Effect of a Mutation at Tyrosine 950 of the Insulin-like Growth Factor I Receptor on the Growth and Transformation of Cells

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ABSTRACT

Recent evidence has shown that the insulin-like growth factor I (IGF-I) receptor plays a major role in the establishment and maintenance of transformation. To identify domains in the IGF-I receptor that are necessary for transformation, the tyrosine at residue 950 of the human IGF-I receptor cDNA was mutated to phenylalanine, and the plasmid expressing the mutant receptor was stably transfected into R~ cells, which are mouse embryo fibroblasts with a targeted disruption of the type I receptor for the insulin-like growth factors. At variance with the wild-type receptor, the Y950 mutant receptor has lost its ability to transmit an IGF-I-mediated mitogenic signal or to transform R~ cells. These experiments show, for the first time, that tyrosine 950 of the IGF-I receptor is necessary for its mitogenic and transforming activities.

INTRODUCTION

Evidence is accumulating rapidly that the IGF-IR plays a crucial role in the establishment and maintenance of the transformed phenotype. Mouse embryo cells with a targeted disruption of the IGF-IR genes cannot be transformed by SV40 T antigen and/or an activated Ha-ras oncogene (1-4), which easily transform embryo cells generated from their wild-type littermates. Expression of an antisense RNA to the IGF-IR RNA in C6 rat glioblastoma cells not only abrogates tumorigenesis in syngeneic rats but also causes complete regression of established wild-type tumors (5). These findings are supported by other reports in the literature, indicating that antibodies to the IGF-IR (6-8); antisense expression plasmids to either IGF-1 (9, 10), its receptor (11), or IGF-II (12); and a dominant negative mutant of the IGF-IR (13) can all reverse the transformed phenotype and/or inhibit tumorigenesis. Conversely, overexpression of the wild-type (but not of a mutant) IGF-IR induces ligand-dependent transformation (4, 14, 15), while overexpression of IGF-II in transgenic mice increases the incidence of certain malignancies (16). It is therefore important to investigate the effect of mutations on the ability of the IGF-IR to stimulate growth and transformation in mammalian cells.

In this report, we have studied the effect of a single point mutation in tyrosine 950 (Y950) of the human IGF-IR on its mitogenic and transforming activities. Although some information is available on the effect of a mutation in the corresponding tyrosine of the IR (for a review see Ref. 17), little is known about the effect of a Y950 mutation in the IGF-IR (see "Discussion"), and most of these data are limited to the metabolic functions of the receptors. To address the problem of mitogenesis and transformation, we used R~ cells (1, 2, 4), in which the IGF-IR genes have been disrupted by targeted homologous recombination (18, 19). The absence of endogenous IGF-IRs in these cells facilitates a mutational analysis. Our experiments show for the first time that the tyrosine 950 residue of the IGF-IR is obligatory not only for its mitogenic effect but also for its transforming activity.

MATERIALS AND METHODS

Plasmids. The Y950 mutant was derived from the wild-type human IGF-IR cDNA (20), as follows: to substitute phenylalanine for tyrosine, the Y950 codon and the unique EcoRI site (multicloning site) of the pBluescript SK IGF-IR were mutated at the same time using a mutagenic primer, 5'-CCGG-AAGTTCTTCGAGGC-3', and a selection primer, 5'-GGGATCCAGAA-AATCGATATCA-3', according to the procedure of a Clontech kit (mutated sites are underlined). EcoRI digestion was performed twice in the procedure before transformation of Escherichia coli to rescue the mutated plasmids. With this mutation, the ScaI site (2970) of the IGF-IR cDNA is removed. The correct mutation was confirmed by digestion patterns including ScaI, and by dyeoxy sequencing. The SalI-BamHI fragment of the mutated plasmid was subcloned into a pCMV-Neo-Bam expression vector (gift of Dr. E. Mercer) to generate the plasmid pCMV F950. Another expression vector, pBPV (Stratagene), was also used. The Smal-HindIII fragment including the F950 site of the mutated pBluescript SK IGF-IR was subcloned into a pBluescript SK Sal-I Bam IGF-IR, while the SalI-NorI fragment of the last plasmid was subcloned into a Xhol-NorI site of the pBPV vector, to generate pBPV F950.

Cell Lines. R~ cells are 3T3-like cells originating from mouse embryos with a targeted disruption of the IGF-IR genes (18, 19). These cells have been described and characterized in detail in previous reports (1, 2, 4). R~ cells (2) are R~ cells stably transfected with a plasmid expressing the wild-type human IGF-IR cDNA under the control of a SV40 promoter (20). The clone used in these experiments is one of several clones with similar properties that were isolated and characterized in a previous report (21). KA cells (2, 4) are R~ cells overexpressing a human IGF-IR cDNA with a point mutation in the ATP-binding site, described in detail by Kato et al. (21).

The establishment of cell lines expressing the Y950 mutant of the IGF-IR was as follows: R~ cells were transfected with pCMV F950 and selected with neomycin. Resultant clones were checked with a 125I-labeled IGF-I-binding assay (22), and positive clones Y950F C11 and C13 were eventually selected and used in this study. For experiments with mixed populations of transfecants expressing a very high number of receptors, pBPV F950 was used. Cells were cotransfected with a pBPV F950 plasmid and pPDV6+, which encodes the puromycin-resistance gene (23, 24), and selected with puromycin (4). Resultant clones were mixed and passaged a few times. After trypanocinization, the cells were washed with PBS and incubated with anti-IGF-IR antibody (1:10) (Oncogene Science) at 4°C for 20 min. After washing with PBS, the cells were incubated with antimaus immunoglobulin G conjugated with FITC (1:50) (Oncogene Science) at 4°C for 20 min. The cells were washed with PBS, and after the intensity distribution of the fluorescence was analyzed by flow cytometry ( Coulter), the upper 5% of the population was sorted under sterile conditions. The sorted cells were washed and allowed to grow in the incubator. After the cells were checked with a binding assay (22), the mixed population was used in experiments.

Internalization. Cells grown on 35-mm dishes were changed from growth medium to serum-free medium (DMEM containing 1 mg/ml of BSA and 25 m Hepes) at least 4 h before the experiments. This medium was then used in all internalization experiments. Cells were incubated with 0.5 ng/ml of 125I-labeled IGF-I (Amersham) for 5 or 6 min with an interval of 1 min at 37°C. After incubation, dishes were transferred on ice and washed three times with cold HBSS. The cells were incubated with cold 0.2 m acetic acid in 0.5 m sodium chloride on ice to strip cell-associated IGF-I (25). After 6 min, this solution was removed and the cells were rinsed with fresh solution. Radioactivity of the combined solutions was measured by an autowell gamma counter.

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3 The abbreviations used are: IGF-IR, IGF-I receptor; IGF-I or IGF-II, insulin-like growth factor I or II; IR, insulin receptor; IRS-1, insulin receptor substrate-1.
Intracellular radioactivity was determined by measuring that of IGF-I-stripped cells lysed by 1 N sodium hydroxide. Nonspecific binding determined by adding 200-fold excess of cold IGF-I was generally less than 10% of total binding. The data were analyzed using internalization plots, by plotting the integral of surface binding from time 0 to the time point against the amount of internalized IGF-I (26).

Tyrosine Phosphorylation. Whole-cell lysates were prepared as described previously (27), and the protein content was determined by Bio-Rad assay. Equal amounts of whole proteins were fractionated by electrophoresis using gradient SDS-polyacrylamide gels (Jule Inc.). For the detection of IRS-1, proteins from whole lysates were electroblotted to nitrocellulose membrane, probed with antiphosphotyrosine antibody (PY20) conjugated with horseradish peroxidase (Transduction Laboratories), and visualized by the enhanced chemiluminescence detection system, following the instructions of the manufacturer (Amersham). Immunoprecipitation of IGF-IR was performed using an mAb to the IGF-IR, nIR3 (Oncogene Sciences, Uniondale, NY), as described previously (27), and autophosphorylation of the IGF-IR was detected after electrophoresis of the immunoprecipitated proteins as described above.

Growth Assay and DNA Synthesis. For the growth assay, cells were plated on 60-mm dishes in growth medium, and 24 h later, the medium was replaced by serum-free medium (DMEM containing 2.5 μM ferrous sulfate and 1 mg/ml BSA) with or without 50 ng/ml of IGF-I. Cell numbers were determined using a hemocytometer 48 h after medium replacement.

For the DNA synthesis assay, cells grown on coverslips were made quiescent in serum-free medium for 4 days and incubated with 50 ng/ml of IGF-I or fresh growth medium and [3H]thymidine (0.5 μCi/ml). After 24 h incubation, the cells were fixed with cold methanol for 20 min, and the percentage of labeled cells was determined by standard autoradiographic techniques.

Scatchard Analysis. This was carried out by standard methods, with the modifications described previously (22).

Soft Agar Assay. This was carried out as described previously (1, 2, 4).

RESULTS

Establishment of Cell Lines. R− cells (1, 2) were transfected with the appropriate plasmids, and eventually selected as described in “Materials and Methods.” A number of clones were examined for expression of the mutant receptor (F950), and two clones were selected for further studies, clone 1 and clone 3. The receptor content of these two clones was determined by Scatchard analysis (Fig. 1), and it is compared to other cell lines in Table 1. The Kd of the mutant receptor (0.47–0.48 nm) was not significantly different from the Kd of the wild-type receptor in R+ cells (0.55 nm). Relevant to the present paper is the determination that clones 1 and 3 (Y950 mutants) have the same number of receptors, or higher than in R+ cells, which are known to grow in serum-free medium supplemented solely with IGF-I and to form colonies in soft agar (2). In turn, all 3 cell lines have approximately 10-fold more receptors than 3T3 or W cells, which cannot grow in IGF-I only, but can grow in platelet-derived growth factor plus epidermal growth factor and IGF-I (2, 22).

Autophosphorylation of the Receptor and IRS-1. White et al. (28) have shown that mutation of the IR at Y960 inhibits signal transmission but does not affect autophosphorylation, and this was confirmed by Yamasaki et al. (29) for the mutation at Y950 of the human IGF-IR. Fig. 2A shows that cells expressing the mutant receptor (clone 3) autophosphorylated the IGF-IR after IGF-I (Fig. 2A, Lanes 3 and 4). The extent of autophosphorylation was perhaps slightly decreased transmission but does not affect autophosphorylation, and this was confirmed by Yamasaki et al. (29) for the mutation at Y950 of the IGF-IR. Fig. 2A shows that cells expressing the mutant receptor (clone 3) autophosphorylated the IGF-IR after IGF-I (Fig. 2A, Lanes 3 and 4). The extent of autophosphorylation was perhaps slightly decreased with respect to R+ cells with a comparable number of receptors (Fig. 2A, Lanes 1 and 2), but the technique is not quantitative, and the only conclusion is that the tyrosine kinase activity is not grossly affected. However, Fig. 2B shows that phosphorylation of IRS-1 was not detectable in clone 3, although it was clearly detectable in R+ cells, thus confirming the results of White et al. (28) for the Y960 mutant of the IR. Although Fig. 2B indicates only the presence of a pl85 band that is tyrosyl-phosphorylated by IGF-I, subsequent experiments with an antibody against IRS-1 (a kind gift of Drs. Lienhard and Keller, Dartmouth Medical School) have confirmed that this band is indeed IRS-1 (not shown).

Internalization of the Mutant Receptor. Yamasaki et al. (29, 30) concluded that a mutation at Y950 of the human IGF-I receptor does not affect internalization. We have confirmed their results with our cells (Fig. 3). To facilitate the measurement of specific internalization, we used cells expressing very high levels of IGF-IR (wild type or mutant), selected by fluorescence-activated cell sorting as described in “Materials and Methods.” These cell populations expressed almost 1 × 107 receptors per cell (see Table 1). The specific internalization rate constant was essentially the same in the cells expressing either the

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Table 1  Receptor content in several mouse embryo cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>No. of receptors/cell</th>
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<tbody>
<tr>
<td>BALB/c 3T3( quiescent)</td>
<td>1.8 × 10^5</td>
</tr>
<tr>
<td>R−</td>
<td>0</td>
</tr>
<tr>
<td>W</td>
<td>2.6 × 10^4</td>
</tr>
<tr>
<td>Y950 clone 1</td>
<td>1.1 × 10^3</td>
</tr>
<tr>
<td>Y950 clone 3</td>
<td>1.6 × 10^3</td>
</tr>
<tr>
<td>Y950 mixed population</td>
<td>9.0 × 10^5</td>
</tr>
</tbody>
</table>

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Fig. 2. Autophosphorylation of wild-type and mutant IGF-IR. A, autophosphorylation of the receptor. Lane 1, R+ cells, no IGF-I. Lane 2, R+ cells 5 min after IGF-I (20 ng/ml). Lane 3, Y950 mutant, clone 3, no IGF-I. Lane 4, Y950 mutant, clone 3, after IGF-I. Arrow, β subunit of the IGF-IR. B, phosphorylation of IRS-1. Lanes, same as in A, Arrow, IRS-1 band. The procedures are described in “Materials and Methods.”
wild-type or the Y950 mutant receptor, and it was 10 times higher than in KA cells, which express the ATP-binding mutant, known to be defective in ligand-induced internalization (29). Thus, it is clear that also in cells without the background of endogenous IGF-IRs, mutation at Y950 does not appreciably affect internalization of the receptor.

Growth Characteristics of the Cells Expressing the Mutant Receptor. We tested our cells for their ability to grow in serum-free medium supplemented solely with IGF-I (50 ng/ml). R− cells, over-expressing the wild-type human receptor, were capable of growing in IGF-I alone (Fig. 4A), but clones 1 and 3, although expressing a similar number of receptors, were incapable of doing so; i.e., they behaved like the parental R− cells (1, 2, 4). In 10% serum, clones 1 and 3 did grow, albeit at a reduced rate in comparison to R− cells (Fig. 4B), again behaving like the parental R− cells, which grow in 10% serum at a rate somewhat lower than that of wild-type cells (2). The experiments in Fig. 4 were repeated several times, with different concentrations of cells; the results were the same (not shown).

It is known that some cells may be incapable of growing under certain conditions, but may still be capable of entering DNA synthesis (Ref. 3, and for a review see Ref. 31). We therefore tested clones 1 and 3 for their ability to enter S phase when stimulated from quiescence. The results are shown in Fig. 5. While 10% serum was almost equally effective in inducing DNA synthesis in R− and Y950 cells, only R− cells entered S phase after stimulation with IGF-I alone; clones 1 and 3 essentially did not respond to stimulation by IGF-I. The experiments with IGF-I were repeated twice, with the same results.

Effect of a Mutation at Y950 of the IGF-IR on the Transformed Phenotype. We have shown previously that R− cells cannot be transformed by the SV40 T antigen (1, 4), by an activated Ha-ras oncogene, or by a combination of the two (2). This inability of R− cells to be transformed by certain oncogenes is promptly abrogated if the cells are stably transfected with a wild-type (but not with a mutant) human IGF-IR cDNA (1, 2, 4). We transfected the Y950 mutant receptor in R− cells (see above). The results are summarized in Table 2. The Y950 mutant receptor was incapable of transforming R− cells, thus behaving like the ATP-binding mutant of the IGF-IR (2, 4). Remarkably, even cells expressing an extremely high number of mutant receptors did not form colonies in soft agar (the only colony was very small, and barely met the criteria used in our laboratory).

DISCUSSION

The original findings in this report are: (a) tyrosine 950 of the IGF-IR is required for its mitogenic effect; and (b) it is also required for its transformation activity. Although it has been reported that mutation in the corresponding tyrosine of the IR decreases thymidine incorporation into DNA, no one has reported previously that this tyrosine residue is also obligatory for transformation. Furthermore,
thymidine incorporation measures, at best, only DNA synthesis (and inaccurately at that); therefore, our experiments also show for the first time that tyrosine 950 is necessary for growth.

On the other hand, our findings on autophosphorylation and internalization of the IGF-IR, and on phosphorylation of IRS-1, are more of a confirmatory nature (29). We believe, however, that these confirmatory results should be included because they are the first to establish a connection between these alterations on the one hand, and mitogenesis and transformation on the other. Using the IR, Tavare and Siddle (17) summarized the results of many investigators; whereas some reports indicated a decrease in autophosphorylation while others found it to be normal, all reports agree that the functions of the IR are markedly decreased. Yamasaki et al. (29, 30) and Prager et al. (32) have studied mutations in the juxtamembrane region of the IGF-IR. Specifically, they found that a mutation at Y950 decreased IGF-I-mediated growth hormone secretion and IRS-1 phosphorylation, and, to a small extent, receptor autophosphorylation, but had little effect on internalization. In the later study from the same laboratory (32), it was reported that more extensive mutations in the juxtamembrane region did affect internalization. These authors did not study the effect of a mutation at Y950 of the IGF-IR on its mitogenic or transforming activities.

We show that the mitogenic signal of the IGF-IR with a mutation at Y950 is grossly impaired. Whereas R- cells overexpressing a wild-type IGF-IR can grow in serum-free medium supplemented solely with IGF-I and can form colonies in soft agar (Refs. 2, 4, and the current study), R- cells overexpressing the Y950 mutant receptor fail both to respond to IGF-I and to grow in soft agar. This is true even with cells expressing extremely high numbers of mutant receptors (see Table 2). It should be noted that W cells (or BALB/c 3T3 cells), expressing a normal number of IGF-IRs, also fail to respond to IGF-I alone or to form colonies in soft agar, both of which properties require an overexpressed receptor (2, 14, 27). In our experiments, the Y950 mutants expressed a large number of receptors, but still completely failed to respond, behaving in fact like the parental R- cells, or the R- cells transfected with a plasmid expressing a mutant receptor, with a mutation at the ATP-binding site (2, 4).

Our experiments showing that both tyrosyl phosphorylation of IRS-1 and mitogenesis are dramatically inhibited in cells expressing the Y950 mutant are in agreement with several reports in the literature on the importance of IRS-1 for signal transmission by either the IR or the IGF-IR (Refs. 33–38; for a review, see Ref. 39). While IRS-1 may not be the sole substrate of the IGF-IR (39, 40), it is clear that it plays a substantial role in its signal transduction. However, we have no evidence, at this point, that IRS-1 may be obligatory for the transduction of the transforming signal.

In the “Introduction,” we mentioned the evidence that the IGF-IR plays an important role in the establishment and maintenance of transformation. Our experiments show for the first time that a mutation at tyrosine residue 950 of the human IGF-IR abrogates the ability of the receptor to transmit a mitogenic signal and to support colony formation in soft agar. These results confirm the importance of a functional IGF-IR for mitogenesis and for establishment and maintenance of the transformed phenotype. As in previous reports (1, 2, 4), the effect of a defective IGF-IR, in either qualitative or quantitative terms, is more profound on transformation than on growth itself (in 10% serum).

REFERENCES


Table 2 Growth in soft agar of several mouse embryo cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>No. of colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-</td>
<td>0</td>
</tr>
<tr>
<td>R+</td>
<td>166</td>
</tr>
<tr>
<td>Y950 clone 1</td>
<td>0</td>
</tr>
<tr>
<td>Y950 clone 3</td>
<td>0</td>
</tr>
<tr>
<td>Y950 mixed population</td>
<td>1</td>
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$5 \times 10^5$ cells were plated in soft agar plates, and colonies more than 125 μm in diameter were counted after 2 weeks. See text for the cell lines.

Fig. 5. DNA synthesis in mouse embryo cells expressing wild-type or mutant IGF-IR. Quiescent cells were stimulated in serum-free medium supplemented by either IGF-I (group 1) or 10% serum (group 2). [3H]thymidine was added at the time of stimulation. Left bars, R- cells; middle bars, clone 1 expressing the Y950 mutant receptor; right bars, clone 3 expressing the Y950 mutant receptor. Results are expressed as percentage increase in labeled cells over that in serum-free medium (which varied, in several experiments, between 6 and 14%).


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