**Different Effects on Mitogenesis and Transformation of a Mutation at Tyrosine 1251 of the Insulin-like Growth Factor I Receptor**

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The wild type insulin-like growth factor I (IGF-I) receptor has both mitogenic and transforming activities. We have examined the effect of point mutations at tyrosine residues 1250 and 1251 on these two properties of the receptor. For this purpose, we stably transfected plasmids expressing mutant and wild type receptors into R- cells, which are 3T3-like cells, derived from mouse embryos with a targeted disruption of the IGF-I receptor genes, and therefore devoid of endogenous IGF-I receptors. A tyrosine to phenylalanine mutation of either the 1250 or 1251 residue, or both, has no effect on the ability of the receptor to transmit a mitogenic signal. However, the tyrosine 1251 mutant receptor and the double mutant have lost the ability to transform R- cells (colony formation in soft agar), even when the receptors are expressed at very high levels, while the Y1250F mutant is fully transforming. These experiments show that the 1251 tyrosine residue is required for the transforming activity of the IGF-I receptor.

Overexpression and constitutive activation of the insulin-like growth factor I receptor (IGF-I-R) in a variety of cell types leads to ligand-dependent growth in serum-free medium and to the establishment of a transformed phenotype (Kaleko et al., 1990; McCubrey et al., 1991; Li et al., 1993a; Sell et al., 1994; Coppola et al., 1994). Conversely, 3T3-like mouse embryo cells with a targeted disruption of the IGF-I-R genes (Li et al., 1993b; Baker et al., 1993) are refractory to transformation by SV40 large T antigen, an activated Ras or a combination of both, that readily transform cells from wild type littermate embryos or other 3T3-like cells (Sell et al., 1993, 1994). The importance of the IGF-I-R in the establishment and maintenance of the transformed phenotype is also supported by other findings, indicating that antisense oligodeoxynucleotides or antisense expression plasmids against IGF-I (Christophori et al., 1994), IGF-I (Trojan et al., 1992, 1993), or the IGF-I receptor (Sell et al., 1993; Baserga et al., 1994; Resnicoff et al., 1994a and 1994b), antibodies to the IGF-I-R (Artaaga, 1992; Kalebic et al., 1992), and dominant negative mutants of the IGF-I-R (Prager et al., 1994; Li et al., 1994), can all reverse the transformed phenotype, inhibit tumorigenesis, and induce loss of the metastatic phenotype (Long et al., 1995).

Our laboratory has been investigating the role that various tyrosine residues of the IGF-IR have on two of its properties, mitogenic activity and ability to transform mouse embryo cells, using R- cells (Sell et al., 1993, 1994; Coppola et al., 1994) established by a 3T3-like protocol from mouse embryos with a targeted disruption of the IGF-I-R genes (Liu et al., 1993b, Baker et al., 1993). The absence of an endogenous background of IGF-IRs in these cells facilitates a mutational analysis. R- cells grow in 10% serum, albeit at a reduced rate in comparison with cells from wild type littermate embryos (W cells), fail to grow in serum-free medium (SFM) supplemented with the growth factors that sustain the growth of other 3T3-like cells (Sell et al., 1994; Valentinis et al., 1994), and, as mentioned above, cannot be transformed by SV40 T antigen and/or an activated and overexpressed Ras. The growth phenotype of R- cells, including their resistance to transformation, returns to normal (i.e. like W cells or other 3T3-like cells) when they are stably transfected with a plasmid expressing a wild type human IGF-I-R cDNA (Ullrich et al., 1986) but not when transfected with a human receptor with a point mutation at the ATP-binding site (Sell et al., 1994; Coppola et al., 1994). Liu et al. (1993a) have previously reported that C-terminal truncations of a constitutively activated Gag-IGF-I-R hybrid construct affected its transforming activity. This was confirmed in a more rigorous way and extended by our own findings that a human IGF-I-R lacking the C-terminal 108 amino acids (Surmacz et al., 1995) and an overexpressed insulin receptor (see below) cannot transform R- cells. We therefore investigated in the present paper the mitogenic and transforming activities of the IGF-I-R with point mutations at tyrosine residues 1250 and 1251 that are absent in the IR (Ullrich et al., 1986). Our principal aim was to determine whether these two tyrosine residues are necessary for mitogenesis and transformation of R- cells. A secondary aim was to determine other properties of the mutant receptors, such as ability to phosphorylate cellular substrates in vivo.

We show that a receptor with a mutation at Tyr-1251, but not at Tyr-1250, cannot transform R- cells, while both mutant receptors are fully mitogenic. Several indications suggest that this preservation of a mitogenic response with loss of transforming activity is not simply due to a quantitative deficit of the Y1251F mutant receptor. The identification of a receptor mutant that is fully mitogenic but nontransforming opens the possibility of identifying new transformation pathways that can be separated from the mitogenic pathways.

**MATERIALS AND METHODS**

Mutagenesis of Human IGF-I-R cDNA—The Tyr-1250, Tyr-1251, and double tyrosine mutants were derived from the wild type human IGF-I-R cDNA with the complete coding sequence (Ullrich et al., 1986). A PCR-aided in vitro mutagenesis method was used to create the tyrosine mutants of the IGF-I-R, in which the tyrosine codon was substituted by the phenylalanine codon.

The first PCR was performed using a pBluescript SK IGF-I-R (a pBluescript SK phagemid (Strategen) in which an XbaI-BamHI fragment including the whole IGF-I-R cDNA was inserted) as a template, a 5'-primer (mutagenic primer), and a 3'-primer as follows. 5'-prime of

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1 The abbreviations used are: IGF-I, IGF-I receptor; IGF-I and IGF-II, insulin-like growth factors I and II, respectively; IR, insulin receptor; IRS-1, insulin receptor substrate-1; SFM, serum-free medium; PCR, polymerase chain reaction.

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(mutagenic primers) were 5'-GGCTGTCCGAGGTCTCTCTTCTTCA CG-3' (1250F), 5'-GGCTGTCCGAGGTCTCTCTTCTTCA CG-3' (1251F), and 5'-GGCTGTCCGAGGTCTCTCTTCTTCA CG-3' (1250F, 1251F); the 3'-primer was 5'-GTAACACGAGCCGCTAG-3' (M13-20 primer, whose sequence is in the pBluescript SK). The first 5'-nucleotide of the mutagenic primers followed a thymine residue in the template sequence to avoid problems with template-independent incorporation of an adenine at the 3'-termini of the amplified products. All three primers were designed to create a NruI site with silent mutations. The mutated sites in the codon corresponding to amino acid position 1250 or 1251, are underlined, while the silent mutations are shown in lowercase. This first PCR product contains an amino acid position 1250 or 1251, are underlined, while the silent mutations. The mutated sites in the codon corresponding to IGF-IR cDNA. The second PCR was performed using the same template to the first PCR, a 5'-primer, and the first PCR product as a 3'-primer (an antisense strand actually serves as a 3'-primer). The 5'-primer was designed to locate beyond the unique HindIII site as follows: 5'-ACACGT GACAGGCGCCGACAG-3'.

The second PCR product was subcloned into a TA cloning vector, pCRTI (Invitrogen), and the correct mutation was confirmed by digestion with NruI and by deoxy sequencing (not shown). A wild type HindIII-BamHI fragment of a pBluescript SK Sal-Bam IGF-IR (a pBluescript SK phagemid containing a Sall-BamHI fragment of the whole IGF-IR cDNA) was replaced by the mutant HindIII-BamHI fragment from the pCR1 containing the second PCR product, and a Sall-NotI site was introduced by mutagenesis. The mutated DNA was subcloned into a Xho-NotI site of the ampicillin-resistant plasmid, pBluescript SK II, resulting in the pCRII-IR clones, R1-IR clones, R2-IR clones, and their parent cells, generating R1, R2, and 2IR clones, respectively.

An Overexpressed Insulin Receptor Does Not Transform R-Cells—Although there is extensive homology between the insulin and the IGF-I receptors, the IGF-I receptor (IGF-IR) is 10 times more mitogenic than the insulin receptor (Lammers et al., 1989). However, an overexpressed insulin receptor (IR) makes 3T3 cells and their progeny immortalized even in the absence of serum (DeAngelis et al., 1994) and induces the transformed phenotype (Giorgini et al., 1991). Since we had shown in previous experiments that overexpressed epidermal growth factor or platelet-derived growth factor receptors cannot transform R- cells (cells with a targeted deletion of the IGF-IR genes), although they can transform wild type cells (Coppola et al., 1994; DeAngelis et al., 1995), we inquired in preliminary experiments whether an overexpressed IR could transform R- cells. For this purpose, R- cells were transfected with a plasmid expressing the human IR (Ullrich et al., 1985), and a number of clones were selected and analyzed for insulin receptor number (Table I) and for ability to grow in soft agar. Confirming the previous report by Randazzo et al. (1990), these clones were capable of growing in SFM supplemented solely with insulin (not shown). However, like the parental R- cells, four different clones of R- cells overexpressing the IR could not form colonies in soft agar (Table I). On the contrary, R- cells, stably transfected with and overexpressing a wild type human IGF-IR (R+ cells), form colonies in soft agar (Table I). Clones 2 and 9 have a number of R1s of 100,000 or 500,000/cell, respectively; at these levels of IGF-IR expression, 3T3-like cells are fully transformed (Sell et al., 1994).
Characterization of Mutants at Tyrosines 1250 and 1251—The preliminary experiments described above, the report by Tartare et al. (1994) that substitution of the IGF-IR C terminus with the IR C terminus decreased its ability to induce DNA synthesis, and the findings by Liu et al. (1995) and by Surmacz et al. (1995) all suggested that the C terminus of the IGF-IR may play an important role in its transforming activity. While there are obviously other candidates, the tyrosine residues at 1250 and 1251 (as mentioned above, absent in the C terminus of the IR), seem to be reasonable candidates for a mutational analysis of function. Mutant receptors, where the tyrosine residue was mutated to phenylalanine by site-directed mutagenesis, as described under "Materials and Methods," were transfected into R(1) cells, and the transfectants were characterized for receptor content. Fig. 1 shows a Scatchard analysis of three cell lines, and Table I summarizes the receptor content of these cell lines. All these cell lines expressed more than 10(6) receptors/cell, more than adequate since, in previous experiments, we had established that 10(6) receptors/cell or more are sufficient for growth in SFM supplemented solely with IGF-I as well as for the establishment of the transformed phenotype (Pietrzowski et al., 1992a; Coppola et al., 1994). Cellular clones with lower numbers of receptors were also established (see below), but most of the subsequent studies were carried out on the cell lines with high receptor numbers.

Mutations at Tyr-1250 and Tyr-1251 Do Not Affect the Mitogenic Signaling—The cell lines listed in Table I were tested for growth in SFM supplemented solely with IGF-I (50 ng/ml). The results of separate experiments are summarized in Fig. 2; R(-) cells, Y1250F cells, Y1251F cells, and the double mutant Y1250F/Y1251F, all grow in SFM plus IGF-I, differences among the cell lines being negligible. These experiments were repeated four times, and because growth of cells often depends on seeding density, we even used different seeding densities (see "Materials and Methods"). In no instance could we detect a statistical difference between R(-) cells (wild type receptor) and the cells expressing the mutant receptors. Therefore, mutations at Tyr-1250 and Tyr-1251 have no significant effect on the ability of the IGF-IR to transmit a mitogenic signal.

A Mutation at Tyr-1251 (but Not at Tyr-1250) Abolishes the Transforming Activity of the IGF-I Receptor—The same cell lines were then tested for their ability to form colonies in soft agar. Fig. 3 shows a microphotograph of the soft agar assay. Following an accepted convention, we counted only colonies >125 μm in diameter; both R(-) cells and Y1250F cells form colonies (panels B and C, respectively). R(-) cells form no colonies and remain as single cells up to the termination of the experiment (panel A). Y1251F cells only formed tiny clusters of cells (panel D), mostly 50–60 μm in diameter, that only occasionally reached the canonical size. The results are summarized in Table III. The ability to form colonies in soft agar is dramatically impaired in Y1251F cells, and in the double mutant Y120F/Y1251F cells, while Y1250F cells are definitely transformed, although the number of colonies in soft agar is somewhat less than with R(-) cells. The finding of the impaired ability of Y1251F cells to grow in soft agar is especially significant, considering the very high number of mutant receptors. In separate experiments, we also tested Y1251F expressing cells with lower receptor numbers (about 10(9)), to rule out a possible paradoxical effect, but these cells, too, failed to form colonies in soft agar (not shown).

The Y1251F Mutant Receptor Acts as a Dominant Negative—Mutants of the IGF-IR have been reported to act as dominant negatives (Prager et al., 1994b; Li et al., 1994) in transformation and/or tumorigenesis. We transfected the Y1251F and

| Cell type | Number of receptors/cell | Kd (nM) |
|-----------|--------------------------|---------|
| R(-)      | 0                        | 0       |
| R(+       | 1.1 × 10(5)              | 1.1     |
| Y1250F    | 9.5 × 10(5)              | 1.0     |
| Y1251F    | 9.7 × 10(5)              | 1.1     |
| Y1250F/Y1251F | 2.4 × 10(4) | 1.0     |

**Table I.**

| Mutant IGF-I Receptor |
|-----------------------|
| Y1250F/Y1251F         |

**Table II.**

**Fig. 1.** Scatchard analysis of cell lines with mutant IGF-I receptor content of various cell lines. R(-) cells stably transfected with the appropriate mutants of the IGF-I receptor were analyzed for receptor content as described under "Materials and Methods." The values given represent the average of three independent experiments.

**Fig. 2.** Growth of R(-)-derived cells in IGF-I. The various cell lines were plated in serum-supplemented medium overnight, then transferred to serum-free medium supplemented solely with IGF-I (50 ng/ml). The cell number was determined 48 h. after changing growth medium to serum-free medium plus IGF-I. The cell lines are described under "Results."
Y1250F mutant receptors in C6 cells, which are rat glioblastoma cells capable of producing colonies in soft agar (Resnicoff et al., 1994a; Coppola et al., 1994). Three clones were selected for each transfection, which expressed an increased number of receptors over the wild type cells (see Table IV), and whose growth in monolayer was not significantly inhibited (data not shown). Their ability to form colonies in soft agar is shown in Table IV. C6 cells expressing the Y1251F receptor formed fewer colonies in soft agar than the parental cells, the inhibition ranging from 75 to 100%. On the contrary, the growth in soft agar of C6 cells expressing the Y1250F mutant was not inhibited; in fact, if anything, the number of colonies was increased over the parent cell line.

Phosphorylation of Mutant Receptors and Shc—There is no a priori reason why the common signal-transducing pathway of the IGF-IR (the so called Ras pathway) should be impaired in cells expressing the two mutant receptors, since these receptors are fully mitogenic in response to IGF-I only. Nevertheless, we inquired whether these receptors had deficits at the very beginning of the pathway. Fig. 4A shows that the mutant receptors are autophosphorylated after stimulation by IGF-I. The technique used is semiquantitative, and the purpose of this experiment is simply to show that mutations at tyrosines 1250 and 1251 do not grossly affect the autophosphorylation of the IGF-IR. No precise conclusion ought to be reached in terms of the contribution of these two tyrosines to the total phosphorylation of the receptor. Fig. 4A also shows that the mutations at 1250 and 1251 do not seem to affect to any significant extent the tyrosyl phosphorylation of the p185 protein, generally accepted as the IRS-1 protein. In other experiments, we have immunoprecipitated IRS-1 from lysates with a specific antibody (a kind gift of Drs. Lienhard and Keller, Dartmouth Medical School), and still we could not find any significant differences in the extent of IRS-1 tyrosyl phosphorylation between wild type and mutant receptors (not shown).

In Fig. 4B, we have examined Shc phosphorylation. In
impairs the transforming activity of the IGF-I-R, while a Y1250F mutation does not. Secondary findings include the fact that 1) there is no gross alteration in the extent of autophosphorylation of the mutant receptors or the tyrosyl phosphorylation of the p185 protein (the mutant receptors are fully mitogenic); and 2) the Y1251F mutant is somewhat less efficient in phosphorylating Shc than the Y1250F mutant after IGF-I stimulation.

The most important finding in the present paper is that the Y1251F mutant, overexpressed, is functional enough to transmit an IGF-I-mediated mitogenic signal but, even at very high levels of receptor expression, does not support transformation, as measured by colony formation in soft agar. A mutation at Tyr-1250 does not affect the transforming ability of the IGF-I-R.

A number of studies have investigated the mitogenicity of the IGF-I-R (Lammers et al., 1989; Kato et al., 1993, Sasaoka et al., 1994; Kato et al., 1994), but until recently, very few studies had addressed its role in transformation (see the Introduction). In the case of transformation, other IGF-I-R mutants have already been shown to have lost the ability to induce IGF-I-mediated mitogenesis and to transform cells, for instance, a triple tyrosine mutant at Tyr-1131, -1135, -1136 (Li et al., 1994), and the Y950F mutant (Miura and Baserga, 1995), indicating that these two domains are required both for cell proliferation and for transformation. Our present results clearly show that neither Tyr-1250 nor Tyr-1251 can be enumerated among the domains of the IGF-I-R necessary for mitogenesis, since, at the receptor levels we have investigated, both mutant receptors are fully mitogenic. However, the Y1251F mutant (but not the Y1250F mutant) has a markedly decreased transforming activity. An important point is whether the failure of the Y1251F mutant to transform mouse embryo cells is due to a qualitative or a quantitative deficit. In the latter case, one could think that a receptor that is weakly active may be sufficient for transmission of a mitogenic signal but not adequate for the establishment of the transformed phenotype; alternatively, the Tyr-1251 residue may be specifically required for transformation, although in association with other domains of the receptor that are needed for the mitogenic signal. Two observations argue against the first alternative: 1) the Y1251F mutant is non-transforming at receptor levels that are 10-fold the levels of wild type receptor sufficient for the establishment and maintenance of the transformed phenotype (Pietrzkowski et al., 1992a, 1992b; Coppola et al., 1994); 2) the Y1251F mutant acts as a dominant negative in a cell type that has 500,000 endogenous IGF-I-Rs. It would be difficult to explain how the additional input from a receptor transmitting a weak but otherwise normal signal could reverse the signal of a wild type receptor. Indeed, the Y1250F mutant receptor not only does not inhibit soft agar growth of C6 cells but actually augments it, as one would expect from an additive effect.

The mechanism is, of course, the next important question. Not surprisingly, our experiments do not show gross differences in tyrosyl phosphorylation of the IGF-I-R or IRS-1, which are involved in insulin and IGF-I-mediated mitogenesis (Waters et al. (1993), Rose et al. (1994), Yamauchi and Pessin (1994), and see review by White and Kahn (1994)). We say not surprisingly, because both mutant receptors are fully mitogenic, and, therefore, there is no a priori reason why the known mitogenic pathways of the IGF-I-R should be affected. For the same reason, we have not explored in this paper the pathways downstream of IRS-1 and Shc. The difference between the wild type receptor and the Y1251F mutant receptor is in their ability to confer a transformed phenotype (colony formation in soft agar). However, soft agar assays are done in 10% serum, which also activates other growth factor receptors that use the Ras pathway, and this precludes a simple analysis at this point. The same criticism can be applied to an explanation involving Shc, which is known to be a transforming protein (Palici et al., 1992; Skolnik et al., 1993; White and Kahn, 1994). Indeed, the Y1251F mutant receptor seems to be somewhat less efficient in its ability to phosphorylate Shc, at least in comparison with the Y1250F mutant (Fig. 4), which makes this explanation a still viable one, especially since we obtained the same results with the C terminus truncated receptor (Surnacz et al., 1995). But again, Shc phosphorylation was determined in serum-free medium supplemented with IGF-I, whereas the transformation assay is done in 10% serum, which contains other growth factors capable of activating Shc (White and Kahn, 1994). Also one cannot ignore some recent reports that there is some kind of balance between IRS-1 and Shc that is important for the stimulation of DNA synthesis by the IR (Yamauchi and Pessin, 1994), and that a transmembrane mutant of the v-ras oncogene that had lost its transforming activity still retained its ability to phosphorylate Shc (Zong and Wang, 1994).

Indeed, the fact that a combination of SV 40 T antigen and an activated, overexpressed Ras fail to transform R-cells (Sell et al., 1994, Surnacz et al. 1995), clearly indicates that one of the transforming pathways of the IGF-I-R is Ras-independent. While there is no question that the Ras pathway is required for transformation (see for instance McCormick (1993) and Medema and Bos (1993)), another pathway that the IGF-I-R does not share with the platelet-derived growth factor and epidermal growth factor receptors has to be hypothesized, a conclusion supported by other independent findings (Falco et al., 1988; Aaronson, 1991; Matuoka et al., 1993; Silvermann et al., 1993). In previous papers (Coppola et al., 1994, DeAngelis et al. 1995), we showed that an overexpressed epidermal growth factor or platelet-derived growth factor receptor cannot transform R cells, although they are capable of transforming wild type cells from littermate mouse embryos. The requirement for a functional IGF-I-R for transformation seems to be true also in the case of the IR (this paper), despite the fact of the extensive homology between the two receptors (Ullrich et al., 1986) and the sharing of signal-transducing pathways (for a review, see Tavare and Siddle (1993) and White and Kahn (1994)). Again, these findings indicate that the IGF-I-R has at least another transforming pathway, which it does not share with the three other receptors.

Our experiments do not tell us whether tyrosine phosphorylation is actually necessary for the transforming activity of the IGF-I-R. We have avoided addressing this question at this point, and we prefer to state that a Tyr to Phe mutation at 1251 (but not at Tyr-1250) almost completely abolishes the transforming activity of the IGF-I-R, leaving unresolved whether this is due to the lack of Tyr-1251 phosphorylation or to a conformational change. Neither do our experiments say that Tyr-1251 is the exclusive depository of the information for a transforming signal. What they say is that a functional Tyr-1251 is required for the transforming ability of the receptor.

The secondary findings have been reported here mainly to indicate that certain functions of the mutant receptors are not grossly impaired, as in the case of the ATP-binding site mutant (Kato et al., 1993), which has lost most of its functions and is neither mitogenic nor transforming (Kato et al., 1993; Sell et al., 1994; Coppola et al., 1994).

In conclusion, our experiments, while confirming the importance of the IGF-I-R in the establishment and maintenance of transformation, have shown that tyrosine residues 1250 and 1251 are not necessary for IGF-I-mediated mitogenesis but that tyrosine 1251 is required for IGF-I-R-dependent transfor-
mation. The dissociation of mitogenesis and transformation at the level of the receptor itself opens intriguing possibilities about the roles of the signal-transducing pathways in the two processes, regardless of whether the dissociation is qualitative, quantitative, or due to a prolongation of the stimulus (Marshall, 1995). Another obvious question to be addressed is whether the transforming activity of the IGF-IR correlates with its ability to protect cells from apoptosis (Harrington et al., 1994; Sell et al. 1995).

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