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# Tumorigenic and Mitogenic Capacities Are Reduced in Transfected Fibroblasts Expressing Mutant Insulin-Like Growth Factor (IGF)-I Receptors. The Role of Tyrosine Residues 1250, 1251, and 1316 in the Carboxy-Terminus of the IGF-I Receptor

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**ABSTRACT**

Regulation of ligand-mediated signal transduction through transmembrane tyrosine kinase growth factor receptors involves phosphorylation of tyrosine residues in the intracellular domain of the receptor. The insulin-like growth factor-I (IGF-I) receptor contains three tyrosine residues in the carboxy-terminal domain at positions 1250, 1251, and 1316. Of these, only the tyrosine at position 1316 is conserved in the homologous position of the insulin receptor. Mutational analysis was used to study the role of these tyrosines in specific outcomes of IGF-I-mediated signal transduction. Mutations in the human IGF-I receptor were either replacement of tyrosines 1250 and 1251 with phenylalanine and histidine (yyFH), respectively, or replacement of the conserved distal tyrosine (position 1316) with phenylalanine (yCF). The yyFH mutation results in an IGF-I receptor with the amino acids found in the homologous position of the human insulin receptor. Cells overexpressing mutated IGF-I receptors were compared with cells expressing only endogenous IGF-I receptors or overexpressing wild-type IGF-I receptors. The ability of yyFH mutant IGF-I receptors to autophosphorylate the β-subunit or phosphorylate insulin receptor substrate-1 was not significantly different from wild-type IGF-I receptors. However, one or both of the proximal tyrosine residues (positions 1250 and 1251) in the carboxy-terminus of the IGF-I receptor are essential for IGF-I-stimulation of mitogenic and tumorigenic pathways. IGF-I-induced mitogenesis, measured as thymidine incorporation and cellular proliferation, was abrogated in cells overexpressing mutant IGF-I receptors with replacement of the proximal double tyrosines (positions 1250 and 1251). Fibroblasts expressing this mutant IGF-I receptor formed fewer tumors than the negative control cells, whereas cells expressing wild-type IGF-I receptors formed large tumors in all recipient mice injected. Conversely, cells expressing mutant IGF-I receptors with only the conserved distal tyrosine (position 1316) replaced had slightly reduced IGF-I-stimulated β-subunit autophosphorylation, thymidine incorporation, and cellular proliferation when compared with cells expressing wild-type receptors. Phosphorylation of insulin receptor substrate-1 by the yCF mutant receptors was not impaired. Despite the ability of these mutant receptors to stimulate mitogenic growth, fibroblasts expressing this mutant receptor were also incapable of forming tumors in recipient nude mice. The distal tyrosine (position 1316) of the IGF-I receptor is crucial for tumor formation but is not essential for IGF-I stimulated mitogenesis. Thus, the tyrosine moieties in the carboxy-terminus of the IGF-I receptor participate in the signal transduction pathways that affect the mitogenic and tumorigenic potentials of cells expressing mutant IGF-I receptors. (*Endocrinology* **137:** 410–417, 1996)

THE INSULIN-like growth factor-I (IGF-I) receptor is a member of the transmembrane growth factor tyrosine kinase receptor family that includes the insulin receptor and insulin-related receptor (1). The IGF-I receptor is known to be involved in the control of normal cellular differentiation and growth, whereas the major physiological function of the insulin receptor is metabolic. The intracellular domain of the IGF-I receptor, in particular, shares homology with the insulin receptor. Within the intracellular domain, several functional domains have been identified. The catalytic (tyrosine kinase) domain shares approximately 85% homology with the equivalent region of the insulin receptor, whereas the juxtamembrane domain and the carboxy-terminal (C-terminal) domain just distal to the catalytic domain are 61% and 44% homologous, respectively. This suggests that the differential physiological function between the IGF-I receptor and insulin receptor may be mediated by the different functional subdomains of the C-termini of these receptors (2).

In addition to the important tyrosine residues in the kinase domain and juxtamembrane region, there are additional tyrosine residues located in the C-terminal domains of both the human IGF-I and insulin receptors (1). In the IGF-I receptor there are two tyrosine residues [Tyr 1250 and 1251, numbering sequence of Ullrich *et al.* (1)] immediately distal to the tyrosine kinase domain that are absent in the insulin receptor. The insulin receptor has two tyrosine residues in the distal C-terminus, of which only one is conserved in the IGF-I receptor (Tyr 1316). Previous studies to delineate the roles of the subdomains of the C-terminal domain of the IGF-I and insulin receptors have used truncation mutants, domain swapping, and antibodies directed to peptides contained within the receptors. Progressive truncation of the C-termi-

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nal domain of a Gag-IGF-I receptor abolished and then restored tyrosine kinase and transforming abilities of the Gag-IGF-I receptor protein (3). An antibody raised to a peptide corresponding to positions 1232–1246 of the IGF-I receptor significantly reduced tyrosine autophosphorylation (4), but an antibody raised against the homologous region of the insulin receptor did not affect *in vitro* tyrosine kinase activity toward an exogenous substrate (5, 6). This peptide corresponds to the region of the IGF-I receptor just proximal to the double tyrosines in the C-terminal domain of the IGF-I receptor.

Removal of the entire C-terminus and a distal portion of the tyrosine kinase domain of the insulin receptor with deletion of 113 (7) or 112 (8) amino acids results in unstable receptors. Deletion of only 82 amino acids produced a stable insulin receptor with significantly decreased autophosphorylation but unchanged insulin-stimulated insulin receptor substrate (IRS)-1 phosphorylation, thymidine incorporation, and 2-deoxyglucose uptake (9). Insulin receptors truncated by 69 amino acids autophosphorylate as well as wild-type receptors when stimulated by insulin but have diminished capacity to phosphorylate exogenous substrates (10). These data suggest that particular subdomains of the C-terminal region of the insulin receptor and, indeed, specific amino acid residues may mediate activation of the signal transduction cascade in a receptor-specific fashion.

We have previously shown that interchanging the C-terminal domains of the insulin and IGF-I receptors resulted in mutant chimeric receptors with altered function (11). Replacement of the insulin C-terminus with that of the IGF-I receptor C-terminus resulted in significantly decreased autophosphorylation, phosphorylation of IRS-1, thymidine incorporation, induction of c-*fos* mRNA, and ornithine decarboxylase (ODC) activity. Unlike the insulin receptor with IGF-I receptor C-terminus, the reciprocal mutant receptor showed no change in those functions except for an increase in ODC activity (11). These results also suggest the importance of the C-terminal domain in the differential functions of the IGF-I and insulin receptors. Tartare and co-workers (12) have reported that fibroblasts expressing chimeric receptors with the distal tyrosine kinase and C-terminal domains of the insulin receptor replacing the homologus domains of the IGF-I receptor had decreased basal autophosphorylation but similar fold IGF-I-stimulation of the β-subunit autophosphorylation and similar levels of phosphorylation of IRS-1 when compared with cells overexpressing wild-type IGF-I receptors. Thymidine incorporation was not significantly different between cells expressing about twice the numbers of the chimeric receptors as compared with those expressing IGF-I receptors, but IGF-I-stimulated glycogen synthesis was increased (12). These results suggest that the C-terminal domains of the IGF-I and insulin receptors mediate separate functions, but identification of specific moieties of the C-terminal domains responsible for various interactions with downstream mediators have not been delineated.

To study the roles of specific tyrosine residues in the C-terminus of the IGF-I receptor, we have mutated both tyrosines (Tyr 1250 and 1251) just distal to the tyrosine kinase domain to amino acids homologous to those in the insulin receptor and, separately, mutated the conserved distal tyrosine residue (Tyr 1316) to phenylalanine. Wild-type and mutant IGF-I receptors were expressed in NIH-3T3 cells. IGF-I-stimulated tyrosine kinase activity was assessed by measuring autophosphorylation of the β-subunit and phosphorylation of IRS-1. The cellular proliferation and tumorigenic potentials for each fibroblast line expressing wild-type and mutant IGF-I receptors were evaluated.

## Materials and Methods

### *Materials and animals*

Restriction endonucleases were purchased from New England Biolabs (Beverly, MA), Boehringer Mannheim (Indianapolis, IN), and Bethesda Research Laboratories (Gaithersburg, MD). Cell culture media and reagents were purchased from Biofluids, Inc. (Rockville, MD) and Advanced Biotechnologies (Columbia, MD). Insulin-free BSA (BSA, fraction V) was obtained from Armour (Kankakee, IL). Recombinant human IGF-I, monoclonal antiphosphotyrosine antibody conjugated to horseradish peroxidase (4G10), and FBS were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Recombinant antiphosphotyrosine RC20H horseradish peroxidase-conjugate was purchased from Transduction Laboratories (Lexington, KY). Monoiodinated [$^{125}$I]-IGF-I, [$^{3}$H]-thymidine, and the enhanced chemoluminescence (ECL) detection kit were purchased from Amersham (Arlington Heights, IL). Prestained high molecular weight protein standards and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO). NIH athymic nude mice were obtained from the Research Resources Units of NIH.

### *Construction of the mutant IGF-I receptor cDNA*

The wild-type human IGF-I receptor expression vector has been previously described (13). Mutations of the human IGF-I receptor cDNA in the pBluescript II plasmid were performed by *in vitro* site-directed mutagenesis using the Transformer Site-Directed Mutagenesis Kit from Clontech (Palo Alto, CA). The sequence of the plasmid primer was 5′-AAAGCTGGGTACCGGTCACCCCCTCGAGGTCGA-3′. The sequence of the mutagenic primer for the mutation designated yyFH was 5′-TTCCGGGAGGTAAGCTTC**TTCCAC**AGCGAGGAGA-3′. This mutation (yyFH) converted the codons encoding the tyrosine residues at positions 1250 and 1251 (1) to phenylalanine and histidine, respectively. The codons for phenylalanine and histidine are shown in bold letters and the introduced *Hind*III restriction site is *underlined*. The phenylalanine and histidine residues are the same as those residues found in the homologous positions of the human insulin receptor. The yCF mutation, converting the conserved distal tyrosine residue (position 1316) to phenylalanine, was generated using a mutagenic primer of 5′-GAGAGA CAGCCT**TTT**GCGCACATGAACGGGGC-3′. The codon for phenylalanine is shown in *bold letters*, and the introduced *Fsp*I site is *underlined*. The cDNA sequences of both mutations were confirmed by restriction enzyme mapping and dideoxysequencing. The mutant cDNAs in pBluescript II were excised with *Sal*I and *Not*I and cloned into a bovine papilloma virus-derived mammalian expression vector (pBPV, Pharmacia, Piscataway, NJ) that had been linearized with *Xho*I and *Not*I.

### *Cell culture and transfection*

All NIH-3T3 cell lines were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 95% air and 5% $CO_2$ at 37 C. NIH-3T3 cells were co-transfected with 20 μg of mutant expression vector or insert-less pBPV plus 1 μg pMC1Neo (Clontech, Palo Alto, CA) in LIPOFECTIN reagent (Bethesda Research Laboratories, Gaithersburg, MD). Selection was carried out as previously described (13). Clones overexpressing IGF-I receptors were selected based on results of IGF-I binding assays as previously described (13). Stably transfected cells were maintained in DMEM supplemented with 10% FBS, antibiotics, and 500 μg/ml G418 (Geneticin; GIBCO, Grand Island, NY). Cells were split for each exper-

iment and cultured in serum-supplemented DMEM without G418. Serum-free medium (SF-DMEM) composed of DMEM with 0.1% BSA, 20 mM HEPES, pH 7.5, and antibiotics was used in assays of IGF-I receptor binding, thymidine incorporation, receptor autophosphorylation, and tyrosine kinase activity. The control cell line (pNeo1) and the cell line overexpressing wild-type IGF-I receptors (NWTb3) have been described previously (14). Other cell lines overexpressing wild-type IGF-I receptors (NWTc34, NWTc43, and NWTc52) were developed in the same manner as the cell line NWTb3.

### *Intact cell tyrosine phosphorylation*

Subconfluent cells in 100-mm plates were serum-starved in SF-DMEM for 16 h, washed twice with SF-DMEM, and then incubated either with or without IGF-I (10 nM) for 1 min at 37 C. The cells were washed rapidly with 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, 100 mM NaCl, 10 mM EDTA, 1% Triton X-100, 4 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium fluoride, 2 μg/ml leupeptin and 2 μg/ml aprotinin). Cell lysates were cleared by centrifugation. Protein content was determined by the method of Bradford using a protein assay kit (Bio-Rad, Richmond, CA). Proteins were stacked through a 4% SDS-polyacrylamide gel and then separated by electrophoresis through a 7.5% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membrane for 4 h at 0.22 A in a tris-glycine buffer with 20% methanol. The amount of IGF-I receptor present on the nitrocellulose membrane was determined by immunoblotting with $Ab_{53}$, a polyclonal antibody that detects the triple tyrosine cluster of the IGF-I receptor. Equivalent amounts of IGF-I receptors were then resolved by SDS-PAGE and transferred to nitrocellulose. Tyrosine-phosphorylated proteins were immunoblotted with a monoclonal antiphosphotyrosine antibody (4G10) conjugated to horseradish peroxidase(dilution 1:1000, UBI) using the ECL detection system. Phosphorylated IRS-1 was detected by immunoblotting with RC20H (dilution 1:2500) followed by treatment of the membrane with the ECL detection system. Radiographic exposures of chemoluminescence-detected immunoblots were scanned densitometrically using the NIH software program Image v1.55. Arbitrary units were normalized to the density of the protein bands from cells overexpressing wild-type IGF-I receptors (NWTb3).

### *Thymidine incorporation*

Subconfluent cell monolayers in 12-well plates were grown to quiescence in SF-DMEM with 0.1% BSA for 24 h. Cells were then incubated for 16 h in DMEM containing either 10% FBS or SF-DMEM with 0.1% BSA plus various concentrations of IGF-I. The medium was replaced with fresh SF-DMEM containing 1 μCi/well of [*methyl*-$^3$H]-thymidine, and the cells were incubated for an additional hour. The cells were rinsed twice with ice-cold PBS, twice with ice-cold 5% trichloroacetic acid, and twice with ice-cold 95% ethanol. The cells were lysed in 0.3 ml of 1 N NaOH, neutralized with 0.3 ml of 1 N HCl, and counted in a liquid scintillation counter.

### *Cellular proliferation*

Cell growth was determined by measuring the colorimetric change of 10% MTT in medium without phenol red after incubation with cells for 4 h at 37 C, followed by lysis of the cells with isopropanol (15, 16). Each cell line was plated in triplicate for each time point. To assess *in vitro* cellular proliferation that would most closely approximate the *in vivo* growth environment, cells were allowed to grow in DMEM supplemented with 10% FBS. To determine the effect of IGF-I on cellular proliferation, cells were grown in DMEM supplemented with 1% FBS and compared with a second set of cells grown in the medium supplemented further with 10 nM IGF-I. Cell number was determined daily from time 0 to 144 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 NWTb3 was subsequently performed with each experiment.

### *Tumor formation in athymic nude mice*

The tumor-forming capacity of parental cells (NIH-3T3), control cells (pNeo 1), and cells transfected with wild-type IGF-I receptors (NWTb3 and NWTc34) or mutant IGF-I receptors (yCFa12, yCFb43, yyFHb1, and yyFHb16) injected sc into recipient animals was measured. In all experiments, male mice, matched for age and weight, were used. Cells ($1 \times 10^7$ cells in 150 μl) were injected sc over the lower dorsal region. Mice were examined for the presence of palpable tumors for a 9-week period. The size of palpable tumors was measured using calipers. Mice were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

### *Statistics*

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

## Results

### *Receptor number of individual clones expressing yyFH and yCF mutant IGF-I receptors*

Individually isolated clones of NIH-3T3 cells transfected with the double tyrosine (yyFH) and distal single tyrosine (yCF) mutant IGF-I receptors were analyzed for cell surface IGF-I receptors by displacement of radiolabeled IGF-I with unlabeled IGF-I. Scatchard analysis was performed using the NIH-Ligand program (17). The receptor number per cell of each cell line is presented in Table 1. The results presented are the mean of at least three measurements. Individual clones expressing mutant IGF-I receptors were matched to NIH-3T3 cells overexpressing similar numbers of wild-type IGF-I receptors. To obtain at least two clones with approximately equal numbers of receptors per cell, two separate transfections for both yyFH and yCF mutant receptors were performed.

### *Receptor autophosphorylation and in vivo tyrosine kinase activity*

Cleared whole cell lysates from cells either not stimulated or stimulated with 10 nM IGF-I were analyzed for phosphorylation of the IGF-I receptor β-subunit and IRS-1. Immunoblots representative of three separate experiments are shown in Fig. 1. IGF-I-stimulated autophosphorylation of the β-subunit of the endogenous IGF-I receptors in the control cells

**TABLE 1.** Transfected cell clones used and number of IGF-I receptors per cell

| Cell line | Overexpressed IGF-I receptor | Receptors/cell ($\times 10^{-3}$) |
|---|---|---|
| NIH-3T3 | None | 19 ± 5 |
| pNeol | None | 19 ± 6 |
| NWTb3 | Wild-type human | 360 ± 22 |
| NWTc34 | Wild-type human | 200 ± 28 |
| NWTc43 | Wild-type human | 370 ± 35 |
| NWTc52 | Wild-type human | 360 ± 118 |
| yyFHb1 | $Tyr^{1250} \Rightarrow$ Phe, $Tyr^{1251} \Rightarrow$ His | 250 ± 43 |
| yyFHb16 | $Tyr^{1250} \Rightarrow$ Phe, $Tyr^{1251} \Rightarrow$ His | 420 ± 68 |
| yCFa12 | $Tyr^{1316} \Rightarrow$ Phe | 180 ± 56 |
| yCFb43 | $Tyr^{1315} \Rightarrow$ Phe | 510 ± 97 |

Receptors/cell were determined by Scatchard analysis by displacement binding of IGF-I. The mean of three determinations ± SEM are listed.

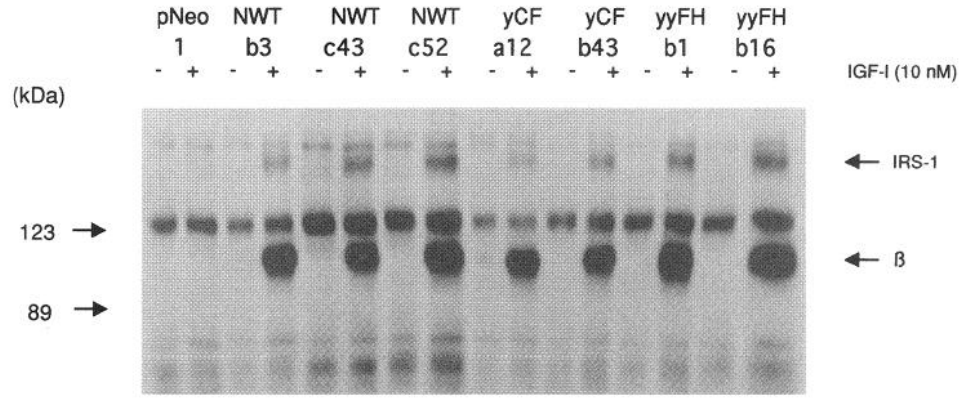


FIG. 1. IGF-I receptor autophosphorylation in intact cells. Whole cells were stimulated with IGF-I (0 nM or 10 nM) as described in *Experimental procedures*. Protein content in cleared whole cell lysates was determined. The positions of protein molecular weight standards are indicated on the left. The position of the β-subunit of the IGF-I receptor is indicated. Equal amounts of receptor, as determined by blotting with Ab 53 in a pilot gel, were separated by 7.5% SDS-PAGE. After transfer to nitrocellulose, the proteins were immunoblotted with a monoclonal antiphosphotyrosine antibody conjugated to horseradish peroxidase (4G10). The immunoblot shown is representative of three separate experiments.

(pNeo1) was barely detectable when 20 μg of cellular protein were subjected to SDS-PAGE and detected with either the monoclonal antiphosphotyrosine antibody 4G10 (Fig. 1) or RC20H (monoclonal antiphosphotyrosine antibody conjugated to horseradish peroxidase, Transduction Laboratories) (data not shown). Autophosphorylation of the the β-subunits of the overexpressed wild-type IGF-I receptors in NWTb3 cells was easily detected after IGF-I stimulation. Similarly, IGF-I-stimulated β-subunit autophosphorylation of overexpressed mutant IGF-I receptors (yyFH and yCF) was easily detected. Densitometric analyses of the phosphorylated β-subunits of mutant IGF-I receptors in multiple experiments were performed and compared with the mean density of the wild-type IGF-I phosphorylated β-subunits. Within each experiment, phosphorylation was normalized for the amount of IGF-I receptor present and then compared with the β-subunit phosphorylation of NWTb3. The average phosphorylation of the β-subunit from cells expressing wild-type IGF-I receptors was 151 ± 6%. Cell lines overexpressing the proximal double tyrosine mutant IGF-I receptors (yyFHb1 and yyFHb16) had equivalent IGF-I-stimulated autophosphorylation as compared with cell lines expressing wild-type IGF-I receptors (154 ± 38% *vs.* 151 ± 6%, respectively). Autophosphorylation of the IGF-I β-subunit was significantly altered in the clones expressing the distal tyrosine mutant IGF-I receptors (yCFa12 and yCFb43) as compared with the autophosphorylation detected in the wild-type clones (89 ± 12% *vs.* 151 ± 6%, $P = 0.03$).

Phosphorylation of IRS-1 in cleared whole cell lysates was detected after IGF-I stimulation of intact cells and blotting of the SDS-PAGE resolved proteins with RC20H (Fig. 2). Densitometric analyses of phosphorylated IRS-1 from cells expressing mutant IGF-I receptors were performed and compared with the phosphorylation of IRS-1 in cells expressing wild-type receptors. Clones expressing the proximal double tyrosine mutant IGF-I receptor (yyFHb1 and yyFHb16) phosphorylated IRS-1 to an equivalent extent as that of cells expressing wild-type receptors (106 ± 37% *vs.* 96 ± 18%, respectively). Similarly, clones expressing the distal tyrosine mutant IGF-I receptor (yCFa12 and yCFb43) phosphorylated

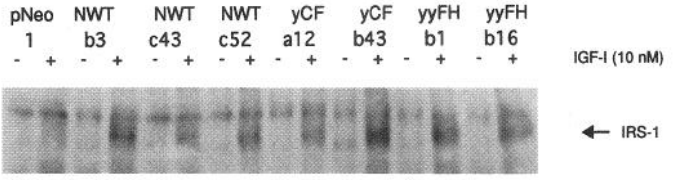


FIG. 2. IGF-I-stimulated phosphorylation of IRS-1 in intact cells. Whole cells were stimulated with IGF-I (0 nM or 10 nM) as described in *Experimental procedures*. Protein content in cleared whole cell lysates was determined. Equal amounts of protein (20 μg) were resolved by 7.5% SDS-PAGE. After transfer to nitrocellulose the phosphorylated proteins were immunoblotted with RC20H (monoclonal antiphosphotyrosine antibody conjugated to horseradish peroxidase). The immunoblot shown is representative of three separate experiments.

IRS-1 slightly less well than cells expressing the wild-type receptor (83 ± 32% *vs.* 96 ± 18%). The difference in phosphorylation of IRS-1 in these clones was not statistically significant when compared with phosphorylation of IRS-1 in cells expressing wild-type IGF-I receptors.

### *Thymidine incorporation*

IGF-I-stimulated thymidine incorporation is shown in Fig. 3. Clones overexpressing the proximal double tyrosine mutant IGF-I receptor (yyFHb1 and yyFHb16) responded to IGF-I stimulation with slightly, but not significantly, greater thymidine incorporation compared with the control cell line (pNeo 1) that expresses only $19 \times 10^3$ receptors/cell. The thymidine incorporation at IGF-I concentrations equal to or greater than 3 nM in either yyFHb1 or yyFHb16 cells was significantly decreased ($P < 0.05$) from the levels seen in cells expressing wild-type IGF-I receptors (NWTb3). The reduced thymidine incorporation was despite both of these cell lines (yyFHb1 and yyFHb16) expressing IGF-I receptors to levels similar to the cell line expressing wild-type receptors (NWTb3) (Table 1). IGF-I-stimulated thymidine incorporation in cells expressing the distal conserved tyrosine mutant IGF-I receptors (yCFa12 and yCFb43) was intermediate between control cells (pNeo 1) and cells expressing the wild-type IGF-I receptor (NWTb3). At all concentrations of IGF-I

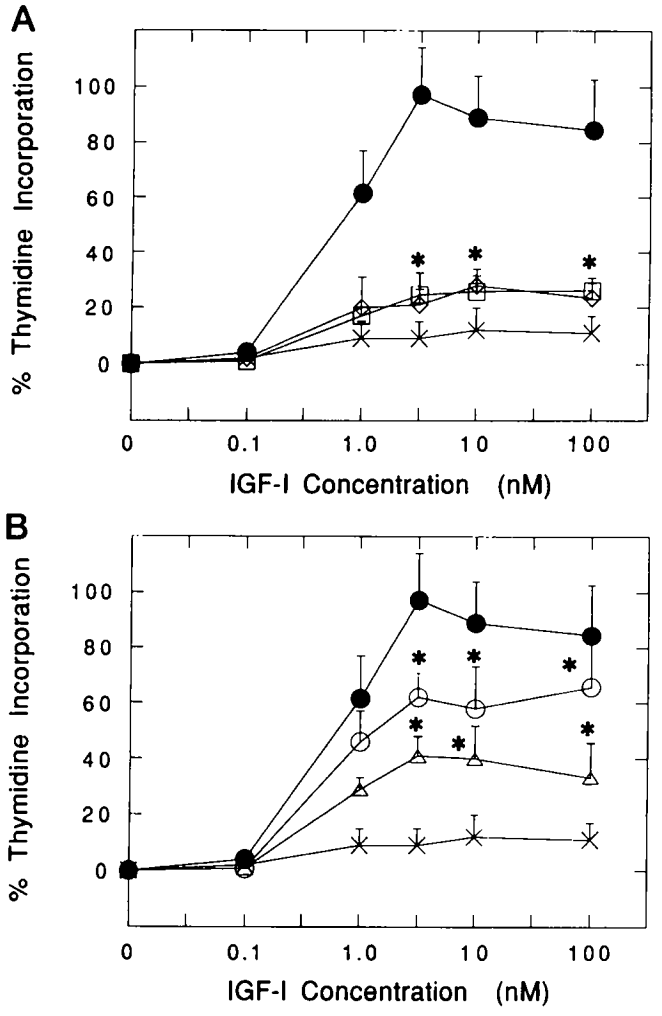


FIG. 3. IGF-I-stimulated thymidine incorporation. Subconfluent monolayers of cells were made quiescent in serum-free medium for 24 h and then stimulated with the indicated concentration of IGF-I or 10% FBS for 16 h at 37 C. [$^3$H] Thymidine (1 $\mu$Ci/ml) was added and the incubation was continued for 1 h. Incorporated counts were measured as described under *Experimental procedures*. All assays were carried out in triplicate and the SEM for each cell line in individual experiments was within 5%. The data shown are the means ± SEM of four independent experiments with the exceptions of yyFHb16 and yCFb43, which are each an average of three experiments. Where not shown, SE *bars* are smaller than the size of the *symbol*. For each cell line, the thymidine incorporation was normalized as follows: % Thymidine Incorporation = (incorporation in the presence of IGF-I - basal incorporation)/(incorporation in the presence of serum - basal incorporation) × 100. Typical cpm incorporated when cells were grown in SF-DMEM: pNeo1, 7770; NWTb3, 5895; yyFHb1, 2800; yyFHb16, 3016, yCFa12, 1196, and yCFb43, 9540. Typical cpm incoporated when cells were grown in 10% FBS: pNeo1, 83,595; NWTb3, 93,851; yyFHb1, 84,836; yyFHb16, 71,707; yCFa12, 73,653 and yCFb43, 129,036. A, Thymidine incorporation of cell lines: pNeo 1(×), NWTb3 (●), yyFHb1 (□), and yyFHb16 (◇). Significant levels as determined by the Student's *t* test are: * averages differ from NWTb3 with $P < 0.05$. B, Thymidine incorporation of cell lines pNeo 1(×), NWTb3 (●), yCFa12 (△), and yCFb43 (○). Significant levels as determined by the Student's *t* test are: * average differs from average of pNeo1 with $P < 0.05$.

equal to or greater than 3 nM, thymidine incorporation in yCFa12 and yCFb43 cells was significantly greater ($P < 0.05$) than thymidine incorporation in pNeo1 cells. Although there was about a 50% decrease in thymidine incorporation in yCFa12 cells as compared with thymidine incorporation in NWTb3 cells, the difference did not reach statistical significance ($P \leq 0.065$ for data points ≥ 3 nM IGF-I). This trend downward occurred in a cell line that expressed fewer IGF-I receptors than did the cell line expressing wild-type IGF-I receptors (Table 1). Therefore, this reduction may only be a reflection of fewer IGF-I receptors per cell.

### *Cellular proliferation*

The cellular proliferation rates in response to 1% FBS plus 10 nM IGF-I of cells expressing either the double tyrosine mutant IGF-I receptors (yyFHb1 and yyFHb16) or the distal tyrosine mutant IGF-I receptors (yCFa12 and yCFb43) were compared with the proliferation rates of cells expressing wild-type IGF-I receptors (NWTb3, NWTc43 and NWTc52) or endogenous mouse receptors (pNeo1). There was only modest growth in all cells grown in 1% FBS without supplementation with IGF-I (data not shown), with none of the cells lines growing to greater than 10,000 cells/well. IGF-I-stimulated cell growth in representative experiments, as determined by the MTT assay, is plotted in Fig. 4. Cells expressing the wild-type receptors grew at a faster rate than did the control cells (pNeo1) (*panels A and B*). Cells expressing the double tyrosine mutant IGF-I receptors (yyFHb1 and yyFHb16) grew similarly to control cell line (pNeo 1) when the growth medium was supplemented with IGF-I (*panel A*), despite mutant receptor numbers of 250,000 and 421,000 receptors/cell, respectively. In *panel B*, one clone expressing the distal tyrosine mutant IGF-I receptor (yCFb43) grew faster than pNeo 1 when grown in the presence of 10 nM IGF-I, but the other clone (yCFa12) did not. Both clones grew less well than cells overexpressing the wild-type IGF-I receptor (NWTb3, NWTc43 and NWTc52) (*panel B*).

Growth rates, expressed as cellular doubling times, were determined for each cell line in three separate experiments. The data are presented in Table 2. Both clones expressing the yyFH mutant receptors grew significantly less well than did cells expressing the wild-type receptors. The doubling time for these clones was not different than that of the cell lines expressing only endogenous mouse IGF-I receptors (pNeo1). Growth rates of cells with yyFH mutant receptors were not increased in the presence of superphysiological concentrations of IGF-I (100 nM) or 10% FBS (data not shown). This indicates that increased doses of IGF-I cannot overcome the growth disadvantage conferred by this mutant IGF-I receptor. One clone expressing the yCF mutant receptor (yCFb43) grew significantly less well than did cells with wild-type receptors; however, the other clone (yCFa12) had more erratic growth in individual experiments and statistically did not differ from cells expressing wild-type receptors. In each experiment, however, the doubling times were more than 1.5-times longer than those for the wild-type receptor clones.

### *In vivo tumorigenicity of transfected NIH-3T3 cells*

Based on preliminary observations that NIH-3T3 cells overexpressing wild-type IGF-I receptors were capable of forming tumors in recipient nude mice, but cells expressing

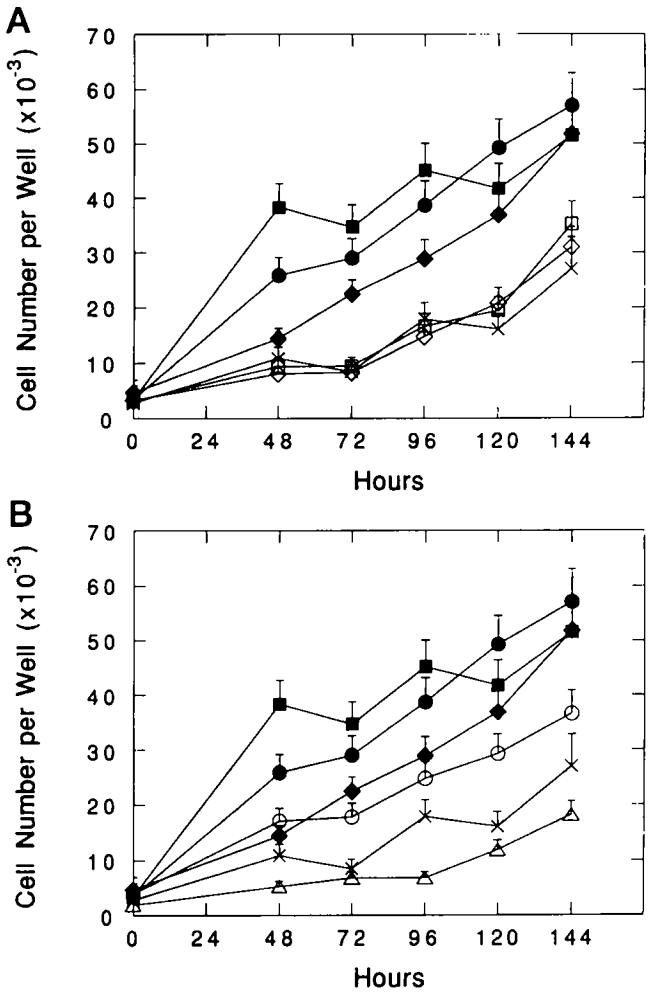


FIG. 4. Cell growth of transfected cell lines in medium supplemented with IGF-I. Cell growth was determined by the MTT assay as described in *Experimental procedures*. Cells were plated at 3000 cells/well in 96-well plates and allowed to grow for 1–5 days in DMEM supplemented with 1% FBS and 10 nM IGF-I. The cells were plated in triplicate. Fresh medium was added at 72 h. The data presented are representative of three experiments. A, Cell lines: pNeo 1(×), NWTb3 (●), NWTc43 (■), NWTc52 (♦), yyFBb1 ([squlo), and yyFHb16 (◇). B, Cell lines: pNeo 1(5), NWTb3 (●), NWTc43 (■), NWTc52 (♦), yCFa12 (△), and yCFb43 (○).

kinase-deficient IGF-I receptors could not induce the same tumor-forming capacity (data not shown), we investigated whether cells expressing IGF-I receptors with mutations in the C-terminal domain were capable of inducing tumors. The control cell lines expressing only endogenous mouse receptors or transfected cells expressing approximately equal numbers of IGF-I receptors were injected into athymic nude mice. The recipient animals were evaluated weekly for the development of tumors. The average tumor volumes as a function of the number of weeks the animals carried the tumors are presented in Fig. 5. Mice injected with the control cell line pNeo1 did not develop tumors until week 7. The average volume of these small tumors was 129 ± 56 mm$^3$ at week 9 with 5 out of 5 mice developing tumors. In contrast, some mice injected with cells expressing wild-type IGF-I receptors (NWTb3 and NWTc34) began developing very small tumors at week 2. By week 4, all these mice had measurable tumors, with an average tumor volume of 130 ± 23 mm$^3$. Thereafter, all mice injected with NWTb3 or NWTc43 cells developed large tumors. In contrast, none of the mice injected with cells expressing the proximal double tyrosine mutant IGF-I receptor (yyFHb1 and yyFHb16) or with cells expressing IGF-I receptors with a mutated single distal tyrosine (yCFa12 and yCFb43) developed palpable tumors by 3 weeks. Small tumors could be detected in 2 out of 15 mice injected with yyFHb1 and 1 out of 5 mice injected with yyFHb16 by week 9. This incidence of tumors was less than that observed with injection of pNeo1 where all mice injected developed small tumors by week 9. Small tumors were also noted in mice injected with yCFb43, and all 5 mice injected with yCFa12 cells survived to 9 weeks with very small tumors.

**TABLE 2.** Doubling times of NIH-3T3 cells transfected with wild-type or mutant IGF-I receptors

| Cell line | Doubling time (h) | | | | *P* value[a] |
|---|---|---|---|---|---|
| | Exp 1 | Exp 2 | Exp 3 | Average | |
| pNeo1 | 48 | 86 | 73 | 69 ± 11 | 0.17 |
| NWTb3 | 46 | 41 | 38 | 42 ± 2 | |
| NWTc43 | 35 | 46 | 40 | 40 ± 3 | 0.81 |
| NWTc52 | 36 | 44 | 46 | 42 ± 3 | 0.96 |
| yyFHb1 | 79 | 63 | 59 | 76 ± 6 | 0.02 |
| yyFHb16 | 84 | 68 | 67 | 73 ± 6 | 0.01 |
| yCFa12 | 115 | 156 | 74 | 115 ± 24 | 0.08 |
| yCFb43 | 56 | 58 | 50 | 55 ± 2 | 0.02 |

[a] Statistical significance of doubling time in hours of cell line compared with the doubling time in hours of NWTb3.

## Discussion

To elucidate the distinct functions of the IGF-I and insulin receptors, we studied human IGF-I receptor mutants that had replacement of the three C-terminal tyrosine residues with either the amino acids found in the homologous positions of the human insulin receptor or, in the case of the conserved distal tyrosine residue, a phenylalanine residue. Although we documented no significant change in autophosphorylation of the β-subunit or phosphorylation of IRS-1 when the tyrosines at positions 1250 and 1251 were mutated to amino acids incapable of being phosphorylated, there were reductions in IGF-I-stimulated thymidine incorporation and cellular proliferation. In particular, the mutant receptors with phenylalanine at position 1250 and histidine at position 1251, confer no increased mitogenic activity to the cell over the basal activity mediated by the cells endogenous receptors. Our results show that mutation of the proximal double tyrosine moiety of the IGF-I receptor totally inactivates the transfected receptors with regard to IGF-I-stimulated thymidine incorporation and cellular proliferation, despite intact tyrosine kinase activity. This suggests that one or both of these tyrosine residues is crucial for mediation of the mitogenic signal after receptor activation by IGF-I. Furthermore, fibroblasts expressing these mutant receptors are incapable of forming tumors when injected into nude mice. These results suggest that this mutation decreases not only the mitogenic capacity but also the tumorigenic potential of transfected fibroblasts. It had previously been shown that

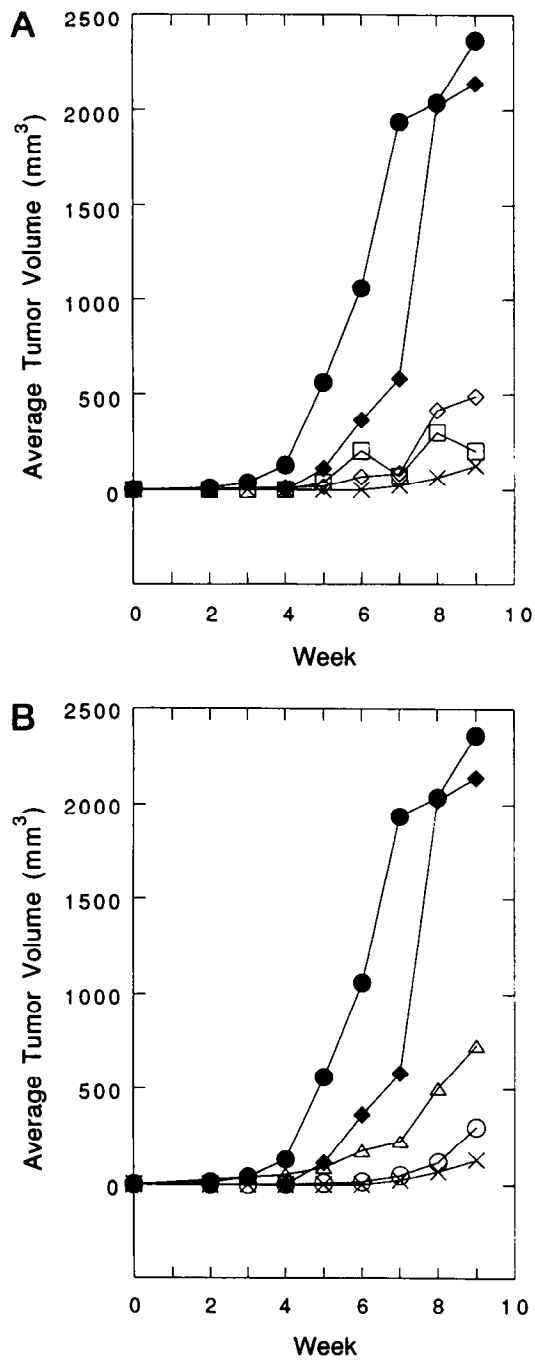


FIG. 5. Tumor growth in athymic nude mice injected with NIH-3T3 cells overexpressing IGF-I receptors. Cell suspensions ($1 \times 10^7$ cells/150 μl) were injected into the lower dorsal region of each nude mouse. The presence of tumors was monitored and tumor size was measured for all palpable tumors. A, Cell lines: pNeo 1(×), NWTb3 (●), NWTc34 (♦), yyFBb1 (□), and yyFHb16 (◇). B, Cell lines: pNeo 1(×), NWTb3 (●), NWTc34 (♦), yCFa12 (△), and yCFb43 (○).

expression of a dominant-negative IGF-I receptor, lacking the majority of the intracellular domain, inhibited tumorigenesis of Rat-1 fibroblasts when injected into recipient animals (18). However, measurement of tumorigenicity of cells expressing IGF-I receptors in which only tyrosine residues in the C-terminal domain have been mutated has not been reported before this study. Of interest, in the current study we demonstrate that tumorigenic potential is reduced in cells expressing a mutant receptor that does not inhibit the tyrosine kinase activity of the endogenous receptor in a dominant-negative fashion. Rather reduced tumor formation was documented in fibroblasts expressing IGF-I receptors with intact tyrosine kinase activity but absent IGF-I stimulated mitogenic activity.

The second IGF-I receptor mutation, in which the distal single tyrosine was mutated to phenylalanine, displayed a different pattern of mitogenic activity and tumorigenic potential. The ability of these mutant receptors to autophosphorylate the β-subunit was slightly reduced, but there was no decrease in phosphorylation of IRS-1. Of note, the mutant cell line expressing fewer receptors than the wild-type overexpressing cell line still retained mitogenic activity. These data suggest that the distal conserved tyrosine residue in the IGF-I receptor is not essential for mitogenic activity, but its presence may contribute to maximal mitogenic effects. In contrast to the maintained mitogenic activity, cells expressing the single distal tyrosine mutant IGF-I receptors are not able to form tumors as readily as cells overexpressing wild-type IGF-I receptors when injected into recipient mice. This mutated IGF-I receptor is the first receptor we have studied in stably transfected NIH-3T3 cells that has a divergence in its ability to transduce mitogenic *in vitro* functions and induce tumors *in vivo*. Although these cells retained mitogenic activity, the trend was a decrease in mitogenic activity as compared with receptor number-matched cells expressing wild-type receptors. Therefore, the inability of these cells to induce tumors may be a reflection of that modest decrease in mitogenic function. Alternatively, the replacement of a specific tyrosine residue in the carboxy-terminal domain of the IGF-I receptor may affect specific divergent pathways leading to activation of mitogenesis or increased tumor formation.

Previous studies of the IGF-I receptor along with studies of other transmembrane tyrosine kinase receptors for insulin, epidermal growth factor, platelet-derived growth factor, and fibroblast growth factor have identified the C-terminal domains of these receptors as important in the process of signal transduction. Differentiation of function of these receptors is thought to be affected by particular and, presumably, unique sites in the intracellular domains. Identification of these sequences has taken on even more importance since the discoveries of various receptor tyrosine kinase substrates. It is known that these various receptors use common downstream adapter and docking proteins in the signal transduction cascade. Thus, attention has been placed on dissecting the differential functions of the IGF-I receptor and insulin receptor. Identification of the primary sequences of the IGF-I receptor and insulin receptor has shown that the amino-acid sequence similarity between these receptors is highest in the tyrosine kinase domain (~85%) with the amino acid sequence of the C-terminal domain the least conserved of all of the intracellular domains (~44%). The tyrosine residues in the IGF-I receptor differ significantly from that of the insulin receptor. The IGF-I receptor has three tyrosyl-residues (positions 1250, 1251, and 1316) whereas the insulin receptor has only two tyrosyl-residues (positions 1316 and 1322) (num-

bering system of Ullrich *et al.*, 1986) (1). Of these, only the most distal tyrosine residue is conserved between the two receptors. In the insulin receptor, the two tyrosine residues are phosphorylated upon ligand binding and most likely play a role in insulin receptor function. Until recently, few studies have been presented to delineate the role of the tyrosine residues in the C-terminal domain of the IGF-I receptor. Results from our studies in NIH-3T3 cells suggest that the distal conserved tyrosine (tyrosine 1316) is not essential for IRS-1 phosphorylation but may contribute to maximal mitogenic activity.

Based on our characterization of these mutant IGF-I receptors, we conclude that mutation of the two tyrosine residues (tyrosine 1250 and 1251) just distal to the tyrosine kinase domain disrupts the mitogenic signaling pathway to a significant extent, and furthermore, this mutation decreases the tumorigenic potential of cells expressing these receptors, whereas cells expressing wild-type IGF-I receptors have both increased mitogenic and tumorigenic potentials. Recently, the transformation and mitogenic properties of R− fibroblasts transfected with mutant IGF-I receptors in which the tyrosine 1251 residue was replaced with a phenylalanine residue alone or in conjunction with a tyrosine 1250 to phenylalanine substitution was reported (19). In a short-term cellular proliferation assay, the cells expressing these mutant receptors did not appear to reduce the mitogenic potential of the cells. These results differ sharply with the decreased mitogenic potential we measured in the yyFH mutant (tyrosines 1250 and 1251 to phenylalanine and histidine, respectively). It is difficult to directly compare the differences in our study with that of Miura *et al.* (19) because of technical differences in three aspects. First, our clones were derived from single stably transfected cells by serial dilution and the clones used were matched for receptor content per cell. The transfected R− cells in their study were selected for highly expressing IGF-I receptors after several passages of the cells. The selection process may have preferentially selected the more rapidly growing clones after the transfection (19). Second, comparison of the mitogenic potential of the NIH-3T3 and R-mutants cannot be done directly because of significant differences in the designs of the cellular proliferation assays (19). Finally, a remote possibility is that there may be significant differences in the physiological responses seen with a phenylalanine substitution (19) as opposed to the histidine substitution of tyrosine 1251 that was used in our studies. In contrast in our studies, mutation of the most distal conserved tyrosine residue (tyrosine 1316) has less of an effect on the mitogenic stimulation of the overexpressed IGF-I receptor but profoundly affects the tumorigenic potential of cells expressing this receptor. Elucidation of the separate signaling pathways by which this receptor mediates its divergent mitogenic and tumorigenic effects should be illustrative in describing the unique physiological function of the IGF-I receptor.

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