MOLECULAR AND CELLULAR BIOLOGY, Mar. 1997, p. 1084–1092 Vol. 17, No. 3
0270-7306/97/$04.00+0
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The Insulin-Like Growth Factor I Receptor as a Physiologically Relevant Target of p53 in Apoptosis Caused by Interleukin-3 Withdrawal

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Received 18 July 1996/Returned for modification 6 November 1996/Accepted 24 November 1996

The wild-type p53 protein is known to modulate apoptosis induced in 32D murine hemopoietic cells by interleukin-3 withdrawal. In 32D cells and in 32D cells constitutively expressing a temperature-sensitive mutant of p53 (32Dtsp53), overexpression of a wild-type (but not a mutant) insulin-like growth factor I receptor (IGF-IR) protects these cells from apoptosis. A tsp53 in its wild-type conformation causes a decrease in the levels of IGF-IRs, and this decrease is accompanied by increased sensitivity of these cells to apoptosis. However, when the expression of the IGF-IR cDNA is regulated by a viral promoter, IGF-IR levels are not decreased by a wild-type p53, and apoptosis does not occur. These findings show that, in 32Dtsp53 cells, the IGF-IR is a physiologically relevant target of p53 in the process of apoptosis.

The p53 protein is a multifunctional protein that plays a major role in apoptosis (see reference 14 for a review). Induction of apoptosis depends often (but not always) on a functional p53 protein (20, 53). The requirement for a wild-type p53 to induce apoptosis in a variety of systems has often been demonstrated by the use of a temperature-sensitive (ts) mouse p53 protein (22, 25), which has a mutation to valine at residue 135. This p53 protein assumes the wild-type conformation at 32°C and assumes a mutant, inactive conformation at 39°C. Blandino et al. (6) have used this mutant to generate stable clones of 32Dtsp53 cells and have shown that the p53 protein in the wild-type conformation accelerates apoptosis caused by interleukin 3 (IL-3) withdrawal. A role of p53 in apoptosis induced by IL-3 withdrawal has also been reported by Canman et al. (8) and by Soddu et al. (39).

The insulin-like growth factor I receptor (IGF-IR) activated by its ligands plays an important role in cell proliferation in at least three ways: (i) it is required, in cooperation with other growth factors, for the optimal growth of cells in vitro (40) and in vivo (3, 21); (ii) it is necessary for the establishment and maintenance of the transformed phenotype, at least in a variety of cell types (see reference 5 for a review); and (iii) it protects cells from apoptosis. The evidence for an antiapoptotic function of the IGF-IR is substantial. Thus, an overexpressed IGF-IR allows the growth of FDC-P1 cells in the absence of IL-3, which is otherwise an obligatory requirement for the survival of these cells of hemopoietic origin (23). These cells can also be protected from apoptosis induced by IL-3 withdrawal by the simple addition of insulin-like growth factor I (IGF-I) (31). IGF-I also protects cells of neuronal origin, injured by various methods (13, 15), as well as cells overexpressing c-*myc* (2), which undergo apoptosis in the absence of other growth factors (17). When the function of the IGF-IR is decreased or otherwise impaired by antisense strategies or by dominant negatives, tumor cells undergo massive apoptosis, which is more prominent in vivo than in vitro (10, 29, 30). Conversely, an overexpressed IGF-IR protects 3T3 cells from etoposide-induced apoptosis (35). Mutational analyses of the IGF-IR have indicated that the three functions mentioned above, mitogenesis, transformation, and protection from apoptosis, map on separate domains of the receptor itself (18, 27a, 41).

Recently, Werner and collaborators have established a relationship between the IGF-IR and p53, by demonstrating that wild-type p53 represses transcription from the IGF-IR promoter, thereby decreasing the level of its expression (50). The biological significance of this effect was not addressed by the authors. In the experiments described here, we have investigated the relationship between p53 and the IGF-IR in apoptosis induced in 32D cells by IL-3 withdrawal; these cells are suitable for such studies because they have a very low level of IGF-IRs (see below). The question we have asked, specifically, is whether the IGF-IR is a physiologically relevant target of p53 in the process of apoptosis in 32D cells.

For this purpose, after establishing in preliminary studies (see below) that the IGF-IR does indeed protect 32D cells from apoptosis caused by IL-3 withdrawal, we stably transfected into 32Dtsp53 cells (6, 39) two different plasmids, both expressing the wild-type human IGF-IR cDNA (43): in one plasmid, the cDNA is under the control of the cytomegalovirus (CMV) promoter, and in the other plasmid, it is under the control of a rat IGF-IR promoter (32, 47, 49). Our rationale was that, according to the findings of Werner et al. (50), at 32°C, the p53, being in wild-type conformation, should reduce the IGF-IR number in cells expressing the IGF-IR under the control of the IGF-IR promoter but not in cells expressing the IGF-IR cDNA under the control of the viral promoter (unresponsive to p53). The question then becomes whether or not the failed down-regulation of the IGF-IR number inhibits the onset of apoptosis in 32Dtsp53 cells.

MATERIALS AND METHODS

Plasmids. pMRIGF1R12, a derivative of CVN-IGF-IR, expresses the human IGF-IR cDNA under control of the rat (−2350/I640) IGF-IR promoter (47, 49) and the neomycin resistance gene (26, 32). Other plasmids included plasmid pcDNA3wt expressing the human IGF-IR cDNA driven by the CMV promoter (CMVp), cloned in pcDNA3 (Invitrogen), which contains the neomycin resistance gene; and plasmid pBPV-KR (9) containing the human IGF-IR cDNA, in

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which the lysine residue 1003 at the ATP binding site has been substituted by arginine (KR mutant). This cDNA was cloned in a bovine papillomavirus-derived mammalian expression vector (pBPV; Pharmacia, Piscataway, N.J.). pPDV6+ plasmid encodes the puromycin resistance gene (12) and was used for selection of transfectants. p53cG carries the ts p53val135 mutant cDNA and the selectable marker Neo (39). The insulin receptor substrate 1 (IRS-1) and Shc plasmids were a kind gift, respectively, of Jaclyn Pierce (National Institutes of Health) and Joseph Schlessinger (New York University Medical School).

Cell lines. 32Dtsp53 clone (cl.) 10 (39) murine hematopoietic precursor cells stably transfected with p53cG were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 10% conditioned medium from the murine myelomonocytic cell line WEHI-3B as a source of IL-3 (6). WEHI-3B cells were cultured in Dulbecco modified Eagle medium or RPMI 1640 supplemented with 10% heat-inactivated FBS.

Growth and survival. 32D cells and derived cell lines were incubated for 24 h at 32 or 39°C, in complete medium, i.e., RPMI 1640 supplemented with both serum and WEHI cell-conditioned medium. They were then washed twice in Hanks balanced salt solution (HBSS) and seeded in RPMI 1640 supplemented with 10% FBS, RPMI 1640 supplemented with 10% FBS and 50 ng of IGF-I (Gibco-BRL) per ml, or RPMI 1640 supplemented with 10% FBS and 10% WEHI cell-conditioned medium as the source of IL-3. Cells were seeded at a density of 2.5×10^4/35-mm-diameter plate in 2 ml of medium. Cell numbers were determined in duplicate after 24 and 48 h (at either temperature) with a hemocytometer. Cell viability was determined by the ability to exclude trypan blue. The values given in the various figures are a combination of cell number and viability. The percent viable cells changed somewhat, if the cells were spun gently before counting, but the proportions among the various cell lines and different conditions remained the same.

DNA transfections and selection. 32Dtsp53 cells (5×10^6) were transfected with pMRIGF1R12 or pcDNA3/IGF-IRwt or pBPV/KR mutant and pcDNA3 empty vector carrying the neomycin resistance gene by electroporation (0.2 V, 960 mF) with a Gene Pulser (Bio-Rad Laboratories Inc., Hercules, Calif.). Following gene transfer, cells were cultured in RPMI 1640 supplemented with 10% FBS and 10% WEHI cell-conditioned medium for 48 h and then cloned by limiting dilution in selection medium containing 800 mg of G418 (Gibco-BRL) per ml for at least 2 weeks. After selection, the clones were maintained in 600 mg of G418 per ml. A similar procedure was used for the transfection of 32D and 32tsp53 cells with plasmids expressing the wild-type human IGF-IR, IRS-1, or Shc. With the latter plasmids, however, selection was carried out with the puromycin resistance gene (12), to generate independent clones.

IGF-I binding assay. IGF-I binding was quantitated with monoiodinated [^{125}I]IGF-I (Amersham). Cells, grown in flasks at 32 or 39°C for 24 h, were washed twice with cold HBSS and reseeded in 1 ml of binding buffer (RPMI 1640 containing 25 mM HEPES [pH 7.4] and 1 mg of bovine serum albumin [BSA] per ml) containing 0.5 ng of [^{125}I]IGF-I for 6 h at 4°C. The cells were washed twice with cold HBBS and lysed in 1 ml of 0.03% sodium dodecyl sulfate for measurement of cell-associated radioactivity. Specific binding was expressed by subtraction of nonspecific binding as determined in the presence of a 400-fold excess of unlabeled IGF-I (Bachem, Torrance, Calif.) from total binding. Radioactivity was measured by an autowell γ counter.

Cell numbers were determined for wells which were treated simultaneously with the experimental wells. Data representing specific binding were analyzed according to the method of Scatchard (34).

The computer software programs used for drawing a line for the plots were the commercially available Microsoft Excel and Cricket Graph; both methods gave the same lines. All binding data represent average counts from duplicate wells.

Western blotting. For the determination of p53 levels, the cells were incubated at 37°C. The cells were lysed in a lysis buffer (50 mM Tris-HCl [pH 8], 5 mM EDTA [pH 8], 50 mM NaCl, 0.5% Nonidet P-40), and 30 μg of the whole lysates were run on a sodium dodecyl sulfate-polyacrylamide gel (4 to 15% gradient) and were transferred to a nitrocellulose membrane. The blot was blocked overnight with 3% BSA in TBST (10 mM Tris-HCl [pH 8], 150 mM NaCl, 0.05% Tween 20), hybridized with anti-p53 (Ab-1 from Oncogene Science), and developed with the enhanced chemiluminescence detection reagents (Amersham). Densitometry was performed on the film with UltroScan XL (Pharmacia LKB).

RESULTS

Role of p53 in IL-3-induced apoptosis of 32D cells. In a previous paper, Blandino et al. (6) had reported that the tsp53 gene product accelerated apoptosis at 32°C and delayed it at 37°C in stably transfected 32D cells. Those experiments were carried out at an optimal concentration of WEHI medium (10%). We wanted to study the effect of different concentrations of IL-3 on the survival of the parental 32D cells and on that of the 32Dtsp53 cells. As a source of IL-3, we used, as is customarily done, WEHI medium (see Materials and Methods). Hereafter, we will refer to the concentration of WEHI medium, for brevity, as the concentration of WEHI–IL-3. We

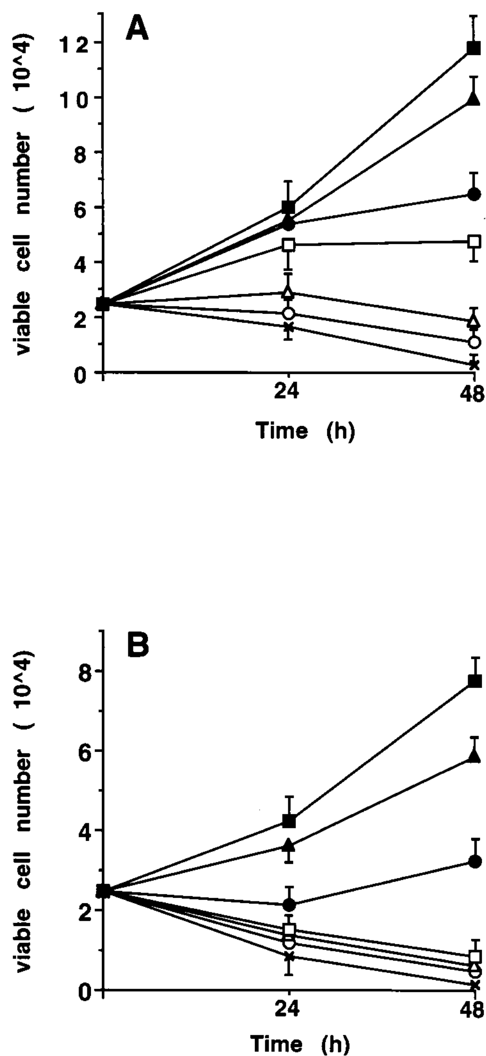


FIG. 1. Effect of WEHI–IL-3 concentration on apoptosis of 32D and 32Dtsp53 cells. The parental cell line 32D (A) and the derived 32Dtsp53 cell line (B) were incubated as described in Materials and Methods, at 32°C, in different concentrations of WEHI–IL-3. Symbols and corresponding concentrations are as follows: closed squares, 10%; closed triangles, 1.5%; closed circles, 1%; open squares, 0.5%; open triangles, 0.2%; open circles, 0.1%; crosses, no WEHI–IL-3.

tested both 32D and 32Dtsp53 cells at 32°C in different concentrations of WEHI–IL-3, ranging from 0 to 10%. The results of a typical experiment are summarized in Fig. 1. At a concentration of 10% WEHI–IL-3, both 32D and 32Dtsp53 cells survive and grow. At a concentration of 0.5%, 32D cells (Fig. 1A) remained stable in number, while 32Dtsp53 cells (Fig. 1B) died. At lower concentrations of WEHI–IL-3, or in the absence of WEHI–IL-3, both cell lines died, although cell death was somewhat more accentuated in the 32Dtsp53 cell line. These experiments confirm previous reports (see above) that p53 plays a role in apoptosis of 32Dtsp53 cells caused by IL-3 withdrawal and that, under appropriate conditions, it can be the determining factor (see also below).

Wild-type human IGF-IR protects 32D cells from apoptosis caused by IL-3 withdrawal. While it is already known that IGF-I protects hemopoietic cells like 32D cells from apoptosis (see Introduction), we still had to establish whether overexpression of the IGF-IR would protect 32D cells from apoptosis caused by IL-3 withdrawal for two reasons. Firstly, the previous

TABLE 1. Effect of an overexpressed IGF-IR and its substrates on the survival of 32D cells[a]

Cell line	No. of cells recovered under indicated condition:		
	10% FBS	10% FBS + IGF-I	10% FBS + IL-3
32D	0.2	0.3	16.0
32D puro	0.9	1.5	20.1
32D/IGF-IR	4.7	5.6	22.1
32D/(1A)IRS-1	0.1	1.0	24.0
32D/(bis)IRS-1	0.1	1.0	32.2
32D/Shc 6	0.1	0.8	17.6
32D/Sch 10	0.2	0.6	17.4

[a] All cell lines were plated at a density of 2.5×10^4 cells/2 ml. The numbers given are the total number of cells (10^4) recovered in the 2 ml after 48 h of incubation under the indicated conditions. IGF-I, 50 ng/ml; IL-3, WEHI medium at a concentration of 5%.

TABLE 2. Clones of 32Dtsp53 cells expressing the human IGF-IR[a]

Clone	No. of IGF-IRs/cell (10^3)		
	37°C	32°C	39°C
32D	2.8		
32D/IGF-IR	34.3		
32Dtsp53		2.0	5.5
32Dtsp53 wp/A		2.0	9.8
32Dtsp53 wp/B		2.8	10.0
32Dtsp53 wp 25		3.5	9.0
32Dtsp53 CMVp/A		10.7	11.8
32Dtsp53 CMVp/B		14.0	16.7
32Dtsp53 CMVp23		14.7	14.7

[a] The number of IGF-IRs was determined by Scatchard analysis in cells grown at 32, 37, or 39°C for at least 24 h. wp, cells transfected with an IGF-IR cDNA under the control of a rat IGF-IR promoter. CMVp, cells transfected with the same cDNA, but under the control of the CMV promoter. These determinations were repeated several times by two operators over a period of 1 year. Ranges of determinations never varied more than 11% on either side of the means given above; in most cases, they varied between 5 and 8%.

experiments showed the protection by the ligand, and we wanted to know whether overexpression of the receptor was also protective. And secondly, the behavior of cells in different laboratories is often variable, even when the cells are supposedly derived from a single source. 32D cells (which have a very small number of IGF-IRs, about 2,800 receptors per cell) were transfected with plasmid pcDNA3/IGF-IR (see Materials and Methods), and clones were selected in puromycin (12). The results obtained with a representative clone are shown in Table 1. The wild-type 32D cells survive and grow only in medium supplemented with IL-3 (Table 1, column 4). 32D cells transfected with the puromycin vector (used for selection) behaved like wild-type 32D cells and survived and grew only when IL-3 was present. 32D cells transfected with the IGF-IR cDNA survived in 10% serum alone and in 10% serum supplemented with IGF-I (Table 1, columns 2 and 3). In fact, under these conditions, they actually grew a little, although not as well as when IL-3 was added to the medium (Table 1, column 4). IGF-I only, without 10% serum, also had a protective effect, but not as pronounced as when added to 10% serum (not shown). It should not be surprising that cells overexpressing the IGF-IR are protected by 10% serum, without IGF-I addition: bovine serum contains substantial amounts of both IGF-I and IGF-II (the two ligands combined are present in plasma at a level of 700 ng/ml) and is known to strongly activate the IGF-IR (38, 44). The data in Table 1 were obtained after 48 h of incubation under the indicated conditions, but the differences were already appreciable at 24 h. Although we have confirmed that withdrawal of IL-3 causes apoptosis of 32D and 32Dtsp53 cells, we have omitted the documentation, which has already been provided repeatedly in other papers (see both Introduction and Discussion). The protective effect of the IGF-IR against apoptosis induced in 32D cells by IL-3 withdrawal was confirmed with several other clones of 32Dtsp53 cells expressing increased levels of the receptor (see below). Similarly, the effect of IGF-I addition on protection was confirmed with 32Dtsp53 cells incubated in 1% serum, also discussed below. Here we simply wish to establish the principle that an overexpressed IGF-IR protects 32D cells from IL-3 withdrawal. 32D cells, besides having a very small number of IGF-IRs, are completely devoid of IRS-1 and IRS-2 (24, 45), which suggests that the IRSs are not an absolute requirement in IGF-IR protection from apoptosis. However, an overexpressed IRS-1 (two different clones) partially protects 32D cells from apoptosis induced by IL-3 withdrawal (Table 1). The extent of protection is not as high as with the wild-type IGF-IR, which fully protects 32D cells, but there is no doubt that an overexpressed IRS-1 increases the survival of 32D cells, especially when IGF-I is added to the medium. On the other hand, Shc proteins fail to protect 32D cells from apoptosis (Table 1); in some experiments, overexpression of Shc actually seemed to accelerate apoptosis. It seems therefore, that the protection from IL-3-induced apoptosis by the IGF-IR follows pathways that are only partially dependent on the two major substrates of the receptor, IRS-1 and Shc.

IGF-IR levels in 32Dtsp53 cells. Having established that p53 has a role in IL-3-induced apoptosis of 32D cells and that an overexpressed IGF-IR can protect these cells from apoptotic injury, we set out to determine the relationship between the two. Since Werner et al. (50) have reported that wild-type p53 represses transcription from the IGF-IR promoter, we first determined the effect of p53 on IGF-IR levels in 32Dtsp53 cells, which express the $p53^{val135}$ protein (25) and still have a requirement for IL-3 for survival (6). 32Dtsp53 cells, like the parental 32D cells, have a small number of IGF-IRs, about 2,800 receptors per cell, at 37°C (for comparison, BALB/c 3T3 cells have from 15,000 to 25,000 receptors per cell, depending on growth conditions). The cells, in growth medium (i.e., including IL-3) were incubated for 24 h at either 32 or 39°C, and the number of IGF-IRs was determined by Scatchard analysis (34). The results are shown in Table 2. At 32°C, the number of IGF-IRs is about 2,000 per cell, and it increases to about 5,500 receptors per cell at 39°C. Thus, in the same cell line, the presence of a p53 protein in a mutant conformation causes an almost threefold increase in the number of endogenous IGF-IRs, in comparison to cells expressing the p53 protein in a wild-type conformation. This effect is not simply due to a temperature effect, since it does not occur in 32D cells (data not shown) and does not occur when the IGF-IR cDNA is under the control of a viral promoter (see below). The determination of IGF-I binding sites (from which one calculates receptor number) is reproducible, provided that the culture conditions are maintained constant, at a low density. Under these conditions, the variations in the number of IGF-I binding sites do not exceed 10%. At high densities, the results can be erratic.

Generation of 32Dtsp53 cells expressing the wild-type human IGF-IR. The original 32Dtsp53 cell line (6, 39) was then transfected with the human IGF-IR cDNA, to obtain appropriate clones. As mentioned above, we used two different constructs: in one case, the human IGF-IR cDNA was under the control of a rat IGF-IR promoter (32, 47, 49), while in the

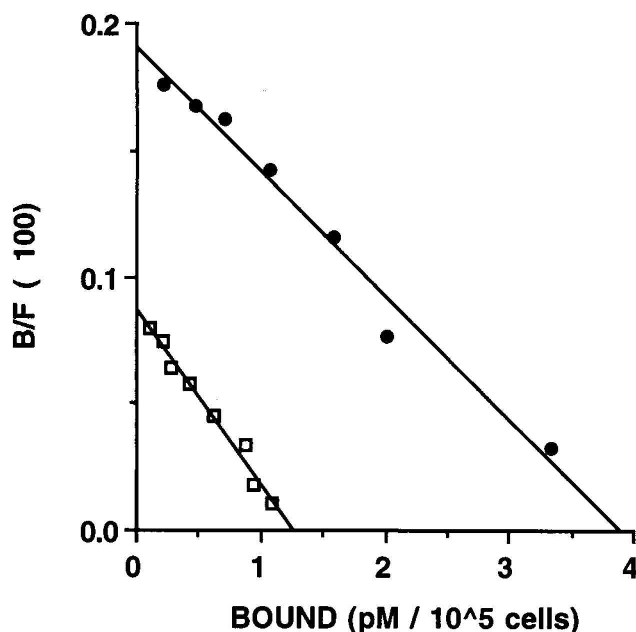


FIG. 2. Scatchard analysis of IGF-I binding sites in 32Dtsp53 cells at 32 and 39°C. The cells used in this experiment were 32Dtsp53 cells stably transfected with a plasmid expressing the human IGF-IR under the control of the IGF-IR promoter. Determination of IGF-I binding sites was carried out as described in Materials and Methods, under the same conditions. However, the cells were previously preincubated for 24 h at 32°C (squares) or 39°C (circles). B/F, bound/free.

second case, the same cDNA was under the control of the CMV promoter. Our strategy was to obtain clones with a sufficient number of IGF-IRs that could protect 32Dtsp53 cells from apoptosis caused by IL-3 withdrawal at 39°C. A very large number of clones was generated, and the number of IGF-IRs expressed was determined, for screening, by a single-point IGF-I binding. The most promising clones were deemed to be those with a moderately increased number of receptors relative to that for 32Dtsp53 cells. These clones were expanded, and the number of IGF-IRs was determined by Scatchard analysis. A representative Scatchard plot, in which we examined one of the clones expressing the human IGF-IR under the control of the rat IGF-IR promoter, is given in Fig. 2. A shift in temperature from 32 to 39°C causes an increase in receptor number from 2.8×10^3 to 10×10^3 receptors per cell. The clones eventually selected for further studies are listed in Table 2, where the numbers of receptors at two different temperatures, 32 and 39°C, are given. 32Dtsp53 cells transfected with the IGF-IR cDNA under the control of the rat IGF-IR promoter showed levels of receptor expression that varied by a factor of three- to fourfold between 39 and 32°C, confirming that the conformational status of p53 can influence IGF-IR levels. However, in cells expressing the IGF-IR cDNA under the control of a viral promoter, the levels of expression of the receptor varied very little between 39 and 32°C, confirming that the change in IGF-IR number is not a simple temperature effect. The receptor number of these clones remained stable throughout several months of experimentation and through the tenure of two different operators. The values given are actually the means of several determinations; since the ranges varied between 5 and 11% of the means, we have omitted them from the table for clarity.

The levels of p53 protein (at 37°C) in these clones were also determined (Fig. 3). As previously reported (6, 22, 25), the tsp53 levels do not vary appreciably at the different temperatures, although the conformational changes cause an almost complete loss of function. Curiously, the three clones transfected with the IGF-IR cDNA under the control of the IGF-IR promoter showed decreased amounts of p53 protein in comparison to those for cells transfected with the IGF-IR cDNA under the control of a viral promoter. By densitometric analysis (see Materials and Methods), the latter cells had about twice as much p53 protein as the former ones. We have not further investigated this point, which does not affect our conclusions.

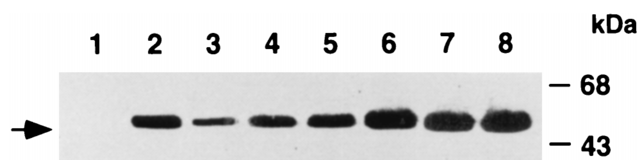


FIG. 3. p53 levels in 32Dtsp53 clones stably transfected with the human IGF-IR cDNA. Levels of p53 protein were determined as described in Materials and Methods. Lanes: 1, 32D cells; 2, 32Dtsp53 cells; 3 to 5, 32Dtsp53 cells stably transfected with a plasmid expressing the IGF-IR under the control of the IGF-IR promoter; 6 to 8, 32Dtsp53 cells stably transfected with a plasmid expressing the IGF-IR under the control of the CMV promoter.

Effect of p53 conformational state on the survival of 32Dtsp53 cells expressing the human IGF-IR. The clones listed in Table 2 were then tested for survival under three different conditions: (i) 10% FBS, (ii) FBS plus IGF-I (50 ng/ml), and (iii) FBS supplemented with IL-3. The experiments were carried out at two temperatures: 32°C (wild-type p53) and 39°C (mutant p53). The results of representative experiments for the cells expressing the IGF-IR (either under the control of the rat IGF-IR promoter or that of a viral promoter) are summarized in Fig. 4 and 5. After incubation at 39°C (Fig. 4), all clones overexpressing the human IGF-IR are protected from cell death (Fig. 4A), especially when IGF-I is added to the medium supplemented with FBS (Fig. 4B). Indeed, there is even an increase in cell number over the number of cells plated, although the increase is in no way comparable to the increase caused by the addition of 10% IL-3 (Fig. 4C). Please note that in Fig. 4, the ordinates for the three panels are different; we have also omitted standard deviations at 24 h that would have made the figures too crowded, but these experiments have been repeated several times, by two different operators, with essentially the same results. The protection in 10% serum alone is due to the presence of both IGF-I and IGF-II in the serum itself. The parental 32Dtsp53 cells in 10% FBS alone die as expected. When IL-3 is added, the growth of the parental cell line and that of the derived clones are essentially similar. The protective effect of the IGF-IR at this temperature, especially in the presence of IGF-I, applies to all clones.

The results with the same clones at 32°C are shown in Fig. 5 (please note that the ordinates are again different). All cell lines grow when IL-3 is added to the medium (Fig. 5C), at a rate that is roughly 40% the rate of growth at 39°C. The 32Dtsp53 cells expressing the IGF-IR under the control of the rat IGF-IR promoter are no longer protected from apoptosis caused by IL-3 withdrawal, whether in the absence (Fig. 5A) or presence (Fig. 5B) of IGF-I, although a few more cells survive in the latter case. But the survival is definitely much lower than at 39°C.

With the cells expressing the IGF-IR under the control of the CMV promoter, the results at 32°C are different. These

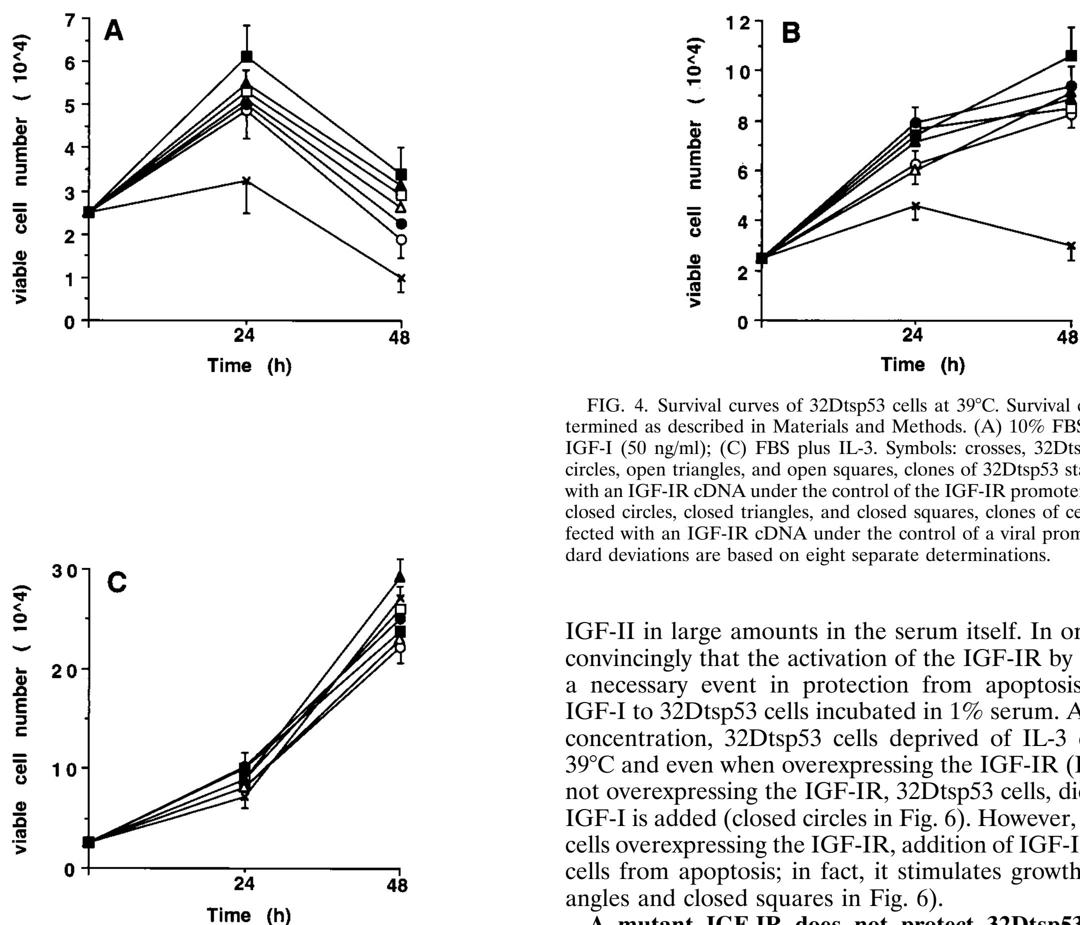


FIG. 4. Survival curves of 32Dtsp53 cells at 39°C. Survival curves were determined as described in Materials and Methods. (A) 10% FBS; (B) FBS plus IGF-I (50 ng/ml); (C) FBS plus IL-3. Symbols: crosses, 32Dtsp53 cells; open circles, open triangles, and open squares, clones of 32Dtsp53 stably transfected with an IGF-IR cDNA under the control of the IGF-IR promoter; clones of cells closed circles, closed triangles, and closed squares, clones of cells stably transfected with an IGF-IR cDNA under the control of a viral promoter. The standard deviations are based on eight separate determinations.

cells survive at 32°C in 10% serum alone, and the survival is increased by the addition of IGF-I. The number of cells recovered is smaller than at 39°C, but it should be noted that at the latter temperature, growth is 2.5-fold the growth at 32°C. The comparison between 32Dtsp53 cells with the rat promoter and those with the CMV promoter is also significant: the latter ones survive at 32°C much better than the cells expressing the IGF-IR cDNA under the control of the rat IGF-IR promoter. In the presence of IGF-I, the CMV clones actually grow. Again, these experiments have been repeated by two operators at different times.

Notice from Table 2 that, with these cell lines, shifting of cells to 32°C does not decrease the IGF-IR number when the promoter is viral, but it does when the promoter is the IGF-IR promoter. It seems, therefore, that unless the IGF-IR number is appropriately decreased, p53 cannot exert its apoptotic effect on 32D cells. On this basis, we can say that (i) the IGF-IR, expressed at a level of approximately 6,000 receptors per cell (or more), is effective in protecting 32Dtsp53 cells from apoptosis, even when the p53 protein is in the wild-type conformation; and (ii) when the wild-type p53 drops the receptor number at levels below 4,000 per cell, protection is no longer afforded or, at any rate, it is at a much lower efficiency.

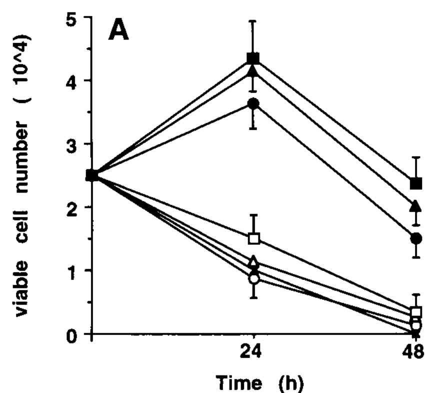
Importance of IGF-I in the protection from apoptosis. All of the previous experiments were done in 10% serum, which is the customary procedure with 32D cells, and we wished to present data that other investigators could compare with their own. Under these conditions, the effect of added IGF-I is obscured, as mentioned above, by the presence of IGF-I and IGF-II in large amounts in the serum itself. In order to show convincingly that the activation of the IGF-IR by its ligands is a necessary event in protection from apoptosis, we added IGF-I to 32Dtsp53 cells incubated in 1% serum. At this serum concentration, 32Dtsp53 cells deprived of IL-3 die, even at 39°C and even when overexpressing the IGF-IR (Fig. 6). Cells not overexpressing the IGF-IR, 32Dtsp53 cells, die even when IGF-I is added (closed circles in Fig. 6). However, in 32Dtsp53 cells overexpressing the IGF-IR, addition of IGF-I protects the cells from apoptosis; in fact, it stimulates growth (closed triangles and closed squares in Fig. 6).

A mutant IGF-IR does not protect 32Dtsp53 cells from apoptosis. We have previously reported that a human IGF-IR cDNA with a point mutation at the ATP binding site (lysine 1003) is essentially an inactive receptor, unable either to transmit a mitogenic signal or to transform (9). We asked here whether this mutant receptor could protect 32Dtsp53 cells from apoptosis. 32Dtsp53 cells were stably transfected with plasmid pBPV-KR (19), and clones were selected as usual. The clone used for Fig. 7 expressed about 10,000 receptors per cell. The results (Fig. 7A) clearly show that this receptor does not protect 32Dtsp53 cells from apoptosis. At both 32 and 39°C, the fraction of surviving cells was essentially the same in the parental cell line and in the cell line stably transfected with the mutant IGF-IR. Note also that, with the mutant receptor, addition of IGF-I has no effect whatsoever on survival (Fig. 7B).

DISCUSSION

We have selected for our studies 32D cells for the following reasons: (i) 32D cells (and also 32Dtsp53 cells) undergo apoptosis after IL-3 withdrawal, a procedure that avoids drugs or toxic agents; (ii) both cell types have often been used for studying apoptosis, which has been documented in detail in several publications (2, 6, 16, 39); and (iii) these cells have very low levels of IGF-IR and no IRS-1 or IRS-2 (24, 52), which makes them ideal for studying the role of the IGF-IR in this model of apoptosis.

Our results can be summarized as follows. (i) p53 plays a role in IL-3-induced apoptosis of tsp53 cells. These cells are resistant to apoptosis when p53 is in a mutant conformation (Fig. 4A). (ii) Overexpression of the IGF-IR protects 32D and



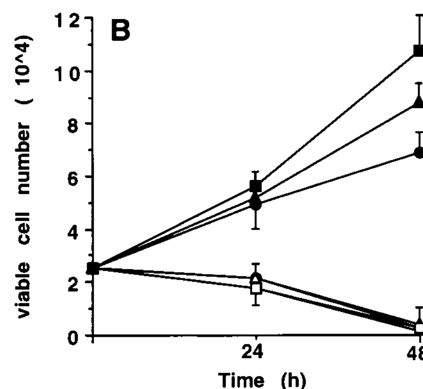
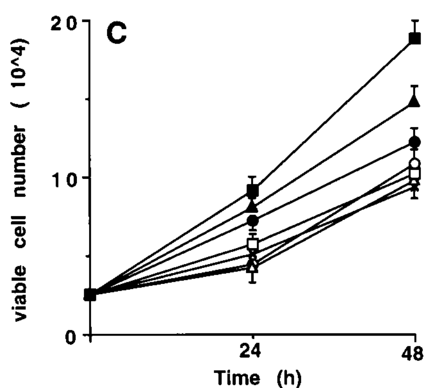


FIG. 5. Survival curves of 32Dtsp53 cells at 32°C. Results shown are for the same experiment as for Fig. 4, except that the cells were incubated at 32°C.



32Dtsp53 cells from apoptosis induced by IL-3 withdrawal. (iii) Wild-type p53 reduces the number of IGF-IRs in 32Dtsp53 cells in which the transfected receptor is under the control of the IGF-IR promoter (confirming the results of Werner et al. [50]). These cells now undergo apoptosis (Fig. 5A and B). (iv) Wild-type p53 fails to reduce the IGF-IR number in 32Dtsp53 cells expressing the receptor under the control of a viral promoter (Table 2). These cells do not undergo apoptosis at 32°C. Our interpretation is that the IGF-IR, at least in these cells, is a physiological target of p53. Failure by p53 to reduce the IGF-IR number results in failure to induce apoptosis in 32Dtsp53 cells.

Several reports have indicated that p53 is involved in the induction of apoptosis caused by IL-3 withdrawal (references 6, 8, and 39 and this paper). The activation of the IGF-IR by its ligands, on the contrary, protects cells from IL-3 withdrawal-induced apoptosis (see Introduction). In those experiments (23, 31), apoptosis was prevented by the addition of IGF-I. Our experiments further confirm those data by showing that overexpression of the IGF-IR protects 32D and 32Dtsp53 cells from apoptosis. That the protective effect is due to the overexpressed and activated IGF-IR is demonstrated by two observations: (i) when the IGF-IR number is decreased, apoptosis occurs; and (ii) in 1% serum (low concentrations of IGF-IR's ligands), protection occurs only if IGF-I is added. The cells are somewhat protected in 10% serum, without the addition of extra IGF-I, because serum contains substantial amounts of both IGF-I and IGF-II. A concentration of 10% serum provides 20 ng of IGF-I per ml, and, if one considers all the various forms of IGF-II (all known to be mitogenic), as much as 50 ng of IGF-II per ml (55), which activate the IGF-IR. Although some of these IGFs are presumably bound by IGF binding proteins, enough is present in bovine serum to activate the IGF-IR (38, 44). At the same time, the fact that these ligands are in part buffered by the IGF binding proteins explains why supplementation with IGF-I does increase survival, even in 10% serum. The overexpression of the IGF-IR does not have to be extraordinary: an increase from 2.0×10^3 to 5.5×10^3 receptors per cell is sufficient to confer a degree of protection. The receptor, though, has to be functional: a receptor with a mutation at the ATP binding site, which inactivates the receptor (9, 19), offers no protection, even though overexpressed.

It may seem puzzling at first glance that such a small increase in IGF-IR number could be sufficient to protect 32Dtsp53 cells from apoptosis caused by IL-3 withdrawal. However, there is evidence that small increases in receptor

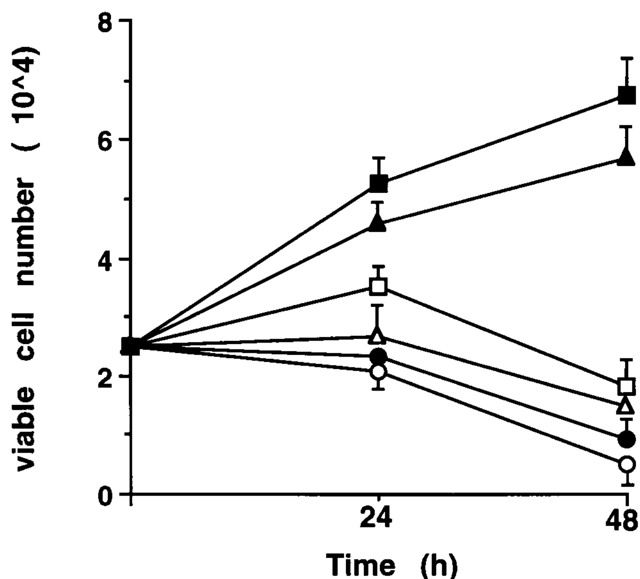


FIG. 6. Effect of IGF-I on survival. Cells were incubated in 1% serum, at 39°C, without IL-3 and plus or minus IGF-I (50 ng/ml). Open symbols, minus IGF-I; closed symbols, plus IGF-I. Circles, 32Dtsp53 cells; triangles, 32Dtsp53 cells expressing the IGF-IR under the control of the receptor promoter; squares, 32Dtsp53 cells expressing the IGF-IR under the control of a viral promoter.

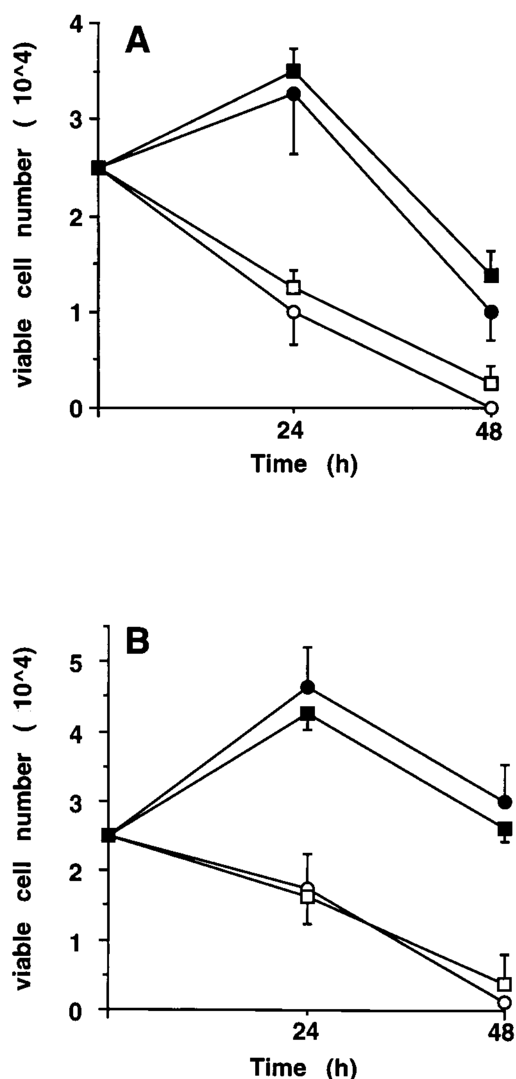


FIG. 7. A mutant IGF-IR does not protect 32Dtsp53 cells from apoptosis. 32Dtsp53 cells were stably transfected with a plasmid expressing a human IGF-IR cDNA with a point mutation at lysine 1003 (see Materials and Methods). Survival curves were determined as described in Materials and Methods. (A) 10% serum; (B) plus IGF-I (50 ng/ml). Symbols: circles, 32Dtsp53 cells; squares, cells with mutant receptor. Closed symbols, 39°C; open symbols, 32°C.

levels can change dramatically the response of cells to IGF-I-mediated mitogenesis. For instance, 3T3 cells with 15×10^3 IGF-IRs per cell do not respond with cell proliferation when stimulated with IGF-I only. A 50% increase in receptor number causes these cells to respond to IGF-I with maximal stimulation (33).

The p53 protein in its wild-type conformation (but not in its mutant conformation) causes a decrease in IGF-IR levels, thus confirming that p53 represses transcription from the IGF-IR gene promoter (50). However, those experiments did not address the issue of the relevance of the p53 effect on the IGF-IR in terms of apoptosis. A similar finding was reported by Webster et al. (46) for the promoter of the highly homologous insulin receptor; these authors also pointed out the high levels of both IGF-IR and insulin receptor in human breast cancers with mutated p53. The decrease in IGF-IR number in our experiments is not due to a simple temperature effect, because the number of receptors does not change appreciably between 32 and 39°C, when the IGF-IR cDNA is under the control of a viral promoter. These conclusions are predicated on the accuracy of IGF-I binding site determinations. In our experience, measurements of IGF-I binding sites (translatable into IGF-IR numbers) are highly reproducible, if the cells are kept at a reasonably low density, with the variations not exceeding 10 to 15%. At high densities, results can vary much more. It is also our experience that, after an initial fluctuation, IGF-IR levels in transfectants remain constant for several years, provided the selectable agent is used in passaging cells. p6 cells (28) and R+ cells (36) have kept constant IGF-IR numbers for periods that have now ranged from 3 to 6 years. The differences in receptor number between 32 and 39°C were also reproducible, and it is indicative that all clones in which the IGF-IR was under the control of a wild-type promoter (including the original 32Dtsp53 cells with endogenous IGF-IRs) showed an appreciable increase when the cells were shifted to 39°C.

It is therefore significant that, under these conditions, 32Dtsp53 cells die when IL-3 is withdrawn at 32°C, but only in those clones in which the IGF-IR levels (controlled by a wild-type IGF-IR promoter) decrease. If the receptor levels are not affected (clones with a cDNA under the control of a viral promoter), the shift to 32°C does not affect survival. While, quite obviously, p53 has many other functions, if any other of these functions were to be solely responsible for apoptosis at 32°C, the 32Dtsp53 cells with the IGF-IR under the control of a viral promoter should have also died at 32°C. We conclude that, in 32Dtsp53 cells, a down-regulation of IGF-IR levels is part of the mechanism(s) for p53-modulated apoptosis. We emphasize part of the mechanism, since p53 is known to act on other molecules that are important in apoptosis; for instance, a tsp53 causes a temperature-dependent decrease in the expression of bcl-2, while at the same time inducing an increase in the expression of bax (26).

IRS-1, IRS-2, and Shc are very important elements in the signal transduction pathway of the IGF-IR and also that of the insulin receptor (1, 27, 45, 51). In our model, IRS-1 partially protects 32D cells from apoptosis by IL-3 withdrawal. The definition of protection can be arbitrary: in comparison to the effect of an overexpressed wild-type IGF-IR, an overexpressed IRS-1 is not quite protecting. However, when compared to untransfected 32D cells, IRS-1 does offer a measure of protection, which was confirmed by repeated experiments. We also overexpressed the Shc protein in 32D cells, but if anything, expression of Shc actually accelerated apoptosis. These results suggest that the protection afforded by the IGF-IR must be exerted not only through IRS-1 but also through another unidentified pathway(s). Jung et al. (19) have reported that the antiapoptotic effect of the activated IGF-IR is due to an inhibition of the ICE pathway, which is apparently involved in many models of apoptosis. Future studies are designed to address the issue of how the IGF-IR connects with the ICE pathway.

It has now been reported that tumor suppressor genes like WT1 (48) and p53 (reference 50 and this paper), as well as interferon (42), cause a decrease in IGF-IR levels, through a repression of transcription from the IGF-IR promoter. p53 has been connected to the IGF system through other findings: it represses transcription from the IGF-II (54) and the insulin receptor (46) promoters and it induces the expression of the IGF-binding protein 3 (7) that antagonizes the effects of IGF-I and IGF-II. It would be interesting to know whether this mechanism applies also to other tumor suppressor genes and to other growth factor receptors (4, 11). The fact that cells without IGF-IRs (by targeted disruption of the IGF-IR genes [3,

21]) are refractory to transformation by several oncogenes (see references 5 and 37 for a review) seems to confirm an essential role of this receptor in transformation and, therefore, a role contrary to that of tumor suppressor genes.

In conclusion, we have demonstrated that a wild-type overexpressed p53 fails to induce apoptosis in 32Dtsp53 cells, when the IGF-IR levels remain relatively high. These findings, together with those of Werner et al. (50), indicate that the IGF-IR is a physiological target of p53 in these cells. While our results are limited to one cell type, we suggest that the IGF-IR may be targeted by p53 in other models of apoptosis.

ACKNOWLEDGMENTS

This work was supported by a grant, GM 33694, from the National Institutes of Health.

The first two authors contributed equally to this work.

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