Insulin-like Growth Factor I Stimulates Tyrosine Phosphorylation of p130Cas, Focal Adhesion Kinase, and Paxillin

ROLE OF PHOSPHATIDYLINOSITOL 3'-KINASE AND FORMATION OF A p130Cas-Crk COMPLEX

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Addition of insulin growth factor-I (IGF-I) to quiescent Swiss 3T3 cells rapidly induced tyrosine phosphorylation of the p130Crk-associated substrate (p130Cas), a novel adaptor protein localized at focal adhesions. Half-maximal effect was obtained at 0.6 nM. IGF-I also promoted the formation of a complex between p130Cas and c-Crk and elicited a parallel increase in the tyrosine phosphorylation of p125Fak and paxillin. IGF-I-induced p130Cas, p125Fak, and paxillin tyrosine phosphorylation could be dissociated from mitogen-activated protein kinase activity (5). The insulin receptor substrates 1 and 2 (IRS-1 and IRS-2) are rapidly phosphorylated on multiple tyrosine residues in response to IGF-I (6, 7) and act as docking proteins that bind, through their phosphorylated residues, SH2 domain-containing proteins which propagate the IGF-I signal (8). These include the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI 3-kinase) and the adaptor proteins Grb-2 and Shc which lead to activation of the Ras/Raf/ERK pathway (5, 9). In addition, IGF-I also promotes cell migration of normal and tumor cells and induces the recruitment of actin into membrane ruffles (10–13).

Changes in the organization of the actin cytoskeleton induced by many extracellular factors are accompanied by striking changes in the tyrosine phosphorylation of several signaling proteins localized at the focal adhesion plaques (14, 15), the distinct sites of the plasma membrane that are in close contact with the extracellular matrix proteins (16). Specifically, the tyrosine phosphorylation of the non-receptor tyrosine kinase p125Fak (17, 18) and of the adaptor proteins p130Cas (19) and paxillin (20, 21) is increased by diverse signaling molecules that regulate cell growth, differentiation, and migration including neurotrophic agonists (22–26), bioactive lipids (26–29), and growth factors (26, 30–32). The increase in the tyrosine phosphorylation of focal adhesion proteins induced by these agents is critically dependent on the integrity of the actin cytoskeleton (24, 27, 28, 30, 33) and is mediated by PI 3-kinase- and PKC-dependent and -independent pathways (24, 27, 31). Tyrosine phosphorylation of p125Fak, p130Cas, and paxillin is also induced by extracellular matrix proteins (17, 34–36) and transforming variants of p60src (37). Thus, the phosphorylation of these focal adhesion proteins represents a point of convergence in the action of growth factors, integrins, and oncogenes (14, 38).

In contrast to other growth factors, insulin has been shown to decrease the level of tyrosine phosphorylation of p125Fak and paxillin in a variety of cell types (39–43) through pathways involving Csk (41), phosphotyrosine phosphatase 1D (43), and integrins (44). The influence of IGF-I on the tyrosine phosphorylation of p125Fak and paxillin has been less studied and is the subject of conflicting reports (12, 44, 45). The effect of IGF-I on the tyrosine phosphorylation of p130Cas, a novel adaptor protein implicated in transformation (19) and recently identified as a critical mediator of cell migration (46, 47), is unknown.

In the present study we report, for the first time, that IGF-I induces a rapid and transient increase in the tyrosine phosphorylation of p130Cas in Swiss 3T3 cells, a useful model system to elucidate signal transduction pathways in the action of growth factors (48). Our results also show that IGF-I promotes the formation of a complex between p130Cas and c-Crk and elicits a coordinate increase in the tyrosine phosphorylation of p125Fak and paxillin. IGF-I stimulates tyrosine phosphorylation of
EXPERIMENTAL PROCEDURES

Cell Culture—Stock cultures of Swiss 3T3 fibroblasts 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, cells were plated in 100-mm dishes at 6 × 10⁵ cells/dish in DMEM containing 10% fetal bovine serum and used after 6–8 days when the cells were confluent and quiescent.

Immunoprecipitation—Quiescent cultures of Swiss 3T3 cells (1–2 × 10⁶) were washed twice with DMEM, treated with IGF-I or other factors in 10 ml of DMEM for the times indicated, and lysed at 4 °C in 1 ml of a solution containing 10 mM Tris/HC1, pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 100 μM Na₃VO₄, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (lysis buffer). Lysates were clarified by centrifugation at 15,000 × g for 10 min, and the supernatants were transferred to fresh tubes for immunoprecipitation. Proteins were immunoprecipitated at 4 °C for 14 h with anti-mouse IgG-agarose-linked mAb directed against anti-p130Cas, anti-c-Crk, anti-p125Fak, or anti-paxillin mAbs, as indicated. Immunoprecipitates were washed three times with lysis buffer and extracted for 10 min at 95 °C in 2 × SDS-PAGE sample buffer (200 mM Tris/HC1, 6% SDS, 2 mM EDTA, 4% 2-mercaptoethanol, 10% glycerol, pH 6.8) and fractionated by SDS-PAGE.

Western Blotting—Treatment of quiescent cultures of cells with growth factors, cell lysis, and immunoprecipitation was performed as described above. After SDS-PAGE, proteins were transferred to Immobilon membranes. The membranes were blocked using 5% non-fat dried milk in phosphate-buffered saline, pH 7.2, and incubated for 2 h at 22 °C with either the anti-Tyr(P) mAb (4G10, 1 μg/ml) or the anti-p130Cas mAb (1 μg/ml), as indicated. Immunoreactive bands were visualized using ¹²⁵I-labeled sheep anti-mouse IgG (1:1000) followed by autoradiography. The autoradiograms were scanned using an LKB Ultrascan XL densitometer, and labeled bands were quantified using an Ultrascan XL internal integrator. The values were expressed as percentages of the maximum increase in tyrosine phosphorylation above control values.

p70S6K Mobility Shift Assay—Activation of p70S6K was determined by the appearance of slowly migrating forms in SDS-PAGE gels, which results from phosphorylation of specific threonine and tyrosine residues within its subdomain VIII. Quiescent cultures of Swiss 3T3 cells were treated with factors as indicated; the cells were lysed in 2 × SDS-PAGE sample buffer and analyzed by SDS-PAGE. Proteins were subsequently transferred to Immobilon membranes which were blocked as described above and incubated for 1 h at 22 °C with a polyclonal p42MAPK antibody (1:1000) in phosphate-buffered saline containing 3% non-fat dried milk. Immunoreactive bands were visualized using iodinated protein A followed by autoradiography.

Materials—IGF-I, bombesin, cytochalasin D, Wortmannin, LY294002, and agarse-linked anti-mouse IgG were obtained from Sigma. Recombinant PDGF (BB homodimer), ¹²⁵I-sheep anti-mouse IgG (15 mCi/mg), and ¹²⁵I-protein A were from Amersham Corp., United Kingdom. The 4G10 anti-Tyr(P) mAb was from Upstate Biotechnology, Inc. (Lake Placid, NY). Rapamycin and PD98059 were obtained from Calbiochem (Nottingham, UK). The mAbs directed against p130Cas, c-Crk, or paxillin were from Transduction Laboratories, and the polyclonal antibodies against p125Fak and p70S6K were from Santa Cruz Biotechnology, Santa Cruz, CA. The polyclonal anti-p42MAPK antibody raised against a COOH-terminal peptide (EETARFPQGYRS) was a gift from Dr. D. Whittet.

RESULTS

IGF-I Stimulates Tyrosine Phosphorylation of the Focal Adhesion Proteins p130Cas, p125Fak, and Paxillin—To determine whether IGF-I induces tyrosine phosphorylation of p130Cas in Swiss 3T3 cells, quiescent cultures of these cells were stimulated with various concentrations of IGF-I for 5 min and lysed, and the extracts were immunoprecipitated with anti-p130Cas antibody. The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting with anti-Tyr(P) mAb. As shown in Fig. 1A, IGF-I induced a marked increase in the tyrosine phosphorylation of p130Cas in a dose-dependent manner. Half-maximum and maximum effects were obtained at 0.6 and 10 nM, respectively.

The kinetics of tyrosine phosphorylation of p130Cas stimulated by IGF-I in Swiss 3T3 cells is shown in Fig. 1B. An increase in tyrosine phosphorylation of p130Cas could be detected as early as 1 min after addition of 10 nM IGF-I, reaching a maximum after 2.5–5 min. Thereafter, tyrosine phosphorylation of p130Cas declined gradually to almost base-line levels after 30 min of incubation. We verified that similar amounts of p130Cas were recovered from lysates of cells treated without or
Role of ERK in IGF-I-induced Tyrosine Phosphorylation of p130Cas, p125Fak, and Paxillin—The Ras-ERK pathway has been implicated in IGF-I-mediated cellular proliferation and tumorigenesis (50). Recent evidence has implicated p42mapk (ERK-2) and p44mapk (ERK-1) in the control of the actin cytoskeleton and cell migration via phosphorylation and activation of the myosin light chain kinase (51). To determine the contribution of the ERK pathway to IGF-I-mediated tyrosine phosphorylation of p130Cas, p125Fak, and paxillin, we used PD98059, a highly specific inhibitor of the ERK-activating enzyme mitogen-activated protein kinase kinase-1 (52). Quiescent cultures of Swiss 3T3 cells were pretreated with 15 μM PD98059 for 2 h and then stimulated for 5 min with either 10 nM IGF-I or 10 nM bombesin (BOM) for 5 min and then lysed. Lysates were immunoprecipitated with either anti-p130Cas mAb or anti-p125Fak mAbs and then analyzed by immunoblotting with anti-Tyr(P) mAb. The positions of p130Cas and p125Fak are indicated by the arrows. The results shown are representative of two independent experiments.

with different concentrations of IGF-I for various times (results not shown).

p130Cas has a cluster of 15 potential SH2-binding motifs, nine of which are sequences that are expected to have a preferential affinity for c-Crk-SH2 domain (19). Consequently, we examined whether IGF-I-induced tyrosine phosphorylation of p130Cas could lead to the formation of a complex between endogenous c-Crk and p130Cas in intact Swiss 3T3 cells. Anti-p130Cas Western blotting of c-Crk immunoprecipitates revealed that IGF-I stimulated an association of p130Cas with c-Crk that was time-dependent and parallel to the IGF-I-induced tyrosine phosphorylation of p130Cas. We verified that similar amounts of c-Crk were recovered from lysates of cells treated without or with IGF-I (result not shown). The association of p130Cas with c-Crk reached a maximum after 2.5–5 min of IGF-I stimulation and then declined (Fig. 2A).

IGF-I also induced a rapid and transient increase in the tyrosine phosphorylation of the non-receptor tyrosine kinase p125Fak and the adaptor protein paxillin which could be detected as early as 1 min after addition of 10 nM IGF-I, reaching a maximum after 5 min. Thereafter, tyrosine phosphorylation of p125Fak and paxillin declined to almost base-line levels (Fig. 2B). Similar amounts of p125Fak and paxillin were recovered from lysates of cells treated without or with IGF-I (results not shown). Thus, IGF-I induces a rapid and parallel increase in the tyrosine phosphorylation of the focal adhesion proteins p130Cas, p125Fak, and paxillin and, concomitantly, promotes association of tyrosine-phosphorylated p130Cas with c-Crk in Swiss 3T3 cells.

Role of ERK in IGF-I-induced Tyrosine Phosphorylation of p130Cas, p125Fak, and Paxillin—The Ras-ERK pathway has been implicated in IGF-I-mediated cellular proliferation and tumorigenesis (50). Recent evidence has implicated p42mapk (ERK-2) and p44mapk (ERK-1) in the control of the actin cytoskeleton and cell migration via phosphorylation and activation of the myosin light chain kinase (51). To determine the contribution of the ERK pathway to IGF-I-mediated tyrosine phosphorylation of p130Cas, p125Fak, and paxillin, we used PD98059, a highly specific inhibitor of the ERK-activating enzyme mitogen-activated protein kinase-1 (52). Quiescent cultures of Swiss 3T3 cells were pretreated with 15 μM PD98059 for 2 h and then stimulated for 5 min with either 10 nM IGF-I or 10 nM bombesin (BOM) for 5 min and then lysed. Lysates were immunoprecipitated with either anti-p130Cas mAb or anti-p125Fak mAbs and then analyzed by immunoblotting with anti-Tyr(P) mAb. The positions of p130Cas and p125Fak are indicated by the arrows. The results shown are representative of two independent experiments.

The Ras-ERK pathway has been involved in IGF-I-mediated cellular proliferation and tumorigenesis (50). Recent evidence has implicated p42mapk (ERK-2) and p44mapk (ERK-1) in the control of the actin cytoskeleton and cell migration via phosphorylation and activation of the myosin light chain kinase (51). To determine the contribution of the ERK pathway to IGF-I-mediated tyrosine phosphorylation of p130Cas, p125Fak, and paxillin, we used PD98059, a highly specific inhibitor of the ERK-activating enzyme mitogen-activated protein kinase-1 (52). Quiescent cultures of Swiss 3T3 cells were pretreated with 15 μM PD98059 for 2 h and then stimulated for 5 min with either 10 nM IGF-I or 10 nM bombesin (BOM) for 5 min and then lysed. Lysates were immunoprecipitated with either anti-p130Cas mAb or anti-p125Fak mAbs and then analyzed by immunoblotting with anti-Tyr(P) mAb. The positions of p130Cas and p125Fak are indicated by the arrows. The results shown are representative of two independent experiments.

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Fig. 2. Time course of IGF-I-induced association of p130Cas with endogenous c-Crk and of IGF-I-induced p125Fak and paxillin tyrosine phosphorylation. A, kinetics of p130Cas-c-Crk complex formation. Cultures of Swiss 3T3 cells (6 × 10^6) were treated for various times either without (−) or with 10 nM IGF-I (as indicated) and lysed. The lysates were then immunoprecipitated with anti-c-Crk mAb and analyzed by immunoblotting with the anti-p130Cas mAb. The results presented are typical of two independent experiments. The position of p130Cas is indicated by the arrow. B, IGF-I induces tyrosine phosphorylation of p125Fak and paxillin. Quiescent Swiss 3T3 cells were treated at 37 °C without (−) or with 10 nM IGF-I for various times as indicated and lysed. The cell lysates were then immunoprecipitated either with the anti-p125Fak mAb or with the anti-paxillin mAb, and the immunoprecipitates were analyzed by immunoblotting with anti-Tyr(P) mAb. The positions of p125Fak and paxillin are indicated by the arrows. The results shown are representative of three independent experiments.

Fig. 3. A, effect of PD98059 on the tyrosine phosphorylation of p130Cas and p125Fak stimulated by IGF-I. Top, quiescent Swiss 3T3 cells were preincubated for 2 h at 37 °C in the absence (−) or presence (+) of 15 μM PD98059. Cells were subsequently incubated without (−) or with either 10 nM IGF-I or 10 nM bombesin (BOM) for 5 min and then lysed. Lysates were immunoprecipitated with either anti-p130Cas mAb or anti-p125Fak mAb and analyzed by immunoblotting with anti-Tyr(P) mAb. The positions of p130Cas and p125Fak are indicated by the arrows. Bottom, in parallel experiments quiescent Swiss 3T3 cells were treated as described above and then lysed in 2× SDS sample buffer. The lysates were then analyzed for ERK activation by the mobility shift assay, as described under “Experimental Procedures.” B, IGF-I-induced tyrosine phosphorylation of p130Cas and p125Fak is independent of PKC activation. Quiescent Swiss 3T3 cells were preincubated either in the absence (−) or presence (+) of 3.5 μM GF109203X for 1 h. The cells were subsequently incubated for another 5 min without (−) or with 10 nM IGF-I, lysed, and immunoprecipitated with either anti-p130Cas mAb or anti-p125Fak mAb and then analyzed by immunoblotting with anti-Tyr(P) mAb. The positions of p130Cas and p125Fak are indicated by the arrows. The results shown are representative of three independent experiments.
duced by IGF-I (results not shown). Thus, IGF-I-induced tyrosine phosphorylation of focal adhesion proteins can be dissociated from the activation of the ERK pathway in Swiss 3T3 cells.

Effect of Wortmannin and LY294002 on IGF-I-stimulated Tyrosine Phosphorylation of p130Cas, p125Fak, and Paxillin—IGF-I induces actin recruitment into membrane ruffles via a PI 3-kinase-dependent pathway (11), but the contribution of this pathway to the tyrosine phosphorylation of focal adhesion proteins induced by IGF-I was unknown. To examine the role of PI 3-kinase in the tyrosine phosphorylation of p130Cas, p125Fak, and paxillin induced by IGF-I in Swiss 3T3 cells, quiescent cultures of these cells were pretreated for 20 min with wortmannin, which binds to and inhibits the catalytic (110 kDa) subunit of PI 3-kinase (54, 55), and then stimulated for 5 min with 10 nM IGF-I. To verify the selectivity of the effect of wortmannin under our experimental conditions, parallel cultures of Swiss 3T3 cells were also stimulated with either PDGF (5 ng/ml) or the G-protein-coupled receptor agonist bombesin which induces p130Cas tyrosine phosphorylation via PI 3-kinase-dependent and -independent pathways, respectively (26). As shown in Fig. 4, A and B, tyrosine phosphorylation of p130Cas stimulated by either IGF-I or PDGF was markedly inhibited by preincubation of the cells with wortmannin up to 60 nM. In contrast, the tyrosine phosphorylation of p130Cas stimulated by bombesin was not inhibited by wortmannin, at identical concentrations.

In order to substantiate the results obtained with wortmannin, we examined if the structurally unrelated compound LY294002, which selectively inhibits PI 3-kinase by a distinct mechanism (56), also prevents p130Cas tyrosine phosphorylation in response to IGF-I. As shown in Fig. 4, A and B, LY294002 also attenuated the increase in the tyrosine phosphorylation of p130Cas in response to either IGF-I or PDGF but not in response to bombesin.

Pretreatment with either wortmannin or LY294002 also inhibited tyrosine phosphorylation of p125Fak (Fig. 5A) and paxillin (Fig. 5B) induced by either IGF-I or PDGF. Thus, IGF-I stimulates p130Cas, p125Fak and paxillin tyrosine phosphorylation through a PI 3-kinase-dependent pathway in Swiss 3T3 cells.

Rapamycin Dissociates IGF-I-induced Tyrosine Phosphorylation of p130Cas, p125Fak, and Paxillin from p70S6K—Rapamycin, which is derived from the fungus Piricularia oryzae, binds to and inhibits the catalytic (285 kDa) subunit of PI 3-kinase (54, 55), and then stimulated for 5 min with 10 nM IGF-I. Consequently, it was important to clarify whether p70S6K lies in the signaling pathway characteristic of the phosphorylated and activated form of the p70S6K. As a consequence of this mobility shift induced by IGF-I but had no effect on IGF-I-stimulated tyrosine phosphorylation of p130Cas, p125Fak, and paxillin in response to IGF-I.

The potent immunosuppressant rapamycin is a selective inhibitor of p70S6K activation in many cell types (57), including Swiss 3T3 cells (58). The phosphorylation of Thr-229 and Thr-389 and Ser 404, which are responsible for the activation and mobility shift of p70S6K, is prevented or reversed by treatment with rapamycin (49). We therefore examined the effect of rapamycin on the IGF-I-stimulated tyrosine phosphorylation of p130Cas, p125Fak, and paxillin. Quiescent cultures of Swiss 3T3 cells were pretreated with 10 nM rapamycin for 1 h and then stimulated with 10 nM IGF-I for 5 min. Cell lysates were then immunoprecipitated with the anti-p130Cas mAb and analyzed by immunoblotting with anti-Tyr(P) mAb. Quantification of p130Cas tyrosine phosphorylation was performed by scanning densitometry. Values shown are the mean ± S.E. of four independent experiments and are expressed as the percentage of the maximal increase in tyrosine phosphorylation of p130Cas above control (unstimulated) values.
IGF-I Stimulates p130Cas and p125Fak Tyrosine Phosphorylation

IGF-I-induced Tyrosine Phosphorylation of p130Cas, p125Fak, and Paxillin Is PKC-independent—The second messengers phosphatidylinositol (3,4,5)-biphosphate and phosphatidylinositol (3,4,5)-triphosphate generated by PI 3-kinase activity have been proposed to activate the novel and atypical isoforms of PKC (59). In addition, phorbol esters have been shown to increase p125Fak and paxillin phosphorylation in response to IGF-I. Quiescent Swiss 3T3 cells were pretreated for 20 min or in the absence (−) or in the presence (+) of 15 μM LY94002 (Ly) for 1 h and subsequently challenged without (−) or with either 10 nM IGF-I or 5 ng/ml PDGF for another 5 min. Cell lysates were then immunoprecipitated with the anti-p125Fak mAb and analyzed by immunoblotting with the anti-Tyr(P) mAb. The position of p125 Fak is shown by the arrows. A, effect of wortmannin and LY94002 on IGF-I-induced tyrosine phosphorylation of p125Fak and paxillin. Quiescent Swiss 3T3 cells were preincubated at 37 °C either in the absence or in the presence of 40 or 60 nM of wortmannin (Wort) for 20 min or in the absence (−) or in the presence (+) of 15 μM LY94002 (Ly) for 1 h and subsequently challenged without (−) or with either 10 nM IGF-I or 5 ng/ml PDGF for another 5 min. Cell lysates were then immunoprecipitated with the anti-paxillin mAb and analyzed by immunoblotting with the anti-Tyr(P) mAb. The position of paxillin is shown by the arrows. B, effect of wortmannin and LY94002 on IGF-I-induced phosphorylation of paxillin. Quiescent Swiss 3T3 cells were preincubated at 37 °C either in the absence or in the presence of 40 or 60 nM of wortmannin (Wort) for 20 min or in the absence (−) or in the presence (+) of 15 μM LY94002 (Ly) for 1 h and subsequently challenged without (−) or with either 10 nM IGF-I or 5 ng/ml PDGF for another 5 min. Cell lysates were then immunoprecipitated with the anti-paxillin mAb and analyzed by immunoblotting with the anti-Tyr(P) mAb. The position of paxillin is shown by the arrows. Quantification of tyrosine phosphorylation of p125Fak and paxillin was performed by scanning densitometry. Values shown are the mean ± S.E. of four independent experiments and are expressed as the percentage of the maximal increase in tyrosine phosphorylation above control (unstimulated) values.

Fig. 5. Wortmannin and LY94002 inhibit IGF-I-stimulated tyrosine phosphorylation of p125Fak and paxillin. A, effect of wortmannin and LY94002 on IGF-I-induced tyrosine phosphorylation of p125Fak. Quiescent Swiss 3T3 cells were preincubated at 37 °C either in the absence or in the presence of 40 or 60 nM of wortmannin (Wort.) for 20 min or in the absence (−) or in the presence (+) of 15 μM LY94002 (Ly) for 1 h and subsequently challenged without (−) or with either 10 nM IGF-I or 5 ng/ml PDGF for another 5 min. Cell lysates were then immunoprecipitated with the anti-p125Fak mAb and analyzed by immunoblotting with the anti-Tyr(P) mAb. The position of p125Fak is shown by the arrows. B, effect of wortmannin and LY94002 on IGF-I-induced phosphorylation of paxillin. Quiescent Swiss 3T3 cells were preincubated at 37 °C either in the absence or in the presence of 40 or 60 nM of wortmannin (Wort.) for 20 min or in the absence (−) or in the presence (+) of 15 μM LY94002 (Ly) for 1 h and subsequently challenged without (−) or with either 10 nM IGF-I or 5 ng/ml PDGF for another 5 min. Cell lysates were then immunoprecipitated with the anti-paxillin mAb and analyzed by immunoblotting with the anti-Tyr(P) mAb. The position of paxillin is shown by the arrows. Quantification of tyrosine phosphorylation of p125Fak and paxillin was performed by scanning densitometry. Values shown are the mean ± S.E. of four independent experiments and are expressed as the percentage of the maximal increase in tyrosine phosphorylation above control (unstimulated) values.

The Integrity of the Actin Cytoskeleton Is Essential for IGF-I-induced Tyrosine Phosphorylation of p130Cas, p125Fak, and Paxillin—Another pathway that lies downstream of PI 3-kinase is the Rac-dependent reorganization of the actin cytoskeleton into membrane ruffles (11, 62–64). Given that tyrosine-phosphorylated p130Cas, p125Fak, and paxillin are found in focal contacts associated with stress fibers and membrane ruffles, we examined whether disruption of the actin cytoskeleton could interfere with the tyrosine phosphorylation of these focal adhesion proteins in response to IGF-I. Quiescent Swiss 3T3 cells were pretreated for 2 h with 1.2 μM cytochalasin D, a concentration that is known to completely disrupt the actin cytoskeleton and the assembly of focal adhesion in Swiss 3T3 cells (24), and then stimulated with 10 nM IGF-I for another 5 min. As shown in Fig. 7A, treatment with cytochalasin D completely blocked IGF-I stimulation of p130Cas, p125Fak, and paxillin tyrosine phosphorylation.

Addition of PDGF, at high concentrations (e.g. 30 ng/ml), also disrupts the organization of the actin cytoskeleton in 3T3 cells (30). As illustrated in Fig. 7A, pretreatment of Swiss 3T3 cells with 30 ng/ml PDGF completely inhibited tyrosine phosphorylation of p130Cas, p125Fak, and paxillin by IGF-I. Thus, the integrity of the actin cytoskeleton is necessary for IGF-I-induced tyrosine phosphorylation of p130Cas, p125Fak, and paxillin.

To assess whether the complex formation between p130Cas and c-Crk induced by IGF-I also depends on the integrity of the actin cytoskeleton, quiescent Swiss 3T3 cells were pretreated for 2 h with or without 1.2 μM cytochalasin D and then stimulated with 10 nM IGF-I. Fig. 7B shows that treatment of Swiss 3T3 cells with cytochalasin D, at a concentration shown in Fig. 7A to inhibit p130Cas tyrosine phosphorylation, prevented the association of p130Cas with c-Crk induced by IGF-I in intact Swiss 3T3 cells.
cells were analyzed directly by immunoblotting for p70S6K mobility shift.

The signal transduction events initiated by the IGF-IR have been extensively investigated, but the pathway(s) leading to the tyrosine phosphorylation of p130Cas, p125Fak, and paxillin independently of ERK activation. In contrast, the structurally unrelated PI 3-kinase inhibitors wortmannin and LY294002 markedly attenuated the increase in the tyrosine phosphorylation of p130Cas, p125Fak, and paxillin induced by IGF-I. Thus, our results identify a PI 3-kinase-dependent pathway in the tyrosine phosphorylation of these focal adhesion proteins in response to IGF-I stimulation.

PI 3-kinase activity is required for the formation of membrane ruffles by several motility-inducing growth factors (11, 65), and the lipid products of this enzyme have been shown to induce actin reorganization and to increase cell migration (64). The small GTP-binding protein Rac is known to mediate the recruitment of actin binding protein Rac is known to mediate the recruitment of actin

The rapidity and the low, physiological concentrations of IGF-I inducing this effect suggest that IGF-I activates a pathway that mediates tyrosine phosphorylation of these focal adhesion proteins. We found that IGF-I stimulated tyrosine phosphorylation of p130Cas, p125Fak, and paxillin independently of ERK activation. The results shown are representative of two independent experiments.

**FIG. 6.** Dissociation of p70^S6K^ activation from the increase in the tyrosine phosphorylation of p130^Cas^, p125^Fak^, and paxillin induced by IGF-I. A, effect of wortmannin on IGF-I-stimulated phosphorylation of p70^S6K^ in Swiss 3T3 cells. Quiescent Swiss 3T3 cells were preincubated in the absence (–) or in the presence (+) of 40 nM wortmannin (Wort.) for 20 min and subsequently incubated for the times indicated either without (–) or with 10 nM IGF-I and then lysed in 2× SDS sample buffer. The cell lysates were analyzed directly by immunoblotting with anti-p70^S6K^ mAb. The positions of p130 Cas, p125 Fak, and paxillin are indicated by the arrows. Bottom, the whole cell lysates from one set of cells were analyzed directly by immunoblotting for p70^S6K^ mobility shift as described under “Experimental Procedures.” B, effect of rapamycin on IGF-I-stimulated tyrosine phosphorylation of p130^Cas^, p125^Fak^, and paxillin and on phosphorylation of p70^S6K^.

Four parallel and quiescent sets of cells were preincubated in absence (–) or in the presence (+) of 10 nM rapamycin (Rap.) for 1 h and subsequently incubated for the times indicated either without (0) or with 10 nM IGF-I and then lysed. Top, the lysates from three sets of cells were immunoprecipitated with either anti-p130^Cas^ mAb, anti-p125^Fak^ mAb, or anti-paxillin mAb and then analyzed by immunoblotting with anti-Tyr(P) mAb. The positions of p130^Cas^, p125^Fak^, and paxillin are indicated by the arrows. Bottom, the whole cell lysates from one set of cells were analyzed directly by immunoblotting for p70^S6K^ mobility shift as described under “Experimental Procedures.” The positions of p70^S6K^ and of the phosphorylated forms of the enzyme (pp70^S6K^) are also indicated in A and B.

**DISCUSSION**

There is increasing evidence indicating that IGF-I signaling is involved in multiple biological processes including cell migration, proliferation, and transformation, but the downstream targets that mediate these effects have not been fully identified. The findings presented here demonstrate that IGF-I induces tyrosine phosphorylation of the adaptor molecule p130Cas in Swiss 3T3 cells. The rapidity and the low, physiological concentrations of IGF-I inducing this effect suggest that this event may be functionally important in the biological action of IGF-I. Our results also demonstrate that IGF-I induces tyrosine phosphorylation of p125Fak and paxillin with kinetics that parallel those of p130Cas tyrosine phosphorylation.

The signal transduction events initiated by the IGF-IR have been extensively investigated, but the pathway(s) leading to tyrosine phosphorylation of the focal adhesion proteins p130 Cas, p125 Fak, and paxillin by IGF-I were unknown. Given that the mitogen-activated protein kinase kinase-1/ERK and PI 3-kinase pathways have emerged as major mediators of the biological effects promoted by IGF-I, we examined their role in IGF-mediated tyrosine phosphorylation of focal adhesion proteins. We found that IGF-I stimulated tyrosine phosphorylation of p130Cas, p125Fak, and paxillin independently of ERK activation. The results shown are representative of two independent experiments.

**FIG. 7.** IGF-I-induced tyrosine phosphorylation of p130^Cas^, p125^Fak^, and paxillin requires the integrity of the actin cytoskeleton. A, effect of high concentrations of PDGF and cytochalasin D on IGF-I-induced tyrosine phosphorylation of p130^Cas^, p125^Fak^, and paxillin. Quiescent Swiss 3T3 cells were pretreated at 37 °C either with 1.2 μM cytochalasin D (Cyt.D) for 2 h or with 30 ng/ml PDGF for 10 min, then cells were incubated for another 5 min without or with 10 nM IGF-I, as indicated. Tyrosine phosphorylation of p130^Cas^, p125^Fak^, and paxillin was analyzed by immunoprecipitation with either anti-p130^Cas^ mAb, anti-p125^Fak^ mAb, or anti-paxillin mAb and then analyzed by immunoblotting with anti-Tyr(P) mAb. The position of p130^Cas^ is indicated by the arrow. The results shown are representative of two independent experiments.

The signal transduction events initiated by the IGF-IR have been extensively investigated, but the pathway(s) leading to tyrosine phosphorylation of the focal adhesion proteins p130 Cas, p125 Fak, and paxillin by IGF-I were unknown. Given that the mitogen-activated protein kinase kinase-1/ERK and PI 3-kinase pathways have emerged as major mediators of the biological effects promoted by IGF-I, we examined their role in IGF-mediated tyrosine phosphorylation of focal adhesion proteins. We found that IGF-I stimulated tyrosine phosphorylation of p130Cas, p125Fak, and paxillin independently of ERK activation. The results shown are representative of two independent experiments.
cytoskeleton is essential for IGF-I-induced tyrosine phosphorylation of p130Cas and p125Fak, and paxillin via the PI 3-kinase pathway. The integrity of the actin cytoskeleton is likely to be required for promoting the assembly of the focal contacts (16), the distinct sites of the plasma membrane where p130Cas•p125Fak•paxillin are recruited and become tyrosine-phosphorylated.

The signaling pathways that lie downstream of PI 3-kinase also include the serine/threonine kinases p70(S6K) (57) and the novel and atypical isoforms of PKC (59). Our results demonstrate that inhibition of either p70(S6K) or PKC activation, with the immunosuppressant rapamycin or the specific PKC antagonist GF 109203X, respectively, did not prevent IGF-I-stimulated tyrosine phosphorylation of p130Cas•p125Fak•paxillin. Thus, IGF-I-mediated tyrosine phosphorylation of focal adhesion proteins is PI 3-kinase-dependent but can be dissociated from activation of either p70(S6K) or PKC.

The results presented here also demonstrate that the tyrosine phosphorylation of p130Cas in response to bombesin is not prevented by either wortmannin or LY294002, at concentrations that virtually abolished the tyrosine phosphorylation of p130Cas induced by IGF-I. These results imply that there is a PI 3'-kinase-dependent and PI 3'-kinase-independent signal transduction pathway stimulating the tyrosine phosphorylation of focal adhesion proteins in the same cells.

The molecular cloning of p130Cas revealed an adaptor protein that contains an SH3 domain, proline-rich regions, and a cluster of SH2 domains; its SH3 domain is involved in the interaction with c-Crk and/or the activity of new downstream effectors in IGF-I signaling (13). Consequently, our results showing that IGF-I mediates a coordinate increase in the level of tyrosine phosphorylation of p130Cas, p125Fak, and paxillin and the formation of a complex between p130Cas and c-Crk through a PI 3-kinase-dependent signaling pathway suggest novel mechanisms of action for this important growth promoting factor.

REFERENCES
1. Baserga, R. (1995) Cancer Res. 55, 249–252
2. Blakesley, V. A., Kaler, T., Helman, L. J., Stannard, B., Farria, T. N., Roberts, C. T., Jr., and LeRoith, D. (1996) Endocrinology 137, 410–417
3. Kulik, G., Klippel, A., and Weber, M. J. (1997) Mol. Cell. Biol. 17, 1595–1606
4. Broga, R., Resnickoff, M., A’Ambrosio, C., and Valentinis, B. (1997) Vitam. Horm. 53, 65–98
5. LeRoith, D. (1996) Bailliere’s Clin. Endocrinol. & Metab. 10, 49–73
6. Myers, M. G., Jr., Grammer, T. C., Wang, L. M., Sun, X. J., Pierce, J. H., and Smith, J. (1994) J. Biol. Chem. 269, 28785–28789
7. Patti, M. E., Sun, X. J., Bruening, J. C., Araki, E., Lipes, M. A., White, M. F., and Kahn, C. R. (1995) J. Biol. Chem. 270, 24670–24673
8. Bruening, J. C., Winney, J., Cheatham, B., and Kahn, C. R. (1997) Mol. Cell. Biol. 17, 1513–1521
9. Kasus-Jacobi, A., Perdereau, D., Tartare-Deckert, S., Van Obberghen, E., Girard, J., and Biron, A. F. (1997) J. Biol. Chem. 272, 17166–17170
10. Borselli, L., Raines, R. W., Grant, L. M., Krebs, E. G., and Ross, R. (1994) J. Clin. Invest. 93, 1266–1274
11. Kotani, K., Yonezawa, K., Kasa, K., Ueda, H., Kitamura, Y., Sakae, H., Ando, A., Chavanieu, A., Calas, B., Gavrilova, P., Nishiyama, M., Waterfield, M. D., and Kasuga, M. (1994) EMBO J. 13, 2313–2321
12. Leventhal, P. S., Shelden, E. A., Kim, B., and Feldman, E. L. (1997) J. Biol. Chem. 272, 2524–2528
13. Brooks, P. C., Klemke, R. L., Schön, S., Lewis, W. J., Schwartz, M. A., and Chernes, D. A. (1997) J. Clin. Invest. 99, 1398–1399
14. Rozengurt, E. (1995) Cancer Surv. 24, 81–96
15. Rozengurt, E., and Rodriguez-Fernandez, J. L. (1997) Essays Biochem. 32, 73–86
16. Burridge, K., and Chrismanowa-Wodnicka, M. (1996) Annu. Rev. Cell Dev. Biol. 12, 463–518
17. Hanks, S. K., Calab, M. B., Harper, M. C., and Patel, S. K. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 8487–8491
18. Schaller, M. D., Bergman, C. A., Cobb, B. S., Vines, R. R., Reynolds, A. B., and Brugge, J. S. (1992) J. Biol. Chem. 267, 5192–5196
19. Sakai, R., Ichimura, A., Hiromi, S., Ogawa, S., Tanaka, T., Mano, H., Yazeaki, Y., and Hirai, H. (1994) EMBO J. 13, 3748–3756
20. Salgia, R., J., L., Le, S. H., Brunkhorst, B., Kang, S. S., Ohshy, E. S., Sun, Y., Pleick, E., Halleck, M., Ernst, T., Tantravahi, R., Chen, L. B., and Griffin, J. D. (1995) J. Biol. Chem. 270, 5039–5047
21. Turner, C. E., and Miller, J. T. (1994) J. Cell Sci. 107, 1543–1551
22. Zachary, J., Sinnett-Smith, J., and Rozengurt, E. (1992) J. Biol. Chem. 267, 19031–19034
23. Zachary, J., Sinnett-Smith, J., Turner, C. E., and Rozengurt, E. (1993) J. Biol. Chem. 268, 22606–22609
24. Sinnett-Smith, J., Zachary, I., Valverde, A. M., and Rozengurt, E. (1993) J. Biol. Chem. 268, 14261–14268
25. Seufferlein, T., Withers, D. J., Broad, S., Herpet, T., Walsh, J. H., and Rozengurt, E. (1995) Cell Growth Differ. 6, 383–393
26. Casamassima, A., and Rozengurt, E. (1997) J. Biol. Chem. 272, 9363–9370
27. Seufferlein, T., and Rozengurt, E. (1994) J. Biol. Chem. 269, 9345–9351
28. Seufferlein, T., and Rozengurt, E. (1995) J. Biol. Chem. 270, 24343–24351
29. Seufferlein, T., and Rozengurt, E. (1994) J. Biol. Chem. 269, 27610–27617
30. Rankin, S., and Rozengurt, E. (1994) J. Biol. Chem. 269, 704–710
31. Rankin, S., Hoshmand-Redi, H., Glasson-Welsh, L., and Rozengurt, E. (1996) J. Biol. Chem. 271, 7829–7834
32. Ojaniemi, M., and Vuori, K. (1997) J. Biol. Chem. 272, 25993–25998
33. Seufferlein, T., Withers, D. J., Mann, D., and Rozengurt, E. (1996) Mol. Biol. Cell 7, 1865–1875
34. Burridge, K., Turner, C. E., and Romer, L. H. (1997) J. Cell Biol. 119, 893–903
35. Lipert, F., Haimovich, B., Schaller, M. D., Cobb, B. S., Parsons, J. T., and Brugge, J. S. (1992) J. Cell Biol. 119, 965–912
36. Vuori, K., and Ruoslabhi, E. (1993) J. Biol. Chem. 268, 21459–21462
37. Guan, J. L., and Shalhawy, D. (1992) Nature 358, 690–692
38. Zachary, I., and Rozengurt, E. (1992) Cell 71, 891–894
