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Anti-apoptotic Signaling of the Insulin-like Growth Factor-I Receptor through Mitochondrial Translocation of c-Raf and Nedd4*

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The type 1 insulin-like growth factor receptor (IGF-IR) sends a strong anti-apoptotic signal by at least three different pathways. By using mutants of the IGF-IR, we showed that one of the pathways depends on residues of the IGF-IR (serines 1280–1283) that interact with 14.3.3 proteins. The result is the activation of Raf-1 and the mitochondrial translocation of both Raf-1 and Nedd4, a target of caspases. A mutant IGF-IR in which the serines at positions 1280–1283 have been mutated to alanine does not protect from apoptosis and fails to translocate Nedd4 or Raf-1 to the mitochondria. This failure is accompanied by a loss of cytochrome *c* from the mitochondria. The 14.3.3/Raf-1/Nedd4 pathway is operative in the presence or absence of the insulin receptor substrate-1.

The type 1 insulin-like growth factor receptor (IGF-IR),[1] activated by its ligands, protects many cell types from a variety of pro-apoptotic agents (1, 2). The protective effect of the IGF-IR against cell death has been confirmed by the finding that down-regulation of the IGF-IR, either by antisense strategies or by dominant negative mutants, causes massive apoptosis of cells (2), especially when the cells are growing in anchorage-independent conditions (3). The pathways by which the IGF-IR protects cells from apoptosis are reasonably well known. The main pathway originates with the interaction of the IGF-IR with one of its major substrates, IRS-1 (4), which activates the phosphatidylinositol 3-kinase pathway (5), which in turn activates the Akt/protein kinase B pathway (6–8). Although it is clear that this pathway is the main pathway by which the IGF-IR exerts its anti-apoptotic effect, there is substantial evidence that the IGF-IR has alternative pathways. One alternative pathway is the mitogen-activated protein kinase pathway (9, 10), originating, at least in part, from another major substrate of the IGF-IR, the Shc proteins (11, 12). A 3rd pathway has been identified by Peruzzi *et al.* (10), which depends on the mitochondrial translocation of Raf-1. All three pathways were shown to lead to BAD phosphorylation and survival (10, 13). In response to survival factors, including IGF-I (13), and the activation of the Akt/protein kinase B pathway (7, 8), BAD is serine-phosphorylated by Akt. It is no longer capable of being heterodimerized with Bcl-X_L at membrane sites (14), is sequestered into the cytosol, bound to 14.3.3, and is inactivated as a cell death-promoting protein (15).

Mutation of the serines 1280–1283 of the IGF-IR does not affect its mitogenicity in mouse embryo fibroblasts, although it abrogates its ability to transform cells (16). Serine 1283 binds isoforms of the 14.3.3 protein (17, 18), and the integrity of serines 1280–1283 is required for the mitochondrial translocation of Raf-1 (10). Targeting of Raf-1 to mitochondria results in inactivation of BAD and inhibition of apoptosis (10, 19, 20). Interestingly, the anti-apoptotic protein Bcl-2 itself can also target Raf-1 to the mitochondria (19). A role of the IGF-IR in the mitochondrial translocation of Raf has received an independent confirmation from Nantel *et al.* (21), who have reported that Raf-1 and Grb10 (a substrate of the IGF-IR) can be co-immunoprecipitated from mitochondrial extracts.

32D cells are a murine hemopoietic cell line (22) that is IL-3-dependent for growth and undergoes apoptosis after IL-3 withdrawal. 32D cells expressing a human IGF-IR can survive in the absence of IL-3, provided the medium is supplemented with IGF-I (10, 23). 32D cells do not express IRS-1 and IRS-2 (24, 25). Since IRS-1 is a powerful mitogen and survival signal that can overwhelm other signals originating from the IGF-IR (23, 26, 27), the use of 32D cells offers a good model to analyze the alternative anti-apoptotic pathways of the IGF-IR. We have taken advantage of the 32D cell characteristics to investigate further the serine 1280–1283 pathway in IGF-I-mediated survival. As mentioned above, serine 1283 has been identified as a binding site for 14.3.3 (17, 18). These proteins are known to interact with several important signaling pathways of the cells, including IRS-1 (17, 28), Raf-1 kinase (29–31), and the death-promoting protein BAD (15, 32).

According to Craparo *et al.* (17), serine 1272 of the IGF-IR also interacts with 14.3.3 proteins. We therefore expected that a serine to alanine mutation in this last serine and/or in serine 1278 would result in an IGF-IR even more defective in its anti-apoptotic signaling. Paradoxically, a double mutation at serines 1272 and 1278 completely reversed the negative effect of the mutations at 1280–1283. The resulting receptor was as good and, perhaps, even marginally better than the wild type receptor in protecting 32D cells from apoptosis induced by IL-3 withdrawal (see below). We have used this contradiction to analyze in more detail the 3rd anti-apoptotic pathway of the IGF-IR. Specifically, we have asked how Raf activation modulates the survival signal of the IGF-IR in 32D cells. Our results show that survival of 32D-derived cells in IGF-I correlates with the mitochondrial translocation of both Raf-1 and Nedd4. Nedd4 is a multifunctional protein that is also a target of caspases (33). This report implicates Nedd4 for the first time as a component in the anti-apoptotic signaling of the IGF-IR.

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[1] The abbreviations used are: IGF-IR, type 1 insulin-like growth factor receptor; IRS-1, insulin receptor substrate-1; FBS, fetal bovine serum; IL, interleukin; MOPS, 4-morpholinepropanesulfonic acid; COX IV, cytochrome oxidase subunit IV.

EXPERIMENTAL PROCEDURES

Plasmids—The wild type IGF-IR and its mutants (4 serine and 6 serine mutants) are described by Romano *et al.* (26) and by Peruzzi *et al.* (10). Additional mutants of the IGF-IR on serines 1272 and 1278 and on single serines at 1280–1283 were generated by site-directed mutagenesis (Quikchange Site-directed Mutagenesis kit, Stratagene) following the manufacturer's instructions. pMSCV-IRS-1 plasmid was described by Valentinis *et al.* (25). The retroviral vectors have been described in detail by Romano *et al.* (26).

Cell Lines and Retroviral Infections—32D clone 3 cells were transduced with a murine leukemia virus-based retroviral vector system (26) to express the wild type IGF-IR or its various mutant. 32D/4 serine (32D 4Ser) cells and 32D d1245 cells (receptor truncated at residue 1245) have been described in previous papers (10, 23, 34). 32D 4Ser cells were transduced with pMSCV puro IRS-1 to generate 32D/4 serine/IRS-1. Mixed populations were selected in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 10% conditioned medium from the murine myelomonocytic cell line WEHI-3B as source of IL-3 (10, 25) in the presence of the appropriate antibiotic.

Survival Assay—Exponentially growing cells were washed three times with Hanks' balanced salt solution and seeded (5×10^4/35-mm plate in 2 ml of medium) in RPMI 1640 supplemented only with 10% FBS; RPMI 1640 supplemented with 10% FBS and 10% WEHI cell-conditioned medium as source of IL-3; RPMI 1640 supplemented with 10% FBS and 50 ng/ml IGF-I (Life Technologies, Inc.). Cell numbers were determined in duplicate after 24 and 48 h, counting only the cells able to exclude trypan blue. For an estimate of the extent of differentiation, we followed the procedure of Valentinis *et al.* (25).

IGF-IR and IRS-1 Expression Levels—The levels of IGF-IR were monitored by Western blot, as described previously (10) using an antibody to the β-subunit of the IGF-IR (Santa Cruz Biotechnology). The detection of IRS-1 in 32D/4 serine/IRS-1 cells was carried out following the same procedure except that the cells were lysed directly in Laemmli buffer (Bio-Rad). The antibody against IRS-1 was from Upstate Biotechnology, Inc.

Detection of Mitochondrial Raf-1 and Nedd4—Cells exponentially growing were washed five times with Hanks' balanced salt solution and seeded (7×10^6) in RPMI 1640 medium supplemented with 10% ΔFBS only, supplemented with 10% ΔFBS and 10% IL-3 (WEHI cell-conditioned medium), or with 10% ΔFBS and 50 ng/ml IGF-I. The protocol used to isolate mitochondria was a modification of those described by Bourgeron *et al.* (35) and Peruzzi *et al.* (10). Cells were harvested after 35 h, washed three times with 10 ml of buffer A (20 mM MOPS, 1 g/liter bovine serum albumin, 1 mM EGTA, 100 mM sucrose), and resuspended in 400 μl of buffer B (buffer A plus 10 mM triethanolamine, 5% Percoll, 0.1 mg/ml digitonin, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 0.2 mM sodium orthovanadate). After homogenization (25 strokes with a Dounce homogenizer B pestle), samples were centrifuged two times (at $2,500 \times g$ for 5 min) to remove the nuclei and centrifuged again at $13,000 \times g$ for 10 min to obtain the heavy membrane pellet. This fraction was resuspended in buffer B and centrifuged again at 13,000 for 10 min. Finally the pellet was resuspended in lysis buffer (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate). Samples were then centrifuged at $13,000 \times g$ for 20 min and measured for protein contents. SDS-polyacrylamide gel electrophoresis and Western blots were performed as described above. The membrane was probed with Raf-1 antibody (Transduction Laboratories). The membrane was subsequently stripped and probed with a cytochrome oxidase subunit IV antibody (Molecular Probes) or with a Nedd4 antibody from Transduction Laboratories (36). We have also used a 2nd procedure for the purification of the mitochondrial and cytosolic fractions (37). The two procedures gave essentially the same results. The actin antibody was from Santa Cruz Biotechnology, and the cytochrome *c* antibody was from PharMingen.

Phosphorylation of Raf-1—Cells exponentially growing were washed three times with Hanks' balanced salt solution and seeded in serum-free medium. After 4 h cells were stimulated with 50 ng/ml IGF-I for the indicated times. Cells were collected and lysed as described above. 30 μg of the whole cell lysates were resolved in a SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose filter. Phosphorylated Raf-1 was detected using an anti-phospho-Raf-1 (S338) from Upstate Biotechnology, Inc. Total Raf-1 was determined using an anti-Raf antibody from Santa Cruz Biotechnology. In Western blots, statistical significance between two measurements was determined by the two-tailed Student's *t* test analysis of variance.

RESULTS

Growth and Survival of 32D Cells Expressing Wild Type and Mutant IGF-I Receptors—The cell lines derived from parental 32D cells (32D) are designated as follows: 32D IGF-IR (expressing a wild type IGF-IR), 32D 4Ser (IGF-IR with serine to alanine mutations at positions 1280–1283), 32D 6Ser (receptor with mutations at serines to alanine 1272, 1278, and 1280–1283), and 32D 2Ser (receptor with mutations at serines 1272 and 1278). In the absence of IL-3, 32D cells rapidly die. The mode of death is apoptosis, which has been repeatedly documented in previous papers from this and other laboratories (23, 25, 38, 39). 32D IGF-IR cells survive in the absence of IL-3, if the medium is supplemented with IGF-I. Under these conditions, 32D IGF-IR cells grow exponentially for 48 h (23, 25). After 48 h, 32D IGF-IR cells stop growing and begin to differentiate along the granulocytic pathway (25, 27). Fig. 1 compares survival and growth of four cell lines as follows: 32D IGF-IR, 32D 4Ser, 32D 6Ser, and 32D 2Ser cells. All cell lines express the IGF-IR, either wild type (32D IGF-IR), or the indicated mutants (Fig. 1, *panel C*). After IL-3 withdrawal, and in the absence of IGF-I, all these cell lines rapidly undergo apoptosis. In the presence of IGF-I, they survive and actually grow for at least 48 h, with one exception. The exception is the 32D 4Ser cell line, which survives for 24 h (*panel A*) and then rapidly dies (*panel B*), as already reported (10). Surprisingly, additional mutations at serines 1272 and 1278 (6-serine mutant receptor) completely restore the ability of the IGF-IR to protect 32D cells from apoptosis. We have repeated this experiment many times, and also with another mixed population, but the 6-serine mutant always protected 32D cells from apoptosis. Indeed, the 6-serine mutant always gave a marginally but reproducibly better survival signal than the wild type receptor (Fig. 1). This was also noticed in mouse embryo fibroblasts undergoing anoikis (26). A mutation limited to serines 1272/1278 resulted in a receptor indistinguishable from the wild type receptor (Fig. 1).

Raf-1 Is Translocated to the Mitochondria in 32D 6Ser Cells—In a previous paper (10), we had shown that the wild type IGF-IR activated Raf-1, causing its translocation to the mitochondria, where it can exert an antiapoptotic effect (19, 20, 40), through the inactivation of BAD (see Introduction). The 4-serine mutant failed to cause mitochondrial translocation of Raf-1, and its phenotype was rescued by the ectopic expression of a mutant Raf that localizes to the mitochondria (20). In Fig. 2, we confirm that, in medium supplemented with IGF-I, the wild type receptor (*2nd lane*), but not the 4-serine mutant (*4th lane*), causes Raf-1 translocation to the mitochondria. The 6-serine mutant, like the wild type receptor, induces translocation of Raf-1 to the mitochondria (*4th lane*). Raf-1 levels in mitochondria are constantly elevated in all cell lines if the cells are growing in IL-3 (*1st, 3rd,* and *5th lanes*). The amounts of cytochrome oxidase (COX IV) in the lysates were used to monitor the amounts of mitochondrial proteins in each lane.

Absence of Nedd4 from the Mitochondria of 32D 4Ser Cells—Grb10 is an adaptor protein that interacts with the IGF-IR (41–43). Grb10 also interacts with Raf-1, and their interaction is accompanied by an anti-apoptotic signal (44). As mentioned in the Introduction, Raf-1 and Grb10 can be co-immunoprecipitated from mitochondrial fractions (21). We could not detect Grb10 in our 32D cells. However, Grb10 is known to interact with Nedd4, both by the yeast two-hybrid system and by co-immunoprecipitation in lysates of mouse embryo fibroblasts (36). We therefore investigated the presence of Nedd4 in the mitochondria of the same three cell lines, after IL-3 withdrawal and supplementation with IGF-I (Fig. 2). Nedd4 is present in the mitochondrial fraction of 32D cells expressing the wild

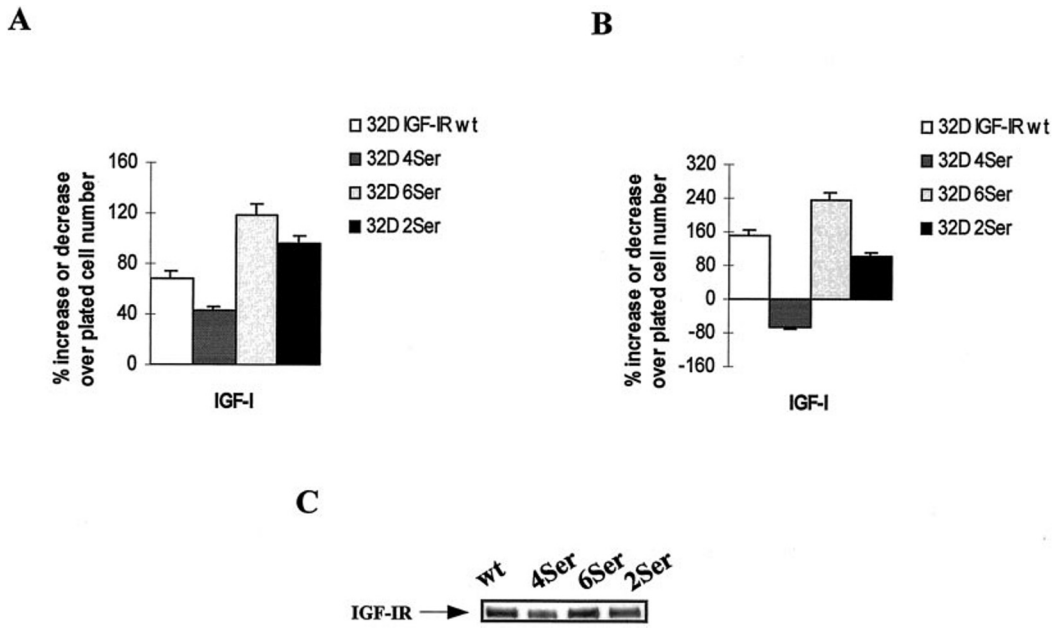

FIG. 1. **Effect of mutations in the serine cluster of the IGF-IR carboxyl terminus on the growth and survival of 32D cells.** The four cell lines (all mixed populations) are indicated as follows: 32D IGF-IR (wild type (*wt*) receptor); 32D 4Ser (IGF-IR with serine to alanine mutations at positions 1280–1283); 32D 6Ser (mutations at positions 1272, 1278, and 1280–1283); and 32D 2Ser (serine to alanine mutations at positions 1272–1278). Cell survival was at 24 (*panel A*) and 48 h (*panel B*) after IL-3 withdrawal and supplementation with IGF-I (50 ng/ml). All cell lines die rapidly in 10% serum without IGF-I. and all grow vigorously in IL-3 (not shown). *Panel C,* levels of expression of the IGF-IR in the four cell lines.

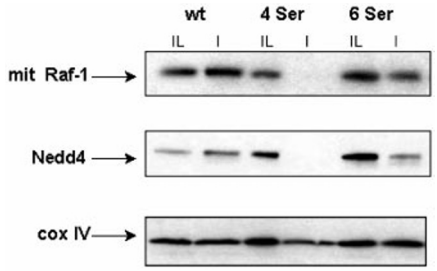

FIG. 2. **The 6-serine mutant receptor allows mitochondrial translocation of Raf-1 and Nedd4.** The three cell lines are indicated *above* the lanes. The *1st row* is a Western blot of Raf-1 levels in purified mitochondrial preparations. *I* indicates supplementation with IGF-I; *IL* indicates supplementation with interleukin-3 (IL-3). The *2nd row* shows the levels of Nedd4 in the same mitochondrial fractions. The gel was stripped and re-probed (*3rd row*) for a mitochondrial specific protein, cytochrome oxidase subunit IV (*COX IV*). *wt*, wild type.

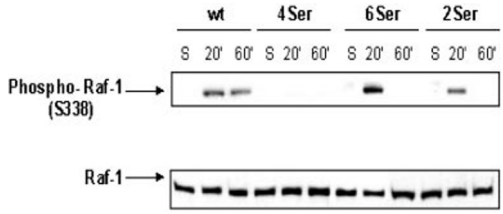

FIG. 3. **Mitochondrial translocation of Raf-1 correlates with phosphorylation at serine 338.** The four cell lines (see Fig. 1 and explanation in the text) tested in these experiments are indicated *above* the respective lanes. The cells were placed in serum-free medium (*S*) for 4 h and then stimulated with IGF-I (50 ng/ml) for the indicated times (in min). Lysates were made and Western blots developed with an antibody to phosphoserine 338 of Raf-1 (see "Experimental Procedures"). The *lower row* gives the total Raf protein in each lane.

type IGF-IR and the 6-serine mutant. Nedd4 was not detectable in the mitochondrial fraction of 32D 4Ser cells in IGF-I, although it was present in the same cells growing in IL-3. Fig. 2 shows a representative experiment, but these experiments have been repeated several times and were reproducible, even when different techniques for the purification of mitochondria were used.

Phosphorylation of Serine 338 of Raf-1—Serines 1272 and 1283 interact with 14.3.3 proteins (17, 18), and 14.3.3 proteins are known to interact with Raf (29, 30, 45–47). We have used antibodies to specific serines of Raf-1 to detect its activation in the lysates from various cell lines, after stimulation with IGF-I. We could not observe any difference among the various cell lines in the extent of phosphorylation of serine 621 or serine 259 (not shown). There were instead clear and reproducible differences in the phosphorylation of serine 338 after stimulation with IGF-I (Fig. 3). Phosphorylation of serine 338 was detectable in the cell lines that survived after IL-3 removal (32D IGF-IR, 32D 6Ser, and 32D 2Ser cells). It was not detectable in the 32D 4Ser cell line. This experiment was repeated at least 3 times, with the same results. Phosphorylation of serine 338 was reproducibly prolonged in 32D IGF-IR cells (still visible at 60 min after IGF-I stimulation). Therefore, it seems that mitochondrial translocation of Raf-1 correlates with its phosphorylation at serine 338.

Nedd4 in the Cytosolic and Mitochondrial Fractions of 32D-derived Cells—The mitochondrial translocation of Nedd4 has never been reported before, and we wanted to confirm its role in another cell line. For this purpose, we used 32D d1245 cells, a mixed population of 32D cells expressing an IGF-IR truncated at residue 1245 (23, 34). At the same time, we asked how much Nedd4 was being translocated to the mitochondria, as a fraction of cytosolic Nedd4. Fig. 4, *panel A*, shows that 32D d1245 cells also fail to translocate Nedd4 to the mitochondria, confirming that a signal originating from the carboxyl terminus of the IGF-IR is required for its mitochondrial translocation. This is important as 32D d1245 cells fail to survive in the absence of IL-3, even when supplemented with IGF-I (34). We confirm that Nedd4 translocates to the mitochondria in 32D 6Ser cells but not in 32D 4Ser cells, when serum is supplemented with IGF-I. Nedd4 is found in mitochondria of all cell lines, when they are grown in IL-3. Fig. 4 also shows that Nedd4 is also predominantly cytosolic. The two panels (mitochondrial proteins and cytosolic proteins) were exposed for 30 min and 20 s,

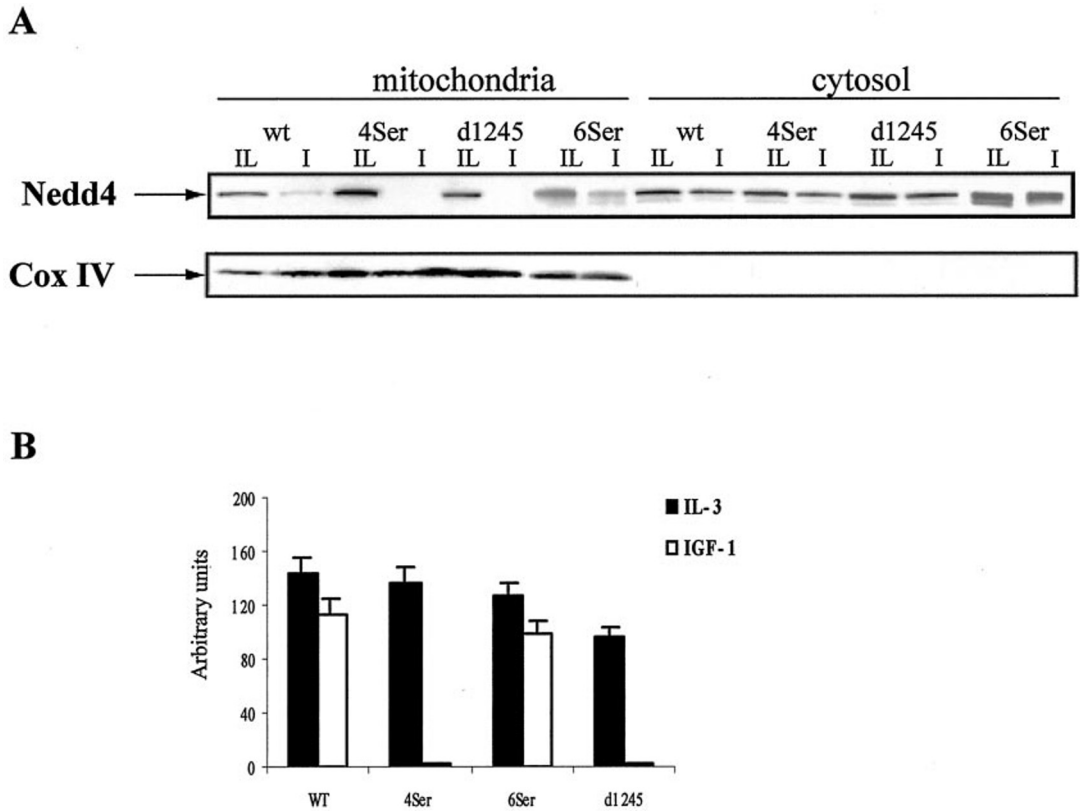

FIG. 4. **Amounts of Nedd4 in the cytosol and mitochondria of 32D-derived cell lines.** *Panel A,* 32D IGF-IR, 32D 4Ser, 32D d1245, and 32D 6Ser cells were used, in serum supplemented with either IL-3 (*IL*) or IGF-I (*I*). The 32D d1245 cells are 32D cells expressing an IGF-IR truncated at residue 1245 (see "Experimental Procedures"). The same amount of protein (40 μg/lane) was applied to each lane. However, the cytosolic fraction was exposed for 20 s, whereas the mitochondrial fraction was exposed for 30 min. Nedd4 is not detectable in the mitochondrial fraction of 32D 4Ser and 32D d1245 cells. The appearance of two isoforms of Nedd4 in the cytosol is discussed in the text. *Panel B,* densitometric analysis of mitochondrial levels of Nedd4 in the cells of *panel A.* Mean and standard deviations of three separate experiments. The only significant differences between IL-3 and IGF-I are found in the cells expressing the 4-Ser and d1245 mutant receptors.

respectively. An approximate calculation suggests that the cytosolic/mitochondrial ratio of Nedd4 (in 32D IGF-IR cells) is about 90:1. These experiments have also been repeated several times, and the purity of the fractions has been constantly monitored (see also below). A densitometric analysis of Nedd4 amounts in the mitochondria is given in Fig. 4, *panel B*, where we show the mean and standard deviations of three separate experiments. Although Nedd4 levels are decreased in all cell lines when they are shifted from IL-3 to IGF-I, the only dramatic differences (roughly a 90% decrease) are noted only in the cell lines expressing the 4Ser and d1245 mutant receptors.

Raf-1 in the Mitochondrial and Cytosolic Fractions of 32D-derived Cells—We have then investigated the fraction of Raf-1 that is translocated to the mitochondria. Fig. 5, *panel B*, shows that the fractionation of the cells into mitochondrial and cytosolic fractions is satisfactory, with the methodology we used. No COX IV is detectable in the cytosolic fraction, and no actin is detectable in the mitochondrial fraction. By using this methodology, we can see that the great majority of Raf-1 is also in the cytosol fraction (Fig. 5, *panel A*). This is true regardless of whether the 32D IGF-IR cells are in IL-3 or in IGF-I. Densitometric measurements of Raf-1 levels in the mitochondria and in the cytosol confirm a decrease of mitochondrial Raf-1 in cells expressing the 4-Ser mutant. Cytosol levels are not significantly different (Fig. 5, *panel E*). A comparison between mitochondria and cytosol indicated that 85–95% of Raf-1 is cytosolic.

Loss of Cytochrome c from the Mitochondria of 32D 4Ser Cells—Apoptosis involving the participation of mitochondria is accompanied by loss of cytochrome *c* from mitochondria and its release into the cytoplasm (48, 49). The mitochondrial fractionation used in this experiment is given under "Experimental Procedures." The purity of the fractions thus obtained is shown in Fig. 5, *panel B*. The failure of the 4-serine mutant receptor to protect from apoptosis and to translocate Raf-1 and Nedd4 to the mitochondria was accompanied by loss of cytochrome *c* from the mitochondria (Fig. 5, *panel C*). There is a corresponding increase in the cytosolic amount of cytochrome *c* (please note that different amounts of protein were needed to visualize cytochrome *c* in the two fractions). Again, in IL-3, the two cell lines behave similarly.

Effect of IRS-1 on Mitochondrial Translocation of Raf-1 and Survival—32D cells do not express IRS-1 or IRS-2 (24, 25). Ectopic expression of IRS-1 in 32D IGF-IR cells inhibits their differentiation (25) and actually transforms them (tumor formation in animals (27)). We expressed IRS-1 in 32D 4Ser cells (see "Experimental Procedures"), as documented in Fig. 6, *panel A*. The cells were then monitored for activation (Fig. 6, *panel C*) and translocation of Raf to the mitochondria (*panel D*). Ectopic expression of IRS-1 in 32D 4Ser cells causes mitochondrial translocation of both Raf-1 and Nedd4 (*panel D*). Finally, we show that 32D 4Ser/IRS1 cells survive, at least up to 48 h after IL-3 withdrawal (*panel B*), whereas the parental 32D 4Ser cells die.

The 6-Serine Mutant Receptor Induces Differentiation of 32D Cells—We have reported previously (25, 27) that the wild type IGF-IR induces the differentiation of 32D cells along the granulocytic pathway. Since the 6-serine mutant receptor gives a survival at least as good as the wild type receptor, we asked whether it also maintained other functions of the IGF-IR, spe-

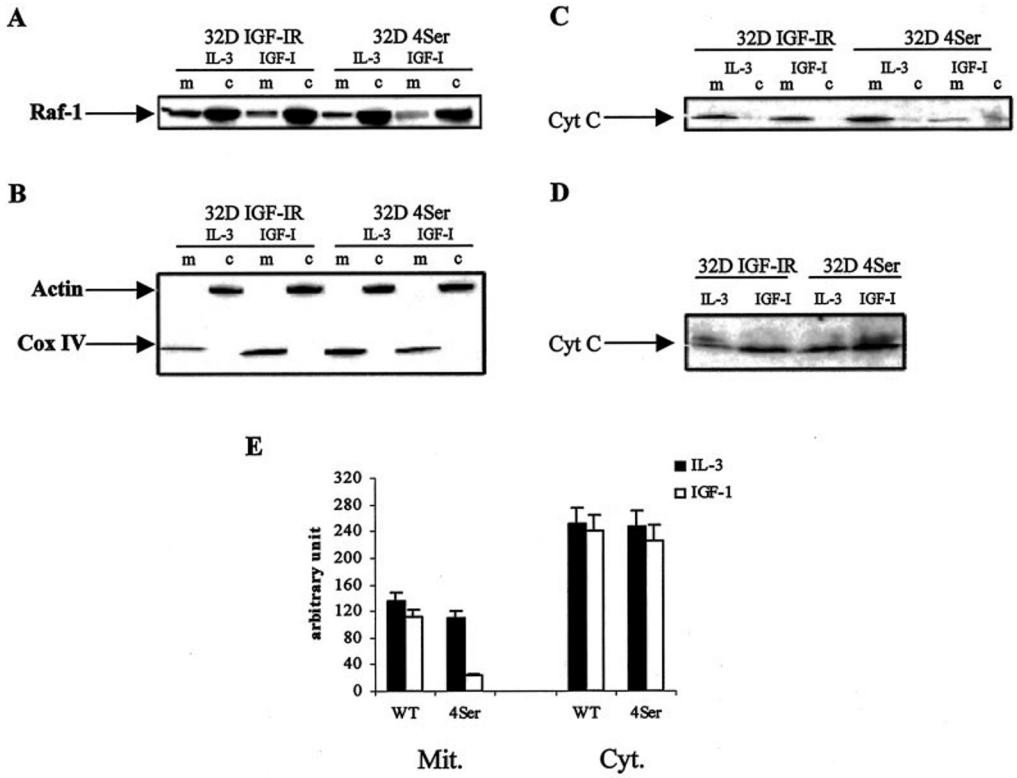

FIG. 5. **Loss of cytochrome *c* from the mitochondria of 32D 4Ser cells.** In *panel A,* we compare the amounts of Raf-1 in the cytosol and mitochondria of the 32D IGF-IR and 32D 4Ser cells. Although the decreased levels of Raf-1 in mitochondria of 32D 4Ser cells is still evident, most of Raf-1 is in the cytosol of both cell lines (40 μg of protein in each lane). *Panel B* shows the purity of the cytosol (*actin*) and mitochondrial (*COX IV*) fractions in these experiments. *Panel C,* cytochrome *c* levels in the mitochondria of the two cell lines (40 μg of protein/lane). *Panel D,* cytochrome *c* levels in the cytosol of the two cell lines (100 μg protein/lane). Each cell line was incubated in either IL-3 or IGF-I. *Panel E,* densitometric analysis of mitochondrial and cytoplasmic levels of Raf-1 (*panel A*). Mean and standard deviations of three separate experiments. The only significant difference between IL-3 and IGF-I was found in the mitochondrial levels of the 4-Ser mutant receptor.

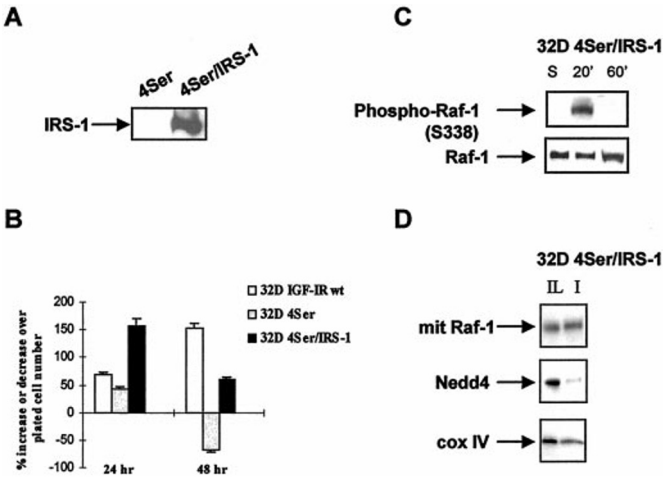

FIG. 6. **Effect of IRS-1 on mitochondrial translocation of Raf-1 and Nedd4.** *Panel A,* expression of IRS-1 in transduced 32D 4Ser cells. *Panel B,* survival of the indicated cell lines in serum supplemented with IGF-I (no IL-3). *Panel C,* phosphorylation of Raf-1 on serine 338 (Raf amounts in the *lower row*). *Panel D,* Raf-1 (*upper row*) in the mitochondrial fraction of 32D 4 Ser/IRS-1 cells. *I* and *IL-3* indicate IGF-1 and interleukin-3, respectively. *Middle row,* same for Nedd4. The levels of COX IV were used to monitor amounts of mitochondrial proteins (*lower row*). The antibodies used were the same as in previous figures.

cifically the ability to induce differentiation of 32D cells. Table I shows that 32D 6Ser cells respond to IGF-I with differentiation, beginning on day 3 after IL-3 withdrawal and IGF-I supplementation. These serines, therefore, are not required for IGF-I-mediated differentiation. It is not possible to determine the effect of a 4-serine mutation on differentiation, because the

TABLE I
The 6-serine mutant of the IGF-I receptor induces differentiation of 32D cells

The percentage of differentiated cells was determined by the methods and the criteria described by Valentinis *et al.* (25). The cells were in medium supplemented with 10% serum and IGF-I. 32D cells expressing the 4-serine mutant receptor were also tested, but all cells were dead by 48 h.

Cell line	Percentage of differentiated cells	
	Day 3	Day 4
32D IGF-IR	55	60.5
32D 6Ser	55	66
32D IGF-IR/IRS1	4	7

cells die within 48 h, and morphological differentiation becomes evident only on day 4 after shifting the cells from IL-3 to IGF-I (25).

Effect of Single Mutations in the 6-Serine Cluster—According to Craparo *et al.* (17) and Furlanetto *et al.* (18) serine 1283 and serine 1272 bind 14.3.3 proteins. We mutated 5 of the 6 serines, singly or in combinations of 2 serines at a time, but the results were disappointing. Only the mutation of the whole serine quartet gave a defective receptor (data not shown). If 14.3.3 proteins are involved in this signaling, the binding in the mammalian cells may be more complex than in the yeast two-hybrid system, perhaps involving conformational changes. The interaction of the IGF-IR with 14.3.3 requires serine phosphorylation (17, 18), an observation we confirmed indirectly. When the serines were mutated to aspartic acid, instead of alanine, the receptor maintained its wild type characteristics (not shown). It should be remembered that the 1280–1283 mutation has little or no effect on phosphorylation of IRS-1 and the

activation of other downstream signaling molecules (16). The mutant receptor is still mitogenic, although it is no longer transforming (16).

DISCUSSION

Our results can be summarized as follows. 1) 32D cells expressing a wild type IGF-IR survive after IL-3 withdrawal, but they die if they express a receptor with a 4-serine mutations (residues 1280–1283). Surprisingly, additional mutations at serines 1272 and 1278 restore a wild type phenotype to the receptor. 2) There is a correlation between survival and mitochondrial translocation of both Raf-1 and Nedd4, a target of caspases (33). 3) Serine 338 of Raf-1 is phosphorylated in 32D cells expressing a wild type IGF-IR or a receptor with mutations at serines 1272 and 1278 and 1280–1283. Serine 338 is not phosphorylated in cells expressing the 4-serine mutant receptor. 4) Failure to translocate Raf-1 and Nedd4 to the mitochondria correlated with leakage of cytochrome *c* from the mitochondria to the cytosol. 5) Ectopic expression of IRS-1 in 32D 4Ser cells restores survival and mitochondrial localization of both Raf-1 and Nedd4.

The most important conclusion is that the 3rd pathway used by the IGF-IR to protect cells from apoptosis correlates with mitochondrial localization of both Raf-1 and Nedd4 and with serine 338 phosphorylation of Raf-1. The fractions of Raf-1 and Nedd4 that translocate to the mitochondria is quite small, in comparison to their amounts in the cytosol. Nevertheless, their absence correlates both with apoptosis and the leakage of cytochrome *c* from the mitochondria to the cytosol.

The mitochondrial translocation of Nedd4 in surviving cell lines is a novel and interesting aspect of these experiments. Nedd4 is a multimodular ubiquitin protein kinase, which displays interaction with several other cellular components, including membranes, protein kinase C, and phospholipids (50). Nedd4 also interacts with the sodium channel (51) and Grb10 (36). Most important in the context of the present experiments is the finding that Nedd4 is a direct target of caspases 1, 3, 6, and 7 (33). Under the usual conditions, Nedd4 is not detectable in the mitochondria of 32D 4Ser cells, but it becomes detectable, although in reduced amounts, when the amount of protein loaded or the length of exposure are increased. There is, however, a constant difference in the mitochondrial amounts of either Raf-1 or Nedd4 between surviving and non-surviving cell lines. Usually only one Nedd4 band is visible, but two bands can be detected in the cytosolic fraction. The presence of more than one isoform of Nedd4 has been reported previously (33, 52, 53).

As mentioned, Nedd4 interacts with Grb10 (36), and Grb10 has been shown repeatedly to partner both the IGF-IR and the insulin receptor (41–43). Grb10 interacts with Raf-1 (44) and co-localizes with it in the mitochondria (21). Despite repeated attempts, we could not obtain convincing evidence of the presence of Grb10 in 32D cells, let alone in mitochondria of 32D cells. We entertained the possibility of ectopic expression of Grb10, but Nantel *et al.* (21) have warned that overexpression of Grb10 alters its intracellular distribution. Although we could not demonstrate the presence of Grb10 in 32D cells, the absence of Nedd4 from the mitochondria of 32D 4Ser cells (in IGF-I and no IL-3) was clear and reproducible.

Since Nedd4 interacts with phospholipids and membranes, and with an ion channel (see above), its presence in the mitochondria of surviving cell lines raises some interesting speculations. The importance of mitochondria and the integrity of their membranes in apoptosis is well established (19, 32) and has been discussed in more than one review (40, 48, 54). It is interesting that the presence of Nedd4 and Raf-1 in the mitochondria correlates with mitochondrial integrity, whereas their absence results in loss of cytochrome *c* to the cytosol. At the moment, one can only speculate on how Nedd4 could participate in the anti-apoptotic action of the IGF-IR. Considering some of interactions of Nedd4 (see above), the role of Ca^{2+} in triggering mitochondrial permeability and the ability of proteins of the Bcl-2 family to form ion channels (48) are possibilities that one should consider. Since Nedd4 can also function as a ubiquitin ligase (see above), an alternative hypothesis is that it may target pro-apoptotic proteins in the mitochondria.

The other interesting aspect of these experiments is the paradoxical effect of the additional mutations at serines 1272 and 1278 on the survival of 32D cells. When the receptor is mutated at all 6 serines in the 1272/1283 region, the receptor returns to normal, both in its anti-apoptotic action as well as in the ability to induce IGF-I-mediated differentiation of 32D cells. It also returns to wild type phenotype in terms of mitochondrial translocation of Raf-1 and Nedd4. As already mentioned, serine 1283 binds 14.3.3 isoforms (17, 18). According to Craparo *et al.* (17), serine 1272 also binds 14.3.3, and all investigators agree that 14.3.3 proteins bind even better to IRS-1 than to the IGF-IR (17, 18, 28) and do not bind at all to the insulin receptor. This may explain why the 4-serine mutant is more incapacitated in 32D cells (no IRS-1) than in mouse embryo fibroblast expressing high levels of IRS-1 proteins (16, 26). Indeed, this mutant receptor is mitogenic and anti-apoptotic in cells expressing IRS-1 (16, 34). We have no explanation why single mutations at serines 1280–1283 have no effect on the IGF-IR survival function. Perhaps, in living mammalian cells, binding of 14.3.3 proteins requires more than one serine. To explain our results with the 4-serine and 6-serine mutant receptors, a reasonable hypothesis could be based on the ambiguous effects of 14.3.3 proteins on the activation of Raf-1. According to Thorson *et al.* (55), 14.3.3 proteins are required for Raf-1 activation, and several papers (45, 46, 56) have shown that 14.3.3 proteins can stabilize Raf-1 in both its inactive and active forms. These contradictory effects have been discussed in reviews by Reuther and Pendergast (57) and by Hagemann and Rapp (47). Hagemann and Rapp (47) have proposed that inactive Raf is bound to 14.3.3 proteins, at both the amino and the carboxyl termini (serines 259 and 621, respectively). When activated by Ras, the 14.3.3 protein at the amino terminus (but not the carboxyl terminus) is released, leading to a change in conformation. The activated Raf-1 is then stabilized again in its active form by binding 14.3.3 to a not yet identified serine between serine 259 and the ATP-binding site of Raf-1 (56). We hypothesize that the 4 serines are required for the first step in the scheme of Hagemann and Rapp (47), *i.e.* release of 14.3.3 from serine 259. When these serines are mutated, serines 1272/1278 freeze Raf-1 in an inactive conformation. Further mutations in these two last serines release Raf-1 from its inactive status and allow its activation (phosphorylation of serine 338 and mitochondrial translocation). In support of this hypothesis is the finding that the mutant Raf that is targeted for mitochondrial translocation lacks serine 259 (20). In fact, we have already reported that this mutant Raf lacking serine 259 rescues 32D 4Ser cells from apoptosis (10). In this model, ectopic expression of IRS-1 also restores survival and mitochondrial translocation of both Raf-1 and Nedd4, presumably because IRS-1 interacts strongly with 14.3.3 proteins, thus replacing the function of the serine quartet.

In conclusion, we would like to propose the following model. The 6 serines in the 1272–1283 region of the IGF-IR modulate Raf-1 activation, as proposed above. A small fraction of the activated Raf-1 translocates to the mitochondria, where it exerts its anti-apoptotic action by interacting (via Grb10?) with Nedd4, a target of caspases. This pathway is a salvage path-

way, which becomes prominent when the cells (like 32D cells) do not express IRS-1. The presence of IRS-1 restores the mitochondrial translocation of Raf-1 and Nedd4, even when the serine quartet is mutated, presumably by its own interaction with 14.3.3 proteins (17, 18, 28). As to the mechanism(s), we already know that mitochondrial translocation of Raf-1 results in the inactivation of BAD (10, 19, 20). Since Nedd4 is a ubiquitin ligase (50), it is tempting to speculate that it may act by targeting BAD or another member of the Bcl-2 family of proteins.

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