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Identification of Domains of the Insulin-Like Growth Factor I Receptor That Are Required for Protection from Apoptosis

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Using a series of insulin-like growth factor I (IGF-I) receptor mutants, we have attempted to define domains required for transmitting the antiapoptotic signal from the receptor and to compare these domains with those required for mitogenesis or transformation. In FL5.12 cells transfected with wild-type IGF-I receptors, IGF-I affords protection from interleukin 3 withdrawal but is not mitogenic. An IGF-I receptor lacking a functional ATP binding site provided no protection from apoptosis. However, receptors mutated at tyrosine residue 950 or in the tyrosine cluster (1131, 1135, and 1136) within the kinase domain remained capable of suppressing apoptosis, although such mutations are known to inactivate transforming and mitogenic functions. In the C terminus of the IGF-I receptor, two mutations, one at tyrosine 1251 and one which replaced residues histidine 1293 and lysine 1294, abolished the antiapoptotic function, whereas mutation of the four serines at 1280 to 1283 did not. Interestingly, receptors truncated at the C terminus had enhanced antiapoptotic function. In Rat-1/c-MycER fibroblasts, the Y950F mutant and the tyrosine cluster mutant could still provide protection from c-Myc-induced apoptosis, whereas mutant Y1250/1251F could not. These studies demonstrate that the domains of the IGF-I receptor required for its antiapoptotic function are distinct from those required for its proliferation or transformation functions and suggest that domains of the receptor required for inhibition of apoptosis are necessary but not sufficient for transformation.

The insulin-like growth factor I receptor (IGF-IR) activated by its ligands (IGF-I, IGF-II, and insulin at supraphysiological concentrations) has been shown to play an important role in the development, growth, and survival of normal cells (1, 2, 12, 25, 42).

IGF-I was first shown to inhibit apoptosis in response to cytokine withdrawal in interleukin 3 (IL-3)-dependent hematopoietic cells (40) and after serum withdrawal in Rat-1/c-MycER cells (20). In the latter system, suppression of apoptosis appeared distinct from any mitogenic activity of IGF-I. Rat-1 cells were also protected in the presence of cycloheximide, indicating that suppression of apoptosis did not require de novo synthesis of proteins.

The demonstration that apoptosis of c-Myc-expressing fibroblasts is suppressed by IGF-I raises the possibility that IGF-IR signaling may play an important role during carcinogenesis by inhibiting apoptosis that is induced by dominant oncogenes, a role distinct from the better-characterized mitogenic activity of IGF-IR. In effect, IGF-IR would fulfill the same role in tumor cells as do antiapoptotic lesions such as deregulated expression of Bcl-2, which synergizes with dominant oncogenes like c-Myc by suppressing apoptosis (5, 14, 47). Antisense inhibition of IGF-IR expression suppresses tumorigenicity of tumor cells introduced into syngeneic or immunocompromised animals, and there is a quantitative relationship among IGF-IR levels, the extent of apoptosis, and the tumorigeneic potential of a rat syngeneic tumor (38, 39). In a transgenic carcinogenesis model involving simian virus 40 T antigen targeted to the β-islet cells, activation of the IGF-IR by IGF-II is a critical determinant of carcinogenic progression (8). Elevated levels of IGF-IR have been detected in human tumors of lung (22, 32), breast (11, 15, 36), and colon (18, 37), and this is often associated with increased levels of circulating IGF-I and/or IGF-II (4, 29). For many such tumors, IGF-I acts as a mitogen in vitro (16, 33), suggesting the involvement of IGF-IR autocrine or paracrine signaling loops in tumors in vivo (25, 48).

Primary fibroblasts derived from IGF-IR-null mice (R^- cells) exhibit a requirement for the IGF-IR in transformation (23, 41) and have been used to map domains in the receptor essential for the IGF-IR's proliferative and transformation activities. Specifically, the C-terminal region of the IGF-IR is required for transformation, and receptors which are truncated to amino acid 1229 fail to transform R^- fibroblasts but retain full mitogenic potential in response to IGF-I (43). Within the C-terminal region, the transforming activity has been further localized to a domain between amino acids 1245 and 1310 (21). However, all of the C-terminal point mutant and truncated receptors retain mitogenic capability. These studies indicate that the mitogenic and transforming functions of the IGF-IR are mediated by spatially distinct domains. Mutations at the kinase domain ATP binding site that ablate kinase activity, at the tyrosine cluster in the kinase domain, or at tyrosine 950 (the major binding site for IRS-I) abolish both proliferation and transformation (10, 17, 26, 30), which demonstrates that these residues are required for both mitogenic and transformation signaling.

In this paper, we have sought to use a series of IGF-IR mutants to map receptor domains involved in mediating cell survival in response to two different apoptotic stimuli. One is cell death induced by IL-3 deprivation in the murine B-cell line FL5.12. These cells have low endogenous levels of IGF-IR, which means that the properties of ectopically expressed IGF-IR mutants can be studied without the confusion of endogenous receptors. The second system uses c-Myc-induced apoptosis in Rat-1 fibroblasts which, despite having apprecia-

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ble levels of endogenous IGF-IR expression, normally utilize IGF-IR signaling for their survival and proliferation. We demonstrate that domains of the IGF-IR required for suppression of apoptosis are distinct from those required for proliferation and overlapping those required for transformation.

MATERIALS AND METHODS

Expression plasmids. The generation of pBPV IGF-IR containing the wild-type (WT) IGF-IR and all of the mutants used in this study have been described elsewhere (21, 24, 26, 27, 30, 31, 43). These IGF-IR cDNA constructs were excised from the shuttle vector SK-IGF-IR (IGF-IR cDNA cloned into pBluescript SK [Stratagene, La Jolla, Calif.]) by digestion with *Sal*I and *Xba*I and cloned into the *Xho*I and *Xba*I sites of the cytomegalovirus enhancer/promoter expression plasmid pcDNA3 (Invitrogen, San Diego, Calif.). The numbering of amino acids in the IGF-IR is that used by Ullrich et al. (45).

Transfection of FL5.12 and Rat-1/c-MycER cells with IGF-IR-containing plasmids. FL5.12 cells were maintained in Iscove's modified defined medium (IMDM) supplemented with 1 mM L-glutamine, 10% fetal bovine serum (FBS), and 10% (vol/vol) conditioned medium from the IL-3-producing cell line WEHI-3B. Cells (5×10^6) were transfected with 20 μg of DNA by electroporation (200 V, 960 μF) or by Lipofectamine (Gibco/BRL, Life Technologies, Inc., Grand Island, N.Y.) with 400 ng of DNA, for 3.5 h. Cells were seeded at 10^5/ml (2 ml/well) in 24-well plates in IMDM–10% FBS supplemented with 10% WEHI CM. G418 (geneticin; Gibco/BRL Life Technologies, Inc.) was added 48 h later to a final concentration of 1 mg/ml. Medium was replenished every 3 to 4 days, and emerging drug-resistant cells were screened for IGF-IR expression by indirect immunofluorescence. Some of the cell lines were subcloned by limiting dilution in 96-well plates.

Rat-1/c-MycER cells (28) were maintained in Dulbecco's modified E4 medium supplemented with 10% FBS and 1 mg of geneticin per ml. The cDNAs encoding the WT and mutant IGF-I receptors were ligated into the Moloney murine leukemia virus-based retroviral vector pLXSN at the polylinker site X. The polylinker includes *Eco*RI and *Bam*HI sites orientated 5′ to 3′. pLXSN has an extended packaging signal for high virus titer as well as a mutated *gag* start codon and shortened envelope region to decrease the risk of helper virus generation. Virus-producing cells were prepared by transfection into the packaging-competent cell line GP+E. Virus-containing supernatant obtained from GP+E cell cultures was used to infect Rat-1/c-MycER cells. One day after infection, the Rat-1/MycER cells were split into medium containing G418 (1 mg/ml). Selected clones were collected, expanded, and assayed for the expression of IGF-IRs at the cell surface by fluorescence-activated cell sorter analysis using the Ab-1 monoclonal antibody (MAb).

Indirect immunofluorescence assays. Cells (2×10^5) were suspended in IMDM containing 25 mM HEPES and 10% human pooled AB serum in 96-well round-bottom plates. Anti-IGF-IR MAb reactive with the human IGF-IR (Ab-1; Oncogene Sciences, Cambridge, Mass.) was added at a final concentration of 1 μg/ml in a final volume of 100 μl and incubated for 1 h at 4°C. Cells were washed three times and exposed to fluorescein-labelled $F(ab')_2$ fragments of goat immunoglobulin to mouse immunoglobulin G for 30 min at 4°C. Cells were again washed twice, and the cell-associated fluorescence was quantified with a FACSCAN flow cytometer (Becton Dickinson, San Jose, Calif.).

Induction of apoptosis by IL-3 withdrawal and cell viability assays. FL5.12 cells that had been transfected with vector or IGF-IR constructs were plated at 3×10^5/ml in medium containing IL-3 for 24 h, washed three times in serum-free medium, and then plated at 5×10^5 cells/ml in IMDM containing 5% FBS (2 ml/well) in 24-well plates. IGF-I (50 ng/ml) or IL-3 (WEHI CM, 10%) was added to triplicate cultures. At the indicated time points, 100-μl aliquots were removed from each well and viability was determined by counting live and dead cells after trypan blue staining. The percentage of viable cells was calculated from the total number of cells per well, and all data represent the mean of triplicate cultures for each condition. Assays were performed in the presence of 5% FBS to retain viability of the cells in the presence of IL-3 but to reduce the amount of endogenous IGF-I or IGF-II that is available in 10% serum, which may mask the effects of exogenously added IGF-I. Cells expressing WT IGF-IRs were also tested for viability in medium containing 1% FBS for comparison to medium containing 5% FBS.

Activation of c-Myc-induced apoptosis and time-lapse video microscopy analysis. Rat-1/MycER cells expressing WT or IGF-IR mutants were seeded into 100-mm petri dishes in the presence of Dulbecco's E4 medium supplemented with 10% FBS. At 24 h, the culture medium was replaced with serum-free medium and c-Myc was activated by addition of 4-hydroxytamoxifen to a final concentration of 100 nM. IGF-I (50 ng/ml) was added, and cells were then observed by time-lapse video microscopy as previously described (13). Each field started with 100 cells, and images were taken at a rate of 12 frames per hour. Apoptotic deaths were recorded by analyzing the film and scoring apoptotic cell death events midway between the last appearance of normality and the point at which the cell became fully detached and fragmented. Data were plotted as cumulative cell deaths against time.

Immune complex kinase assays and Western blotting. FL5.12 cells (2×10^7) transfected with WT IGF-IR or the Y1250/1251F receptor were washed in serum-free medium, divided into aliquots, and either stimulated with IGF-I (50 ng/ml) for 5 min or not. Cells were lysed in ice-cold lysis buffer (1% Nonidet P-40, 10 mM Tris, 50 mM NaCl, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, protease inhibitors, and 1 mM sodium orthovanadate) at 4°C for 15 min. Nuclei and cell debris were removed by centrifugation at 16,000 × *g* for 20 min at 4°C. IGF-IRs were immunoprecipitated by incubation with the anti-IGF-IR MAb, Ab-1, followed by sheep anti-mouse antibody-coated protein G agarose beads. Immunoprecipitates were washed three times with lysis buffer, and an aliquot of beads was removed from each sample for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting.

Immune complex kinase assays were performed by resuspending immunoprecipitates in 30 μl of kinase reaction mix (10 mM Tris [pH 7.4], 10 mM $MnCl_2$, 1 mM Na_3VO_4, and 10 μCi of [γ-^{32}P]ATP) for 10 min at room temperature. Reactions were terminated by washing with ice-cold lysis buffer followed by resuspension in 30 μl of SDS-PAGE loading buffer. Proteins were eluted from beads by boiling for 5 min and separated in 4 to 12% gradient gels before autoradiography using XRP film (Eastman Kodak, Rochester, N.Y.). For immunoblotting, immunoprecipitated proteins were separated by SDS-PAGE and transferred to Immobilon P membranes (Millipore) and the membranes were blocked by incubation for 1 h in a 5% milk solution in Tris-buffered saline. Incubation with primary antibody, a polyclonal antiserum to IGF-IR C-terminal peptide (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.), for 1 h was followed by secondary antibody (peroxidase-conjugated sheep anti-rabbit immunoglobulin G [Jackson Laboratory]) for an additional 1 h. Detection was by enhanced chemiluminescence (Amersham).

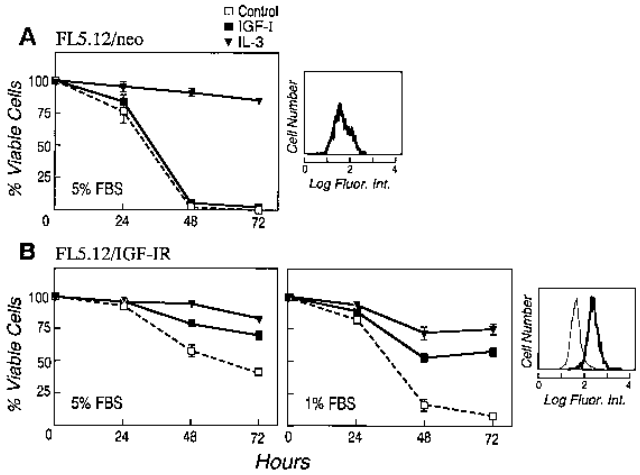

FIG. 1. Viability of FL5.12 cells stably transfected with IGF-IR. Cells were cultured at 5×10^5/ml in medium containing either 5% FBS or 1% FBS (Control), containing FBS plus IGF-I (50 ng/ml), or containing FBS plus IL-3 (WEHI CM, 10%), and the viability was monitored at the indicated time points by trypan blue exclusion. (A) Survival curves for FL5.12/neo cells; (B) survival curves for FL5.12/IGF-IR cells. Data points represent the means and standard deviations of cell viability derived from triplicate cultures. To the right of panels A and B are the IGF-IR expression levels as determined by staining with the Ab-1 MAb directed to the human IGF-IR where the thin line represents staining obtained with the negative control (no primary antibody) and the thick line represents staining with the Ab-1 MAb.

RESULTS

IGF-I inhibits apoptosis in FL5.12 cells stably transfected with a WT IGF-IR. FL5.12 cells are derived from a murine B lymphoblastoma and are dependent on IL-3 for proliferation and survival in culture. FL5.12 cells were transfected with a human IGF-IR-containing plasmid under the control of the cytomegalovirus enhancer/promoter. Cells expressing IGF-IRs were selected by indirect immunofluorescence staining with an anti-IGF-IR MAb (Ab-1). Demonstration by flow cytometry of surface expression of IGF-IR on the surface of the cells is shown in Fig. 1B. This IGF-IR expression is equivalent to approximately 10,000 receptors per cell as determined from ^{125}I-IGF-I binding analysis (not shown).

FL5.12/IGF-IR cells were then analyzed for their viability when IL-3 was withdrawn in the presence of medium contain-

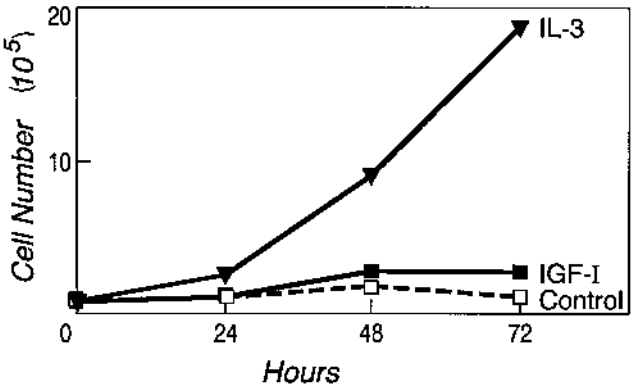

FIG. 2. Proliferation of FL5.12/IGF-IR cells in the presence of IL-3 or IGF-I. Cells were seeded at 10^5/ml in IMDM containing 5% FBS (Control), 5% FBS plus IGF-I (50 ng/ml), or 5% FBS plus IL-3 (WEHI CM, 10%). At the indicated time points, an aliquot was removed and the cell number was determined.

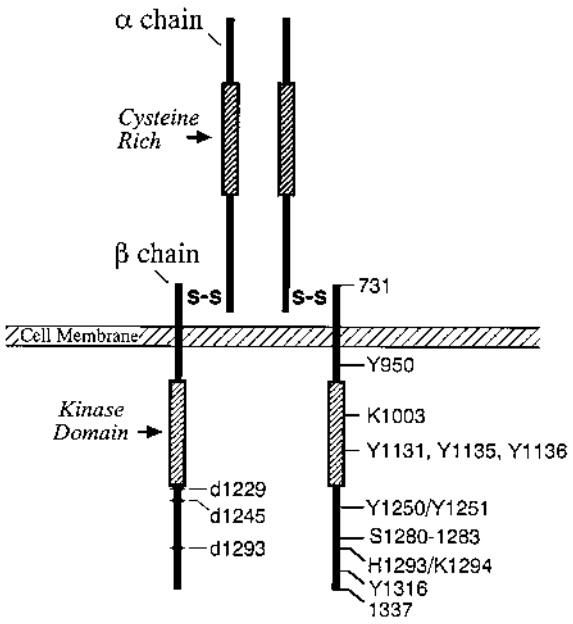

FIG. 3. Schematic representation of mutations in IGF-IR structure. With the amino acid numbering from the work of Ullrich et al. (45), the predicted structure of the IGF-IR is shown, indicating the approximate location of the various β chain mutants used in this study. The positions at which deletion mutations were made are indicated on the left side of the receptor, and the residues that were point mutated are indicated on the right side. The first and last amino acids in the β chain at positions 731 and 1337 are also indicated.

ing 5% FBS or 5% FBS plus IGF-I. Control cells expressing only neomycin resistance died rapidly upon IL-3-withdrawal (Fig. 1A). Compared with Neo^r cells, the FL5.12/IGF-IR cells demonstrated greater viability in the presence of 5% FBS alone (>50% at 48 h) (Fig. 1B). In the presence of 5% FBS plus IGF-I, these cells exhibited sustained viability (>70%) over 72 h, comparable to that conferred by IL-3 (Fig. 1B). When the assay was performed in the presence of medium containing 1% FBS, the viability decreased to <20% at 48 h, comparable to Neo^r cells (Fig. 1B). This indicates that the enhanced survival of FL5.12/IGF-IR cells in 5% FBS alone is due to activation of the IGF-IR by FBS. Again, addition of exogenous IGF-I provides a sustained survival response over 72 h that is comparable to that provided by IL-3 (60% viability), although the viability in the presence of both IL-3 and IGF-I is slightly lower compared to 5% FBS. For this reason, subsequent assays were performed in the presence of 5% FBS. IGF-I and IGF-II have been reported to be present in serum at a combined concentration of 70 ng/ml (46). We conclude that apoptosis is very effectively suppressed in FL5.12 cells that overexpress the WT IGF-IR and that the IGF-IR-mediated protection is manifested in two ways: first by increased survival in the presence of 5% FBS and second by increased survival in response to exogenously added IGF-I. Moreover, the degree of protection from IL-3 withdrawal could be correlated with the level of IGF-IR expression. FL5.12 cells expressing different levels of IGF-IR were isolated by fluorescence-activated cell sorter analysis and assayed as described above. Cells with higher IGF-IR expression showed greater and more persistent protection from IL-3 withdrawal in the presence of IGF-I (data not shown).

IGF-I promotes survival but not proliferation in FL5.12/IGF-IR cells. IL-3 acts as both a mitogen and a survival factor for FL5.12 cells. We were, therefore, interested in determining whether IGF-I could also act as a mitogen and a survival factor in IL-3-deprived FL5.12/IGF-IR cells. Proliferation of FL5.12/IGF-IR cells was measured by counting the total number of cells in cultures seeded at 10^5 cells/ml in the presence of either IGF-I (50 ng/ml) or IL-3 (WEHI CM, 10%), conditions under which cell death is minimal. Cells proliferated in the presence of IL-3 as demonstrated by an increase in cell number. In contrast, no proliferation was evident in the presence of IGF-I (Fig. 2). This was confirmed by flow cytometric analysis of the cell cycle distribution of FL5.12/IGF-IR cells maintained in the presence of either IL-3 or IGF-I, which demonstrated that FL5.12/IGF-IR cells cultured in IL-3 are distributed throughout the cell cycle whereas cells viably maintained in IGF-I became arrested in the G_0/G_1 phase of the cell cycle (data not shown). Thus, in FL5.12 cells, activation of the IGF-I/IGF-IR signaling pathway specifically regulates cell survival and has no observable mitogenic effect. Therefore, these cells provide a system in which IGF-IR mutants can be specifically assayed for their antiapoptotic activities.

Analysis of point mutants of the IGF-IR for their ability to protect FL5.12 cells from IL-3 withdrawal. Using site-directed mutagenesis, we sought to determine which domains of the IGF-IR were responsible for mediating the antiapoptotic effects of IGF-I. In particular, we were interested in determining whether the antiapoptotic signal is mediated by regions of the receptor that differ from those previously shown to be required for its transforming or mitogenic actions.

The location of the various mutations in the IGF-IR is depicted in Fig. 3. Plasmids directing expression of each of the IGF-IR mutants were transfected into FL5.12 cells, and clones expressing the mutant receptors homogeneously and at approximately equal levels were selected by indirect immunofluorescence analysis with the MAb Ab-1 (Fig. 4A). These clones were then analyzed for their ability to confer IGF-I-dependent survival on FL5.12 cells following IL-3 withdrawal. Viability of the cultures was monitored over 72 h as described for Fig. 1. The data are summarized in Table 1, where they are also compared with published data on the proliferative and transforming activities of each mutant.

Mutant Y950F (30) ablated tyrosine residue 950, a residue critical for interaction with IRS-1 and SHC (19, 44) and necessary for both mitogenic and transforming functions of the receptor (26). The Y950F-expressing cells demonstrated survival in the presence of 5% FBS and 5% FBS plus IGF-I that

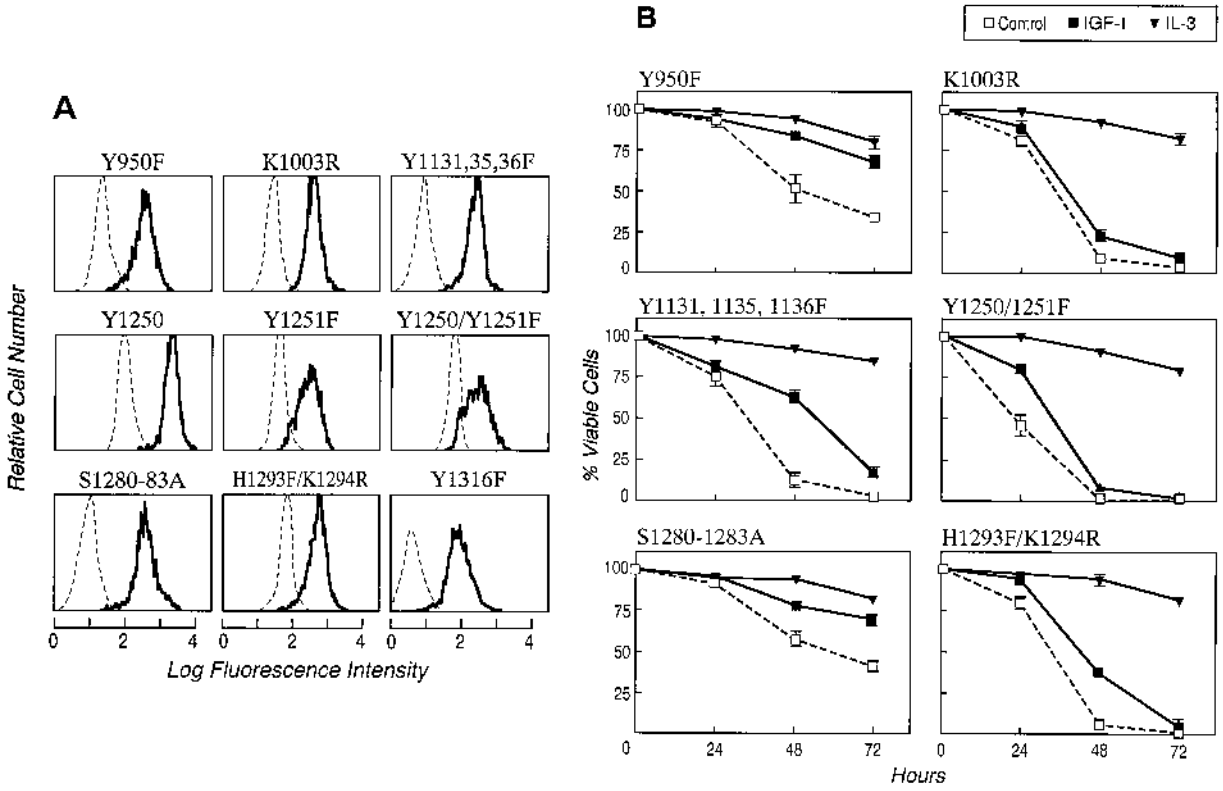

FIG. 4. (A) Expression levels of IGF-IR mutants after stable transfection in FL5.12 cells. Cells were assayed by indirect immunofluorescence for binding of the Ab-1 MAb directed against the human IGF-IR. The dashed line represents staining obtained with the negative control (no primary antibody), and the solid line represents Ab-1 binding. The name of each mutant expressed is indicated above the box. (B) Protection from IL-3 withdrawal in FL5.12 cells expressing point mutants of the IGF-IR. The panel shows survival curves for FL5.12 cells expressing the K1003R mutant IGF-IR, the Y950F mutant, the Y1131, 1135, and 1136F mutant, the Y1250/1251F mutant, the S1280-1283A mutant, and the H1293F/K1294R mutant. Cells were cultured at 5×10^5/ml in medium containing 5% FBS (Control), 5% FBS plus IGF-I (50 ng/ml), or 5% FBS plus IL-3 (WEHI CM, 10%). Cell viability was monitored by trypan blue exclusion at 24, 48, and 72 h, and the data are presented as the means and standard deviations determined from triplicate cultures.

was quite comparable to that of FL5.12 cells expressing WT IGF-IRs. Cells are approximately 75% viable at 72 h in the presence of 5% FBS plus IGF-I (Fig. 4B). This suggests that interaction with IRS-1 or SHC via tyrosine 950 is not required for IGF-IR inhibition of apoptosis.

TABLE 1. Summary of mitogenic, transforming, and antiapoptotic function of IGF-IR mutants

Receptor	Function		
	Mitogenic[a]	Transforming[a]	Antiapoptotic[b]
WT	+	+	+
Y950F	−	−	+
K1003R	−	−	−
Y1131, 1135, 1136F	−	−	+
Y1250F	+	+	+
Y1251F	+	−	−
Y1250/1251F	+	−	−
S1280-1283A	+	−	+
H1293F/K1294R	+	−	−
Y1316F	+	+	+
d1229	+	−	+
d1245	+	−	+
d1293	+	−	+

[a] Data with R^- cells derived from references 10, 21, 26, 27, 30, 31, and 43.
[b] Data summarized from IGF-I-mediated protection afforded by mutant IGF-IRs in IL-3 withdrawal assays with FL5.12 cells.

To assess the requirement for kinase function in signaling survival, two mutations in the kinase domain were tested. One is the K1003R mutant, in which the critical ATP binding lysine residue within the kinase active site has been ablated (24). In the other mutant, a cluster of tyrosines at residues 1131, 1135, and 1136 are all changed to phenylalanine (26). In fibroblasts, both the kinase-inactivating K1003R mutant and the tyrosine cluster mutant are completely inactive for both mitogenesis and transformation (26). FL5.12 cells expressing the receptor that was mutated at lysine 1003 exhibited negligible FBS- or IGF-I-mediated protection from IL-3 withdrawal; at 48 h they exhibited only 25% viability in the presence of added IGF-I (Fig. 4B). In contrast, the tyrosine cluster triple mutant provided effective IGF-I-dependent protection up to 48 h: 65% of the cells retained viability, compared to 20% with the K1003R mutant. However, by 72 h this effect had diminished to 18% viability, which indicates that this mutant is less effective than WT or Y950F receptors at promoting sustained survival.

The C-terminal region of the IGF-IR has regions of sequence that have little homology with the insulin receptor. Therefore, we next examined a series of C-terminal IGF-IR point mutants. Tyrosines 1250 and 1251 were mutated singly or together to phenylalanine (31). All of these mutants retained mitogenic activity in fibroblasts, although only Y1250F remained active in transformation assays (31). Cells expressing the Y1250F receptor demonstrated IGF-I-mediated protection from IL-3 withdrawal similar to that provided by WT receptors

(Table 1), with >75% viability in the presence of IGF-I at 48 h (not shown). In contrast, cells expressing either the Y1251F mutant alone or the Y1250/1251F double mutant exhibited a much-diminished IGF-I protection from IL-3 withdrawal. This was most pronounced in the double mutant receptor (Fig. 4B). The viability of cells expressing Y1250/1251F in the absence of IL-3 was less than 10% after 48 h. Thus, Y1251 is required for the survival function.

A further C-terminal IGF-IR mutant was derived by replacing all four serines at 1280 to 1283 with alanines. This mutant had previously been shown to be mitogenic, although it had no transforming function. Mutant S1280-1283A retained the ability to promote survival in the presence of IGF-I, producing 70% viability at 72 h in the presence of 5% FBS plus IGF-I (Fig. 4B). In mutant H1293F/K1294R, two amino acids that are situated at the beginning of an 8-amino-acid stretch of basic residues, a sequence which is not shared with the insulin receptor, are replaced. Mutant H1293F/K1294R was active mitogenically but could not support cell transformation (21). It also retained a very weak ability to promote IGF-I-dependent survival upon IL-3 withdrawal (Fig. 4B): approximately 30% of cells survive at 48 h, but all cells are dead by 72 h. This suggests that residues H1293 and K1294 contribute to IGF-IR-mediated inhibition of apoptosis. The final point mutant within the C terminus to be analyzed was Y1316F, which provided protection from IL-3 withdrawal (Table 1), although this was less than that of WT receptors, with 60% of the cells being viable in 5% FBS plus IGF-I at 72 h (not shown).

Taken together, analysis of the C-terminal point mutants in FL5.12 cells suggests that domains required for inhibition of apoptosis partially overlap with those required for transformation.

Analysis of truncation mutants of IGF-IR for protection from IL-3 withdrawal in FL5.12 cells. To examine further those domains of the IGF-IR involved in survival, a series of C-terminal truncation mutants of the IGF-IR were expressed in FL5.12 cells and analyzed for their ability to protect FL5.12 cells from IL-3 withdrawal. One mutant was truncated immediately after the kinase domain (d1229), one was truncated six amino acid residues before the Y1251 residue (d1245), and one was truncated immediately before the H1293/K1294 residues (d1293). As discussed above, the level of IGF-I-mediated protection from apoptosis is correlated with the levels of WT IGF-IR expression. Levels of expression of the truncated receptors d1229 and d1245 were never as high as those for any of the other mutants or for the WT receptors (compare Fig. 4A with Fig. 5). For this reason, efforts were made to obtain higher levels of expression of the d1229 and d1245 mutants by transfection or subcloning, but these efforts were unsuccessful. Nonetheless, several clones of FL5.12 cells expressing each truncated receptor were analyzed for IGF-I-mediated protection from IL-3 withdrawal.

IL-3 withdrawal assays were performed as described above, and the survival curves for representative clones are shown in Fig. 5. All three of the truncated receptors showed protection from IL-3 withdrawal in the presence of 5% FBS or in 5% FBS plus IGF-I. This was surprising given that the Y1251F and H1293F/K1294R point mutants lie within the deleted portion of the d1245 and d1293 IGF-IRs, respectively, and inactivate the receptor for survival (Fig. 4B). Given their low levels of expression in FL5.12 cells, the truncated receptors appear to be at least as effective as the WT receptor in promoting survival and may even have enhanced survival activity.

Analysis of IGF-IR mutants for protection from c-Myc-induced apoptosis. IGF-I effectively suppresses c-Myc-induced apoptosis in fibroblasts (20) and vascular smooth muscle cells

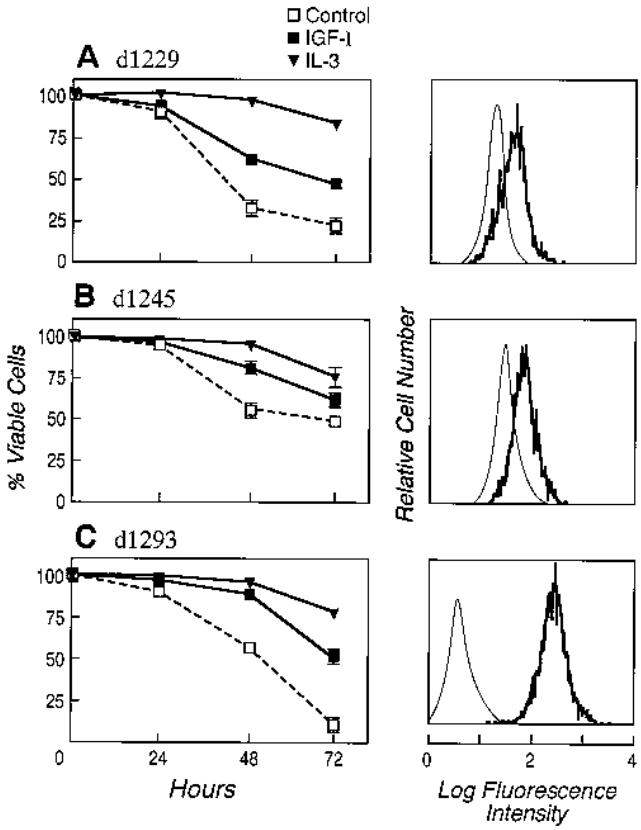

FIG. 5. Protection from IL-3 withdrawal in FL5.12 cells expressing truncated IGF-IRs. Cells were incubated in medium containing IL-3 for 24 h, washed extensively, and cultured at 5×10^5/ml in medium containing 5% FBS (Control), 5% FBS plus IGF-I, or 5% FBS plus IL-3. Cell viability was monitored by trypan blue exclusion at 24, 48, and 72 h, and the data are presented as percent viability of total cells plotted against time. Each point represents the mean and standard deviation of triplicate cultures. Shown are results for FL5.12 cells expressing the d1229 mutant IGF-IR (A), the d1245 mutant IGF-IR (B), and the d1293 mutant IGF-IR (C). Expression levels of the truncated IGF-IRs were determined flow cytometrically and are shown to the right of each panel.

(3), both of which are cells of mesenchymal origin expressing endogenous IGF-IRs. Because of this and the fact that existing studies of the mitogenic and transforming potential of IGF-IR mutants were conducted in fibroblasts, we were interested in testing the abilities of IGF-IR mutants to suppress apoptosis induced by c-Myc. Mutants and WT IGF-IRs were expressed in Rat-1/c-MycER cells that express a 4-hydroxytamoxifen-dependent c-Myc protein and that die by apoptosis when c-Myc is activated in the absence of serum or IGF-I (28). Rat-1 clones expressing equivalent levels of the various IGF-IRs were selected by flow cytometry. Expression levels for selected clones expressing WT, Y950F, Y1131, 1135, 1136F, and Y1250/1251F mutant IGF-IRs are shown in Fig. 6. Cultures of each mutant clone were assayed by time-lapse video microscopy for apoptosis following activation of c-Myc, either in the presence or in the absence of IGF-I.

Rat-1/c-MycER cells transfected with control Neo^r vector exhibited significant IGF-I-dependent protection from apoptosis due to the presence of endogenous IGF-IR (Fig. 6A). However, transfection of Rat-1/c-MycER cells with an expression vector directing elevated expression of WT IGF-IR increases the protection afforded by exogenous IGF-I (Fig. 6B). This is typically evident as a longer delay before the onset of apoptosis within the culture. Thus, the protective effect of ectopically

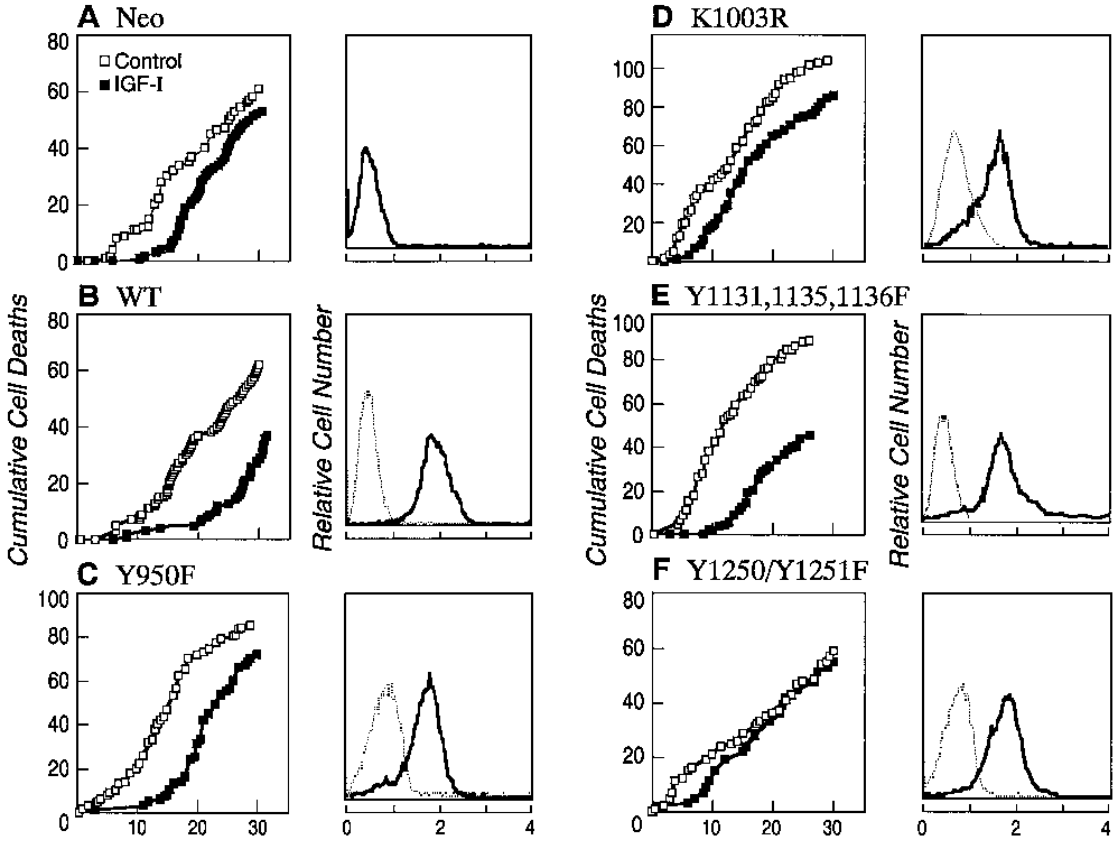

FIG. 6. Protection from c-Myc-induced death in Rat-1/c-MycER cells. Cells were seeded in 100-mm petri dishes in medium containing 10% FBS for 24 h. At this time, medium was replaced with serum-free medium (Control) or with medium containing IGF-I (50 ng/ml), and 4-hydroxytamoxifen (100 nM) was added to activate c-Myc. Cells were then monitored by time-lapse video microscopy for 30 h, and the number of apoptotic deaths from a field of 100 cells was scored. (A) Apoptotic cell deaths for Rat-1/c-MycER cells expressing neomycin resistance; (B) Rat-1/c-MycER cells transfected with WT IGF-IR; (C) Rat-1/c-MycER cells expressing the Y950F mutant; (D) Rat-1/c-MycER cells expressing the K1003R mutant; (E) Rat-1/c-MycER cells expressing the Y1131, 1135, 1136F mutant; (F) Rat-1/c-MycER cells expressing the Y1250/1251F mutant. Expression levels of human IGF-IR on the cells, as determined flow cytometrically with the Ab-1 MAb, are shown to the right of each panel; the dotted line depicts staining obtained with cells that received no primary MAb, and the solid line represents staining with the Ab-1 MAb. The time-lapse data shown are the result of a single experiment. Identical experiments gave the same results. The *x* axes for lettered panels are time in hours and for unlettered panels are log fluorescence intensity.

expressed IGF-IR can be clearly detected in the presence of endogenous IGF-IR expression.

We next analyzed the survival of Rat-1/c-MycER cells expressing the IGF-IR mutant Y950F, the K1003R mutant, and the tyrosine cluster mutant, Y1131, 1135, 1136F (Fig. 6C, D, and E). Cells expressing Y950F, K1003R, and Y1131, 1135, 1136F die more rapidly in the absence of serum than do their counterparts that express WT receptors. The K1003R mutant exhibits an IGF-I-mediated response analogous to that evident in the Neo cells, indicating no added protection from the overexpressed kinase-dead receptor (Fig. 6D). However, IGF-I exerts a protective effect against c-Myc-induced apoptosis, in the Y950F and Y1131, 1135, 1136F receptors, which indicates that the receptor can still activate a ligand-dependent survival signal. The protective action of the Y1131, 1135, 1136F mutant is comparable to that of WT receptors (Fig. 6D), and the Y950F mutant has reduced survival function relative to the WT receptor (Fig. 6C). However, the antiapoptotic activity of both of these mutants is noteworthy given that each one is completely dead for mitogenic signaling in fibroblasts. In contrast to the Y950F and the tyrosine cluster mutants, the Y1250/1251F mutant provides no significant protection from c-Myc-induced apoptosis in response to IGF-I (Fig. 6E). This is a striking result because it indicates that the protective effect of endogenous receptors is suppressed by the mutant receptor, which suggests that it may exert a dominant negative function.

Two of the IGF-IR C-terminal truncation mutants, d1245 and d1293, were expressed in Rat-1/c-MycER cells and analyzed for survival function. Consistent with their enhanced activity in FL5.12 cells, the truncated mutant receptors appeared to be constitutively active for survival (24a). Thus, the behavior of IGF-IR mutants in Rat-1 fibroblasts undergoing c-Myc-induced apoptosis follows the same pattern as that seen in FL5.12 cells undergoing apoptosis in response to IL-3 withdrawal. Once again, domains required for inhibition of apoptosis appear distinct from, if overlapping with, those required for transformation.

The mitogenic potential of the IGF-IR mutants was also examined in Rat-1/MycER cells by counting the cell divisions occurring during the time-lapse analysis. This revealed that the WT, Y1250/1251F, and truncation mutants had similar proliferation rates, whereas the Y950F, K1003R, and tyrosine cluster mutants had reduced rates of proliferation (data not shown). Therefore, the various IGF-IR constructs demonstrate the same mitogenic potential in Rat-1 cells as they do in R^- cells (summarized in Table 1).

In vitro kinase activity of WT and mutant Y1250/1251F receptors. One of the mechanisms that could account for our

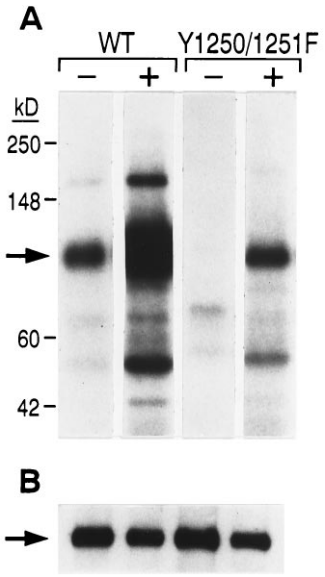

FIG. 7. Autophosphorylation of WT and Y1250/1251F mutant IGF-IRs in vitro. FL5.12 cells expressing WT or mutant IGF-IRs were washed in serum-free medium, stimulated with IGF-I (+) or not (−) for 5 min, and immunoprecipitated with the Ab-1 MAb. (A) In vitro kinase assays were performed on the immunoprecipitates as outlined in Materials and Methods followed by SDS-PAGE in 4 to 12% polyacrylamide gels and autoradiography. (B) Immunoblot of immunoprecipitates with a polyclonal antibody to the IGF-IR.

results with the deletion mutants and point mutant receptors is that the C terminus of the IGF-IR has a regulatory function on the kinase activity of the receptor that affects the survival function. To address this, we sought to determine if there were differences in autophosphorylation activity between WT receptors which demonstrated an active survival function and a mutant receptor without survival function. In vitro kinase assays were performed with WT and Y1250/1251F receptors expressed in FL5.12 cells. Cells were stimulated with IGF-I, the receptors were immunoprecipitated, and kinase activity was measured by determining the extent of autophosphorylation of the receptor beta chain as well as phosphorylation of coprecipitating proteins. The WT IGF-IR becomes heavily phosphorylated (Fig. 7A) in response to IGF-I stimulation, and some coprecipitating bands are also phosphorylated. However, the Y1250/1251F receptor shows an increase in phosphorylation in response to IGF-I but incorporates significantly less labelled phosphate. This difference is more than one would expect for a receptor lacking two tyrosines, and there is much less incorporation of ^{32}P into coprecipitating proteins than with the WT receptor. The level of receptor in the immunoprecipitates for each cell line is equivalent as shown by immunoblotting of IGF-IR in the immunoprecipitates (Fig. 7B). This result may indicate that the mutation at Y1250/1251 has a negative effect on the kinase activity of the receptor expressed in FL5.12 cells. Since we have demonstrated that kinase activity is required for survival function, one might expect that reduced kinase activity may contribute to loss of survival activity.

DISCUSSION

The IGF-IR and its ligands have well-described signal transduction roles in the suppression of apoptosis in diverse cell types, including cells derived from mesenchyme, epithelium, and central nervous system (1, 20, 34, 40). The IGF-IR signaling pathway also appears to play an important role in the establishment and progression of neoplasia. Thus, IGF-IR signaling is mitogenic in certain cell types, it is required for the transformation of fibroblastic cells in vitro (41, 43), it suppresses apoptosis induced by a number of mitogenic oncogenes in a manner analogous to the antiapoptotic oncogene Bcl-2 (20), and it is essential for neoplastic progression in at least one well-characterized transgenic model of multistage carcinogenesis (8). Three attributes have been assigned to IGF-IR signaling: mitogenesis, transformation in vitro, and suppression of apoptosis. However, it is not clear whether each of these three attributes contributes to neoplasia, whether they are independent functions, or whether they are merely different aspects of the same underlying signal transduction pathway. For this reason, we have attempted using site-directed mutagenesis to assign domains of the receptor to each function.

The IL-3-dependent cell line FL5.12 was used to study the ability of the IGF-IR to inhibit apoptosis induced by withdrawal of IL-3, because it provides an unambiguous system in which to study the antiapoptotic function of the IGF-IR. FL5.12 cells are dependent on IL-3 for both survival and growth and express very low numbers (<1,000 per cell [unpublished observations]) of endogenous IGF-IR. However, FL5.12 cells transfected with WT IGF-IR are protected by FBS and IGF-I from apoptosis following IL-3 withdrawal, which indicates that IGF-IR can activate a preexisting survival signaling pathway in these cells. We also conducted parallel studies in fibroblastic cells which possess a bona fide IGF-IR signaling pathway, although these cells express a significant level of endogenous IGF-IRs. In both cell systems, our results are similar.

We examined the antiapoptotic potential of a series of IGF-IR mutants that had been previously characterized for their proliferative and transforming activities in IGF-IR-null fibroblasts. All mutants were analyzed in IL-3-deprived FL5.12 cells, and some of these were further tested in Rat-1/c-MycER fibroblasts. In both cell models, inactivation of the critical ATP-binding lysine residue K1003 completely ablated survival, as it does proliferation, transformation, and all other functions associated with the receptor (10, 24). Thus, the ATP binding site is required for IGF-IR inhibition of apoptosis. In contrast, ablation of tyrosine 950, a residue involved in interaction with the IRS-1 docking protein and SHC (19, 35), or of the tyrosine cluster (Y1131, Y1135, Y1136), required to maximize the interaction with IRS-1 (19), had no discernible effect on IGF-IR survival signaling in FL5.12 cells and Rat-1/MycER cells. Both mutations have no mitogenic and transforming functions in IGF-IR-null cells (26, 30). These data indicate that a domain of the IGF-IR that interacts with IRS-1 is not required for inhibition of apoptosis but is essential for both proliferative and transforming functions. This result is also supported by our unpublished observation that IGF-IR elicits a survival signal when transfected into the IL-3-dependent myeloid cell line, 32D, which does not express IRS-1 or IRS-2.

The two C-terminal point mutations in IGF-IR, Y1251F and H1293F/K1294R, abolish IGF-I-mediated protection from IL-3 withdrawal. These mutants are also inactive in transformation, although they retain mitogenic activity when expressed in IGF-IR-null fibroblasts (21, 31). The S1280-1283A IGF-IR mutant is similarly active mitogenically and inactive in transformation in IGF-IR-null fibroblasts (27). In contrast to Y1251F and H1293F/K1294R mutants, however, mutant S1280-1283A remains competent for survival signaling in FL5.12 cells. Mutants in which the entire C terminus of the receptor is truncated, for example, mutants d1229 and d1245,

retain both their antiapoptotic and mitogenic (43) activities, although they are inactive in transformation. Taken together, these data indicate a degree of overlap between the transforming and antiapoptotic functions of the receptor with regard to their requirement for certain residues in the C-terminal region of the receptor. Suppression of apoptosis appears necessary for IGF-IR-mediated cell transformation but is clearly not sufficient. Whereas all mutants inactive in suppressing apoptosis are also inactive for cell transformation, some mutants inactive in transformation remain active in suppressing apoptosis (Table 1). In addition to the suppression of apoptosis, transformation thus requires additional signals arising from the IGF-IR, such as those possibly mediated by the serine residues at 1280 to 1283.

Our data also suggest that the regions of the IGF-IR required for inhibition of apoptosis and for mitogenesis are distinct. Mutants such as Y950F and Y1131, 1135, 1136F are inactive for mitogenesis yet retain antiapoptotic activity. Conversely, mutants such as Y1250F/Y1251F and H1293F/K1294R cannot promote survival yet remain mitogenic.

One somewhat paradoxical result is that the IGF-IR C-terminal truncation mutants d1229 and d1245 IGF-IRs retain antiapoptotic activity while point mutations within the same C-terminal region, such as Y1251F and H1293F/K1294R, ablate it. There are two possible explanations for this. One is that the C-terminal domain of IGF-IR is a killer domain that acts directly to promote cell death, although it does not resemble known killer domains, like those within the Fas/TNF/FADD/TRADD receptor family (reviewed in reference 9) or the BH3 domains of Bax, Bak, or Bik (6, 7). Residues Y1251 and H1293/K1294 would presumably mitigate the action of this putative killer domain, since their ablation generates a receptor that is inactive for survival signaling. A second possibility is that the IGF-IR C terminus acts as an intrinsic inhibitory domain that suppresses the survival signal, possibly by modulating phosphorylation or dephosphorylation of other receptor residues involved in the survival signal. In this scenario, the critical residues at Y1250/Y1251 and H1293/K1294 would presumably be involved in neutralizing the inhibitory action of the C-terminal domain, and C-terminal truncation mutants lacking this inhibitory domain might be superactive.

It has been noted that cells expressing IGF-IRs with C-terminal truncations or point mutations exhibit hyperphosphorylation of IRS-1 and SHC (43). It has also been reported that in protein interaction studies in yeast, C-terminally truncated IGF-IRs and those with mutations in the C terminus had enhanced interaction with both SHC and IRS-1 (44). However, our own observations that a mutant that is truncated at residue 1245 and contains the Y950F mutation retains survival function (28a) argue against the notion that the enhanced activity of the truncated receptors is due to increased signaling through IRS-1 or SHC. Nonetheless, analogous mechanisms might exist for other IGF-IR substrates. A role for the C terminus in regulating the kinase activity of the receptor is also suggested by the demonstration that the Y1250/1251F mutant has less autophosphorylation and kinase activity in FL5.12 cells. We are currently extending our investigation of this observation.

In conclusion, we have demonstrated that the antiapoptotic action of the IGF-IR utilizes residues and domains that are discrete from those involved in mitogenic signaling and that partially overlap with those mediating cell transformation. All receptor mutants that retain transforming activity are active in survival signaling. This suggests that inhibition of apoptosis is an essential component of transformation. However, not all antiapoptotic mutants are active in transformation, which indicates that transformation requires other functions provided by the receptor. In addition, the C terminus of the IGF-IR exerts an overall proapoptotic function, which may be mediated directly or through negative regulation of other regions of the receptor. Such regulatory interactions at the level of the receptor could provide a specific target for efforts to manipulate IGF-IR-mediated survival in different cells.

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