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# Dissociation Between Resistance to Apoptosis and the Transformed Phenotype in IGF-I Receptor Signaling

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**Abstract** Programmed Cell Death (PCD) is known to play an important role in both the development and the growth rate of human tumors. It has in fact been suggested that suppression of the apoptotic pathway is a requirement for the establishment of the transformed phenotype. In order to elucidate the relationship between resistance to apoptosis and transformation, we have asked in this investigation whether or not the two processes can be directly correlated. For this purpose, we have used mouse embryo fibroblasts (MEF) expressing either the wild-type or several mutants of the type 1 insulin-like growth factor receptor (IGF-IR). The wild-type IGF-IR has both transforming and anti-apoptotic activities, and we have asked whether these two activities can be or not separated in mutant receptors. Using this well-defined system, our results show that certain mutants of the IGF-IR that have strong anti-apoptotic and mitogenic activities, are incapable of transforming MEF (colony formation in soft agar). We have, instead, a good correlation between mitogenic and anti-apoptotic activities, suggesting the possibility that the two processes may share similar signaling pathways from the IGF-IR. On the other hand, our results indicate that transformation requires an additional signal, above and beyond the mitogenic and survival signals. Our conclusion is that, at least in this system, the establishment of the malignant phenotype and resistance to apoptosis can be dissociated, implying the possibility of separate targeting. J. Cell. Biochem. 72:294–310, 1999. 

**Key words:** programmed cell death; transformed phenotype; mouse embryo fibroblasts

It is well-established that an increase in cell number of any given cell population (in vivo or in vitro, normal or abnormal) depends on three parameters [Baserga, 1985]: 1) the length of the cell cycle. A shortening in the length of the cell cycle will cause cells to divide more frequently, resulting in an increase in cell number; 2) the growth fraction, i.e., the fraction of cells in a population that are actively in the cell cycle [Mendelsohn, 1962]. In many cell populations, a sizable fraction of cells is in G/0, in which the cells are in a sort of hibernation, from which they can be rescued by appropriate stimuli. An increase in the growth fraction, i.e., a decrease in the G/0 fraction, will also result in an increased production of cells; and 3) a decrease in the rate of cell death, whether by apoptosis or necrosis. This third parameter plays a role as important as the rate of cell proliferation (first two parameters) in determining the growth rates of human tumors [Bresciani et al., 1974]. Indeed, it has often been proposed that resistance to apoptosis *per se* can be at the basis of tumor development in humans. The best known example is the association between overexpression or activation of the anti-apoptotic protein Bcl-2 and the development of lymphomas in man [Tsujimoto et al., 1985, Tsujimoto and Croce, 1986]. A similar situation has been described for chronic myelogenous leukemia, where enhanced cell survival, rather than proliferative advantage, might be the primary and most direct consequence of BCR/ABL expression in progenitor cells [Kantarjan et al., 1993; McGahon et al., 1994]. Other reports in the literature have emphasized this inverse relationship between susceptibility to apoptosis and tumor development [McCarthy et al., 1994; Kitada et al., 1998], summarized in the state-

Abbreviations used: FBS, fetal bovine serum; IGF-I, insulin-like growth factor 1; IGF-IR, type 1 IGF receptor; IR, insulin receptor; MEF, mouse embryo fibroblasts; SFM, serum-free medium.

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ment by Boehm et al. [1998] that ". . . Suppression of the apoptotic pathway is a requirement for carcinogenesis." Howes et al. [1994] went even farther by concluding that the alternative to retinoblastoma development was apoptosis of photoreceptors.

While there is no question that, in some cases, inhibition of PCD may favor tumor development, it would be desirable to know whether, in a defined system, there is a direct correlation between resistance to apoptosis and the transformed phenotype. A good model to answer this question is that of the type 1 insulin-like growth factor receptor (IGF-IR), which is known to act in at least four different ways in controlling the size of a cell population [Baserga et al., 1997]. It is mitogenic, it sends a powerful anti-apoptotic signal, it is quasi-obligatory for the transformation of cells, and it can also modulate cell differentiation. In this paper, we focus on the two properties of the IGF-IR relevant to the question we have posed above: the ability to induce transformation and the capacity to increase cell survival.

It is known that overexpression and/or constitutive activation of IGF-IR in a variety of cell types leads to ligand-dependent growth in serum-free medium and to the establishment of a transformed phenotype; i.e., ability to form colonies in soft agar and/or to produce tumors in mice [Kaleko et al., 1990; McCubrey et al., 1991; Pietrzkowski et al., 1992; D. Liu et al., 1993; Sell et al., 1994; Coppola et al., 1994]. This property is shared with several other growth factor receptors, and, indeed, with many other cellular products. A unique characteristic of the IGF-IR is that $R^-$ cells (i.e., mouse embryo fibroblasts with a targeted disruption of the IGF-IR genes) are refractory to transformation by several viral and cellular oncogenes that readily transform mouse embryo fibroblasts with a physiological number of IGF-IR, like 3T3 cells of various origins. The list of oncogenes that fail to transform R-cells include the SV40 large T antigen [Sell et al., 1993], an activated ras or a combination of T antigen and ras [Sell et al., 1994], the bovine papilloma virus E5 protein [Morrione et al., 1995], the human papilloma virus [Steller et al., 1996], an activated c-src [Valentinis et al., 1997], the EWS/FLI-1 oncogene [Toretsky et al., 1997], and overexpressed growth factor receptors, such as the EGF receptor [Coppola et al., 1994], the PDGF β receptor [DeAngelis et al., 1995], and the insulin receptor [Miura et al., 1995a].

The evidence for an anti-apoptotic function of the IGF-IR is also substantial. Thus, an overexpressed IGF-IR allows the growth of FDC-P1 cells in the absence of interleukin-3 (IL-3), which is otherwise an obligatory requirement for the survival of these cells of hemopoietic origin [McCubrey et al., 1991]. These cells can also be protected from apoptosis induced by IL-3 withdrawal by the simple addition of IGF-I [Rodriguez-Tarduchy et al., 1992]. IGF-I also protects cells overexpressing c-myc, which undergo apoptosis in the absence of growth factors [Harrington et al., 1995], as well as human cancer cells from cell death induced by diverse anticancer drugs [Dunn et al., 1997]. An overexpressed and functional IGF-IR protects cells from apoptosis induced by etoposide [Sell et al., 1995], tumor necrosis factor α [Wu et al., 1996], transforming growth factor β [Hsing et al., 1996], p53 [Prisco et al., 1997; Ohlsson et al., 1998], ionizing and non-ionizing radiations [Kulik et al., 1997; Turner et al., 1997; Nakamura et al., 1997], okadaic acid [D'Ambrosio et al., 1997], and IL-3 withdrawal [O'Connor et al., 1997; Zhou-Li et al., 1997; Dews et al., 1997]. Conversely, when the function of the IGF-IR is decreased or otherwise impaired by antisense strategies or by dominant negative mutants, or by triple-helix formation, tumor cells undergo massive apoptosis, resulting in a dramatic inhibition of tumorigenesis [D'Ambrosio et al., 1996; Prager et al., 1994; Resnicoff et al., 1994a, 1994b; Shapiro et al., 1994; Neuenschwander et al., 1995; Lee et al., 1996; Pass et al., 1996; Rininsland et al., 1997], and metastases [Long et al., 1995; Burfeind et al., 1996; Dunn et al., 1998]. Thus, in the case of IGF-IR targeting, induction of apoptosis influences tumor growth. However, that does not mean that the signals for survival and transformation are necessarily correlated. A reasonable question at this point is whether IGF-IR-mediated survival and transformation depend on the same or different signaling pathways. A first step approach would be to test whether these two functions of the IGF-R are separable or not at the receptor level. For this purpose, we have analyzed in mouse embryo fibroblasts (MEF) how mutations in the β subunit of the IGF-IR affect its transforming activity and its ability to protect cells from apoptosis. For the purpose of these investigations, we define transformation as it was origi-

nally defined by Macpherson and Montagnier [1964], as ability to form colonies in soft agar. This is a stricter definition than focus formation in monolayer cultures, and it correlates well with tumor formation in animals [Aaronson and Todaro, 1968]. To measure resistance to apoptosis, we have selected anoikis [Frisch and Francis, 1994; Khwaja et al., 1997; Day et al., 1997], a form of apoptosis that occurs when cells are denied attachment to a substratum. Since the IGF-IR seems to be especially important in protection from apoptosis when the cells are placed in anchorage independent conditions [Baserga et al., 1997; Baserga, 1997], anoikis is a suitable model to test the anti-apoptotic effect of the IGF-IR. Another advantage of anoikis is that our MEF undergo apoptosis when incubated in serum-free medium in suspension, but survive, without even entering S phase, when IGF-I is added to the medium [Valentinis et al., 1998]. It is therefore possible in this model to study the survival signals of the IGF-IR, independently of its mitogenic signals.

We used for this purpose R-cells [Sell et al., 1993, 1994; Coppola et al., 1994] that are 3T3 cells originating from mouse embryos with a targeted disruption of the IGF-IR genes [Baker et al., 1993; J Liu et al., 1993]; these cells were transduced with retroviral vectors carrying either the wild-type or various mutants of the IGF-IR. We find that certain mutations in the β subunit of the IGF-IR that do not impair the mitogenic response nor survival in suspension cultures, completely abrogate colony formation in soft agar. We conclude that, at least in the case of the IGF-IR, the establishment of a transformed phenotype requires additional signals beyond the mitogenic and survival signals.

## MATERIALS AND METHODS

### Plasmids

The retroviral vector MSCV.neoEB was kindly provided by Dr. R.G. Hawley (University of Toronto, Canada), and is described elsewhere [Hawley et al., 1994]. Plasmids pHIT60 and pHIT123 were a gift of Dr. A. Kingsman (University of Oxford, UK), and are described elsewhere [Soneoka et al., 1995]. pHIT60 contains the murine leukemia virus (MLV) *gag-pol* cassette under the control of the human cytomegalovirus immediate early (hCMVi.e.) promoter, whereas pHIT123 contains the hCMVi.e. driven MLV ecotropic envelope. Both plasmids carry the SV40 origin of replication in their backbone.

Plasmid pGR15 contains the human wild-type receptor for the insulin-like growth factor-1 (IGF-1R) under the control of the MLV 5′-long terminal repeat (LTR). The wild type IGF-1R cassette was excised by XbaI-BamHI digestion from a CVN expression vector [Ullrich et al., 1986], ligated into XbaI-BamHI digested and dephosphorylated pUC19 cloning vector, re-excised by SalI-BamHI digestion and finally ligated into XhoI-BglII digested and dephosphorylated MSCV.neoEB retroviral vector.

Plasmids pGR35, pGR46, pGR47, and pGR48 were generated by placing various mutant IGF-IR cassettes in the MSCV.neoEB retroviral vector, as described for pGR15. All these mutants derive from the human wild type IGF-IR cDNA [Ullrich et al., 1986], and have been previously described. The numbering of the IGF-IR amino acids residues follows the system described by Ullrich et al. [1986], in which the 30 amino acids of the signal peptide are omitted. Briefly, pGR35 contains a mutant IGF-IR, in which four serines at residues 1280, 1281, 1283, and 1284 were changed to four alanines [Li et al., 1996]. pGR46 contains a mutant IGF-IR in which a tyrosine at residue 950 was changed to phenylalanine [Miura et al., 1995b]. pGR47 contains a mutant IGF-IR in which the histidine at residue 1293 was changed to phenylalanine, and the lysine at residue 1294 was changed to leucine [Hongo et al., 1996]. pGR48 contains an IGF-IR cassette with a triple tyrosine mutation at residues 1131, 1135, and 1136 to phenylalanine [Gronborg et al., 1993; Li et al., 1994].

The other mutant receptors have not been previously described. Plasmid pGR18 contains an MLV 5′-LTR driven IGF-IR cDNA, in which the tyrosines at residues 950, 1131, 1135, and 1136 were changed to phenylalanine. This plasmid was generated by placing the XbaI-HindIII cDNA fragment of mutant (Y950F) IGF-IR and the HindIII-BamHI cDNA fragment of mutant (Y1131F, Y1135F, and Y1136F) IGF-IR in XbaI-BamHI digested and dephosphorylated pUC19 cloning vector. The four tyrosines IGF-IR mutant was then excised by SalI-BamHI digestion, and ligated into XhoI-BglII cloning sites of MSCV.neoEB.

Plasmid pGR25 contains an MLV 5′-LTR driven IGF-IR cDNA, in which four basic aminoacids were mutated. These are the arginine at

residue 1289, which was changed to phenylalanine, the histidine at residue 1290, which was changed to leucine, the histidine at residue 1293, which was changed to phenylalanine, and the lysine at residue 1294, which was changed to leucine. All these mutations were made by polymerase chain reaction (PCR), following a mutagenesis strategy described by Valsesia-Wittman et al. [1994]. Briefly, two sets of primers were designed. One set was wild-type, the other was mutagenic. The wild-type set had a sense primer which anneals to a sequence of IGF-1R upstream of HindIII restriction site [Ullrich et al., 1986], and an antisense primer which anneals to the 3′-end of IGF-IR sequence, in correspondence of BamHI restriction site [Ullrich et al., 1986]. The sequence of the wild-type sense primer was 5′-CAGTGAACGAGGCCGCAAGCATGCG-3′ (positions 3101 to 3126, the genome coordinates are given beginning from ATG start codon). The sequence of the wild-type antisense primer was 5′-GCACACGTTACTGTTTGCACAGATTCAGGATCCAAGGATCAGCAGGT- 3′ (positions 4142 to 4096, the sequence of BamHI restriction site is underlined). The set of mutagenic primers was designed in correspondence of the residues that have to be changed. These primers contains some nucleotides mismatches, they are sense and antisense, and have overlapping sequences. The sequence of the sense mutagenic primer was 5′-CGACttcctgTCAGGAttcctgGCCGAGAACG-3′ (positions 3951 to 3983, the nucleotides that have been mismatched are indicated in lower case letters), whereas the sequence of the antisense mutagenic primer was 5′-GGCcaggaaTCCTGAcaggaaGTCGGGCAGTG-3′ (positions 3976 to 3944, the mismatched nucleotides are indicated in lowercase letters). Two PCR reactions were then set up. The template in both cases was the wild type IGF-1R. One PCR mixture contained the sense wild-type primer and the antisense mutagenic primer, whereas the other PCR mixture contained the wild-type antisense primer and the sense mutagenic primer. The PCR reaction conditions were as follows: a denaturation step of the template at 94°C for 1 min, followed by 1 min interval at 52°C to allow the annealing of the primers to the template, and a 3 min incubation at 72°C for polymerase elongation of the primers. This cycle was repeated 35 times. At the end of these 35 cycles, an additional incubation at 72°C for 15 min was included to allow completion of the amplification. The PCR products were electrophoresed on a 1% agarose gel. The correct size products were excised from the gel and purified with a gel extraction kit (Qiagen, Santa Clarita, CA), following manufacturer's recommendations. The end PCR products were in 50 µl of water. A second round of PCR was then set up, by mixing 10 µl of each PCR product, the two wild-type primers and the two mutagenic primers. The PCR conditions were the same as above. At the end of the PCR reaction, the PCR product was purified as already described, digested with HindIII and BamHI, and ligated in the pUC19 cloning vector together with the XbaI-HindIII wild type IGF-1R cDNA. The HindIII-BamHI fragment was then sequenced to check for mutations. The mutant IGF-IR cDNA was then excised by SalI-BamHI digestion, and ligated into XhoI-BglII cloning sites of MSCV. neoEB.

Plasmid pGR39 contains an MLV 5′-LTR driven mutant IGF-IR cDNA, in which six serines were changed to alanines. The mutations were made by PCR in the following amino acid residues: 1272, 1278, 1280, 1281, 1282, and 1283. The set of wild type primers was the same as above described. The sequence of the sense mutagenic primer was 5′-GAGAACATGGAGgcCGTCCCCCTGGACCCCGCGGC-3′ (positions 3892 to 3927, the mismatched nucleotides are indicated in lower case letters). The sequence of the antisense mutagenic primer was 5′-CAGGGGGACGgcCTCCATGTTCTCTGGCTCCAGGT-3′ (positions 3915 to 3881, the mismatched nucleotides are indicated in lower case letters). The conditions for the PCR reactions were the same as for plasmid pGR25, except for the DNA template, which in the case of plasmid pGR39 was the IGF-IR cDNA with four serine mutations to alanine at amino acid residues 1280, 1281, 1282, and 1283.

Plasmid pGR72 contains an MLV 5′-LTR driven mutant IGF-IR cDNA, in which two arginines at residues 708 and 709 were changed to histidines. These mutations abolish the proteolytic cleavage between the α and β subunits of IGF-IR. These mutations were also made by PCR, as mentioned above [Valsesia-Wittman et al., 1994]. The wild-type set of primers had a sense primer that anneals to a sequence of IGF-IR upstream of KpnI restriction site [Ullrich et al., 1986], and an antisense primer that anneals downstream of ClaI restriction site [Ullrich et al., 1986]. The sequence of the wild-type sense primer was 5′-GGACATAAACACCAGGAACAACG-3′ (positions 1424 to 1447). The

sequence of the wild type antisense primer was 5′-GGAATGTCATCTGCTCCTTCTG-3′ (positions 2504 to 2482). The sequence of the sense mutagenic primer was 5′-TGAAAGGAAGCaccatGATGTCATGCAAGTG-3′ (positions 2204 to 2235, the nucleotides that have been mismatched are indicated in lower case letters). The sequence of the antisense mutagenic primer was 5′-ATGACATCatgtgGCTTCCTTTCAGGTCTG-3′ (positions 2229 to 2199, the nucleotides that have been mismatched are indicated in lower case letters). The conditions for PCR reactions were the same as for plasmid pGR25. The final PCR product was then digested with KpnI and ClaI, gel purified as above described, and inserted into a δKpnI-ClaI and dephosphorylated IGF-1R cDNA, which was previously inserted into XbaI-BamHI cloning sites of pSP64 cloning vector (Promega, Madison, WI). The KpnI-ClaI fragment was then sequenced to check for mutations. The mutated IGF-IR cDNA was re-excised by SalI-BamHI digestion, gel purified and ligated into XhoI-BglII cloning sites of MSCV.neoEB.

### Cell Lines, Transfections, and Retroviral Transductions

R-neoS cells [Sell et al., 1993, 1994] were grown in DMEM supplemented with 10% fetal bovine serum and 2 mM L-Glutamine. After retroviral transduction, R-cells expressing the various IGF-IR mutants and wild-type IGF-IR were grown in DMEM supplemented with 10% fetal bovine serum, 2 mM L-Glutamine, and 1 mg/ml G418 (Gibco BRL, Gaithersburg, MD).

The fetal human kidney carcinoma 293T cell line was produced in Dr. David Baltimore's laboratory [Pear et al., 1993] and was purchased from ATCC upon authorization of Rockefeller University. 293T cells were grown in DMEM supplemented with 10% fetal bovine serum and 2 mM L-Glutamine.

Transient calcium phosphate DNA transfection of 293T cells were carried out as described [Pear et al., 1993]. MLV retroviral vectors were harvested and utilized for transducing target cells as described elsewhere [Soneoka et al., 1995; Romano et al., 1997].

### IGF-IR Expression Levels

Cells were seeded in 100 mm dishes in 10% serum and grown until 80% confluent then lysed in lysis buffer (HEPES 50 mM pH 7.5, NaCl 150 mM, $MgCl_2$ 1.5 mM, EGTA 1 mM, glycerol 10%, Triton X-100 1%, NaF 100 mM, Na-Pyrophosphate 10 mM, Na-Orthovanadate 0.2 mM, PMSF 1 mM, Aprotinin 10 mg/ml). Fifty μg of whole cell lysate were resolved on a 4–15% SDS-PAGE (Bio-Rad, Richmond, CA) and transferred to a nitrocellulose filter. After blocking with 5% nonfat milk in TBS-T buffer (Tris-HCl pH 7.5 10 mM, NaCl 150 mM, Tween 0.1%) filters were probed with an antibody to the β subunit of the IGF-IR (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Detection was carried out with ECL (Amersham Life Science, Arlington Heights, IL).

### Growth in Monolayer

Cells were seeded at a density of $3 \times 10^4$/35 mm dishes in 2 ml of DMEM supplemented with 10% FBS to let them attach to the plate (usually a few h). Cells were then washed three times with Hank' S BBS buffer and starved for 48 h in SFM. The cells were then left either in SFM, or stimulated with 50 ng/ml of IGF-I (Gibco), or complete growth medium (10% serum). Cell number was determined after 48 h with the hemocytometer.

### Survival in Poly(HEMA) Plates

Thirty-five mm bacterial dishes (Fisher Biotech, Orangeburg, NY) were coated with poly-(HEMA) as described previously [Folkman and Moscona, 1978; Valentinis et al., 1998]. Exponentially growing cells were washed three times with Hanks' solution and treated with Versene to detach them from the plate. Cells were resuspended in SFM, counted, and seeded at the density of $5 \times 10^4$/2 ml (unless otherwise noted) in DMEM supplemented with 10% FBS, or SFM, or SFM supplemented with IGF-I (Gibco) at a concentration of 20 ng/ml. Viable cells (by trypan-blue exclusion) were counted after 24 h with a hemocytometer.

### Soft Agar

Anchorage-independent growth was determined by a soft agar assay as described previously [Sell et al., 1993, 1994]. One $\times 10^3$ cells in growth medium (DMEM supplemented with 10% FBS) containing 0.2% agarose (Difco Laboratories, Detroit, MI) were plated in 35 mm dishes with growth medium containing 0.4% agarose underlie. Cells were allowed to grow in soft agar for 3 weeks. Anchorage-independent

growth was assessed by scoring the number of colonies larger than 125 μm.

### IRS-1 Phosphorylation

Cells were plated in 100 mm dishes in DMEM + 10% FBS and grown until 80% confluence. Then cells were washed three times with Hank's and switched to SFM for 48 h. Cells then were switched to SFM, SFM + 50 ng/ml of IGF-I (Gibco BRL), or complete medium, for 10 m and then lysed. Three hundred μg of protein lysate were immunoprecipitated overnight at 4°C with anti-IRS-1 antibody (Upstate Biotechnology, Inc., Lake Placid, NY) and protein A agarose (Oncogene Science, Manhasset, NY). After resolution on a 4–15% SDS-PAGE (Bio-Rad) and transfer to a nitrocellulose filter, the membrane was probed with an anti-Phosphotyrosine antibody (Transduction Laboratories). Detection was carried out with ECL (Amersham Life Science, Arlington Heights, IL). After membrane incubation with stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 7.6) at 50°C for 30 m, membrane was washed twice with TBS-T buffer blocked overnight in 5% nonfat milk in TBS-T buffer and probed with anti IRS-1 antibody (Upstate Biotechnology, Inc).

## RESULTS

The main purpose of this investigation was to compare the ability of various mutant IGF-IR to transform MEF and to protect them from anoikis. Some of these mutants have already been studied as plasmids in stably transfected clonal cell lines [Hongo et al., 1996]; other mutants are new. The inclusion of some mutants previously tested has two purposes: to validate anoikis as a model of apoptosis and to monitor whether the mutant receptors behave in the same way, when expressed from a plasmid or from a retroviral vector. All the cell lines used in these experiments were derived from a neo-sensitive variant of R-cells [Sell et al., 1993, 1994], and consist of mixed populations selected in G418 after infection with the appropriate retrovirus. There are two advantages in using retroviral vectors: the selection of mixed populations avoids clonal variations, and the number of receptors expressed is consistently high and within a two-fold range (see below). Table I gives the list of the retroviral vectors used, and their IGF-IR inserts. The new mutants are marked with an asterisk. The mutation at residues 1289/90/93/94 (GR25) involves four basic aminoacids that are absent in the C-terminus of the insulin receptor [Ullrich et al., 1986, whose numbering we are following]. GR 18 is a double mutant, resulting from the fusion of two previous mutants, with mutations at Y950 and the three tyrosine residues in the tyrosine kinase domain. GR39 derives from GR35, by mutation of two additional serines at residues 1272 and 1278. GR72 has a mutation that abrogates the cleavage of the receptor into α and β subunits (see below). The cell lines are indicated with the same designation as the retroviral vectors.

### Levels of Expression of the Transduced IGF-I Receptors

Figure 1 shows a Western blot of the various cell lines. We have added for comparison the lysates from two cell lines, in which IGF-IR's levels were determined by Scatchard analysis: R600 cells [Rubini et al., 1997] with $30 \times 10^3$ receptors/cell, and p6 cells [Pietrzkowski et al., 1992] with $5 \times 10^5$ receptors/cell. Although there is some variability from one mixed population to another, all cell lines express high levels of IGF-IR, that can be roughly approximated at between 1 and $3 \times 10^5$ receptors/cell. The GR72 mutant, as expected, produces only the proreceptor (Fig. 1B), while R-cells are completely negative. In previous papers [D'Ambrosio et al., 1997; Rubini et al., 1997; Valentinis et al., submitted], we have shown that $30 \times 10^3$ receptors/cell are sufficient for IGF-I-mediated mitogenesis, protection from apoptosis and colony

**TABLE I. Retroviral Vectors Expressing the IGF-I Receptors Used in These Experiments**[a]

| Code | Mutations |
|---|---|
| GR15bis | wild type receptor |
| *GR18 | tyrosines 950 and 1131, 1135 and 1136 to phenyalanine |
| *GR25 | residues 1289/90/93/94 to alanine |
| GR35 | serines at 1280–1283 to alanine |
| *GR39 | serines 1272, 1278, and 1280–83 to alanine |
| GR46 | tyrosine 950 to phenylalanine |
| GR47 | residues 1293/94 to alanine |
| GR48 | tyrosines 1131, 1135, and 1136 to alanines |
| *GR72 | proreceptor |

[a]The following mutants have been previously described in plasmids: GR35 [Li et al., 1996]; GR46 [Miura et al., 1995b]; GR47 [Hongo et al., 1996]; and GR48 [Li et al., 1994]. The generation of the other mutants (*), and the insertion into retroviruses are described in Materials and Methods.

formation in soft agar. Therefore, the levels of expression of the receptors in our cell lines seem to be more than adequate for the proposed experiments.

### Growth in Monolayer

All the cell lines tested grow very well in 10% serum (not shown), a finding that is not surprising, since R-cells themselves can grow in serum-supplemented medium [Sell et al., 1993, 1994]. We next tested these cell lines for growth in serum-free medium or in serum-free medium supplemented solely with IGF-I. Figure 2 shows the results, expressed as percentage increase over serum-free medium. Most of the receptors tested allowed cells to respond to IGF-I with growth in cell number. The 3YF mutant (GR48) shows a very small increase (25%), confirming previous results in the literature [Gronborg et al., 1993; Li et al., 1994; Hernandez-Sanchez et al., 1995], that this mutant receptor's function is severely impaired. However, the double mutant GR18 did not grow at all, behaving in this respect like the K1003 mutant (ATP-binding site), which is totally unresponsive to IGF-I [Coppola et al., 1994; Kato et al., 1993]. The Y950F mutant grew very well in IGF-I, in fact, as well as the cells with the wild-type receptor. We will return to this particular mutant later (see below). The Proreceptor mutant is also totally inactive: like the parental R-cells, cells expressing the proreceptor grow in serum, but fail to grow in SFM supplemented with IGF-I. Of the other mutants, it is clear that mutations in the C-terminus do not affect IGF-I-mediated mitogenesis, as already reported (see references in Table I). Thus, of the various cell lines tested, only R-cells, GR72, and GR18 cells are totally insensitive to IGF-I-mediated mitogenesis, with GR48 being a borderline case.

### The IGF-I Receptor and Anoikis

Over-expressed insulin [Prisco et al., in press] and IGF-I receptors [Valentinis et al., 1998]

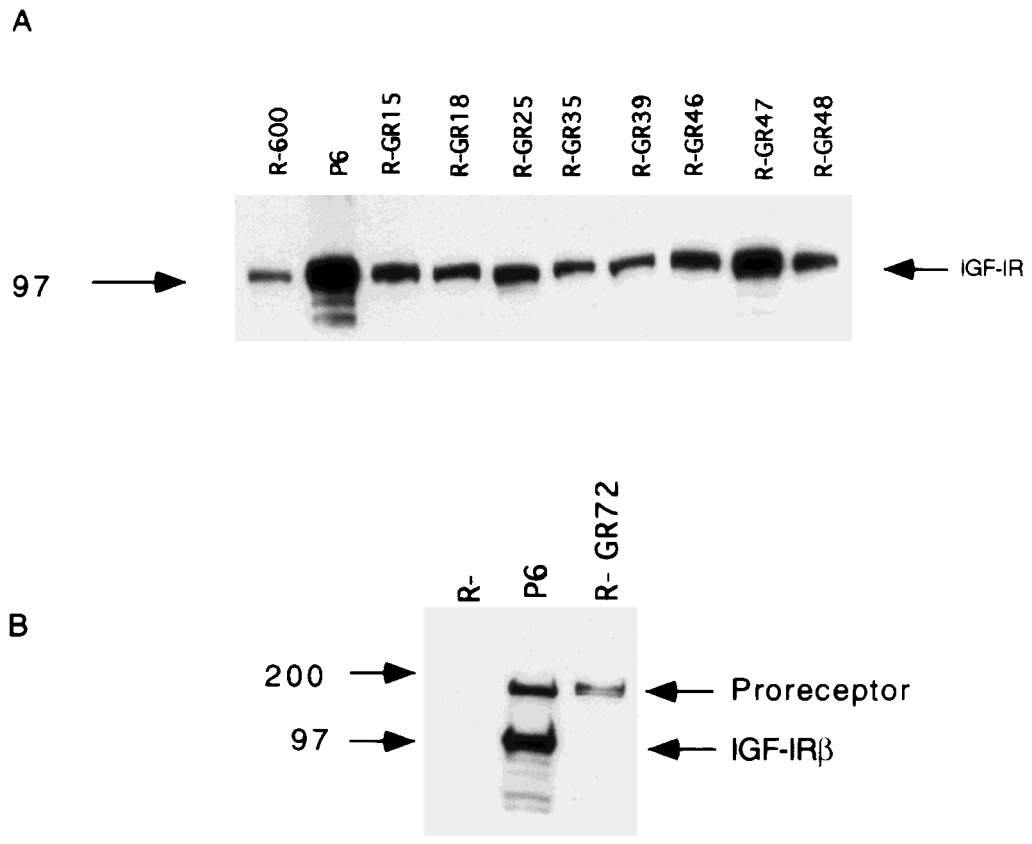


**Fig. 1.** Expression of wild-type and mutant IGF-I receptors stably transduced into R-cells. **A:** Western blots of lysates from the various cell lines, as indicated. The antibody and the methods use are given in Materials and Methods. **B:** The proreceptor (GR72) is not processed into a β subunit, and gives again p6 cells as control, as well as R-cells, that are completely negative.

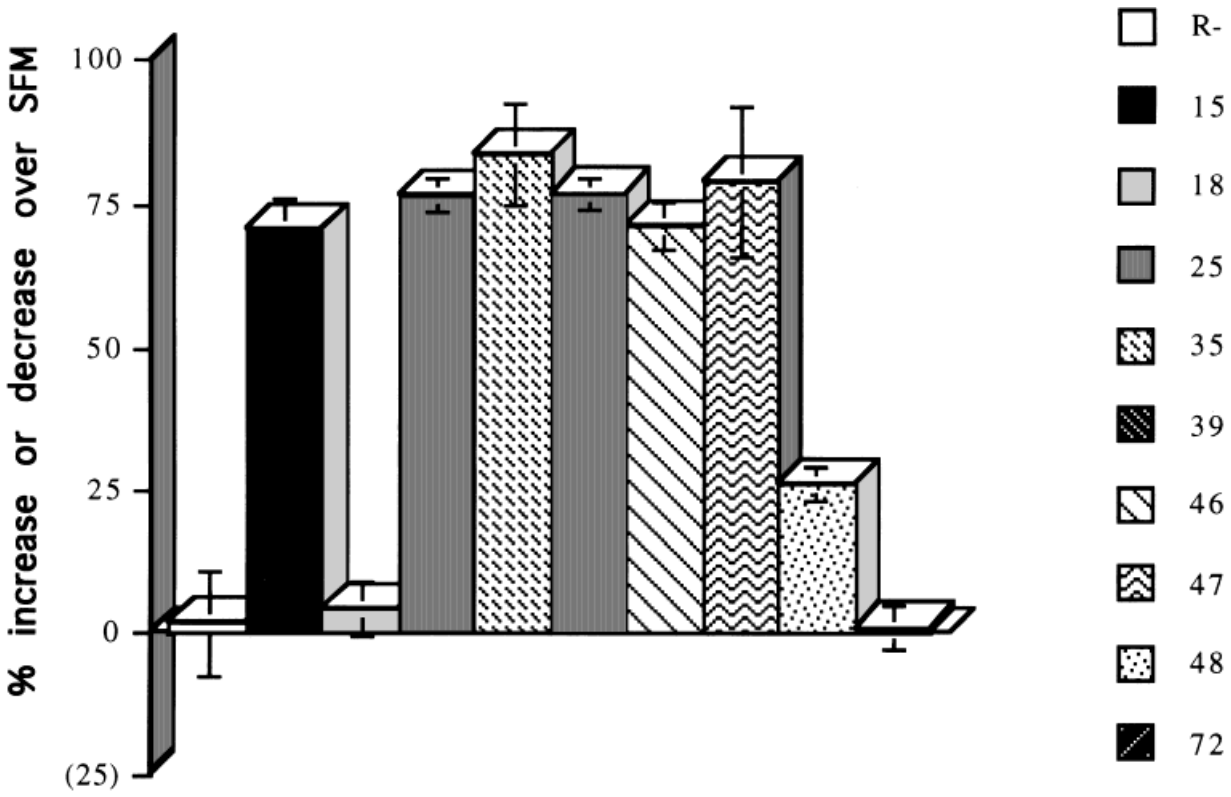


**Fig. 2.** Growth in monolayer cultures of various R-derived cell lines. The mixed populations listed in Table I were tested for their ability to grow in SFM or in SFM supplemented solely with IGF-I (see Materials and Methods). The percentage increase (or decrease) in cell number by IGF-I is expressed as percent over the controls in SFM, after 48 h incubation. Scale bars give standard deviations.

protect R-derived cells from apoptosis induced by detachment from the extra-cellular matrix (ECM), referred to as anoikis (see Introduction). When plated on polyHEMA plates, MEF undergo apoptosis in SFM. In a previous paper [Valentinis et al., 1998], we have given the documentation that cell death of MEF in poly-HEMA plates is due to apoptosis, and that addition of IGF-I can protect cells without actually inducing mitogenesis or even DNA synthesis. Apoptosis due to anoikis was demonstrated by three different methods. However, MEF seeded on polyHEMA plates, grow in 10% serum, and survive, but do not grow in SFM plus IGF-I [Valentinis et al., 1998]. Resistance to anoikis requires not only IGF-I, but also a functional IGF-IR and at least $22 \times 10^3$ receptors/cell [Valentinis et al., submitted]. Figure 3 gives the survival of the various R-/ derived cell lines after 24 h in polyHEMA-coated plates. As expected from fibroblast cell lines, all cell lines survive and grow in 10% serum, even when denied attachment to a substratum. All cell lines die in SFM, again as expected [Valentinis et al., 1998]. When IGF-I is added, the wild-type receptor (GR15) and certain mutant receptors protect R- derived cells from anoikis. These mutants are GR39 (6-serine mutant), GR46 (Y950F) and GR47 (1293/94 mutant). Three other mutant receptors, GR35 (4-serine mutant), GR25 (four basic amino-acids), and GR48 (3YF) offer only partial protection from anoikis; the number of cells is lower than the number of cells plated, but survival is significantly increased when survival in IGF-I is compared to survival in SFM. This indicates that these three mutant receptors respond to IGF-I, albeit not sufficiently to completely protect the cells from anoikis. The two mutant receptors that behave like the parental R-cells are GR 18 (Y950F and 3YF) and GR72 (the proreceptor mutant). These two mutants were also the ones that were totally unresponsive to IGF-I-mediated growth in monolayer (see Fig. 2), confirming a correlation between mitogenicity and protection from apoptosis (see Discussion). An important point is that the mutant receptors previously tested in two other models of apoptosis [D'Ambrosio et al., 1997;

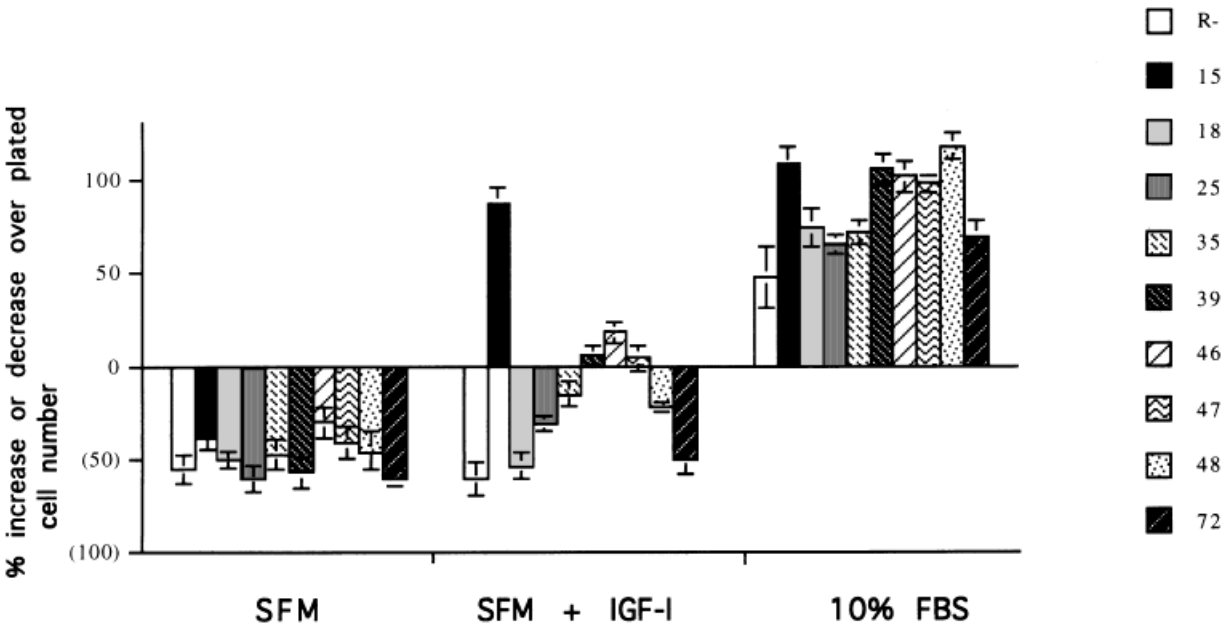


**Fig. 3.** Survival of R-derived cell lines in polyHEMA-coated plates. Survival was determined as surviving cells after 24 h in polyHEMA cultures, under the conditions given on the abscissa and in Materials and Methods. SFM, serum-free medium; IGF-I, 20 ng/ml. Scale bars indicate standard deviations.

O'Connor et al., 1997] behave in the same way in the anoikis assay, thus validating both the latter model and the use of retroviruses instead of plasmids.

### Colony Formation in Soft Agar

Colony formation in soft agar in a reasonable measure of an intermediate stage of transformation of cells cultured in vitro [Ponten, 1971]. It is generally considered as evidence for a transformed phenotype [Baserga, 1997], and it correlates well with tumorigenesis in nude mice [Aaronson and Todaro, 1968]. Table II shows colony formation in soft agar of selected cell lines, under the conditions given in Experimental Procedures. The table gives a representative experiment, but these experiments have been repeated several times, even with different concentrations of cells, yielding the same results. Actually, the experiments shown in Table II were done without the addition of IGF-I to the medium, but, in other experiments, IGF-I was added with the same results, as expected, since $3 \times 10^3$ wild-type receptors/cell are sufficient for colony formation in soft agar in the absence of IGF-I.

The only cell lines that give a number of colonies above background are GR15 (the wild-type receptor), and the 1293/94 mutant (GR47). Interestingly, if the other two basic amino-acids

**TABLE II. Colony Formation in Soft Agar of Selected Cell Lines**[a]

| Cell line | Number of colonies, mean (range) |
|---|---|
| R- | 2.5 (1–4) |
| GR15bis | 42.0 (39–44) |
| GR18 | 0.2 (0–1) |
| GR25 | 4.1 (3–5) |
| GR35 | 3.5 (0–6) |
| GR39 | 4.1 (1–7) |
| GR46 | 2.5 (2–3) |
| GR47 | 32.0 (29–35) |
| GR48 | 0.3 (0–1) |

[a]The assay was carried out following the methods described in Materials and Methods. The number of colonies in soft agar was determined after 3 weeks. Repeated experiments have yielded similar results.

in that region (1289/90) are also mutated (GR25), then the receptor loses completely its transforming activity. These experiments confirm that several residues in the C-terminus and the C-terminus itself are required for the transforming activity of the IGF-IR [Liu et al., 1993; Miura et al., 1995a; Li et al., 1996; Hongo et al., 1996; Surmacz et al., 1995]. The tyrosines at 950 and the kinase domain are also required for transformation, as previously shown [Miura et al., 1995b; Li et al., 1994]. It should be noted, however, that the TK domain is also required for IGF-I-mediated mitogen-

esis, whereas the C-terminus is dispensable for that function [Hongo et al., 1996; Surmacz et al., 1995].

### The Y950 Mutant

As mentioned above, the Y950F mutant (GR46) grew quite well in SFM supplemented with IGF-I. The retroviral transduction was repeated twice, mixed populations were selected twice, and each mixed population gave the same results: a mutation at Y950 did not affect IGF-I-mediated mitogenesis. This was surprising, since a previous report from our laboratory indicated that a Y950F mutation inhibited IGF-I-mediated mitogenesis [Miura et al., 1995b]. We directly sequenced the β subunit of the IGF-IR insert used in the construction of the retrovirus; the Y950 residue was mutated to F, and the rest of the sequence was normal. In comparison to the data base, there were three conservative mutations at positions 2298 (T instead of C, amino acid T 736), 3511 (G instead of C, amino acid G 1140), and 3643 (A instead of G, amino acid Q 1184); these substitutions were conservative, resulting in the same amino acids, did not affect the restriction map and were present in all of our constructs that we checked. They may represent single nucleotide polymorphisms, although we have no proof for it. At any rate, the sequence of the β subunit of the IGF-IR does not explain the difference between the present results and those of Miura et al. [1995b]. Since there were slight differences in the procedures used, we re-examined the IGF-I-mediated growth of R-/Y950F cells reported by Miura et al. [1995b], observing the same conditions used in both the previous and the present experiments.

The results are given in Figure 4. The clone described by Miura et al. [1995b] and the new mixed population (this paper) actually behaved in the same way. When grown in SFM supplemented with ferrous sulfate [Miura et al., 1995b], both cell lines responded to IGF-I with a nonsignificant increase. However, if ferrous sulfate was replaced by transferrin, both cell lines showed a clear response to IGF-I.

### IRS-1 Phosphorylation

In previous papers [Hongo et al., 1996; Surmacz et al., 1995], we have shown that truncated IGF-IR, including the δ1245, and various mutants of the C-terminus, phosphorylate normally on tyrosines both IRS-1 and Shc proteins. Since we have some additional mutants, and since the Y950 mutant has a different growth behavior, we show here a summary of IRS-1 phosphorylation by the receptors examined in

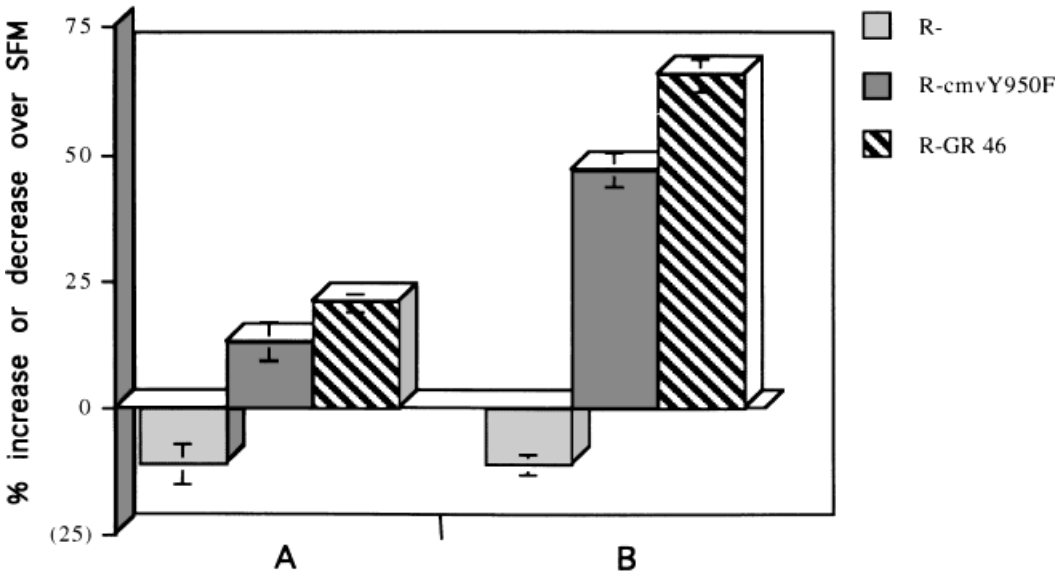


**Fig. 4.** Growth of R-cells expressing the Y950 mutant of the IGF-I receptor. Cells were grown in monolayers under two different conditions: with ferrous sulfate or with transferrin. Three cell lines were used: the parental R-cells, a cell line R-/Y950 described by Miura et al. [1995b], and a mixed population of R-/Y950 generated by transduction with a retrovirus expressing the Y950 mutant of the IGF-IR (this paper). Growth in IGF-I is expressed as percent increase or decrease over SFM, after 48 h incubation.

this paper. Figure 5 shows the tyrosyl phosphorylation of IRS-1 in selected cell lines. Although the amounts of IRS-1 that were immunoprecipitated varied among the various cell lines, the results are clear. IGF-I causes an increase in tyrosyl phosphorylation of IRS-1 in all cell lines except three: GR18 (double mutation at 3Y and Y950), GR48 (3Y), and GR72 (the proreceptor). There is a weak signal in GR18, but it is not increased by IGF-I; the weak basal phosphorylation could be due to activation of IRS-1 by integrins [Vuori and Ruoslahti, 1994]. The results with IRS-2 were the same (not shown). Interestingly, the amount of IRS-2 that was immunoprecipitated was at least as much as the amount of IRS-1, but the extent of tyrosyl phosphorylation was much lower, an observation that has been recently reported in breast cancer cells (Jackson et al., 1998).

## DISCUSSION

As stated in the introduction, a decrease in PCD is considered by many investigators as a pre-requisite for tumor development in humans. As summarized by Kitada et al. [1998], "Dysregulation of programmed cell death (PCD) can play an important role in the pathogenesis of human malignancies through several mechanisms. . . ". The purpose of this investigation was to determine whether there was a direct correlation between the transforming function and the anti-apoptotic function of the IGF-R. Specifically, we wished to know whether the same domains of the IGF-IR control both functions or not, and whether an anti-apoptotic signaling inevitably resulted in a transformed phenotype. Our results can summarized as follows (for convenience, we have grouped the results with the various receptors in Table III): 1) our data establish clearly for the first time in the same cell type that, in the case of the IGF-IR, resistance to apoptosis (anoikis) and transforming activity can be dissociated. There are at least two mutant receptors that confer complete resistance to anoikis but do not transform R-cells: GR39 (six serines), and GR46 (Y950). In addition, GR35 (serines at 1280–1283) and GR48 (3Y) offer partial protection from anoikis, but lack transforming activity; 2) mutation of the four basic amino-acids in the 1289/94 region completely abrogates transforming activity, whereas a mutation limited to 1293/94 does not; 3) the six serine mutant, in which all binding sites for 14.3.3 [Craparo et al., 1997] have been mutated, maintains its protective effect against anoikis. Curiously, the four-serine mutant is less effective than the six-serine mutant in conferring resistance to apoptosis; 4) in these experiments, Y950F is mitogenic (after IGF-I stimulation) and confers full resistance to anoikis, while remaining non-transforming [Miura et al., 1995b]; 5) while the 3YF mutant (tyrosine kinase domain) has a modest protective effect against anoikis, a combination of mutations at both Y950 and the tyrosine kinase domain (GR18) results in a receptor that has completely lost the three functions studied in these experiments; and 6) a mutant at the cleavage site between the two subunits (GR72) makes a proreceptor, which joins the double mutant GR18 (double mutation at Y950 and the tyrosine kinase domain), and the ATP-binding mutant at lysine 1003 [Coppola et al., 1994; Kato et al., 1993], as receptors that are essentially inactive when

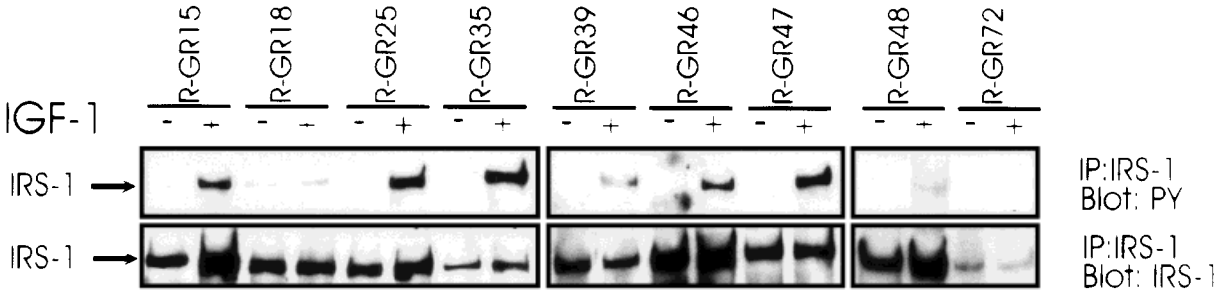


**Fig. 5.** Tyrosyl phosphorylation of IRS-1 in selected cell lines. IRS-1 was immunoprecipitated from lysates as described in Materials and Methods and the membrane was then blotted with an anti-phosphotyrosine antibody. + or − indicates whether the cells were stimulated or not with IGF-I. The cell lines are indicated; the immunoblots were stripped and blotted with an anti-IRS-1 antibody to determine the amounts of IRS-1 that had been immunoprecipitated.

**TABLE III. Summary of Results With Mutant IGF-I Receptors**

| Cell line | Mitogenic response to IGF-I | Survival in poly HEMA | Colony formation in soft agar |
|---|---|---|---|
| Gr15bis | +++ | +++ | +++ |
| GR47 | +++ | ++ | +++ |
| GR39 | +++ | ++ | no |
| GR46 | +++ | ++ | no |
| GR35 | +++ | + | no |
| GR25 | +++ | +/− | no |
| GR48 | + | + | no |
| R- | no | no | no |
| GR18 | no | no | no |
| GR72 | no | no | no |

transfected into R- cells. These novel findings are discussed separately.

In the first place, on the basis of our findings, it is reasonable to conclude that resistance to anoikis and transforming activity of the IGF-IR can be dissociated at the receptor level. Resistance to anoikis requires a combination of both Y950 and the tyrosine kinase domain, the latter one being the most important, at least in this system. The C-terminus of the IGF-IR had already been shown in other models (IL-3 withdrawal and okadaic acid) to be dispensable for protection from apoptosis [D'Ambrosio et al., 1997; O'Connor et al., 1997; Dews et al., 1997]. Thus, the finding that several mutants of the C-terminus can still protect from anoikis is compatible with the results obtained in other models of apoptosis.

As we have mentioned above, protection from apoptosis by the IGF-IR seems to correlate with its mitogenicity [D'Ambrosio et al., 1997; O'Connor et al., 1997; and this paper], as the C-terminus of the IGF-R is not required for IGF-I-mediated mitogenesis [Hongo et al., 1996; Surmacz et al., 1995]. This is remarkable inasmuch as in anoikis, the IGF-IR does not send a mitogenic signal [Valentinis et al., 1998], and yet the receptors that can send an IGF-I-mediated mitogenic signal in monolayer cultures are the same that protect cells from anoikis. It should be added at this point that our results with anoikis are in agreement with those obtained by O'Connor et al. [1997] in murine hemopoietic cells undergoing apoptosis after IL-3 withdrawal, and by D'Ambrosio et al. [1997] in okadaic acid-induced apoptosis in MEF. The same mutant receptors that protected in those situations also protected MEF from anoikis; the dissociation between resistance to apoptosis and transformation, therefore, cannot be attributed to a peculiarity of the anoikis model. This is true also of GR48: in the IL-3 withdrawal experiments of O'Connor et al. [1997], the 3Y mutant conferred partial protection against apoptosis, at least for the first 24 h, although, admittedly, its protective effect declined at later times. For transformation, Y950 and the tyrosine kinase activity are still required: obviously, a nonmitogenic receptor could hardly promote anchorage-independence. But, in addition to the mitogenic signaling, the IGF-IR, for transformation, needs an additional signal or signals, that originate from the C-terminus, and are not required for protection from apoptosis. In previous papers [Miura et al., 1995a; Li et al., 1996; Hongo et al., 1996], we had shown that at least three domains in the C-terminus were necessary for transforming activity: the tyrosine residue at 1251, the serine residues at 1280–1283, and the region around 1289–1294. Interestingly, when only 1293/1294 were mutated, the loss of transforming activity was only partial [Hongo et al., 1996], repeated experiments often giving ambiguous results. This mutant is not ambiguous in the present experiments: it transforms, but when the other two basic amino-acids at 1289–1290 are also mutated, then the receptor is no longer transforming.

Although we have tried by various methods, we have not been able yet to identify a substrate that binds to the 4 basic amino-acids around 1289–1294. A protein binding to the phosphorylated tyrosine 1251 has been reported by Dumenil et al. [1997], but its significance has yet to be determined. However, the protein binding to the serines of the C-terminus is known, and it is the 14.3.3 protein [Craparo et al., 1997; Furlanetto et al., 1997], that also binds IRS-1 [Craparo et al., 1997; Kosaki et al., 1998], and has been recently tied to cell cycle progression and the activation of cdc2 kinase [Weinert, 1997], and to the stabilization of the phosphorylated form of the Bad protein [Zha et al., 1996; Datta et al., 1997]. But there is a curious observation here. The 4-serine mutant (1280–1283) is slightly deficient in protection from anoikis, whereas the 6-serine mutant (1272, 1278 and 1280–1283) is fully protective. According to Craparo et al. [1997], binding of 14.3.3 to the IGF-IR requires both 1272 and 1283, but this observation has not been con-

firmed by other laboratories, who believe that the most important serine is the one at 1283 [Furlanetto et al., 1997]. If Furlanetto et al. [1997] are correct, then it would seem that a mutation that inhibits the binding of 14.3.3 to serine 1283 partially affects the anti-apoptotic signaling of the IGF-IR, while a second mutation at serine 1272 compensates for it and restores full activity. Incidentally, the same difference between the 4-serine and the 6-serine mutants has also been observed in 32D cells [Peruzzi et al., in preparation]: indeed, in those cells that have no IRS-1 nor IRS-2 [Zhou-Li et al., 1997; Zamorano et al., 1996; Wang et al., 1993], the incapacity of the 4-serine mutant to protect from apoptosis is even more dramatic. One would like to speculate that the milder effect in MEF (that have IRS proteins) may be due to the fact that 14.3.3 can still bind to IRS-1.

In these experiments, we have confirmed that the Y950F mutant loses its ability to transform cells [Miura et al., 1995b]. However, it is capable of protecting MEF from anoikis (albeit not as efficiently as the wild-type receptor) and to elicit an IGF-I-mediated growth response. The protection from anoikis by the Y950 mutant is compatible with a recent report by Boehm et al. [1998] with the insulin receptor. Using a chimeric receptor, these authors showed that a deletion of 12 amino-acids around Y960 did not affect the protective effect of the receptor against staurosporine-induced apoptosis. They concluded that the anti-apoptotic effects of this receptor can be mediated by signaling pathways distinct from those requiring IRS-1 and Shc. Similarly, Chen et al. [1995] reported that an over-expressed IRS-1 could rescue cells transfected with a Y960 mutant of the insulin receptor. Our experiments also indicate that the Y950F mutant can still render R-cells capable of IGF-I-mediated mitogenesis. A possible explanation may be found in the fact that IRS-1 binds to the autophosphorylated IGF-IR by a mechanism that does not require the NPX(p)Y motif [He et al., 1996], an explanation that finds support in our finding that IRS-1 is tyrosyl phosphorylated by IGF-I in cells expressing the Y950F mutant. Indeed, Yenush et al. [1996] have shown that, at high levels of expression of the insulin receptor, both the Pleckstrin domain and the PTB domain of IRS-1 bind to it, whereas at low levels of receptor expression, the PTB domain is inefficient, and the Pleckstrin domain becomes more important. These authors, in fact, reported that a mutation in the PTB domain of IRS-1 did not affect its tyrosyl phosphorylation, nor its signaling, a result that is perfectly compatible with our results obtained with the Y950F mutant. It seems that, with our over-expressed Y950 mutant, we have enough weak binding to send a moderate mitogenic signal. It may be relevant to mention here the experiments of Tartare-Deckert et al. [1995] that IRS-1 may also bind directly to the tyrosine kinase domain of the IGF-IR; our finding that the double mutant Y950F/3YF is completely inactive is compatible with the observation of these authors.

Another possibility should be considered. Several investigators have repeatedly demonstrated a requirement for IRS-1 for insulin-mediated mitogenesis, or, at least, DNA synthesis [Myers et al., 1993; Waters et al., 1993; Rose et al., 1994; Yamauchi et al., 1994], and we have indirectly confirmed it here by showing that all mitogenic receptors cause tyrosyl phosphorylation of IRS-1. However, it is also known that over-expression of the IR in 32D cells, that have neither IRS-1 nor IRS-2 [Wang et al., 1993; Yenush et al., 1996] is not sufficient for insulin stimulation of growth in the absence of IL-3 [Wang et al., 1993]; but when 32D cells over-express the IGF-IR, they can grow even in the absence of IL-3 [Prisco et al., 1997; Zhou-Li et al., 1997; Dews et al., 1997]. It indicates that the IGF-IR has an additional mitogenic pathway that is IRS-1-independent. For the moment, we will limit ourselves to state that the anti-apoptotic and mitogenic effects of the IGF-IR do not require Y950, and, in fact, may not require IRS proteins at all [Prisco et al., 1997; Zhou-Li et al., 1997; Dews et al., 1997].

This, of course, immediately brings up the possibility that the IGF-IR may be signaling through Shc for mitogenesis and anti-apoptotic function. Olefsky and collaborators [Sasaoka et al., 1994a,b] have emphasized the role played by Shc in insulin- and IGF-I-mediated mitogenesis, and activation of the ras pathway. We have looked into the role of Shc in our cells, but they express such low levels of Shc proteins, that have made it difficult to ascertain convincingly their phosphorylation status. Interestingly, when we immunoprecipitated Shc proteins after IGF-I stimulation, we could demonstrate Grb2 in the immunoprecipitates, and that the amount of Grb2 was higher in cells with the

wild type receptor than in cells with the Y950F receptor (data not shown). However, in the absence of a satisfactory demonstration of Shc phosphorylation levels, we prefer to leave the matter unresolved, for the moment.

Finally, we should consider the fact that the mitogenicity of the Y950 mutant receptor is at variance with a previous report from our own laboratory [Miura et al., 1995b]. We have resolved the discrepancy (see Results) and, although this finding is not really a crucial part of the present communication, we would like to present it here, because it makes a most important point. The point is that the conditions in which the same cells are cultured can make a difference, indeed a very dramatic difference. Traditionally, it is said that the addition of transferrin to SFM can be replaced by ferrous sulfate, and we used to follow that recommendation, since ferrous sulfate is less expensive than transferrin. More recently, we had noticed that some cell lines grew better when transferrin was used instead of ferrous sulfate, and we therefore switched our routine to the constant use of transferrin. The fact that both cell lines, one established in 1994 and one in 1997, respond equally to the same conditions, confirm the reproducibility of the mutant receptors. However, in one condition (ferrous sulfate), both cell lines would be judged as non-responding, while in the other condition (transferrin), one would conclude that they are responding to IGF-I. Clearly, this experiment ought to warn all of us about the necessity of carefully controlling the medium conditions in all our growth assays. As a possible explanation, we may offer the reports in the literature that the levels of transferrin receptor are increased by growth factor stimulation [Miskimins et al., 1986; Hirsch et al., 1995]. Perhaps, IGF-I stimulation increases the number of transferrin receptor, thus increasing its effect, whereas it would have no effect on the uptake of ferrous sulfate.

The proreceptor mutant (GR72) and the double mutant GR18 (Y950F and 3YF) are by far the most inactive; indeed, the cells expressing the proreceptor grow slowly even in 10% serum, suggesting that this mutant may actually act as a dominant negative. The unresolved question of this paper is the explanation of why a given receptor can send both a mitogenic and survival signal, but not a transformation signal. This question is, of course, actively studied in several laboratories, including ours. The only clue we have is that R- cells, refractory to transformation by a variety of viral and cellular oncogenes (see Introduction), can be transformed by v-src, but not an activated c-src [Valentinis et al., 1997]. We would like to speculate that transformation requires the mitogenic pathway *plus* another pathway, originating from the C-terminus, that may perhaps mimic the transforming mechanism(s) of v-src.

In conclusion, we have shown unequivocally that certain IGF-IR mutants can still transmit a mitogenic and/or survival signals, while incapable of sending a transforming signal. The dissociation between these activities should caution about resistance to apoptosis with the transformed phenotype.

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