Replacement of Tyrosine 1251 in the Carboxyl Terminus of the Insulin-like Growth Factor-I Receptor Disrupts the Actin Cytoskeleton and Inhibits Proliferation and Anchorage-independent Growth*

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Insulin-like growth factor (IGF)-I signaling through the IGF-I receptor modulates cellular adhesion and proliferation and the transforming ability of cells overexpressing the IGF-I receptor. Tyrosine phosphorylation of intracellular proteins is essential for this transduction of the IGF-I-induced mitogenic and tumorigenic signals. IGF-I induces specific cytoskeletal structure and the phosphorylation of proteins in the associated focal adhesion complexes. The determination of the exact pathways emanating from the IGF-I receptor that are involved in mediating these signals will contribute greatly to the understanding of IGF-I action. We have previously shown that replacement of tyrosine residues 1250 and 1251 in the carboxyl terminus of the IGF-I receptor abrogates IGF-I-induced cellular proliferation and tumor formation in nude mice. In this study, replacement of either tyrosine 1250 or 1251 similarly reduces the cells ability to grow in an anchorage-independent manner. The actin cytoskeleton and cellular localization of vinculin are disrupted by replacement of tyrosine 1251. Tyrosine residues 1250 and 1251 are not essential for tyrosine phosphorylation of two known substrates; insulin receptor substrate-1 and SHC, nor association of known downstream adaptor proteins to these substrates. In addition, these mutant IGF-I receptors do not affect IGF-I-stimulated p42/p44 mitogen-activated protein kinase activation or phosphatidylinositol (PI) 3'-kinase activity. Thus, it appears that in fibroblasts expressing tyrosine 1250 and 1251 mutant IGF-I receptors, the signal transduction pathways impacting on mitogenesis and tumorigenesis do not occur exclusively through the PI 3'-kinase or mitogen-activated protein kinase pathways.

Insulin-like growth factor-I (IGF-I) and -II (IGF-II) are ligands of the type I insulin-like growth factor receptor (IGF-I receptor). Binding of these mitogenic ligands to this transmembrane receptor results in autophosphorylation of tyrosine residues of the β-subunit and activation of its intrinsic tyrosine kinase activity, which leads to the activation of multiple intracellular signaling pathways (1, 2). The importance of a functional IGF-I receptor in normal mammalian development is highlighted by the abnormal phenotype of knockout mice developed in the laboratory of Efstratiadis (3, 4). These mice have poor intrauterine growth and die shortly after birth. The IGF-I receptor has also been shown to be important in conferring a transformed phenotype to cells. Signaling through the IGF-I receptor is involved in several spontaneous malignancies (for a review see Blakesley et al. (5)), presumably via an autocrine/paracrine mechanism. Growth of tumor cells in culture can be inhibited by blocking the expression of the IGF-I receptor using antisense strategies (6, 7) or using antibodies that bind to and reduce ligand-stimulated activation of the receptor (8–10). Furthermore, interference of the IGF-I receptor by these methods results in inhibition of tumor formation when these cells are injected in athymic mice (6, 9–15). Overexpression of the IGF-I receptor in nontransformed NIH-3T3 fibroblasts increases both the IGF-I-stimulated mitogenesis and tumorigenicity of these cells (16–19). Expression of kinase-deficient and dominant negative mutant IGF-I receptors in NIH-3T3 or Rat-1 fibroblasts, however, abrogated IGF-I-stimulated thymidine incorporation and cellular proliferation and inhibited anchorage-independent growth in soft agar and tumor formation in nude mice (5, 16, 20). The above data are consistent with the hypothesis that a fully functional IGF-I receptor capable of transducing the IGF-I mitogenic signal is essential for normal embryonic development. Furthermore, the receptor is capable of initiating and/or maintaining a transformed phenotype in cells in which it is overexpressed.

Mutant IGF-I receptors have been used to investigate the specific signaling pathways emanating from the receptor that are responsible for transducing the mitogenic and tumorigenic signals of IGF-I. In particular, the tyrosine residues in the COOH terminus have been mutated to determine if these residues are important in signaling. We and others have previously shown that replacing specific tyrosine residues (tyrosines 1250 and 1251) in the COOH terminus of the receptor reduced both the mitogenic and tumorigenic potential of the mouse fibroblasts in which the receptor was expressed, while replacement of tyrosine 1316 did not change the mitogenic potential of the cells but did abrogate the transformed phenotype (19, 21). It has been shown that IGF-I-stimulated IGF-I receptor autophosphorylation and phosphorylation of insulin receptor substrate-1 (IRS-1) were unaffected by these tyrosine residue sub-
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stitions (19). To further investigate the role of the tyrosine residues in the COOH terminus of the IGF-I receptor in mediating mitogenic and tumorigenic signals, we studied NIH-3T3 cells stably expressing IGF-I receptors with substitutions, singly and in combination, of tyrosines 1250, 1251, and 1316.

**EXPERIMENTAL PROCEDURES**

**Materials and Animals—**Restriction endonucleases were purchased from New England Biolabs (Beverly, MA), Boehringer Mannheim, and Life Technologies, Inc. Cell culture media and reagents were purchased from Life Technologies, Inc. (Rockville, MD) and Advanced Biological Technology, Inc. (Rockland, ME). Bovine papilloma virus (BoPv) DNA was obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Recombinant human IGF-I, monoclonal anti-phosphotyrosine antibody conjugated to horseradish peroxidase (4G10), and fotal bovine serum were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Monoclonal anti-growth factor receptor-bound protein 2 (Grb2) antibody, monoclonal and polyclonal anti-SH2 antibodies, monoclonal anti-PTP1D/Syp, and recombinant anti-phosphotyrosine RC20H horseradish peroxidase-conjugated antibodies were purchased from Transduction Laboratories (Lexington, KY). Polyclonal anti-extracellular signal-regulated kinase-1 (Erk-1) and polyclonal anti-Erk-2 antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal antibody to IRS-1 was the kind gift of J. Pierce (NCI, National Institutes of Health). Phospho-specific MAP kinase antibody that detects phosphorylation of the kinase tyrosine 185 was purchased from New England Biolabs, Inc. Monoclonal anti-Erk-2 and polyclonal anti-MAP kinase antibodies were obtained from Zymed Laboratories (San Francisco, CA). Fluorescein-labeled goat anti-mouse antibodies were purchased from Kirkegaard and Perry, Inc. (Gaithersburg, MD). Full-length myelin basic protein was obtained from Life Technologies. Monodeiodinated 125I-IGF-I and the complimentary primer was 5'-GTT CTTC TCT CTG GTA GAA GAA GCT TAC CGG CAA GCC A-3'. The mutated bases sequences encoding the full-length cDNA for the mutated IGF-I receptors have been previously described (19). The wild-type IGF-I receptors yNF (tyrosine 1250 mutated to phenylalanine) and yNH (tyrosine 1251 mutated to histidine) were expressed from pBPV plasmids containing the wild-type IGF-I receptor expression vector has been previously described (16). Oligonucleotides encoding the mutant receptors with tyrosines 1250 and 1251 mutated to phenylalanine and histidine, respectively (yNFyNH), or tyrosine 1316 mutated to phenylalanine (yCF) were subcloned after incubation with cells for 4 h at 37 °C, followed by lysis of the cells with isopropl alcohol (22, 23). Each cell line was plated in triplicate for each time point. To determine the effect of IGF-I on cellular proliferation, cells were grown in DMEM with 1% FBS and supplemented with 10 nM IGF-I. Cell number was measured daily from time 0 to 96 h. The medium was replenished at 72 h to maintain exponential growth. Each cell line was tested for cellular proliferation in three separate experiments. Standard curves correlating cell number and absorbance were performed for all cell lines and found to give comparable results; therefore, one standard curve using NIH3T3 cells was subsequently performed with each experiment.

**Construction of the Mutant IGF-I Receptor cDNA—**The wild-type human IGF-I receptor expression vector has been previously described (16). Oligonucleotides encoding the mutant IGF-I receptors with tyrosines 1250 and 1251 mutated to phenylalanine and histidine, respectively (yNFyNH), or tyrosine 1316 mutated to phenylalanine (yCF) have been previously described (19). The mutant IGF-I receptors yNF (tyrosine 1250 mutated to phenylalanine) and yNH (tyrosine 1251 mutated to histidine) were expressed from pBPV plasmids containing cDNA encoding mutant sequences generated by site-specific mutagenesis using mutagenic primers in a PCR-based mutagenesis strategy. Briefly, segments of the cDNA encoding the mutant IGF-I receptor were subcloned from pBluescript II into a second vector. Complimentary primer sequences were chemically synthesized to include the mutated triplet codon of interest. The direct mutant primer for yNF was 5'-TCC CGG GAG GTA AGC TCC TTT TAC AGC GAG GAC AAC A'-3', and the complimentary primer was 5'-GTT CTC CTC CTC GCT GTA GAA GAA GCT TAC CGC CAA GCC A-3'. For yNH, the direct mutant primer was 5'-TCC CGG GAG GTA AGC TCC TTT TAC AGC GAG GAC AAC A'-3', and the complimentary primer was 5'-GTT CTC CTC CTC GCT GTG GTA GAA GCC CAG TAC CGC CAA GCC A-3'. The mutated bases are underlined and the introduced HindIII sites are in italics. The mutated codons are presented in boldface type; in yNF TTC is the codon for phenylalanine, and in yNH CAC is the codon for histidine. After excision of the mutant sequences with restriction endonucleases, the overlapping cDNA fragments were ligated into pBluescript. The sequences encoding the full-length cDNA for the mutated IGF-I receptors in pBluescript II were excised with SalI and NotI and cloned into a bovine papilloma virus-derived mammalian expression vector (pBPV; Amersham Pharmacia Biotech) that had been linearized with XhoI and NotI.

**Cell Culture and Transfection—**All NIH-3T3 cell lines were cultured in Dulbecco’s modified Eagle’s medium with 4.5 g/liter glucose with added 2 mM glutamine (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO2 at 37 °C. NIH-3T3 cells were co-transfected with 20 μg of mutant expression vector or insertless pBPV plus 1 μg of pMC1Neo (CLONTECH, Palo Alto, CA) in Lipofectin reagent (Life Technologies). Clones stably expressing IGF-I receptors were selected in DMEM supplemented with 500 μg/ml G418 (Geneticin; Life Technologies) as described above (18). Clones overexpressing IGF-I receptors were selected based on results of IGF-I binding assays and were selected as described previously (16). Stably transfected cells were maintained in DMEM supplemented with 10% FBS, antibiotics, and 500 μg/ml G418 (Geneticin; Life Technologies). Cells were split for each experiment and cultured in serum-supplemented DMEM without G418.

**Cell Proliferation—**Cells were harvested after incubation with cells for 4 h at 37 °C, followed by lysis of the cells with isopropl alcohol (22, 23). Each cell line was plated in triplicate for each time point. To determine the effect of IGF-I on cellular proliferation, cells were grown in DMEM with 1% FBS and supplemented with 10 nM IGF-I. Cell number was measured daily from time 0 to 96 h. The medium was replenished at 72 h to maintain exponential growth. Each cell line was tested for cellular proliferation in three separate experiments. Standard curves correlating cell number and absorbance were performed for all cell lines and found to give comparable results; therefore, one standard curve using NIH3T3 cells was subsequently performed with each experiment.

**Cellular Adhesion Assay—**Fibroblasts, grown to subconfluence, were treated with trypsin (2 mg/ml) and immediately dislodged from the tissue culture flask by brisk shaking. The cells were resuspended in DMEM with 10% FBS and allowed to recover for 20 min at 37 °C in a polypropylene conical centrifuge tube. The cells were washed twice with prewarmed SP-DMEM and then plated onto fibronectin-coated 96-well plates at a density of 104 cells/well in 20 μl of DMEM supplemented with 10% FBS with or without 10 nM IGF-I were overlaid on a base of 0.8% agarose in DMEM supplemented with 10% FBS in 35-mm tissue culture dishes. All cell lines were plated in triplicate. Cells were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO2. The number of colonies with a greater than 125-μm diameter was scored at the end of 3 weeks.

**Immunofluorescence Assays—**For each cell line, in each of five separate experiments, the total number of cells plated in duplicate wells was determined by proceeding with fixation and staining of the cells without aspiration of unbound cells. The relative amount of adherent cells was normalized by expressing absorbance of bound cells divided by absorbance of total plated cells.

**In Situ Staining of Tyrosine-phosphorylated Proteins and Cytoskeletal Proteins—**Fibroblasts were plated on glass coverslips in six-well plates to achieve 30% confluency. Medium was changed to DMEM with 0.5% FBS for 24 h, and cells were treated with 10 nM IGF-I for 8, and 15 min at 37 °C. Cells were fixed in 4% PFA in PBS at 4 °C for 15 min at room temperature, permeabilized in 0.4% Triton X-100 for 5 min, and blocked in 10% FBS in PBS. Detection of cytoskeletal proteins or tyrosine-phosphorylated proteins was accomplished by treatment of primary antibody for 1 h at room temperature followed by secondary antibody conjugated with fluorescein for 1 h at room temperature. The primary antibodies used were monoclonal anti-paxillin antibody (5 μg/ml)

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ml) and monoclonal anti-vinulcin antibody (5 μg/ml). Following aspiration of the primary antibody and three washes with PBS, secondary antibody was applied as goat anti-mouse antibody conjugated with fluorescein (6.25 μg/ml) along with rhodamine phallolidin 0.67 unit/ml, which labels F-actin. Coverslips were affixed to slides using Biomedica gel and mounted with Vectorshield. Immunofluorescent slides were examined by fluorescence microscopy for staining patterns.

**Intact Cell IGF-I Stimulation and Analysis of the Phosphorylation State of Cellular Proteins**—Confluent cells in 100-mm plates were serum-starved in SF-DMEM for 20 h, washed twice with SF-DMEM, and then incubated either without or with IGF-I (10 nm) at 37 °C for various times as indicated for each experiment. At the times indicated, cells were solubilized in the presence of ice-cold PBS. The cells were then lysed in the presence of 350 μl of freshly prepared lysis buffer (50 mM Hepes, pH 7.9, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 4 mM sodium pyrophosphate, 2 mM sodium orthovanadate (Na3VO4), 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium fluoride, 2 μg/ml leupeptin, and 2 μg/ml aprotinin). Cell lysates were assayed in a Triton-insoluble material by centrifugation. Protein content was determined by the method of Bradford (24) using a protein assay kit (Bio-Rad). Protein samples were then loaded onto 4% SDS-polyacrylamide gels and electrophoresed through a 9% SDS-polyacrylamide gel. Proteins were transferred by electrotransferring through a 9% SDS-polyacrylamide gel and then separated by electrophoresis through a 9% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membrane for 5 h at 0.22 amperes in a Tris-glycine buffer with 20% methanol. The amount of IGF-I receptor present on the nitrocellulose membranes was determined by immunoblotting with a rabbit antibody that detects the triple tyrosine cluster of the IGF-I receptor. Equivalent amounts of IGFR-I receptors were then resolved by SDS-PAGE and transferred to nitrocellulose. Tyrosine-phosphorylated proteins were immunoblotted with a monoclonal anti-phosphotyrosine antibody (4G10) conjugated to horseradish peroxidase (1:1000 dilution, Upstate Biotechnology Inc.), and antibody-bound proteins were visualized using ECL according to the manufacturer's specifications. Tyrosine-phosphorylated IRS-1 was detected by immunoblotting with RC20H (1:2500 dilution) followed by treatment of the membrane with the ECL detection system. Erk-1 and Erk-2 were detected by immunoblotting with anti-phosphotyrosine MAP kinase antibody (1:1000 dilution), which specifically binds to Erk-1 and Erk-2, which are phosphorylated on tyrosine 185, followed by a secondary antibody horseradish peroxidase conjugate and detection as described above.

Immunoprecipitation studies were done on cleared whole cell lysates prepared from cells grown to confluency, serum-starved overnight, stimulated, and lysed as described above for measurements of tyrosine-phosphorylated proteins. IGF-I stimulation of cells was for 1 min. For IRS-1 studies or 3 min for SHC studies. Immunoprecipitations of 600 μg of cellular protein were carried out overnight with rotation at 4 °C in the same buffer in a total volume of 600 ml with either polyclonal anti-IRS-1 antibody (7 μl) or polyclonal anti-SHC antibody (7 μl). Immunoprecipitates were captured by incubation at 4 °C for 4 h. with 50 μl of 10% (w/v) Protein A-Sepharose beads (Amersham Pharmacia Biotech) in 50 ml Tris-HCl (pH 7.0) followed by three 1-ml washes in ice-cold PBS, and incubated for 24 h in SF-DMEM. Cells were then incubated in 1 ml of prewarmed (37 °C) SF-DMEM without or with 10 nm IGF-I. Cells were washed twice with ice-cold PBS and twice with freshly prepared wash buffer (20 ml Tris (pH 7.5), 100 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, and 100 μM Na3VO4). Cells were lysed in 500 μl of wash buffer containing 1% Nonidet P-40, 10% glycerol, and 2 ml phenylmethylsulfonyl fluoride for 10 min on ice. Lysates were centrifuged for 10 min at 13,000 × g for 4 °C, and supernatants were frozen. Protein content was measured by the method of Bradford as indicated above. Extracts (600 μg of protein) were immunoprecipitated overnight at 4 °C with 7 μl of monoclonal anti-phosphotyrosine antibody (clone 4G10) with the addition of 30 μl of protein A-Sepharose for the last 4 h. The complexes were then recovered with 50 μl of Protein A-Sepharose beads complexes were then resuspended in 40 μl of ice-cold 10 mM Tris, pH 7.5, 100 mM NaCl, 100 μM Na3VO4. Samples were incubated for 10 min with 10 μl of 100 mM MnCl2, 10 μl of 2 μg/ml phosphatidylinositol, and 10 μl of 440 μM ATP containing 40 μl of [32P]ATP. The reaction was stopped with the addition of 20 μl of 8 HCl and 160 μl of CHCl3/methanol (1:1). The organic phase was extracted, and chromatography was carried out as described (16) using a total of 241 ml of chromatography gel. Detection of the reaction was detected by autoradiography and quantitated using NIH Image version 1.55 after the signal was digitalized. Phosphatidylinositol 4-monophosphate was used as a marker and visualized with iodine vapor.

**Statistics**—Data are presented as the mean ± S.E. Statistical significance between groups was tested using Student's t test.

**RESULTS**

**Cellular Proliferation in Response to IGF-I**—Control cells (pNeo1) and all cell lines overexpressing wild-type (NWT) or mutant IGF-I receptors (yyFH/FI, yNF, or yNH) did not grow in the presence of 1% FBS (data not shown). It has already been shown that cells expressing wild-type IGF-I receptors grow in response to 1% FBS supplemented with 10 μM IGF-I, whereas the negative control cell line pNeo1 and cells expressing the double tyrosine mutant IGF-I receptor (yyFH) do not proliferate under those growth conditions (19). To determine if this absence of IGF-I-stimulated growth response was mediated through either tyrosine 1250 or 1251, NIH-3T3 cells stably expressing mutant IGF-I receptors (yyFH) do not proliferate under those growth conditions (19).

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**Measurement of MAP Kinase Activity**—Subconfluent cells in 100-mm plates were serum-starved in SF-DMEM for 16 h, washed twice with SF-DMEM, and then incubated either without or with IGF-I (10 nm) at 37 °C for 8 min. The cells were then solubilized in the presence of 400 μl of freshly prepared ice-cold lysis buffer (20 ml Tris (pH 7.5), 137 mM NaCl, 10% glycerol, 25 mM β-glycerophosphate, 2 mM EDTA, 1% Triton X-100, 2 mM sodium pyrophosphate, 1 mM Na3VO4, and 1 mM phenylmethylsulfonyl fluoride). Cell lysates were centrifuged by clarification. Protein content was determined as described above. The MAP kinase assay was performed as described from the Krebs laboratory (25) after immunoprecipitation of Erk-2 with a polyclonal antibody. Proteins were separated by discontinuous reducing SDS-PAGE (15% polyacrylamide gel), and the gels were fixed in acetic acid/isopropyl alcohol (12.5%:25%) and transferred to 1.4-mm thick X-OMAT film to determine the relative positions of the radioactive products. These products were quantified by PhosphorImager analysis (Molecular Dynamics, Inc., Sunnyvale, CA).

**Anchorage-independent Growth in Soft Agar**—To test the capability of cells expressing either wild-type IGF-I receptors or mutant IGF-I receptors to grow in an anchorage-independent manner, the cells were grown in soft agar. The abilities to
form colonies in DMEM supplemented with 10% FBS and 10 nm IGF-I were measured. As shown in Fig. 2 both clones expressing wild-type human IGF-I receptors (NWTb3 and NWTc43) formed significantly more colonies than did the negative control NIH-3T3 cells expressing only endogenous mouse IGF-I receptors (pNeo1). Cells expressing the double tyrosine mutation at positions 1250 and 1251 (yyFH) and yNF did not form colonies when grown under these conditions. The ability of these overexpressed receptors to transduce an IGF-I-initiated signal, resulting in a transformed phenotype.

Effect of Mutant IGF-I Receptor on Cellular Adhesion to Fibronectin—Substitution of tyrosine 1251 with a histidine residue was noted to affect cellular morphology and the ease of detaching the fibroblasts from tissue culture flasks. We hypothesized that changes in the function of the IGF-I receptor affected cellular adhesion to the extracellular matrix (ECM). Cellular adhesion of fibroblasts expressing wild-type receptors or mutant receptors was measured after growth of cells in 10% FBS. Analysis of variance, comparing the cellular adherence of each cell line in five separate experiments, revealed that there were no statistical differences between the cell lines due to some variability in the results. For example, in one representative experiment, the relative proportion of cells adhering to fibronectin during the first 30 min following plating was 54, 48, 45, and 48% for NWT, yyFH, yNF, and yNH cells, respectively. We conclude that replacement of both tyrosines 1250 and 1251 was important for the ability of stably transfected NIH-3T3 cells to form colonies in soft agar, cells expressing IGF-I receptors with only one of the tyrosine residues replaced were tested under the same experimental conditions. Cells expressing mutant receptors with replacement of either tyrosine 1250 (yNFa38 and yNFa45) or tyrosine 1251 (yNHa8 and yNHc5) did not form significant numbers of colonies (Fig. 2). Thus, replacement of either tyrosine 1250 or 1251 in the COOH terminus of the IGF-I receptor significantly reduced the ability of these overexpressed receptors to transduce an IGF-I-initiated signal, resulting in a transformed phenotype.

Effect of Expressed Mutant IGF-I Receptors on the Actin Cytoskeleton—Adherence of cells to the ECM is mediated through the integrin receptors. A second requirement for proper adherence of a cell to the ECM is an actin cytoskeleton that is capable of appropriate assembly and disassembly. Fluorescence microscopic examinations of control pNeo1 cells, cells expressing wild-type IGF-I receptors (NWTb3), and cells expressing mutant IGF-I receptors were done following staining for the actin cytoskeleton and for proteins (vinculin and paxil-
lin) in the associated focal adhesion plaque. As shown in Fig. 3, staining of the actin cytoskeleton was normal for NWTb3 cells (upper panel A), pNeo1 cells (data not shown), and cells expressing the 1250 mutant receptor (yNFa45) (upper panel B). These cell lines showed linear actin staining with termination of the actin strands in discrete regions primarily within the distal poles of the cells. The actin cytoskeleton was disrupted in cells expressing the 1251 mutant receptor (yNHc5) such that...

**Fig. 2** IGF-I-stimulated effects on the actin cytoskeleton and distribution of vinculin in NIH-3T3 cells expressing wild-type or mutant IGF-I receptors. Cells preincubated in DMEM supplemented with 0.5% FBS were stimulated with 10 nM IGF-I for 0, 8, and 15 min. Fixed and permeabilized cells were treated with a 5 μg/ml concentration of mouse monoclonal anti-vinculin antibody at room temperature for 1 h, followed by 1 h with 6.25 μg/ml anti-mouse antibody conjugated with fluorescein. Rhodamine phalloidin (0.67 units/ml) was added simultaneously with the second antibody. Upper panels (actin) and lower panels (vinculin) are representative of seven similar experiments using multiple clones. A, NWTb3; B, yNFa45; C, yNHc5; D, yyPHb1. Results are shown for the 8-min time point.
there is no prominent staining of linear actin strands (upper panel C). The actin staining is concentrated in long filopodial structures. In addition, the sizes of the cells are increased (the size may be better appreciated in lower panel C) as compared with NWTb3 cells. The actin cytoskeleton of cells expressing the 1250/1251 mutant receptor (yyFHb1) was less well organized (upper panel D) than that of NWTb3 cells but more organized than that of yNHc5 cells. The actin staining of each cell line was not dependent on IGF-I stimulation, at least under the conditions of this study. To assess if disruption of the actin cytoskeleton occurred in concert with disruption of focal adhesion plaques that are associated with the cytoskeleton, the same cells were simultaneously stained and examined for vinculin (Fig. 3, lower panel). The vinculin staining of each cell line with IGF-I stimulation (8 or 15 min) was similar to that of cells grown in the absence of IGF-I. Vinculin was localized in a punctate pattern, concentrated at the periphery of cells. The concentration of such foci was greatest in NWTb3 (lower panel A), yNFa45 (lower panel B), yyFHb1 (lower panel D), and pNeo1 cells (data not shown). In contrast, there is a lack of punctate staining in cells expressing the 1251 mutant IGF-I receptor (yNHc5 cells) (lower panel C). Paxillin stained in a pattern similar to vinculin in each of the cell lines (data not shown).

These results show that overexpression of IGF-I receptors (NWT cells) changes neither the pattern of the actin cytoskeleton nor the distribution of certain proteins in the focal adhesion plaques from that seen in cells expressing endogenous levels of IGF-I receptors (pNeo1). The 1250 mutant IGF-I receptor, when overexpressed, also does not change the actin cytoskeleton or the patterns of vinculin and paxillin when the cells are grown in the absence of IGF-I. We conclude that overexpression of a functional IGF-I receptor does not significantly change the actin cytoskeleton when fibroblasts are grown in the absence of IGF-I. Changes in these structural proteins are seen, however, in cells expressing the 1251 mutant receptor when grown under the same conditions. In the time frame of this experiment, stimulation with IGF-I does not further affect the structure of the actin cytoskeleton in the fibroblasts expressing endogenous IGF-I receptors or overexpressing wild-type (NWT) or mutant (1250 and/or 1251 substitutions) receptors.

**IGF-I-stimulated Autophosphorylation**—We considered the possibility that the mutant IGF-I receptors were abnormally phosphorylated on tyrosine residues in response to IGF-I treatment. Autophosphorylation of tyrosine residues in the β-subunit of the IGF-I receptor following IGF-I stimulation for 1 min at 37 °C was measured in cleared whole cell lysates from cells expressing only endogenous mouse IGF-I receptors (pNeo1) or cells expressing wild-type human or mutant human IGF-I receptors. A representative experiment measuring β-subunit phosphorylation is shown in Fig. 4. The nitrocellulose membrane was stripped and rebotted with antibody 1–2 to detect the total amount of IGF-I receptor applied to the gel (data not shown). The amount of phosphorylated β-subunit was normalized to the amount of IGF-I receptor. There were no significant differences in the level of IGF-I-stimulated β-subunit tyrosyl phosphorylation of mutant IGF-I receptors (yNF or yNH clones) when compared with the level of phosphorylation of wild-type IGF-I receptors (NWTb3 or NWTc43 cells). We had previously shown that the level of β-subunit phosphorylation of the mutant IGF-I receptors yyFH and yCF did not differ significantly from wild-type receptors (19). The results demonstrated in Fig. 4 are consistent with the previous findings that replacement of tyrosine residues in the COOH terminus of the IGF-I receptor does not affect the overall phosphorylation of tyrosine residues in the remainder of the β-subunit in response to IGF-I.

**IGF-I-stimulated Activation of IRS-1 and SHC Pathways**—To begin to elucidate the intracellular signal transduction pathways that may mediate the IGF-I signal, resulting in appropriate cellular adherence, proliferation, and transformation, we evaluated the level of tyrosyl phosphorylation in cellular immunoprecipitates of IRS-1 and SHC, both known positive effectors of the IGF-I receptor-mediated signal (27). A representative experiment is shown for each immunoprecipitation. We have previously shown that cells expressing the double tyrosine mutant receptor (yyFH) or the tyrosine 1316 mutant receptor (yCF) had levels of IGF-I-stimulated IRS-1 tyrosyl phosphorylation that were similar to that of cells expressing wild-type IGF-I receptors (19). As shown in Fig. 5, IGF-I-stimulated phosphorylation of IRS-1 in immunoprecipitates of Triton-soluble cellular proteins from cells expressing either tyrosine 1250 (yNF) (A) or 1251 (yNH) (B) mutant IGF-I receptors was approximately equivalent with that of cells expressing wild-type receptor (NWTb3 and NWTc43). Also shown for comparison are immunoprecipitates from yCF (A) and yyFH (B). When these membranes were stripped and rebotted for IRS-1, approximately equal amounts of IRS-1 were immunoprecipitated and loaded in each lane (data not shown). Although there was no diminution in IGF-I-stimulated total tyrosyl phosphorylation of IRS-1 in cells expressing mutant receptors, we postulated that these mutant receptors may reduce the ability of phosphorylated IRS-1 to form intact complexes with downstream adapter proteins. To further investigate the ability of the mutant receptors to mediate the IGF-I signal via the IRS-1 pathways, we investigated the association of known adapter molecules with phosphorylated IRS-1. Grb2, a 24-kDa adapter protein, could be detected in immunoprecipitates of IRS-1 and associated with IRS-1 from unstimulated
and stimulated cells. There was no increased association in the pNeo1 control cells following IGF-I stimulation (A). Grb2 association with IRS-1 was increased in IGF-1-stimulated cells, whether the cell was expressing wild-type or mutant IGF-I receptors (A and B). In fact, IRS-1 immunoprecipitates from IGF-I-stimulated cells expressing the mutant IGF-I receptors (yNF, yNH, yyFH, and yCF) contained approximately the same amount of Grb2 as did immunoprecipitates from IGF-I-stimulated NWTb3 and NWTc43. To investigate whether dephosphorylation might play a role in the signaling via IRS-1, we analyzed the amount of PTP1D/Syp in the immunoprecipitates (Fig. 5). There was some variability in the amount of PTP1D/Syp detected from one experiment to another. There was no increase in PTP1D/Syp association with IRS-1 in pNeo1 cells stimulated with IGF-I. The amounts of PTP1D/Syp protein associated with phosphorylated IRS-1 from cells expressing mutant receptors (yNF, yNH, yyFH, or yCF), as compared with cells expressing wild-type receptors, was the same in two other separate experiments (data not shown). It appears that replacement of tyrosines 1250, 1251, or 1316 does not affect IGF-I-induced activation of IRS-1-Grb2-PTP1D complexes.

Similarly, IGF-I stimulation of cells expressing the mutant receptors (yNF, yNH, yyFH, or yCF) yielded equivalent amounts of tyrosine-phosphorylated SHC immunoprecipitated with a polyclonal anti-SHC antibody as compared with SHC immunoprecipitates from cells expressing wild-type receptors (NWTb3 and NWTc43) (Fig. 6, A and B). The p52 isoform of SHC was phosphorylated in cells in the basal state and stimulated states. The phosphorylation did not increase in pNeo1 cells following stimulation with IGF-I (A). When the membrane was stripped and reblotted with a monoclonal antibody to SHC, both isoforms p46 and p52 could be detected (data not shown). In this particular experiment, the amounts of SHC in the stimulated cell lysates of yNFa38 (A) and yyFHb1 and yyFHb16 (B) were decreased. In three other experiments, these cell lines had significant increases in phosphorylation of SHC(p52) following IGF-I stimulation. We considered the possibility that replacement of the tyrosines in the COOH terminus of the IGF-I receptor, while not affecting the overall tyrosyl phosphorylation level of SHC, could reduce activation of the SHC signaling pathway. We examined the ability of SHC to form complexes with Grb2 in cells expressing wild-type and mutant IGF-I receptors in response to IGF-I (Fig. 7). There was no detectable Grb2 associated with SHC in any of the cells in the unstimulated state (Fig. 7, A and B). Grb2 was not seen in the immunoprecipitate of pNeo1 cells following IGF-I stimulation. Grb2 association with SHC increased following IGF-I stimulation of the cells and correlated with the amount of phosphorylated SHC(p52) in the immunoprecipitates. In three
other experiments (data not shown) it could be demonstrated that IGF-I stimulated Grb2 association with phosphorylated SHC in yNFa38 and yyFHb1. The results shown in Fig. 6 are consistent with the hypothesis that replacement of tyrosine residue 1250, 1251, or 1316 does not affect IGF-I-induced phosphorylation of SHC or activation of the SHC-Grb2 complexes.

**IGF-I-induced MAP Kinase Activation**—The possibility was considered that any changes in the association of immediate downstream substrates with the IGF-I receptor may have been below the detection limits of our studies. If in fact the mutant IGF-I receptors mediated signals that only modestly affected some of these proximal IGF-I-induced complexes, it remained possible that activities of downstream proteins in the signaling cascade could be significantly reduced. In particular, since both activated IRS-1 and SHC complexes result in activation of the MAP kinase pathway, we examined the phosphorylation of Erk-1 and Erk-2 in response to IGF-I and correlated those results with the IGF-I-induced activation of Erk-2 using myelin basic protein as a substrate. It has been shown that phosphorylation of threonine 183 and tyrosine 185 of both Erk-1 and Erk-2 is indicative of fully stimulated MAP kinase activity (28, 29). As shown in Fig. 7A, IGF-I stimulated tyrosine phosphorylation of two proteins around 44 kDa, presumably the tyrosine 185-specific phosphorylated forms of Erk-1 and Erk-2 (Fig. 7A) in cells expressing the wild-type IGF-I receptor. There was no increase in phosphorylation of Erk-1 or Erk-2 in the negative control cell line pNeo1 in response to IGF-I. The identity of these tyrosine-phosphorylated bands was confirmed when the membrane was stripped and immunoblotted with an antibody that recognizes both Erk-1 and Erk-2 (Fig. 7B). Approximately similar amounts of the MAP kinases were immunoprecipitated. The lack of detectable tyrosine-phosphorylated Erk-1 and Erk-2 was not due to the lack of Erk-1 or Erk-2 in the immunoprecipitates. Cells expressing mutant IGF-I receptors (yNHa8 and yyFHb1) contained IGF-I-stimulated tyrosine-phosphorylated Erk-1 and Erk-2 in proportions that were similar to those of cells expressing wild-type receptors (NWTc43) (Fig. 7A). The activity of MAP kinases in an Erk-2 immunoprecipitate was measured using myelin basic protein as a substrate (Fig. 8). IGF-I induced a small increase in MAP kinase activity in the control pNeo1 cells, most likely via the low number of endogenous mouse receptors. Overexpression of wild-type human receptors greatly augmented this response (NWTc43).

Overexpression of the mutant receptors with replacement of either tyrosine 1250 (yNFa38 and yNFa45) or 1251 (yNHa8 and yNHa5) also significantly increased IGF-I-induced MAP kinase activation. These results indicate that the IGF-I-induced signals that converge by activating the MAP kinase pathway were not affected by replacement of either tyrosine 1250 or 1251 of the receptor.

**IGF-I-induced PI 3'-Kinase Activation**—The second major pathway that is activated by IGF-I stimulation is PI 3'-kinase. Anti-phosphotyrosine immunoprecipitates from cells either unstimulated or stimulated with 10 nM IGF-I were assayed for PI 3'-kinase. A representative assay is shown in Fig. 9. A summary of several assays is presented in Table I. Similar to previously shown data (16), NIH-3T3 cells overexpressing wild-type IGF-I receptors had approximately a 6.4-fold increase in PI 3'-kinase activity in response to IGF-I, whereas the negative control cell line (pNeo1) had only an 1.8-fold increase. Cells expressing mutant IGF-I receptors with either tyrosine 1250 (yNF) or 1251 (yNH) replaced had IGF-I-induced PI 3'-kinase activation that was as good as that of wild-type receptors. Both clones of yNF displayed an increase in activity that was significantly greater than in the pNeo1 cells, with yNFa38 at 10.9-fold and yNFa45 at 5.3-fold stimulation. The clones of yNH also had IGF-I-induced PI 3'-kinase stimulation significantly higher than that of the pNeo1 cells, with yNHa8 at 6.6-fold and yNHa5 at 7.1-fold stimulation. These results indicate that replacement of tyrosine 1250 or 1251 does not inhibit the IGF-I-induced PI 3'-kinase activity.

**DISCUSSION**

IGF-I signaling through the IGF-I receptor is important in the rearrangement of the cytoskeleton, adherence to ECM proteins, stimulation of cellular proliferation, and induction/maintenance of a transformed phenotype. IGF-I induces specific rearrangements of the cytoskeleton in several cell types (30–33). The rapid rearrangement of the cytoskeleton is necessary for cellular motility and cellular division (31, 32). We have shown that replacement of tyrosine 1251 in the IGF-I receptor affects cellular morphology and cellular proliferation. Cellular
morphism, adherence to ECM proteins, and proliferation are dictated by the coordination of the focal adhesion complex, consisting of the integrin-ECM protein receptors and proteins of the focal adhesion plaque on the intracellular face of the plasma membrane, and the actin cytoskeleton (34, 35). Proteins located in the focal adhesion plaque include vinculin, paxillin, α-actinin, and talin (36, 37). These proteins and their phosphorylation states are critical for the regulation of actin organization (34, 38). Vinculin binds paxillin and co-localizes in the focal adhesion complex with talin, α-actinin, F-actin, and the β1-subunit of the integrin-ECM receptor in fibroblasts (36, 39–41). Vinculin has been implicated in the control of adhesion and motility in fibroblasts (42, 43), PC12 cells (44), and chick neuronal cells (45). Filopodia and lamellipodia, structures important in cell morphology and function, are less stable in vinculin-deficient PC12 cells than in control cells (44). Vinculin-deficient F9 embryonic carcinoma cells are capable of forming filopodia but do not form functional lamellipodia (41). Transient inactivation of vinculin in chick neuronal cells results in structurally abnormal filopodia, consistent with the hypothesis that vinculin provides structural integrity to the filopodia (45). Depending on the cell type studied and whether the removal of vinculin is permanent or temporary, the effect on cellular adhesion varies. In our studies, we have shown that the 1251 mutation of the IGF-I receptor changes the location of vinculin within the cell similar to that of vinculin-deficient cells wherein there was a concentration of vinculin in filopodia but a paucity of vinculin at the end of the actin stress fibers. In fact, specific perturbation of the IGF-I receptor also results in disorganization of the actin cytoskeleton. Fibroblasts expressing the 1251 mutant IGF-I receptor demonstrate this disorganized cytoskeleton when grown in the absence of IGF-I. As expected for the time course of the experiment, acute stimulation with IGF-I did not affect the actin cytoskeleton. Nor did acute IGF-I stimulation alter the distribution of vinculin. As discussed below, we could demonstrate no changes in the IGF-I-induced phosphorylation/dephosphorylation patterns of several known proteins in the IGF-I signaling cascade in fibroblasts expressing the 1251 mutant receptor to account for this perturbation of structural proteins. The phenomenon of chronic disruption of the actin cytoskeleton in cells with the 1251 mutant receptor suggests that this receptor may be associated with a change in the level of expression of one or more proteins. Whether this is a direct effect of the IGF-I receptor signaling cascade or whether it is by a perturbation in cross-talk with other receptor signaling cascade systems is unknown at this time. In studies reported here, fibroblasts expressing the 1251 mutant IGF-I receptor had equal or increased adhesion to fibronectin compared with cells expressing wild-type receptors. This is in contradistinction to the reduced adherence seen with the vinculin-deficient cell lines and cells from mice in which vinculin was deleted by homologous recombination (46, 47). The reasons for these discrepancies are unclear at this point, but one possibility is that aberrant IGF-I signal transduction perturbs one or more proteins that modulate cellular adhesion in connection to vinculin. Published studies of endothelial cells and chondrocytes suggest that IGF-I stimulation increases the expression of specific cellular adhesion molecules, such as intracellular adhesion molecule-1, vascular cell adhesion molecule-1, and α,β1 integrin (48, 49). Antibodies to E-cadherin prevented the cell-cell adhesion of human breast cancer cells overexpressing IGF-I.

![Image](image_url)

**FIG. 9.** IGF-I-stimulated PI 3'-kinase activity in NIH-3T3 cells expressing either wild-type or mutant receptors. Confluent monolayers of cells were serum-starved overnight and then either not stimulated (−) or stimulated (+) with 10 nM IGF-1 for 3 min. Preparation of the cell lysates, immunoprecipitation of tyrosine-phosphorylated proteins with the antibody 4G10, and the assay of PI 3'-kinase activity using phosphatidylinositol (PIP) as a substrate were performed as described under “Experimental Procedures.” The autoradiograms of the products separated by thin layer chromatography of a single experiment are shown. The position of PIP loaded as a control is indicated on the left.

### TABLE I

| Cell line Stimulation | yNHa8 | yNHc5 | NWTo43 |
|-----------------------|-------|-------|--------|
| pNeo1                 |       |       |        |
| 1 b                   | 39    |       |        |
| 1 c                   | 49    |       |        |
| 1 yNF (y1250F)        | 10.90 |       |        |
| 1 a38                 | 6.55  |       |        |
| 1 a45                 | 5.94  |       |        |
| 1 yNH (y1251H)        |       |       |        |
| 1 a8                  |       | 6.55  |        |
| 1 c5                  |       | 5.94  |        |

Mean-fold stimulation of phosphatidylinositol 3'-kinase activity in cells expressing wild-type or mutant IGF-I receptors is shown. Values presented are the ratio of signal in the phosphatidylinositol 3'-monophosphate in p85 immunoprecipitates of cells stimulated with 10 nM IGF-I for 1 min over the signal from unstimulated cells.
receptors (50). We propose that even in the presence of vinculin, aberrant signaling from the IGF-I receptor disrupts the function of the focal adhesion complex and maintenance of actin stress fibers. We postulate that tyrosine 1251 of the IGF-I receptor is important in the formation and maintenance of the cytoskeleton.

Tyrosine 1250 in the IGF-I receptor is also important in cellular proliferation and induction/maintenance of a transformed phenotype despite the relatively normal cellular adhesion of fibroblasts expressing this mutant IGF-I receptor. We considered the possibility that the known IGF-I signaling pathways were perturbed by replacement of tyrosine 1250 or 1251 in the receptor. The fully activated wild-type IGF-I receptor is capable of associating with downstream substrates with subsequent tyrosyl phosphorylation of these substrates. Phosphorylation of these “docking” proteins results in the stimulation of several intracellular signaling cascades that have been partially described. At present, it is known that the stimulation of cells with IGF-I results in the phosphorylation of members of the IRS family of proteins (51–53). Tyrosine-phosphorylated IRS-1 binds to the regulatory subunit (p85) of PI 3’-kinase (51), the tyrosine phosphatase PTP1D/Syp (52, 54–56), and Grb2 (57, 58), which then binds through its Src homology domain to the GTP-GDP exchange protein son of sevenless (mSOS) (57, 58). The interaction of Grb2 with mSOS induces Rac activation by exchanging GTP for Ras-bound GDP (59, 60) and the stimulation of the MAP kinase kinase (or Erk) pathway (61, 62). MAP kinases/Erkks have many substrates including transcription factors, thereby transducing the growth factor signal to the nucleus. Phosphorylation of the IGF-I receptor also results in phosphorylation of SHC on tyrosine residues that act as binding sites for Grb2, association of mSOS with Grb2, and activation of the MAP kinase pathway via activation of Ras (61, 63–67). In breast cancer cells, it has been shown that normal levels of SHC are necessary for appropriate cell-cell interactions (68). We considered the possibility that SHC phosphorylation and/or association with downstream proteins could have been reduced in those cells demonstrating abnormal cell morphology and adherence. Studies described here of NIH-3T3 cells overexpressing the tyrosine 1251 mutant IGF-I receptor indicate that normal levels of SHC and association of tyrosine-phosphorylated SHC with Grb2 are not adequate for normal cellular cytoskeleton, cellular adhesion, or cellular proliferation.

In the studies of NIH-3T3 cells overexpressing IGF-I receptors presented here, it is shown that IGF-I stimulation of the cells resulted in phosphorylation of the MAP kinases Erk-1 and Erk-2 and activation of Erk-2 enzyme activity over that observed in the cell line expressing only endogenous receptors. Replacement of tyrosine residue 1250 or 1251 did not affect the total level of phosphorylation of Erk-1 and Erk-2 or the ability of Erk-2 to phosphorylate the substrate myelin basic protein in response to IGF-I. Because several pathways have been shown to converge or integrate their signals via the MAP kinase pathway, we conclude that tyrosines 1250 and 1251 are not essential for the activation of this pathway. Furthermore, the replacement of tyrosines 1250 and 1251, even in combination, did not affect the IGF-I-stimulated phosphorylation of IRS-1 or the formation of the IRS-1-Grb2 complex, which is known to activate the MAP kinase pathway via mSOS, Ras, Raf-1, and MEK activation. The second pathway known to integrate its signal through the MAP kinase pathway was also not affected. IGF-I stimulation of SHC phosphorylation and the formation of the SHC/Grb2 complex was the same in cells expressing the IGF-I receptor with tyrosines 1250 and 1251 replaced, singly or in combination, as in cells expressing wild-type IGF-I receptors. Thus, not only do tyrosines 1250 and 1251 not appear to be essential for activation of the MAP kinase pathway; these residues do not appear to be essential for signaling via the IRS-1-Grb2 and SHC-Grb2 activation complexes. These results are consistent with the known binding sites for IRS-1 and SHC in the protein-tyrosine kinase receptors. Both IRS-1 and SHC are known to bind to NPY-containing peptides whose sequences are found in the juxtamembrane domains of the these receptors (69, 70). This is not to say that changes in the COOH terminus of the IGF-I receptor do not affect the binding and/or subsequent activation of substrates that principally interact with other portions of the receptor. Rather, it is necessary to perform further studies to determine if tyrosines in the COOH terminus of the IGF-I receptor modulate binding of specific substrates. At this point in time, it is not clear which substrates are primarily affected by replacement of tyrosines 1250 or 1251, such that the cytoskeleton is disrupted and IGF-I-stimulated cellular proliferation is inhibited.

Several lines of evidence have demonstrated the importance of functional signaling through the IGF-I receptor in initiating or maintaining a transformed phenotype. We and others have shown previously that functional IGF-I receptors are necessary for fibroblast xenografts to be capable of forming anchorage-independent colonies in soft agar and tumors in athymic mice (5, 18–20). Constitutive activation of the MAP kinase/Erk pathways has been associated with promotion and/or maintenance of a tumorigenic phenotype (71–74). In the studies presented here, however, cells that overexpressed receptors with replacement of the COOH-terminal tyrosines 1250 and 1251 could transduce an augmented signal resulting in increased MAP kinase activity, but this increased activity did not translate into an increase in mitogenesis or transformation of the cells such that these cells could grow in an anchorage-independent manner. We propose that overexpression of components such as IRS-1, SHC, Ras, Raf-1, or MEK of the MAP kinase pathway can increase signaling through the pathway that is unopposed by any regulatory pathways in the cell. However, overexpression of COOH-terminal mutant IGF-I receptors can result in activation of the same MAP kinase pathway; but at the same time a second, as yet undefined pathway, which is essential for initiation and/or maintenance of the transformed phenotype, has been altered. We considered that the PI 3’-kinase pathway may have been affected by replacement of the tyrosines in the COOH terminus of the receptor. Although there have been no reports of constitutively active PI 3’-kinase conferring a transformed phenotype to cells, it has been shown that expression of constitutively active p110 protein in fibroblasts resulted in growth factor-independent enhancement of transcription from the c-fos promoter, and this enhancement could be blocked by co-expression of dominant negative Ras (75). Therefore, we tested the possibility that the COOH-terminal mutants might have affected the activity of the PI 3’-kinase. IGF-I-stimulated PI 3’-kinase activity was equal in cells expressing the mutant receptors to cells expressing wild-type receptors. It thus appears that tyrosines 1250 and 1251 of the IGF-I receptor are not essential for PI 3’-kinase activation by IGF-I. These results are not surprising, since previous studies have shown that the p85 subunit of PI 3’-kinase binds to tyrosine 1316 (76). Therefore, of the mutant IGF-I receptors studied, we would have predicted that the yCF (tyrosine 1316 substitution) IGF-I receptor would have had the greatest effect on PI 3’-kinase activity. However, both clones expressing this mutant receptor displayed equivalent PI 3’-kinase in response to IGF-I as did the wild-type clones. In this system, it appears that the major signaling via the IGF-I receptor is not directly to the p85 subunit but rather via IRS-1, which is known to interact with both the receptor and p85 (77–79). We cannot
discount, however, the possibility that PI 3'-kinase can associate with other motifs of the IGF-I receptor not specifically affected by the mutations we studied. Studies of the interactions between these two molecules in the yeast two-hybrid system indicate that regions other than the motif surrounding tyrosine 1316 in the COOH terminus of the receptor may modulate the interaction (75). However, unlike interactions with IRS-1 and SHC, tyrosine 950 in the juxtamembrane domain does not appear to be involved in the interaction of the receptor with the p85 regulatory subunit of PI 3'-kinase (80).

Overexpression of mutant IGF-I receptors with the replacement of tyrosines 1250 and 1251 in fibroblasts significantly affects the phenotype that is transduced by overexpression of wild-type IGF-I receptors in these same cells. We propose that this effect is similar to the abrogation of mitogen-stimulated cellular proliferation and transformation that was observed when the action of any of the signaling molecules discussed above was blocked. However, in the system presented here we are able to show that the IGF-I mutant receptors have normal activation of the MAP kinase and PI 3'-kinase pathways. These results are in agreement with the conclusion of Sell et al. (81) that signals mediated by the IGF-I receptor resulting in cellular transformation could be via a Ras-independent pathway. We extend this conclusion to state that such a pathway could also be PI 3'-kinase-independent.

Taken together, the results reported here and previously (19) indicate that replacement of specific tyrosine residues in the COOH terminus of the IGF-I receptor significantly reduces mitogen-stimulated cellular proliferation. Furthermore, whereas overexpression of wild-type IGF-I receptors transforms fibroblasts into cells with a high tumorigenic potential, overexpression of receptors with these specific tyrosine residues replaced abolishes this tumorigenic potential. Although previous studies have strongly implied that constitutive activation of the MAP kinase pathway induces a transformed phenotype, in our hands cells rendered nontumorigenic by way of mutated IGF-I receptors did not show a diminution in MAP kinase activation. Furthermore, we could document no decrease in the acute activation of other signaling proteins (IRS-1, SHC, and PI 3'-kinase) that have been implicated in potentiating cellular proliferation and initiating and/or maintaining a transformed phenotype. We are able to show that tyrosine 1251 in the COOH terminus of the IGF-I receptor is critical for the appropriate configuration of the actin cytoskeleton and the presence of at least one of the known proteins in the focal adhesion plaque through which the cytoskeleton interacts with the plasma membrane and extracellular attachment sites. In conclusion, we have shown that both tyrosines 1250 and 1251 are involved in signaling from the IGF-I receptor that impacts the cytoskeletal architecture, cellular proliferation, and the transforming ability of the cells. This altered phenotype cannot yet be correlated with alterations in the IGF-I stimulation of known signaling pathways emanating from the IGF-I receptor.

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