Effect of Mutations at Serines 1280-1283 on the Mitogenic and Transforming Activities of the Insulin-like Growth Factor I Receptor*

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The insulin-like growth factor I receptor (IGF-IR) controls the extent of cell proliferation in a variety of cell types by at least three different ways: it is mitogenic, it causes transformation, and it protects cells from apoptosis. Previous reports indicated that certain domains in the C terminus of the IGF-IR transmitted a transforming signal that is additional to and separate from the mitogenic signal. We have now mutated the four serine residues at 1280–1283 of the IGF-IR, and transfected the mutant receptor into R3T3 cells. Cells expressing the mutant receptor are fully responsive to IGF-I-mediated mitogenesis, but are not transformed (no colony formation in soft agar). Several downstream signal transducers are not affected by the mutation, again suggesting a separate pathway for transformation. The mutant receptor can act as a dominant negative for growth, but cannot induce apoptosis in cells with endogenous wild-type receptors.

The IGF-IR belongs to the family of tyrosine kinase growth factor receptors (1), and is 70% homologous to the IR (2). For many years, the IGF-IR activated by its ligands (IGF-I, IGF-II and insulin at supraphysiological concentrations) has been known to transmit a mitogenic signal to cells in culture (3,4). The importance of the IGF-IR in cell growth has been confirmed in vivo by the finding that mouse embryos with a targeted disruption of the IGF-IR and IGF-II genes have a size at birth that is only 30% the size of wild-type littermates (5,6). The finding that mouse embryos with a targeted disruption of the IGF-IR and IGF-II genes have a size at birth that is only 30% the size of wild-type littermates (5,6).

The important role of the IGF-IR in the establishment and maintenance of the transformed phenotype is also supported by other findings. Antisense oligodeoxynucleotides or antisense expression plasmids against IGF-II (19,20), IGF-I (21), or the IGF-IR (8,22–25), antibodies to the IGF-IR (26,27), and dominant negative mutants of the IGF-IR (28–30), can all reverse the transformed phenotype, inhibit tumorigenesis, and induce loss of the metastatic phenotype (31).

We have recently reported that R3T3-like cells, stably transfected with an IGF-IR truncated at residue 1229, are fully responsive to IGF-I-mediated mitogenesis but are not transformed (18). Subsequently, we reported that a point mutation at tyrosine 1251 (from tyrosine to phenylalanine) also resulted in a receptor that could not transform R3T3 cells, but made them responsive to IGF-I with growth (12). Interestingly, a tyrosine to phenylalanine mutation at residue 1250 had no effect, the receptor maintaining both its mitogenic and transforming activities.

This dissociation between mitogenicity and transforming activity is of considerable interest because it suggests that, for transformation, the IGF-IR uses an additional pathway which is not required for mitogenic signaling, and that this pathway originates from the C terminus. In the case of the Y1251F mutant, however, the transfectants, although they could not make bona fide colonies in soft agar, were capable of generating tiny clusters that did not attain the size traditionally acceptable for scoring a colony (12). These data, and those of Liu et al. (17) with chimeric Gag-IGF-IR constructs, suggested that more than one residue in the C terminus was necessary to account for the full transforming activity of the IGF-IR. In the present article, we have investigated the role of a serine quartet, at residues 1280–1283, on the mitogenic and transforming activities of the IGF-IR. Mutation at these four serines again results in the remarkable dissociation between mitogenicity and transforming activity. And, as in previous cases (12,18), the tyrosyl phosphorylation of IRS-1 and Shc, and the activities of several downstream signal transducers are not affected, suggesting that the transforming signal either follows an additional pathway totally separate from the mitogenic signaling, or, alternatively, that it causes in the known substrates more subtle changes that can be revealed by Western blotting.

MATERIALS AND METHODS

IGF-IR Expression Plasmids—The wild-type IGF-IR expression vector used in this study is pBPV/IGF-IR (a kind gift of Dr. LeRoith, J.L. Burgaud and R. Baserga, submitted for publication.)
Mutant IGF-1 Receptor

National Institutes of Health), which contains the complete coding sequence of the wild-type human IGF-IR cDNA (2). A PCR-assisted in vitro mutagenesis method was used to create the quartet serine mutant of the IGF-IR, in which the codons specifying serine residues 1280, 1281, 1282, and 1283 were replaced with alanine codons. The mutagenesis procedure included two PCR reactions. The first PCR reaction was performed using primer containing dIIISite. The primers used for the second PCR product were then cloned into a pCR II vector (Invitrogen, San Diego, CA). The sequence of the whole region derived from PCR was confirmed by sequencing. The 980-bp base pair HindIII BamH1 fragment containing four serine mutations was then cloned into pBTV mammalian vector (Pharmacia, Piscataway, NJ) via a shuttle vector SK-IGF-IR (IGF-IR cDNA cloned in pBluescript SK, Stratagene). 

Cell Culture and Transfection—R cells, which are IGF-IR null fibroblasts derived from mouse embryos with a targeted disruption of the IGF-IR genes (5, 6), were routinely cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum. Transfections were performed using a standard calcium-phosphate precipitation method. The cells R 4A were obtained by cotransfecting R cells with pBTV/IGF-IR-4A, encoding a quartet serine mutant receptor cDNA, and pRSVNeo (32), encoding the neomycin resistance gene. Cell lines C6/4A were obtained by cotransfecting C6, a rat glioblastoma cell line, with pBTV/IGF-IR-4A and pRSVNeo. R 4D cells were previously established by stably transfecting R cells with the wild-type human IGF-IR cDNA under the control of an SV40 promoter (7). 

Scatchard Analysis—The number of IGF-IR in each cell type was determined by Scatchard analysis following the ligand replacement protocol described by Yamazaki et al. (33) with modifications. Briefly, cells were plated at a density of 1 × 10^5/cm^2 in DMEM containing 10% fetal bovine serum. After 24 h, cells were washed twice with buffered saline. Binding was carried out by incubating cells at 4 °C for 6 h with 1 ml of binding buffer (DMEM plus 0.2 M Hepes pH 7.4 and 1 mg/ml bovine serum albumin) containing 1 ng of 125I-IGF-1 (Amersham) and increasing amounts of unlabeled IGF-1, ranging from 0 to 40 ng. After incubation, cells were washed twice, lysed with 1 ml of 0.03% SDS, and the cell-associated radioactivities were measured in a γ-counter. Specific binding was expressed by the subtraction of nonspecific binding as determined in the presence of 400 ng/ml (400-fold excess) unlabelled IGF-1 from total binding. The replacement data were converted to Scatchard plots assuming that 125I-labeled and unlabeled IGF-1 have the same affinity for the IGF-IR. 

Growth Assays—All growth assays were performed as follows. Cells were plated at a density of 10 × 10^3 per well in DMEM supplemented with 10% serum. Twenty-four hours after plating, cells were washed twice with buffered saline and placed in serum-free medium; i.e. DMEM supplemented with 1 μM ferrous sulfate and 0.1% bovine serum albumin (Sigma). Recombinant human IGF-I (Life Technologies, Inc.) was then added to the medium at the concentrations indicated in the legend to the figures. Cells were incubated at 37 °C for 48 h. Cell numbers were determined at the end of the assay period by adding 1 ml of 0.03% SDS, resuspending in 1 ml of serum-containing medium and counting in a hemocytometer. The data were analyzed statistically by the Student’s t test. 

Anchorage-independent Growth Assay—The ability of various cell lines to grow in soft agar was determined as described by Sel et al. (7, 8). Cells were plated in DMEM containing 10% serum and 0.2% agarose (with 0.5% agarose underlayer). The cells were allowed to grow in soft agar for up to 2 (C6-derived cells) or 3 (R-derived cells) weeks at 37 °C. Anchorage-independent growth was assessed by scoring the number of colonies larger than 125 μm, which formed in soft agar during the assay period. 

Phosphorylation of IGF-IR, Insulin Receptor Substrate-1 (IRS-1), and Shc—Receptor autophosphorylation was measured as described previously (7, 8). Quiescent cells were stimulated with IGF-1 at 20 ng/ml for 10 min. The cells were then rinsed and lysed with lysis buffer. The protein concentration in the clarified lysate was determined by Bio-Rad protein assays (Bio-Rad). Cell lysates containing equal amounts of protein were subject to immunoprecipitation with a monoclonal anti-human IGF-IR antibody (AB-1, Oncogene Science Inc., Manhasset, NY). The precipitated proteins were resolved on a 4–15% gradient SDS-PAGE. The separated proteins were electrophroted to nitrocellulose filters using an electrophoretic transfer apparatus. The filters were then incubated with a monoclonal anti-phosphotyrosine antibody conjugated with horseradish peroxidase (Transduction Laboratories, Lexington, KY), or an anti-phosphoserine antibody (Sigma), followed by an anti-mouse IgG conjugated with horseradish peroxidase (Oncogene Science, Inc.). Detection of bound phosphoantibodies was carried out by using the ECL detection system (Amersham). 

Immunoprecipitation and Western Blotting—Cell lysates were collected and the total protein level was determined as described in the above paragraph. Equal amounts of protein were subject to immunoprecipitation with the indicated antibodies. The precipitated proteins were resolved on SDS-PAGE and electrophroted to a nitrocellulose filter. The filters were then blocked with 5% nonfat milk in TBST buffer (10 mM Tris, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) and incubated with antibodies indicated in the figure legends. Detection of bound antibodies was carried out by using a secondary antibody (anti-mouse or rabbit IgG conjugated with horseradish peroxidase) and ECL detection system. 

Antibodies used in these experiments include: anti-Grb-2 monoclonal antibody and anti-Stat-1 monoclonal antibody from Transduction Laboratory; and anti-J AK polyclonal antibody from UBI. 

PI-3 Kinase Assay—PI-3 kinase activity associated with IRS-1 was determined following the procedure recommended by the manufacturer of an anti-IRS-1 antibody (UBI). Briefly, cells were stimulated for 10 min with 20 ng/ml IGF-1, lysed, and equal amounts of protein from cell lysates were immunoprecipitated with the polyclonal antibody to rat IRS-1 (UBI). The precipitates were incubated in vitro in the presence of inositol and [γ-32P]ATP for 20 min. The products of the kinase reaction were analyzed by thin-layer chromatography. Radioactive spots of PI-3 phosphate were visualized by autoradiography and quantitated by excising the spots from chromatography plates and counting the radioactivity in a scintillation counter. 

MAP Kinase Assay—The levels of MAP kinase (p42 and p44) activation were determined by a method described previously (34) with modifications. Quiescent cells were treated with IGF-1 (20 ng/ml) for 15 min, or left untreated, before the cells were lysed. Cell lysates containing equal amounts of protein were subject to immunoprecipitation with an anti-ERK2 AC (agarse conjugated; Santa Cruz Biotechnology, Santa Cruz, CA). The precipitates were washed three times with kinase buffer containing 10 mM Tris, pH 7.4, 150 mM NaCl, 0.5 mM MgCl2, and 0.5 mM dithiothreitol and re-suspended in kinase buffer containing [γ-32P]ATP and myelin basic protein (Sigma). The kinase reaction was allowed to proceed for 20 min at 30 °C and terminated by adding SDS-PAGE sample buffer. The samples were then boiled for 5 min and separated on a 12% SDS-PAGE. The gel was dried, exposed to Kodak X-Omat AR film, and bands corresponding to myelin basic protein were excised from the gel and counted in a scintillation counter. 

Diffusion Chambers—Diffusion chambers (23) were used to test apoptosis of cells in vivo. Wild-type C6 cells or C6 cells stably transfected with the serine mutant receptor (5 × 10^3 cells per chamber) were placed into the chambers after a 24-h preincubation in vitro. The chambers were loaded with wild-type or A45S and Shc were detected by the same method described above, except, cell lysates were subjected to immunoprecipitation with a polyclonal anti-IRS-1 antibody (Upstate Biotechnology Inc. (UBI), Lake Placid, NY), or a polyclonal anti-Shc antibody (Transduction Laboratories) before applying to a SDS-PAGE. To determine the amount of IRS-1, immunoprecipitates were blotted with anti-IRS-1 antibody. 

RESULTS 

Characterization of R-derived Cell Lines—A number of clones were screened for the expression of the mutant receptor, and, eventually, 5 clones were selected for detailed studies. The number of IGF-IRs was determined by Scatchard analysis, and the cell lines used in these experiments are summarized in Table I. One of the clones (A45S) has a number of receptors only 40% above the levels of R^+ cells; our previous experience has
been that, in 3T3-like cells, 100,000 receptors per cell are sufficient for ligand-dependent transformation. The other clones were purposely selected with higher receptor numbers. All these cell lines, including the parental R- cells, grow in 10% serum (not shown).

Mitogenic Activity of the Mutant Receptor—The ability of the serine mutant receptor to respond to the mitogenic effect of IGF-I is shown in Fig. 1. This assay is done on cells in monolayer in SFM, and asks the very simple question whether IGF-I can stimulate their growth under these conditions. As usual, R- cells did not grow in SFM whether or not supplemented with IGF-I. All other cell lines transfected with either wild-type or mutant receptor responded to IGF-I, at two different concentrations (20 or 50 ng/ml) with an increase in cell number over SFM, the higher concentration being in most cases slightly more effective than the lower concentration. The increase over SFM was statistically significant at p < 0.05 for all clones expressing the mutated receptor (but not R- cells), and so was the difference in IGF-I-mediated stimulation between R- cells and 4A clones (Fig. 1). These results were reproducible. The mutation in the four serines at 1280–1283 of the IGF-IR, therefore, does not seem to impair its ability to transmit an IGF-I-mediated mitogenic signal.

Colony Formation in Soft Agar—We tested a total of 5 clones expressing the serine mutant receptor for their ability to form colonies in soft agar. The results are summarized in Table I. R- cells do not form colonies in soft agar, while R- cells give many colonies, as already repeatedly reported (7, 10, 12, 29, 35). The R- cells stably transfected with the serine mutant do not form colonies in soft agar, or form very few colonies compared to the wild-type R- cells. Four of these clones have substantially more receptors than R- cells (clone 4A19 has 5 million receptors per cell), and yet they do not form colonies in the soft agar assay. Some clones made tiny clusters, which did not qualify for colony formation units, the values for R-, R-4A5, R-4A17, and R-4A19 were, respectively, 3.8, 3.4, 3.3, and 3.1. Similar results were obtained with Shc (Fig. 3b): after immunoprecipitation with a Shc-antibody and blotting with an anti-phosphotyrosine antibody (Fig. 2b), the IGF-I-R band was actually more prominent in the mutant clones than in R- cells.

Although there is no a priori reason why the tyrosyl phosphorylation of IRS-1 and Shc should be affected (the mutant receptor is fully mitogenic), we investigated it in selected clones. Fig. 3 (panel a) shows that there is no impairment of IRS-1 tyrosyl phosphorylation in the clones expressing the serine mutant in response to IGF-1 stimulation; indeed, the signal is increased in the 3 mutant clones in respect to R- cells, but this could be accounted by the difference in the number of receptors (see Table I). The cell lines of Fig. 3 had similar amounts of IRS-1, by Western blotting: in arbitrary densitometry units, the values for R-, R-4A5, R-4A17, and R-4A19 were, respectively, 3.8, 3.4, 3.3, and 3.1. Similar results were obtained with Shc (Fig. 3b): after immunoprecipitation with a Shc-antibody and blotting with an anti-phosphotyrosine antibody, the signals were perhaps slightly increased in the mutant receptor clones. These increases will be taken into consideration under "Discussion."

PI-3 Kinase Activity—PI-3 kinase activity was determined and compared between wild-type and serine mutant receptor transfected cell lines. Because of the way the experiment was performed (see "Materials and Methods"), we are determining only the PI-3 kinase activity associated with IRS-1. As shown in Table II, mutations in the serine residues of the IGF-1 receptor do not impair the ability of the receptor to stimulate PI-3 kinase. In fact, there is an increase in PI-3 kinase activity in the mutant cell lines compared to wild-type cell line R-. In R- cells, there is a slight increase of PI-3 kinase activity upon IGF-1 stimulation. This may be due to the cross-reaction of IGF-1 with the IR.

![Figure 1](image)

**Fig. 1. Effect of IGF-I on the proliferation of cells overexpressing wild-type or mutant IGF-IR.** Cells were plated in serum-containing medium. After 24 h, the growth medium was removed and replaced with SFM with or without IGF-I (20 or 50 ng/ml). Cell numbers were determined after another 48 h. Each data point represents the mean of triplicate cultures of the same condition. The data were statistically analyzed and the growth of receptor-transfected cell lines (wild-type or mutant), but not R- cells, were significantly increased when stimulated with IGF-I (20 or 50 ng/ml.), p < 0.05. SFM; II, IGF-I, 20 ng; III, IGF-I, 50 ng.

**Table I**

| Mutant IGF-1 Receptor | Number of IGF-1 binding sites and colony formation in soft agar of R- derived cell lines |
|-----------------------|------------------------------------------------------------------------------------------------|
| Cell line No. of receptors \times 10⁶ | No. of colonies at 1 week | 2 weeks | 3 weeks |
| R-  | 0  | 0  | 0  |
| R-4A5  | 1.4  | 0  | 0  | 0  |
| R-4A6  | 4.0  | 0  | 0  | 2  |
| R-4A8  | 3.7  | 0  | 0  | 1  |
| R-4A17  | 2.8  | 6  | 5  | 3  |
| R-4A19  | 5.3  | 0  | 0  | 0  |

The number of IGF-I receptors in each cell line and colony formation in soft agar were determined as described under "Materials and Methods." R- cells have no IGF-I receptors; all other cell lines are derived by stable transfection of R- cells with either a wild-type human IGF-1R cDNA (R+ cells), or a receptor with serines 1280-1283 mutated to alanine (4A clones).
Downstream Signaling—We asked whether differences between the wild-type and the mutant receptors could be detected downstream from the immediate substrates of the IGF-IR. We first investigated the association between Shc and Grb-2, by immunoprecipitating lysates with an anti-Shc antibody and blotted with an anti-Grb-2 antibody. Fig. 4 shows that there is no impairment in the mutant receptor clones in the ability of Shc to bind and co-precipitate Grb-2.

We then examined the mitogen-activated protein (MAP) kinase activity, which is essentially the effector in the receptor tyrosine kinase/Ras/MAP kinase pathway. As shown in Table II, we were again unable to identify any impairment in the MAP kinase activity in cell lines expressing the serine mutant receptor, when compared with cells expressing the wild-type receptor.

Since the Stat-JAK pathway has been implicated in the signaling by the polypeptide growth factors (36–39), we have examined the status of Stat-1 in these cells. Immunoprecipitation with an antibody to Stat-1 and blotted with anti-phosphotyrosine antibody indicated no difference between R1 cells and the cells expressing the mutant receptor (not shown). We also investigated the association between the IGF-IR and JAK2, based on the reports which have shown that JAK2, another component in the JAK/Stat pathway, associated with the angiotensin II AT1 receptor (40) or the growth hormone receptor (41) upon ligand binding. JAK2 was detectable in lysates after immunoprecipitation with an antibody to the IGF-IR, but the amount that co-precipitated was the same in R1 and mutant cells, and did not change whether the cells were or not stimulated with IGF-I (data not shown).

Serine Mutants Can Act as Dominant Negatives for Transformation—It has been reported that some mutant IGF-IR can act as dominant negatives, especially in transformation and/or tumorigenesis (28–30). We tested the serine mutant receptor for its ability to act as a dominant negative in C6 rat glioblastoma cells. For this purpose, we transfected into C6 cells the serine mutant receptor and tested selected clones in soft agar. The selected clones have many more receptors than the wild-type cells (Table III), indicating that the number of mutant receptors outweigh the number of endogenous wild-type receptors. It should be remembered that the results in this kind of experiment are complicated by the fact that the mutant receptors can form hybrid receptors with endogenous ones or can be transactivated by the endogenous receptors (see “Discussion”). Yet, C6 cells stably transfected with the serine mutant recep-

TABLE II

| Cell line | PI-3 kinase fold stimulation | MAP kinase |
|-----------|-----------------------------|------------|
| R+        | 12.7                        | ND*        |
| R1        | 45.7                        | 1.5        |
| R1/A15    | 164.0                       | 2.5        |
| R1/A19    | ND                          | 3.0        |

* ND, not determined.

Fig. 2. In vivo IGF-I stimulation of the IGF-IR autophosphorylation. Tyrosine and serine phosphorylation of the IGF-IR b-subunit was determined in cells expressing the wild-type and mutant IGF-IR after a 10-min IGF-I stimulation. The same amounts of proteins from stimulated or unstimulated cells were immunoprecipitated by an anti-IGF-IR antibody, separated by a SDS-PAGE, transferred to nitrocellulose filters, and blotted with an anti-phosphotyrosine antibody (panel a) or an anti-phosphoserine antibody (panel b) as described under “Materials and Methods.” The size of the proteins were determined using a molecular weight marker. The arrows indicate the position of the IGF-IR b-subunit.

Fig. 3. Tyrosyl phosphorylation of IRS-1 and Shc in various clones. Cell lysates extracted from IGF-I stimulated or unstimulated cells were prepared as described under “Materials and Methods.” Lysates containing equal amounts of protein were immunoprecipitated with an anti-IRS-1 antibody, or an anti-Shc antibody, and then applied to the gel. Phosphotyrosine-containing proteins were detected as described under “Materials and Methods.” Panel a, phosphorylation of IRS-1; panel b, phosphorylation of Shc.
tors form fewer colonies in soft agar than wild-type parental C6 cells (Table III). In this sense, the serine mutant receptor acts as a dominant negative for transformation, like the Y1251F mutant receptor (12). Transfection of wild-type receptor or the Y1250F mutant in C6 cells does not alter their ability to form colonies in soft agar, in fact, the number of colonies is often increased (30).

Interestingly, though, the serine mutant receptor did not induce apoptosis, as an antisense expression plasmid to the IGF-IR can do (23). The C6 cells of Table III were tested in the diffusion chamber in vivo (23). This assay, in which the cells are bathed in plasma and have to be anchorage-independent to grow, measures a totally different property from the mitogenic assay (in SFM, with cells attached to a plastic surface). The recovery of 4A3 cells (the clone that gave the lowest number of colonies in soft agar) from the chamber after 24 h was 92%, compared to 220% for the wild-type C6 cells, and 200% with the other C6 clone expressing the mutant receptor. When we measured recovery of 4A3 cells after 48 h, the recovery was 208%. Therefore, it seems that the clones 4A3 cells grew slower than wild-type C6 cells in vivo, or, alternatively, that only a fraction of them underwent apoptosis.

**DISCUSSION**

The IGF-IR activated by its ligands plays an important role in cell proliferation for several types of cells in at least 3 different ways: it participates in the mitogenic signaling, it is required for the establishment and maintenance of transformation, and it protects cells from apoptosis both in vitro and in vivo (for a review, see Ref. 42). Although the two receptors share many common responses, the IGF-IR is 10 times more mitogenic than the IR (43), and is capable of transforming cells (see Introduction), whereas the IR, in the absence of an IGF-IR, cannot do so (12). A number of mutations in the IGF-IR have been shown to affect both its mitogenicity and its transforming activity. These include mutations at tyrosine 950 (35), in the 3 tyrosine residues of the kinase domain (29, 44) and at the ATP-binding site (10, 45). These mutations, however, have profound effects on the various functions of the IGF-IR, and equivalent mutations in the IR have similar crippling effects (for a review, see Ref. 46). Conversely, there are mutations that have no effect on either the mitogenic or transforming signaling (12, 29).

The C terminus of the IGF-IR is a logical domain to investigate differences with the IR in terms of mitogenesis and transformation because the homology with the IR, in this region, is considerably lower than in the rest of the subunits. Liu et al. (17) has shown that terminal truncations of the IGF-IR affected its transforming activity, but they used a chimeric receptor, with a Gag protein in lieu of the subunit, that was constitutively activated, and did not test for IGF-1-mediated mitogenicity (the IGF-IR is not obligatory for growth in 10% serum, as demonstrated by R− cells). Suriacz et al. (18) were the first to show that the IGF-IR-mediated mitogenic response and transforming activity of the IGF-IR could be dissociated. They used for this purpose a receptor with a deletion of the last 108 amino acids at the C terminus, transfected into R− cells; this receptor transmitted a full mitogenic signal, but could not make cells form colonies in soft agar. Subsequently, Miura et al. (12) showed that a chimeric receptor, where the C terminus of the IGF-IR had been replaced by the C terminus of the IR (47), had lost most of its transforming activity. They also showed that a point mutation at tyrosine residue 1251 also caused abrogation of the transforming activity of the IGF-IR, without affecting its IGF-I-mediated mitogenicity. This tyrosine residue was chosen because tyrosines 1250 and 1251 are absent from the IR: interestingly, a single point mutation at 1250 gave a receptor that was both mitogenic and transforming (12).

The dissociation between IGF-I-mediated mitogenicity and transforming activity has important implications (see below), and for this reason, we have continued our investigations to define the domains in the IGF-IR C terminus that are required for transformation. Among the candidates are the region around residues 1293–1294 (absent from the IR), and the serine quartet at 1280–1283 (there are only two serines in a similar position in the IR). In this article, we present our results with the serine mutations, and these are clear: mutation of the serine quartet at 1280–1283 results in a receptor that is fully mitogenic, but has lost its ability to transform R− cells (colony formation in soft agar). A reasonable criticism that can be leveled at the dissociation between mitogenicity and transforming activity is that a slightly defective receptor (for instance, the receptor truncated at 1229 (18), the Y1251F mutant (12) and the serine mutant of this paper) can be enough for mitogenicity but not enough for transforming activity; i.e. the dissociation is purely a question of quantitative signaling. We have dealt with this criticism before (12), and we confirm here that two observations militate against this interpretation: 1) some of the clones express extremely high numbers of receptors, up to 50 times the number of IGF-IRs that are sufficient to induce transformation, and 2) both the Y1251F and the serine mutant act as dominant negatives for transformation (see below), while transforming receptors do not (30).

Although the serine mutant receptor acts as a dominant negative, reducing the number of colonies in soft agar, when transfected into C6 cells, a very aggressive rat glioblastoma cell line (22), it failed to induce apoptosis of C6 cells. We obtained the same results with other IGF-IR mutants (30), and, so far, the only mutant IGF-IR that inhibits tumorigenesis seems to be the truncated receptor described by Prager et al. (28). The modesty of the dominant negative effect that several of the mutant IGF-IRs have can be explained in part by the fact that growth factor receptors readily form hybrid receptors or transactivate each other (48–54). It is this problem that makes experiments with R− cells (devoid of endogenous receptors) more informative. It remains the fact that some mutant receptors that are dominant negative for transformation do not induce apoptosis (Ref. 30 and this article), suggesting that the two processes can be clearly separated. It is very important to realize, that mitogenicity, colony formation in soft agar, and growth in vivo are radically different assays that test diverse properties. In the mitogenic assay, cells are attached to a plastic surface and in vivo, and are asked to respond to IGF-I. In soft agar (10% serum) and in vivo, cells have to be anchorage-independent to grow: indeed, this is why the two properties can be dissociated at the level of the receptor, they involve two different cellular responses.

Tyrosyl phosphorylation of IRS-1 and Shc are not grossly impaired, and PI-3 kinase stimulation is perhaps increased, not surprisingly, since this receptor is fully mitogenic, and these substrates are part of the IGF-IR mitogenic signaling
(Refs. 55–57, and for review, see Ref. 58). There is in fact a suggestion that tyrosyl phosphorylation of IRS-1 and Shc and PI-3 kinase activity may be increased, as we had already observed with other non-transforming receptors (12, 18). We would like to postpone a quantitative discussion of the signaling pathway; the yeast two-hybrid system is a better method to quantitate interaction between the IGF-1R and its substrates (59). With the methods used in this article, which are the methods mostly used in the literature, and with the differences in the number of receptors, a precise quantitative assessment is not warranted. Our present experiments should simply be taken as indicating that there is no impairment of the known signaling pathway in our serine mutant.

To understand fully the implications of these and previous findings, we should remember that the wild-type IGF-1R, overexpressed, is fully mitogenic and transforming and that neither IRS-1 (60) nor Shc, by themselves, or in combination, can transform R- cells. Also, tyrosyl phosphorylation of either IRS-1 or Shc, and PI-3 kinase activity are not decreased in the receptor truncated at 1229 (18), in the Y1251 mutant (12), and in the serine mutant (this article), again, not surprisingly, since these receptors are fully mitogenic.

It seems, therefore, that the C terminus of the IGF-1R sends a transforming signal that is additional to and separate from the mitogenic signal, and that can be localized between residues 1245 and 1310 (61). Within these boundaries, the serine quartet at 1280–1283 (this article), tyrosine 1251 (12), and the 1293–1294 region (61) act as the major sites for the transforming activity. It is possible that this signal is mediated through an unknown, or at least unidentified, substrate that interacts directly with the transforming domains of the IGF-1R. Alternatively, the C terminus may cause subtle changes in IRS-1 (or Shc) that are not detectable by a semi-quantitative assay for tyrosyl phosphorylation, changes that, in turn, could start a Stat pathway is not the transforming pathway we are searching for.

In conclusion, we have identified in the four serines at 1280–1283 a third, important domain of the IGF-1R that is required for its transforming activity. The fact that this mutant receptor, like the Y1251F mutant (12) and the 1293–1294 mutant (61), is fully mitogenic, clearly indicates that the IGF-1R has a transforming pathway, which is in addition to and separate from the mitogenic pathway. This dissociation has implications at both the basic and the applied levels. On one side, it suggests experiments to identify the additional transforming pathway, while at the same time it offers the possibility of interfering with transformed cells (apoptosis?) without greatly affecting the proliferation of normal cells.

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[References to scientific articles and publications are included, likely detailing experimental methods, results, and conclusions regarding the study of IGF-1R signaling and transformation.]

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