suspension were treated as described in B. The cells were lysed, and the extracts were incubated with anti-FAK Ab C-20, and the immunoprecipitates were analyzed by Western blotting with anti-FAK-Tyr(P)-577 Ab. In all cases, the membranes were analyzed further by Western blotting with anti-FAK Ab. The positions of FAK-Tyr(P)-397, FAK-Tyr(P)-577, and FAK are indicated by arrows. The autoradiograms shown are representative of at least three independent experiments. Quantification of FAK phosphorylation at Tyr-397 and Tyr-577 was performed by densitometric scanning of the bands. Values shown are the mean ± S.E. of at least three independent experiments and are expressed as the percentage of the maximal increase in FAK tyrosine phosphorylation value above control (unstimulated) values.
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15,000 rpm for 10 min. Supernatants were transferred to fresh tubes, and proteins were immunoprecipitated at 4 °C for 4 h with protein A-agarose linked to polyclonal anti-FAK (C-20) antibody, as described previously (10, 11). Immunoprecipitates were washed three times with RIPA buffer and extracted in 2× SDS-PAGE sample buffer (200 mm Tris-HCl, pH 6.8, 1 mM EDTA, 6% SDS, 4% 2-mercaptoethanol, 10% glycerol) by boiling 10 min and resolved by SDS-PAGE.

Western Blotting—After SDS-PAGE, proteins were transferred to Immobilon membranes. After transfer, membranes were blocked using 5% nonfat dried milk in PBS, pH 7.2, and incubated overnight at 4 °C with the anti-FAK-Tyr(P)-397 Ab (0.1 μg/ml) or anti-FAK-Tyr(P)-577 Ab (0.5 μg/ml), as indicated. The membranes were washed three times with PBS, 0.1% Tween 20 and then incubated with secondary antibodies (horseradish peroxidase-conjugated donkey antibodies to rabbit, NA934) for 1 h at 22 °C. After washing three times with PBS, 0.1% Tween 20, the immunoreactive bands were visualized using ECL detection reagents. Autoradiograms were scanned using the GS-710 Calibrated Imaging Densitometer (Bio-Rad), and the labeled bands were quantified using the Quantity One software program (Bio-Rad).

Materials—Bombesin, endothelin, bradykinin, vasopressin, LPA, bovine fibronectin, and poly-L-lysine were obtained from Sigma. Horseradish peroxidase-conjugated donkey antibodies to rabbit, NA934, and ECL reagents were from Amersham Pharmacia Biotech. PP-2 and PP-3 were obtained from Calbiochem-Novabiochem. FAK polyclonal Ab C-20 was from Santa Cruz Biotechnology (Santa Cruz, CA). The phosphospecific polyclonal Ab to Tyr-397 and Tyr-577 of FAK was obtained from BIOSOURCE International (Camarillo, CA). All other reagents used were of the purest grade available.

RESULTS AND DISCUSSION

The Role of Src Family Kinase Activity in FAK Phosphorylation at Tyr-397 in Response to Fibronectin—In order to examine the role of Src family kinases in integrin-mediated phosphorylation of FAK at Tyr-397, we determined the effect of the pyrazolopyrimidine PP-2, a selective inhibitor of Src family kinase members (56, 57), on the phosphorylation FAK at Tyr-397 induced by fibronectin. Previously, we demonstrated that this compound, at concentrations that completely inhibited the catalytic activity of Src family kinases, did not interfere with FAK autophosphorylation activity (54, 57). Cultures of Swiss 3T3 cells placed in suspension were treated in the absence or in the presence of increasing concentrations of PP-2 (0.1–10 μM) and then plated onto dishes coated with either fibronectin or poly-L-lysine for 30 min. Cell lysates were immunoprecipitated with anti-FAK Ab, and the immune complexes were analyzed by SDS-PAGE followed by Western blotting with a phosphospecific antibody directed against the autophosphorylation site of FAK (anti-FAK-Tyr(P)-397 Ab). As shown in Fig. 1A, treatment of cells with PP-2 strikingly inhibited the phosphorylation of FAK at Tyr-397 in a concentration-dependent fashion. Maximal inhibition was obtained at 10 μM. In contrast, treatment with 10 μM PP-3, a structurally related but inactive analog of PP-2, did not interfere with integrin-mediated FAK phosphorylation at Tyr-397 (Fig. 1B).

FAK autophosphorylation at Tyr-397 creates a high affinity binding site for the SH2 domain of Src, and Src associated with FAK is thought to phosphorylate FAK at additional sites including Tyr-576 and Tyr-577, which are located in the kinase catalytic domain of FAK. Treatment with 10 μM PP-2 completely blocked the phosphorylation of FAK Tyr-577 induced by fibronectin (inset, Fig. 1B, right). These results suggest that Src family kinases are required for fibronectin-induced FAK phosphorylation at both activation loop and autophosphorylation sites.

FAK Phosphorylation at Tyr-397 in Response to Fibronectin Is Greatly Diminished in Cells Deficient in Src, Yes, and Fyn (SYF Cells)—Recently, Klinghoffer et al. (58) reported that integrin-induced tyrosine phosphorylation of FAK was markedly reduced in SYF cells, but the phosphorylation of specific sites was not analyzed. In order to substantiate that Src family kinases are required for FAK Tyr-397 phosphorylation induced by fibronectin, as suggested by the pharmacological results shown in Fig. IA, we examined integrin-induced FAK Tyr-397 phosphorylation in SYF cells and in cells deficient in Yes and Fyn but not in Src (YF cells). As illustrated in Fig. 2, plating YF cells (which express c-Src) onto fibronectin induced a marked increase in FAK Tyr-397 phosphorylation as compared with either parallel cultures plated on poly-L-lysine or to SYF cells plated on fibronectin. The increase in FAK Tyr-397 phosphorylation induced by fibronectin in YF cells was virtually abrogated by treatment with 10 μM PP-2. In contrast, plating SYF cells on fibronectin induced only a small (but measurable) increase in FAK Tyr-397 phosphorylation, as compared with SYF cells plated on poly-L-lysine. Interestingly, the small increase in fibronectin-induced FAK Tyr-397 phosphorylation in the absence of Src, Yes, and Fyn was not affected by treatment with 10 μM PP-2. Taken together, the results presented in Figs. 1 and 2 indicate that Src family kinases are required for maximal integrin-induced FAK autophosphorylation and substantiate the specificity of PP-2 as an Src family inhibitor since this agent did not exert any inhibitory effect on FAK Tyr-397 phosphorylation in cells lacking Src, Yes, and Fyn.

FIG. 2. FAK phosphorylation at Tyr-397 in response to fibronectin is greatly diminished in cells deficient in SYF cells. SYF and YF cells held in suspension were treated for 15 min in the absence (−) or in the presence (+) of 10 μM PP2. Cells were then plated on either poly-L-lysine or fibronectin-coated dishes for 30 min, as indicated. The cells were then lysed, and the extracts were incubated with anti-FAK Ab C-20 followed by Western blotting with anti-FAK-Tyr(P)-397 Ab. The membranes were analyzed further by Western blotting with anti-FAK Ab. The positions of FAK-Tyr(P)-397 and FAK are indicated by arrows. The autoradiograms shown are representative of at least three independent experiments. Quantification of FAK phosphorylation at Tyr-397 was performed by densitometric scanning of the bands. Values shown are the mean ± S.E. of at least three independent experiments and are expressed as the percentage of the maximal increase in FAK tyrosine phosphorylation value above control (unstimulated) values.

The Role of Src Family Kinase Activity in Integrin-mediated Stimulation of FAK—Several laboratories have suggested that integrin activation by fibronectin induces FAK phosphorylation at Tyr-397 in Swiss 3T3 cells, quiescent cultures of these cells were treated with 10 nM bombesin for various times and lysed. The extracts were im-
munoprecipitated with a polyclonal anti-FAK Ab, which recognizes the C-terminal sequence of FAK, and the immune complexes were analyzed by SDS-PAGE followed by Western blotting using a site-specific antibody (anti-FAK-Tyr(P)-397) that recognizes the phosphorylated state of FAK at Tyr-397.

As shown in Fig. 3A (upper panel), bombesin stimulation of Swiss 3T3 cells induced a rapid and marked increase in the phosphorylation of FAK at Tyr-397. Densitometric scanning showed that the phosphorylation of this residue reached a maximum 2 min after the addition of bombesin to intact cells. Immunoblotting with anti-FAK antibody of FAK immunoprecipitates verified that similar amounts of FAK were recovered after different times of bombesin treatment (Fig. 3A, lower panel). These results demonstrate that bombesin induces FAK phosphorylation at Tyr-397, the major autophosphorylation site of FAK that plays a critical role in FAK signaling.

To determine whether Src family kinase activity is required for FAK phosphorylation at Tyr-397 induced by bombesin, cultures of Swiss 3T3 cells were treated in the absence or in the presence of increasing concentrations of PP-2 and then stimulated with this agonist. As shown in Fig. 3B, exposure to PP-2 did not reduce the level of phosphorylation at Tyr-397 induced by bombesin stimulation, even at a concentration (10 μM) that prevented integrin-induced phosphorylation of FAK at Tyr-397 and Tyr-577 (Fig. 1). In fact, the level of bombesin-induced FAK Tyr-397 phosphorylation in cells treated with PP-2 was indistinguishable from that obtained in control (untreated) cultures or in cultures treated with the inactive analog PP-3 (Fig. 3B, lower panel).
at least three independent experiments. Quantification of FAK-Tyr(P)-397 was performed by scanning densitometry of the bands. Values shown in suspension were treated for 15 min in the absence (−) or in the presence (+) of 10 μM PP-2 and then plated on poly-L-lysine-coated dishes (P-LYS) for 40 min or on fibronectin-coated dishes (FIB) for various times, as indicated, and subsequently lysed. FAK phosphorylation at Tyr-397 was analyzed by immunoprecipitation (IP) using anti-FAK antibody C-20 followed by Western blotting with anti-FAK-Tyr(P)-397. In all cases, the membranes were analyzed further by Western blotting using anti-FAK Ab. The positions of FAK-Tyr(P)-397 and FAK are indicated by arrows. The autoradiograms shown are representative of at least three independent experiments. Quantification of FAK-Tyr(P)-397 was performed by scanning densitometry of the bands. Values shown are the mean ± S.E. of at least three independent experiments and are expressed as the percentage of the maximal increase in FAK-Tyr(P)-397 value above control (unstimulated or plated on poly-L-lysine) values.

**Fig. 4.** Kinetics of FAK phosphorylation at Tyr-397 in response to bombesin or fibronectin in Swiss 3T3 cells. A, Swiss 3T3 cells held in suspension were treated for 15 min in the absence (−) or in the presence (+) of 10 μM PP-2 and then plated on poly-L-lysine-coated dishes (P-LYS) for 40 min or on fibronectin-coated dishes (FIB) for various times, as indicated, and subsequently lysed. FAK phosphorylation at Tyr-397 was analyzed by immunoprecipitation (IP) using anti-FAK antibody C-20 followed by Western blotting with anti-FAK-Tyr(P)-397. B, confluent and quiescent Swiss 3T3 cells were treated for 15 min in the absence (−) or in the presence (+) of 10 μM PP-2 and then stimulated with 10 nM bombesin (BOM) for various times as indicated and subsequently lysed. FAK phosphorylation at Tyr-397 was analyzed by immunoprecipitation using anti-FAK antibody C-20 followed by Western blotting with anti-FAK-Tyr(P)-397. In all cases, the membranes were analyzed further by Western blotting using anti-FAK Ab. The positions of FAK-Tyr(P)-397 and FAK are indicated by arrows. The autoradiograms shown are representative of at least three independent experiments. Quantification of FAK-Tyr(P)-397 was performed by scanning densitometry of the bands. Values shown are the mean ± S.E. of at least three independent experiments and are expressed as the percentage of the maximal increase in FAK-Tyr(P)-397 value above control (unstimulated or plated on poly-L-lysine) values.

We verified that similar amounts of FAK were recovered after treatment with or without bombesin and with or without PP-2 and PP-3 (Fig. 3B, lower panels).

Western blotting of FAK immunoprecipitates with the site-specific antibody anti-FAK-Tyr(P)-577 revealed that bombesin stimulation also induced phosphorylation of FAK at Tyr-577 in Swiss 3T3 cells (Fig. 3C). Treatment of Swiss 3T3 cells with increasing concentrations of PP-2 prevented phosphorylation of FAK at Tyr-577 induced by bombesin in a concentration-dependent manner. Half-maximal and maximal inhibitory effects were achieved at 2 and 10 μM, respectively. We verified that similar amounts of FAK were recovered after treatment with increasing concentrations of PP-2 (Fig. 3C). Thus, PP-2, at a concentration that completely blocked FAK activation loop phosphorylation (Fig. 3C), did not prevent FAK autophosphorylation at Tyr-397 stimulated by bombesin (Fig. 3B).

**Differential Contribution of Src Family Kinase Activity to FAK Phosphorylation at Tyr-397 in Response to Bombesin and Fibronectin—**The results presented in Figs. 1–3 indicate that Src family kinases are required for fibronectin but not for bombesin receptor stimulation of FAK phosphorylation at Tyr-397. In order to substantiate further the existence of Src-dependent and -independent pathways leading to phosphorylation of FAK at Tyr-397, we examined the effect of PP-2 on the phosphorylation of FAK at Tyr-397 in Swiss 3T3 cells stimulated with either bombesin or fibronectin for various lengths of time.

As shown in Fig. 4A, exposure to PP-2 markedly attenuated the phosphorylation of FAK at Tyr-397 in cells plated onto fibronectin-coated dishes at all the times examined, supporting the participation of Src in FAK autophosphorylation induced by adhesion-dependent signals. In contrast, treatment with PP-2 did not affect the phosphorylation of FAK at Tyr-397 in response to bombesin stimulation for various times (Fig. 4B). We verified that similar amounts of FAK were recovered after different times of exposure to fibronectin, bombesin, or PP-2 (Fig. 4, lower panel). These results substantiate further the notion that Src family kinases are required for fibronectin but not for bombesin receptor stimulation of FAK phosphorylation at Tyr-397.

**Role of Src in FAK Phosphorylation at Tyr-397 and Tyr-577 in Response to Vasopressin, Bradykinin, LPA, and Endothelin—**The preceding results with bombesin prompted us to determine the role of Src family kinase activity in the phosphorylation of FAK at Tyr-397 and Tyr-577 induced by activation of other endogenously expressed GPCRs in Swiss 3T3 cells. Cultures of these cells were treated in the absence or in the presence of 10 μM PP-2 and then stimulated with 20 nM vasopressin, 20 nM bradykinin, 2 μM LPA, or 20 nM endothelin for 10 min and lysed. The lysates were immunoprecipitated with anti-FAK Ab, and the immune complexes were analyzed by SDS-PAGE followed by Western blotting with either anti-FAK-Tyr(P)-397 or anti-FAK-Tyr(P)-577 Abs. As shown in Fig. 5, cell stimulation with these agonists induced a marked increase in the phosphorylation of FAK at both Tyr-397 and Tyr-577. Treatment with 10 μM PP-2 completely prevented the increase in the phosphorylation of FAK at Tyr-397 (Fig. 5A) but did not affect the phosphorylation of FAK at Tyr-397 in response to vasopressin, bradykinin, LPA, or endothelin (Fig. 5B). We verified that similar amounts of FAK were recovered after treatment with these GPCR agonists with or without PP-2.

Since previous results indicated that SYF and YF cells express receptors for LPA (58), we also examined whether this agonist induces FAK Tyr-397 phosphorylation in these cells. As
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Differential role of Src family kinase activity in FAK phosphorylation at Tyr-397 and Tyr-577 induced by GPCR agonists in Swiss 3T3 cells. A, confluent and quiescent Swiss 3T3 cells were treated for 15 min either in the absence (−) or in the presence (+) of 10 μM PP-2. Cells were then incubated for a further 10 min in the absence or in the presence of 20 nM vasopressin (VAS), 20 nM bradykinin (BK), 2 μM LPA, or 20 nM endothelin (END), as indicated. The cells were then lysed, and the extracts were incubated with anti-FAK antibody C-20, followed by Western blotting with anti-FAK-Tyr(P)-397 Ab. B, confluent and quiescent Swiss 3T3 cells were treated as described in A. The cells were then lysed, and the extracts were incubated with anti-FAK antibody C-20, followed by Western blotting with anti-FAK-Tyr(P)-577 Ab. In all cases, the membranes were analyzed further by Western blotting with anti-FAK Ab. The positions of FAK-Tyr(P)-397, FAK-Tyr(P)-577, and FAK are indicated by arrows. The autoradiograms shown are representative of at least three independent experiments. Quantification of FAK-Tyr(P)-397 and FAK-Tyr(P)-577 was performed by scanning densitometry of the bands. Values shown are the mean ± S.E. of at least three independent experiments and are expressed as the percentage of the maximal increase in FAK-Tyr(P)-397 or FAK-Tyr(P)-577 value above control (unstimulated or plated on poly-ι-lysine) values.

Concluding Remarks—A rapid increase in the overall tyrosine phosphorylation of the non-receptor tyrosine kinase FAK has been extensively documented as an early event in the action of multiple extracellular stimuli that modulates cell growth, motility, differentiation, and apoptosis in a variety of cell types. It is increasingly recognized that the function of FAK in signal transduction depends on the phosphorylation of specific residues in this enzyme. In particular, autophosphorylation of FAK at Tyr-397 has emerged as a crucial event in the activation of multiple effector pathways.

The best characterized function of FAK autophosphorylation at Tyr-397 is the creation of a high affinity binding site for the SH2 domain of Src family members. Given that competition for the SH2 and/or SH3 domains of Src by high affinity allosteric ligands promotes enzymatic activation of this kinase (64, 65), the association of Src with FAK should lead to the formation of a molecular complex in which Src kinases are activated. A model has recently been proposed that envisages reciprocal catalytic activation of FAK and Src family kinases in response to adhesion-dependent signals (32). In the framework of this model, Src activated by binding to FAK Tyr-397 phosphorylates FAK at Tyr-576 and -577 which are located in the activation loop of the kinase catalytic domain of FAK (42, 53) and thereby promotes maximal FAK catalytic activity. Because FAK mutants in which Tyr-576 and Tyr-577 were substituted by phenylalanines show substantially reduced FAK Tyr-397 phosphorylation, relative to that of FAK, it has been proposed that activation loop phosphorylation FAK by Src family kinases stimulates intermolecular phosphorylation of FAK at Tyr-397, thereby leading to signal amplification at sites of integrin-mediated cell adhesion (32, 45). The results presented here demonstrate that treatment with the selective Src family kinase inhibitor PP-2, at concentrations that suppress activation loop phosphorylation, markedly attenuates FAK Tyr-397 phosphorylation in Swiss 3T3 cells plated onto fibronectin-coated dishes. Moreover, FAK phosphorylation at Tyr-397 in response to integrin engagement is drastically reduced in triple mutant cells lacking Src, Yes, and Fyn. Taken together, these findings are consistent with a model in which integrin-induced FAK autophosphorylation at Tyr-397 requires Src family kinase function.

Despite its importance, little is known about the effect of GPCR agonists on the phosphorylation of FAK at specific tyrosines, especially Tyr-397, the major site of autophosphorylation. Our results demonstrate that stimulation of intact Swiss 3T3 cells with bombesin induces a rapid increase in FAK phosphorylation at Tyr-397. Stimulation with other GPCR agonists...
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In conclusion, the results presented here indicate that phosphorylation of FAK at Tyr-397 induced by bombesin and other GPCRs is Src-independent, whereas the phosphorylation of this residue promoted via integrin-mediated cell adhesion requires Src family kinase function. Our results identify, for first time, the existence of distinct pathways leading to FAK phosphorylation at Tyr-397 stimulated by GPCR agonists and adhesion-dependent signals in the same cell.

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