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Signaling Differences from the A and B Isoforms of the Insulin Receptor (IR) in 32D Cells in the Presence or Absence of IR Substrate-1

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The A isoform of the insulin receptor (IR) is frequently overexpressed in cancer cells and is activated by IGF-II as well as by insulin, whereas the B isoform is predominant in differentiated tissues and responds poorly to IGF-II. The IR substrate-1 (IRS-1), a docking protein for the IR, is known to send a mitogenic signal and to be a powerful inhibitor of cell differentiation. We have investigated the biological effects of the two IR isoforms in parental 32D hemopoietic cells, which do not express IRS-1, and in 32D-derived cells in which IRS-1 is ectopically expressed. The effects of the two isoforms on cell survival, differentiation markers and nuclear translocation of IRS-1 were compared. The results confirm that the A isoform responds to IGF-II and preferentially sends mitogenic, antiapoptotic signals, whereas the B form, poorly responsive to IGF-II, tends to send differentiation signals. (*Endocrinology* 144: 2650–2658, 2003)

THE INSULIN RECEPTOR (IR) and the type I IGF receptor (IGF-IR) share a high degree of homology. After ligand binding, the receptors activate common intracellular mediators involved in the regulation of cell metabolism, proliferation and survival (1–3). According to classical view, the IR predominantly mediates anabolic effects, whereas the IGF-IR predominantly mediates antiapoptotic, mitogenic, and transforming effects (4, 5).

However, several lines of evidence also support a mitogenic and transforming role of the IR (6, 7). The IR is overexpressed in a variety of malignancies including breast, thyroid, ovary, renal, gastrointestinal, brain tumors, and leukemia (8–10). The IR can be activated by IGF-II, a cytokine frequently expressed in cancer (see below). In mouse embryo fibroblasts (MEF) devoid of IGF-IR, IGF-II sends a proliferative signal through the IR (11). Genetic studies have also shown that IGF-II can stimulate mouse embryo growth through the IR (12). IGF-II binds with higher affinity to the isoform A of the IR (IR-A) than to isoform B (IR-B). IR-A, generated by exon 11 skipping, is characterized by the absence of 12 amino acid residues at the carboxyl terminus of the IR α-subunit and is predominantly expressed in fetal tissues and cancer cells (13–15). In contrast IR-B, containing the 12 amino acid residues encoded by exon 11, binds insulin well and IGF-II poorly, and is predominantly expressed in adult differentiated cells (13, 16).

Limited data are available with regard to functional differences between IR-A and IR-B. IR-A binds insulin with a slight higher affinity, whereas IR-B elicits a slightly stronger kinase activation (17, 18). IR-A appears more efficient in mediating receptor endocytosis and insulin degradation (19). Recently, it has been suggested that the two IR isoforms have somewhat different signaling pathway, thus providing a mechanistic basis for selective insulin action (20). Our previous data indicate that different ligands may affect IR-A biological effects. In mouse fibroblasts devoid of IGF-IR and transfected with the IR-A, insulin has a more pronounced metabolic effect, whereas IGF-II has a more pronounced mitogenic effect (13). This predominant mitogenic effect in response to IGF-II may be relevant to tumor progression, as many malignancies overexpress IR-A and produce IGF-II (21, 22).

To further investigate the differences between the two receptor isoforms in survival, differentiation and intracellular signaling we have extended our studies to 32D cells. 32D cells are a murine hemopoietic cell line, which has an absolute requirement for IL-3 and undergoes massive apoptosis upon IL-3 withdrawal (23, 24). Parental 32D cells have low levels of both insulin and IGF-I receptors, and do not express IRS-1 or IRS-2 (25, 26). Ectopic expression of IRS-1 in 32D cells delays but does not prevent apoptosis (27, 28). When the levels of IGF-IR are increased by transfection with a wild-type human IGF-IR, the cells (32D IGF-IR cells) survive the shift from IL-3 to IGF-I (29) but eventually differentiate (26). Introduction of IRS-1 in 32D IGF-IR cells inhibits differentiation, and transforms the cells, which form tumors in mice (30). Overexpression of the IR in parental 32D cells has little or no effect on survival (25, 31). Overexpression of the IR combined with ectopic expression of IRS-1, however, results in survival after IL-3 withdrawal (25, 31). A reasonable explanation is that IGF-I- or insulin-mediated survival of 32D cells requires at least two of three signaling pathways (32):

Abbreviations: IGF-IR, Type I IGF receptor; IR, insulin receptor; IR-A or -B, isoform A or B of the IR; IRS-1, IR substrate-1; MEF, mouse embryo fibroblasts; MPO, myeloperoxidase; SV40, simian virus 40; UBF, upstream binding factor.

the IRS-1/Akt.p70^{S6K,} the Y950 (Y960 in the IR)/Shc/Ras/MAPK pathway, and a third pathway that depends on the integrity of a serine quartet at 1280–1283 of the IGF-IR (31, 33). The IR (that does not have the serine quartet) has only two pathways for an antiapoptotic signal, IRS-1 and the Y960 pathways. In the absence of IRS-1, the IR has only one pathway and cannot protect 32D cells from apoptosis (32).

32D and 32D-derived cell lines are an attractive model to study mitogenic and differentiation signals of the two isoforms in the presence or absence of IRS-1. As end points of signaling, we have taken: 1) cell survival after IL-3 withdrawal, and supplementation with ligands (IGF-II or insulin); 2) the induction of differentiation markers, like myeloperoxidase (MPO) mRNA (30) or the levels of Id2 proteins. Id2 proteins are induced by IGF-I (34–36) and inhibit differentiation (37–39); 3) because the proliferative stimulus of IRS-1 may be associated with its nuclear translocation (40–42), we have investigated whether either isoform of the IR can induce nuclear translocation of IRS-1. Finally, 4) we have determined the expression of lipocalin 24p3, a glycoprotein induced by IL-3 withdrawal in a variety of IL-3-dependent cell lines, including 32D cells (43).

Materials and Methods

Plasmids

pMSCV-IRS-1 was generated from pMSCV-pac retroviral vector by fusing the wild-type mouse IRS-1 sequence. The pNTK2 expression vectors containing the cDNA for either IR-A (Ex11−) or IR-B (Ex11+) were kindly provided by Axel Ullrich (Munich, Germany; Ref. 13).

Cell lines

32D IR-A and 32D IR-B cells were derived from the 32D murine hematopoietic cell line clone 3, stably transfected with a plasmid expressing the human cDNA for either IR-A (Ex11−) or IR-B (Ex11+; Ref. 44). 32D IRS-1 cells were generated by retroviral transduction of 32D cells with an MSCV-IRS-1 retroviral vector carrying puromycin resistance. 32D IRS-1 IR-A and 32D IRS-1 IR-B cells are 32D IRS-1 cells stably transfected with either IR-A or IR-B plasmids. All these cell lines are mixed populations. Cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Inc., Gaithersburg, MD), 10% WEHI cell-conditioned medium (as a source of IL-3), 2 mM L-glutamine (Life Technologies, Inc.), and the required antibiotic to maintain the selective pressure (600 μg/ml G418 for 32D IR-A and 32D IR-B cells, 600 μg/ml G418 plus 1 μg/ml puromycin for 32D IRS-1 IR-A and 32D IRS-1 IR-B cells). For brevity, the WEHI cell-conditioned medium will be referred to as IL-3.

Transfection

Cells were transfected by electroporation. Briefly, 10×10^6 cells were suspended in electroporation buffer which consisted of a mixture of 20 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM D-glucose, buffered to pH 7. The plasmids containing the IR-A or IR-B cDNA (20 μg of DNA) were mixed with the cell suspension and placed in a 4 mm-gap electroporation cuvette (Bio-Rad Laboratories, Inc., Hercules, CA). Cells were electroporated at a fixed capacitance of 960 μF and 0.32 kV using a Bio-Rad Laboratories, Inc. Gene Pulser instrument Bio-Rad Laboratories, Inc. The electroporated cells were transferred to a 75-cm^2 flask containing complete medium and placed in the incubator. The medium was replenished after 24 h. The antibiotic selection was added after 48 h.

Survival

Exponentially growing cells were washed 3 times with HBSS and seeded 5×10^4/ml in IL-3-free medium (RPMI 1640 medium containing 10% heat-inactivated FBS) supplemented with insulin (Sigma, St. Louis, MO) or IGF-II (Life Technologies, Inc.), 10 nM or 10% WEHI cell conditioned medium. Cells were counted by standard procedures (31, 36). Statistical analysis was carried out as previously described (31).

Northern blots

Cells were washed three times and seeded under the same conditions used for growth analysis. Cells incubated with IL-3 served as controls. At the indicated time points, cells were collected, and total RNA was extracted using the RNeasy kit (QIAGEN Inc., Valencia, CA) following the manufacturer's instructions. Ten micrograms of total RNA for each sample were run on 1% agarose-formaldehyde gel, blotted onto a nylon membrane, and hybridized with a 1.45-kb myeloperoxidase cDNA fragment obtained from pUC19-MPO6 plasmid (a kind gift from Dr. M. Valtieri) or with a full-length 24p3 mouse cDNA (41).

Immunoprecipitation and immunoblots

For IR and IRS-1, cells were lysed with lysis buffer (50 mM HEPES, pH 7.5; 150 mM NaCl; 1.5 mM $MgC1_2$; 1 mM EGTA; 10% glycerol; 1% Triton X-100; 100 mM NaF; 10 mM Na-pyrophosphate; 0.2 mM Na-orthovanadate; 1 mM phenylmethylfulfonyl fluoride; 10 μg/ml Aprotinin). Fifty to 100 μg of proteins were resolved on 4–15% SDS-PAGE and after transfer, the nitrocellulose membrane was probed with a rabbit polyclonal anti-IR-β subunit (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or with a rabbit polyclonal anti-IRS-1 antibody (Upstate Biotechnology, Inc., White Plains, NY). For Id2 detection, exponentially growing cells were washed in Hanks' balanced solution to remove IL-3 and incubated for the indicated times in medium with 10% heat-inactivated fetal bovine serum plus insulin or 10 nM IGF-II. Western blotting for the Id2 and Grb2 protein was carried out by standard techniques as previously described (34). All the experiments were repeated at least three times to monitor the reproducibility of our results.

Immunohistochemistry

32D and 32D-derived cells were washed three times with Hanks' buffer and seeded at a density of 5×10^4/2ml of RPMI supplemented with 10% heat-inactivated FBS plus or minus the growth factors, as indicated. Cells were harvested after 16 h and cytospins prepared. After fixing in 3.7% formaldehyde solution in PBS and permeabilization with 0.2% Triton X-100 in PBS, the immunostaining was carried out using the Histomouse SP Kit (95-9541, Zymed Laboratories, Inc., South San Francisco, CA) following the manufacturer's protocol.

Antibodies

The antibodies used included: a rabbit polyclonal anti IR-β subunit (Santa Cruz Biotechnology, Inc.), and a rabbit polyclonal anti-IRS-1 antibody (Upstate Biotechnology, Inc.). The antibody for the Id2 protein (Santa Cruz Biotechnology, Inc.) was diluted 1:500 in TBS-T/5% milk). The anti-Grb2 antibody was a mouse monoclonal antibody from Transduction Laboratories, Inc. (Lexington, KY) and was used at 1:1000 in TBS-T/5% milk.

Results

Cell lines

From parental 32D cells, we generated mixed populations of cells overexpressing either the A or the B isoforms of the IR. Figure 1 shows that the populations transfected with the IR's isoforms overexpress the IR (compare lanes 3 and 4 to lane 5). A weak band of IR can be also detected in parental 32D cells as also shown by Peruzzi *et al.* (31). From 32D IRS-1 cells, we obtained two other mixed populations by transfection with the same plasmids. These latter cell lines (again mixed populations) express high levels of IR and IRS-1. The A isoform cells express somewhat more IRS-1 than the isoform B cells, which, in turn, express slightly higher levels of

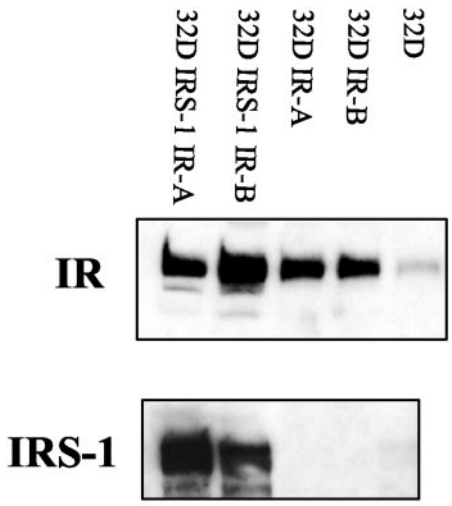

FIG. 1. Expression of IR and IRS-1 in 32D-derived cells. Parental 32D cells were transfected with plasmids expressing either the IR-A or IR-B isoforms, to generate mixed populations overexpressing either form of the IR. Each of these mixed populations was subsequently transduced with a retroviral vector expressing IRS-1, and new mixed populations were selected. Levels of receptor and IRS-1 were determined in cell lysates by Western blot with the appropriate antibodies (see *Materials and Methods*). The *right lane* is a lysate from parental 32D cells that express low levels of IR.

IR (Fig. 1, lanes 1 and 2). We believe these differences are not crucial, as the IR is highly expressed in both cell lines (approximately 10^5 receptors/cell). The difference in IRS-1 expression is also not crucial. When single cell clones are generated from mixed populations, the levels of expression of IRS-1 vary greatly from one clone to another (45), yet all clones grow equally well. In fact, even low levels of IRS-1 expression can make 32D IGF-IR cells tumorigenic in mice (30). In subsequent experiments, the cell populations will be designated as indicated in Fig. 1.

Cell survival

Like all 32D-derived cell lines, these cell lines grow very well in serum supplemented with IL-3 and die promptly in serum deprived of IL-3 and not supplemented with growth factors. In the following figures, we show only the growth in IL-3 of 32D IR-A and 32D IR-B cells (Fig. 2A), but all cell lines grew in IL-3. The cells overexpressing the IR isoforms and no IRS-1 die when IL-3 is withdrawn, even when the serum is supplemented with insulin or IGF-II (Fig. 2A). They die so rapidly that by 24 h most of the cells are dead, like parental 32D cells (31). For comparison, we show that 32D cells expressing the IGF-IR grow well in IGF-I, at least for 48 h, confirming that, in the absence of IRS-1, the IGF-IR has other antiapoptotic pathways not shared with the IR (33). The modality of cell death is by apoptosis, as repeatedly shown

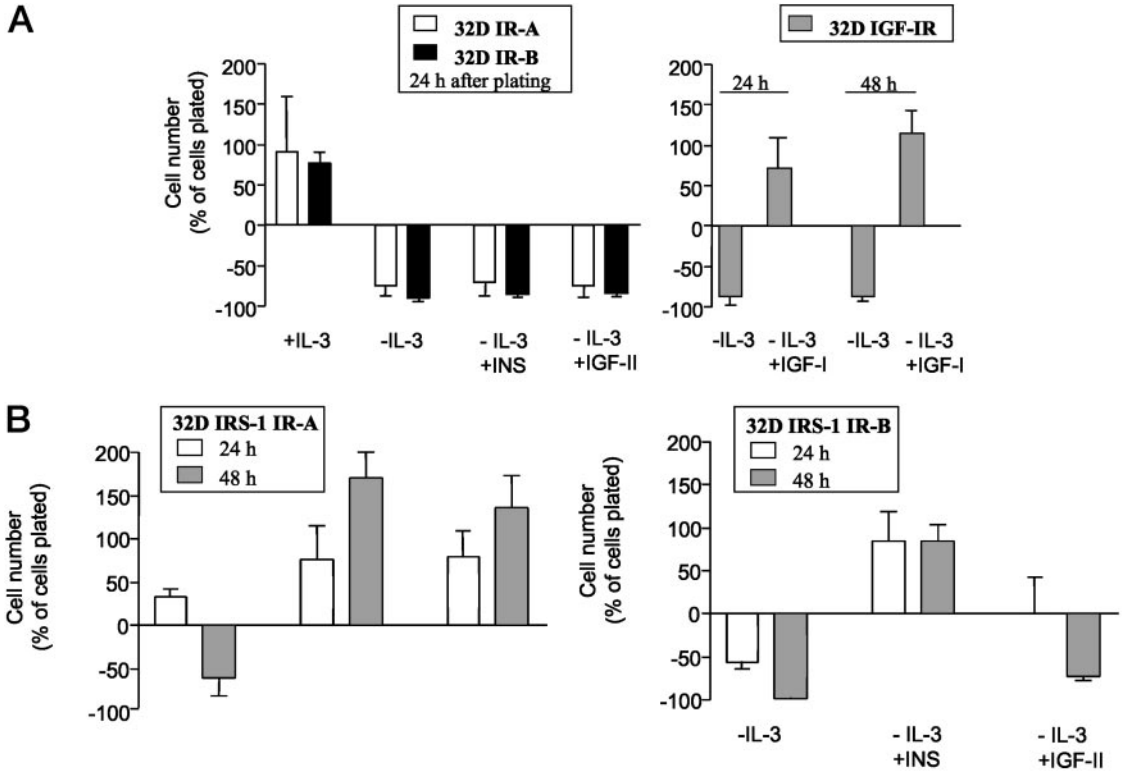

FIG. 2. Survival of 32D-derived cells after IL-3 withdrawal. The cell lines used are those described in Fig. 1. Survival was determined at 24 and 48 h after IL-3 withdrawal, or supplementation with either insulin (10 nM) or IGF-II (10 nM). The number of cells was counted, and the results are expressed as percentage increase (or decrease) over cells plated. A, Survival of 32D-IR-A and 32D-IR-B cells. No 48-h count could be made because most of the cells were dead by 24 h. Both cell lines grow in IL-3. To the *right*, we show the growth of 32D IGF-IR cells, in the absence or presence of IGF-I after IL-3 withdrawal. B, Survival of 32D IRS-1 IR-A and 32D IRS-1 IR-B under the same conditions and up to 48 h. Both cell lines survive in insulin, but only the 32D IRS-1 IR-A cells survive and grow in IGF-II. The data given are the results of four different experiments.

in several papers from this and other laboratories (24, 26, 31, 32). When 32D IR-A cells express IRS-1, they now survive and grow in both insulin and IGF-II. 32D IRS-1 IR-B cells grow in insulin, but die in IGF-II, albeit more slowly than in 10% serum. These experiments were repeated several times and confirm previous reports indicating a difference between the two isoforms in their ability to respond to IGF-II (13, 14). They also confirm that in cells expressing IRS-1, the IR is as effective as the IGF-IR in protecting cells from apoptosis (25, 46).

Expression of MPO mRNA

In 32D IGF-IR cells, the receptor sends a differentiation signal that can be detected in the first 24 h after IL-3 is replaced by IGF-I. The marker of differentiation is MPO mRNA, which can be detected even while the 32D IGF-IR cells are still growing exponentially, in the first 48 h after shifting from IL-3 to IGF-I (26). This differentiation program is extinguished by the expression of IRS-1 (30). The MPO marker is very convenient as it is detectable at an early stage; therefore, it can be used to find out whether a differentiation program has been established even in cells programmed for eventual apoptosis (Ref. 35; and see *Discussion*). We determined the expression of MPO mRNA in the four cell lines at 24 h after shifting the cells from IL-3 to different growth conditions (Fig. 3). MPO mRNA is clearly present in 32D IR-B cells regardless of the presence or absence of ligands. It is present, but weakly, in 32D IR-A cells. It suggests that IR-B, like the IGF-IR, can initiate a differentiation program in 32D cells, presumably by default, as this program is also activated in cells stimulated with IGF-II. The differentiation program is interrupted by the premature death of cells not expressing IRS-1. The presence of IRS-1 decreases or even abrogates the induction of MPO mRNA. Thus, ectopic expression of IRS-1 in these cells induces survival (see Fig. 2) and abolishes the differentiation program (Fig. 3). Similar results were obtained with the IGF-IR, where ectopic expression of IRS-1 inhibits differentiation (26, 30).

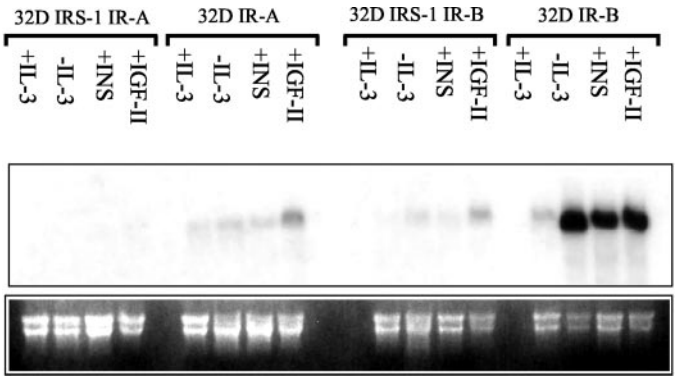

FIG. 3. Expression of MPO mRNA in 32D-derived cells. The four cell lines are the same as described in Fig. 1. The levels of MPO mRNA were determined by Northern blots as described in *Materials and Methods*, at 24 h after IL-3 withdrawal and supplementation with the indicated growth factors. Repeated experiments gave the same results.

Expression of Id2 protein

Id2 protein levels are usually increased in proliferating cells, and decrease markedly in differentiated cells (37, 38, 47, 48). An increase in Id2 mRNA and protein caused by the activation of the IGF-IR has been reported from one of our laboratories (34, 35). In those experiments, the expression of Id mRNA and proteins was markedly increased by the presence of IRS-1 (34). We have determined the effect of the two isoforms of the IR on the expression of Id2 proteins in 32D-derived cells, and the results are shown in Fig. 4, A–C. Id2 protein levels increase in all four cell lines, when IL-3 is replaced by either insulin or IGF-II. The increase is temporary, as protein levels decrease after 12 h. The high level of Id2 in panel C for the cells expressing IRS-1 and the IR-B isoform at 72 h is largely due to the amount of protein in that lane (see the Grb2 control). The increase in Id2 expression is unaffected by the presence of IRS-1. This is true for both receptors and is at variance with the results obtained with the IGF-IR. In 32D-derived cells expressing the IGF-IR, IRS-1

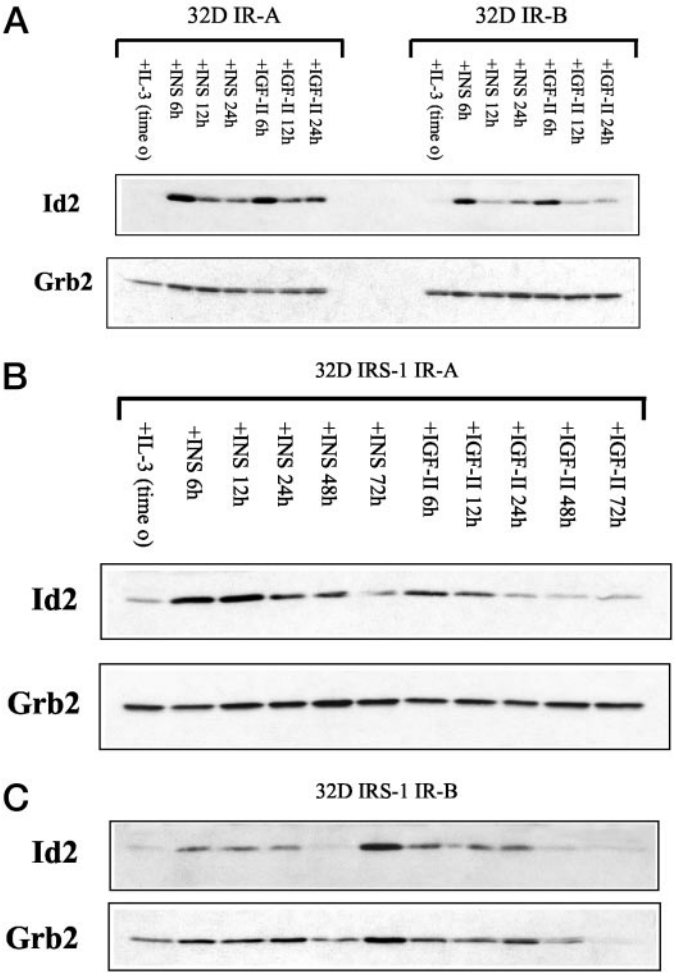

FIG. 4. Up-regulation of Id2 gene expression in 32D-derived cell lines. Levels of Id2 proteins were determined by Western blots using the antibodies described in *Materials and Methods*. A, 32D IR-A (*left*) and 32D IR-B (*right*). B, 32D IRS-1 IR-A. C, 32D IRS-1 IR-B. Treatment is indicated *above the lanes*. The amounts of protein in each lane were monitored with an antibody to Grb2. These experiments have been repeated several times, with similar results.

strongly increased Id2 protein levels (34). These experiments have been repeated several times. They show that the IR, like the IGF-IR, can induce Id2 gene expression. At variance with the IGF-IR, IRS-1 has little effect on the levels of Id2 proteins.

Nuclear translocation of IRS-1

We have previously demonstrated, in different cell lines, that IRS-1 is tyrosyl phosphorylated by both isoforms of the IR (13). We have confirmed in 32D-derived cells that IRS-1 is tyrosyl phoshorylated by both forms (not shown). More recently, Reiss and co-workers (40) and one of our laboratories (41, 42) have reported that IRS-1 can translocate to the nuclei of cells stimulated with IGF-I or transformed by oncogenes, like viral T antigens and v-src. We investigated by histochemistry the subcellular localization of IRS-1 in 32D-derived cell lines. A representative experiment is shown in Fig. 5, where immunohistochemistry was carried out on 32D IRS-1 IR-A and 32D IRS-1 IR-B cells. The cells were shifted from IL-3 to either insulin or IGF-II, fixed after 16 h, stained for IRS-1, and counter-stained with hematoxylin. Translocation of IRS-1 into the nuclei is detected by the change in the color of the nuclei (42). IRS-1 is mostly, if not completely, cytoplasmatic in unstimulated cells and in 32D IRS-1 IR-B cells stimulated with IGF-II. Insulin causes nuclear translocation of IRS-1 in both cell lines, and IGF-II in 32D IRS-1 IR-A cells. The difference in size of the 32D IRS-1 IR-B cells, unstimulated or stimulated with IGF-II was also reproducible (compare with the cells in insulin). These results were confirmed by confocal microscopy (not shown, see *Discussion*).

Expression of 24p3

Devireddy *et al.* (43) have reported that IL-3 withdrawal causes the expression of a lipocalin, designated as 24p3. This lipocalin is secreted into the medium and according to Devireddy *et al.* (43), causes apoptosis of IL-3-dependent cells. IGF-I inhibits the transcription of 24p3 (43), but the presence of IRS-1 is crucial (41). We tested the expression of 24p3 mRNA in our selected cell lines, under different growth conditions. We are presenting only the data obtained up to 16 h after IL-3 withdrawal, because with some of these cell lines, cells start dying at 16 h (see Fig. 2). Figure 6, A and B, shows that 24p3 is induced in 32D IR-A and 32D IR-B cells, regardless of the growth factors added, and provided that IL-3 is withdrawn. This result suggests that 24p3 induction is caused not by the ligands but simply by IL-3 withdrawal. When 32D IR-B cells express IRS-1, 24p3 is poorly induced. In 32D IRS-1 IR-A, no or very little 24p3mRNA is detectable under these conditions. These experiments confirm that IRS-1 inhibits 24p3 mRNA induction after IL-3 withdrawal. A reasonable explanation is that IRS-1 also plays a major role in inhibiting 24p3 transcription in cells expressing the IR. Figure 6C confirms that the IGF-IR does not need IRS-1 to inhibit 24p3 induction, as 24p3 is not induced (or barely so) in 32D IGF-IR cells, up to 24 h after shifting from IL-3 to IGF-I.

Discussion

The roles of the IGF-IR and IRS-1 in the growth, survival, and transformation of 32D cells have been studied in several

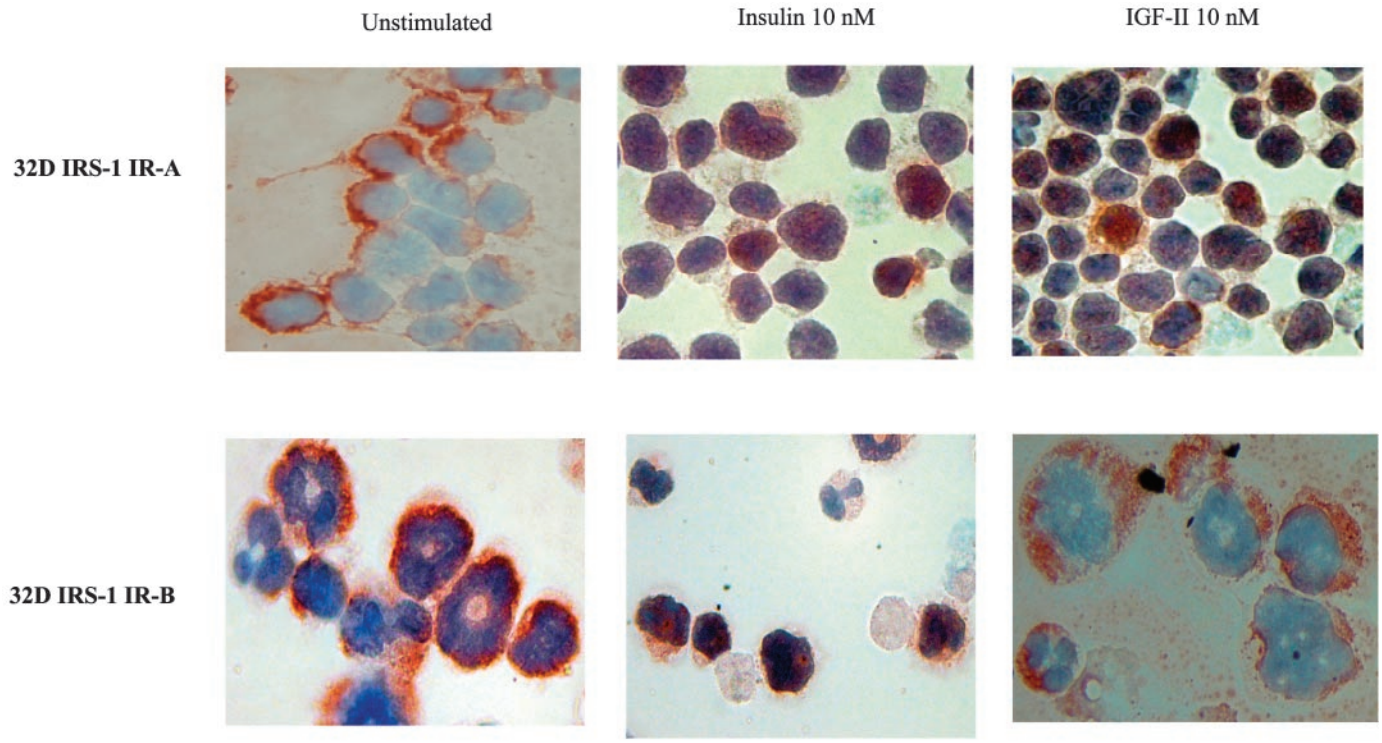

FIG. 5. Immunohistochemistry of 32D IRS-1 cells expressing either the A or the B isoforms of the IR. The cells were stained with an antibody to IRS-1 and counterstained with hematoxylin (42). *Upper panels* are 32D IRS-1 IR-A cells, *lower panels* 32D IRS-1 IR-B cells, 16 h after shifting from IL-3 to either insulin or IGF-II. The panels, in order from the *left*, are: no ligands, insulin, IGF-II. IRS-1 is translocated to the nuclei by both ligands in the IR-A cells, but only by insulin in cells with the B isoform. Magnification, ×1000.

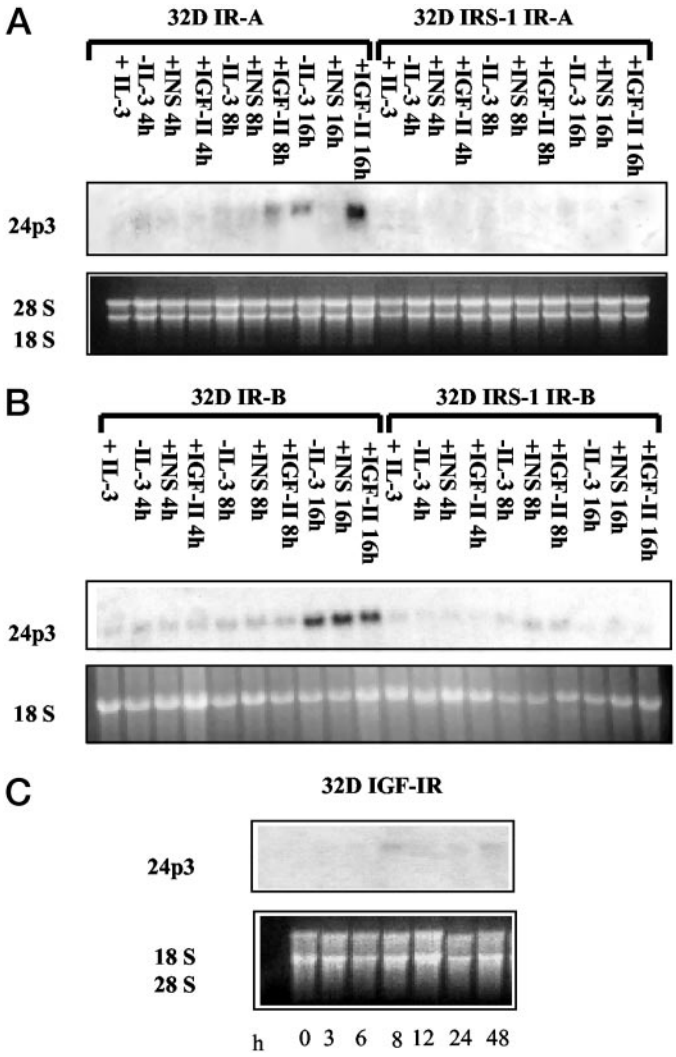

FIG. 6. Expression of 24p3 in 32D-derived cells. The levels of 24p3 mRNA were determined by Northern blot in the various cell lines as indicated and up to 16 h after IL-3 withdrawal. Growth factors' supplementation is indicated *above the lanes*. A, 32D IR-A and 32D IRS-1 IR-A cells. B, 32D IR-B and 32D IRS-1 IR-B cells. C, 32D IGF-IR cells 24 h after shifting from IL-3 to IGF-I.

papers from one of our laboratories (26, 30–32, 36, 41, 49). In this paper, we have examined the effect of the two isoforms of the IR on 32D and 32D-derived cells. There are two interesting comparisons to be made in the discussion. The first is the comparison between IGF-II and insulin stimulation on the two isoforms of the IR. The second interesting comparison is between the two IR and the IGF-IR.

32D cells are a good model for studying IGF-I and IR signaling because of the various permutations they offer. The absence of expression of IRS-1 does not make 32D cells more artificial than other cells in culture. During differentiation, the expression of IRS-1 is modulated. Cell types prone to differentiation often do not express IRS-1 or express very low amounts. This is true of hemopoietic cell lines (25), especially myeloid cell lines (50), but also of neuronal cells (51) and myoblasts (52). Furthermore, in cells with low levels of IRS-1, induction of differentiation causes a further decrease in IRS-1 expression (53, 54). A convincing demonstration is offered by skeletal muscle. IRS-1 is barely detectable in skeletal muscle, is highly expressed in growing myoblasts, and markedly decreases when myoblasts differentiate into myotubes (52). The absence of IRS-1 in 32D cells, therefore, should not be considered as a unique case. On the contrary, absence of IRS-1 in 32D and other cell lines should be considered a model for cell differentiation.

With this premise, we can summarize our results as follows: 1) Both isoforms, overexpressed, fail to protect parental 32D cells from apoptosis caused by IL-3 withdrawal, regardless of the growth factor added (insulin or IGF-II). 2) If 32D cells overexpress both the IR and IRS-1, the results after IL-3 withdrawal are different. The cells expressing the A isoform and IRS-1 survive if the medium is supplemented with either insulin or IGF-II. The cells expressing the B isoform and IRS-1 survive in insulin, but not in IGF-II. 3) A differentiation program, as monitored by MPO expression, is strongly activated by the B isoform, and more weakly by the A isoform. In both instances, IRS-1 expression abrogates the differentiation program, as it does with the IGF-IR (26). 4) At variance with the IGF-IR, IRS-1 does not increase further the activation of Id2 gene expression by the two isoforms of the IR in the absence of IL-3. 5) Nuclear translocation of IRS-1 is detectable when either isoform is stimulated by insulin, but only the IR-A isoform induces translocation with IGF-II. 6) 24p3 mRNA is induced by IL-3 withdrawal. Induction of 24p3 is inhibited by the IGF-IR (alone) or by either isoform of the IR in combination with IRS-1. This strongly suggests that IRS-1 plays a major role in suppressing the induction of 24p3. For the convenience of the reader, a comparison between the two isoforms of the IR and the IGF-IR are summarized in Table 1.

These experiments confirm that the IR, without IRS-1, cannot protect 32D cells from apoptosis, whereas the IGF-IR is fully protective by itself (31). This is true of either isoform of the IR. Ectopic expression of IRS-1 rescues the antiapoptotic activity of the IR. The requirement for IRS-1 is confirmed by the observation that in MEF, that express endogenous IRS-1, the antiapoptotic activities of the IGF-I and IRs are only marginally different (46). This is due to the fact that the IGF-IR has alternative pathways for survival, not shared with the IR (31, 33). Although both isoforms protect 32D cells from apoptosis when expressing IRS-1 and stimulated with insulin, only the A isoform does so, when the cells are stimulated with IGF-II. Thus, we confirm in 32D cells the different response of the two isoforms to IGF-II (see *Introduction*).

The IGF-IR is known to send a differentiation signal, which is abrogated by IRS-1 (reviewed in Ref. 55). This is especially apparent in 32D IGF-IR cells, where the shifting of cells from IL-3 to IGF-I causes, after a 48-h period of exponential growth, differentiation along the granulocytic pathway (26). While these cells are growing exponentially, early markers of differentiation are already apparent, such as an increase in MPO mRNA levels (26, 35). These markers are extremely useful to detect the induction or the lack of induction of a differentiation program in those 32D-derived cells that die before granulocytes can be morphologically detected (usually 4 d after shifting from IL-3 to IGF-I). Using this approach, we show here that, in the absence of IRS-1, the IR sends in

TABLE 1. Comparative effects of the IGF-1 and insulin receptors on 32D cells

Receptor	IRS-1	Ligand	Survival and growth	MPO	Id2	24p3	Nuclear IRS-1
IRA	−	IGF-II	−	+	+	+	NA
IRA	−	Insulin	−	−	+	−	NA
IRB	−	IGF-II	−	+++	+	++	NA
IRB	−	Insulin	−	+++	+	++	NA
IGF-IR	−	IGF-I	+++	+++	+	−	NA
IRA	+	IGF-II	+++	−	+	−	+++
IRA	+	Insulin	+++	−	+	−	+++
IRB	+	IGF-II	−	+	+	+	−
IRB	+	Insulin	++	−	+	−	+
IGF-IR	+	IGF-I	++++	−	+++	−	+++

Data compiled from this paper and previous ones (26, 30, 31).

32D cells a differentiation signal, stronger with the B than with the A form. As with the IGF-IR, ectopic expression of IRS-1 abrogates the differentiation program.

A negative marker of differentiation is instead the increase in Id2 gene expression. The Id proteins are generally transcription factors that play a role in the differentiation of a variety of cell types (37, 39, 56). Id gene expression is markedly increased in proliferating cells and tumor cell lines (39, 57, 58). High levels of Id gene expression inhibit differentiation (35, 37, 38). Although there is an increase in Id2 gene expression after insulin or IGF-II and with both isoforms, the increase is modest and tends to decrease rapidly afterwards. The IGF-IR is a stronger inducer of Id gene expression (34, 35). The most interesting observation, though, is the little effect that expression of IRS-1 has on the levels of expression of Id2 protein in 32D cells overexpressing the IR, regardless of the isoform. As mentioned, Id2 expression is dramatically increased by IRS-1 in 32D IGF-IR cells (34). We really have no plausible explanation on why IRS-1 increases Id2 gene transcription when activated by the IGF-IR and not when activated by the IR. However, in MEF, Id gene expression is regulated in part by two domains of the IGF-IR. These two domains are the serine quartet at 1280–1283 and a group of six amino acids, four of which are basic amino acids, at residues 1289–1294 (59). Interestingly, the IR lacks these two domains, indeed, two of the basic amino acids in the second domain are replaced by glutamic acid. The absence of these two domains may explain why the IR (both isoforms) is a weak inducer of Id gene expression, even in 32D cells expressing IRS-1. Nevertheless, these results were reproducible.

We and others (40–42) have recently reported that IRS-1 can translocate to the nuclei of cells stimulated by IGF-1 or expressing oncogenes like the simian virus 40 (SV40) and JCV T antigens, or v-src. This has been shown to occur in MEF, medulloblastoma cells, and 32D-derived cells. In addition, nuclear IRS-1 has been reported in tissue sections of human medulloblastoma (40), human breast cancer (60) and rat liver (61). The evidence for nuclear (and nucleolar) translocation of IRS-1, as well as IRS-2 has been rigorously documented in previous papers (40, 41, 42, 62) and needs not to be reiterated here. Suffices to say that it was documented by immunohistochemistry, confocal microscopy, subcellular fractionation, the use of IRS-1 mutants and of FLAG-tagged IRS proteins. In those experiments, it was possible to show that IRS-1 was cytosolic in quiescent cells and moved to the nucleus after stimulation with IGF-I. Cells transformed by the SV40 T antigen and v-src (42) and by the JCV T antigen (40) show nuclear IRS-1 regardless of the stimulation with growth factors. Nuclear IRS-1 binds the upstream binding factor (UBF), which is a regulator of RNA polymerase I activity (42, 62, 63). The binding of IRS-1 to UBF results in an increase in rRNA synthesis (62), as one would expect from an activation of UBF. This observation constitutes the first molecular link between IRS-1 and the ribosomal DNA transcriptional machinery, which controls cell size. Indeed, 32D IGF-IR cells expressing IRS-1 are twice as large as 32D IGF-IR cells, even when the cells are growing exponentially (30).

We have also reported that the A isoform (but not the B isoform) of the IR can cause nuclear translocation of IRS-1 in MEF, whether stimulated by insulin or IGF-II (63). The translocation is significantly less than with the IGF-IR, but clearly detectable. In that paper, we used confocal microscopy, as well as immunohistochemistry and subcellular fractionation to confirm the nuclear translocation of IRS-1 by the IR. The present results confirm that IGF-II sends a mitogenic signal in 32D IRS-1 IR-A cells, where most of the cells show nuclear localization of IRS-1. Although an occasional 32D IRS-1 IR-B shows nuclear IRS-1, most of them show a cytosolic localization after IGF-II stimulation.

The two isoforms inhibit the expression of 24p3 lipocalin, but only in the presence of IRS-1. The lipocalin protein family is a large group of small extracellular proteins. Among the members of the lipocalin protein family are the retinol-binding protein, the retinoic acid binding protein, apolipoprotein D and a prostaglandin D synthase (reviewed in Ref. 64). However, the lipocalin that is relevant to this discussion is a glycoprotein designated as the 24p3 gene product (65). When IL-3 is withdrawn from certain IL-3-dependent hemopoietic cell lines, the 24p3 protein is transcribed and secreted into the medium (43). 24p3 is also secreted by 32D cells after IL-3 withdrawal, and its transcription is inhibited by addition of IGF-I (43). IL-3 withdrawal induces 24p3 in cells expressing either isoform of the IR, but this induction is abrogated by the presence of IRS-1 expression. The IGF-IR does not need IRS-1 to inhibit 24p3 expression (see Fig. 6C). On the other side, the effect of IRS-1 on 24p3 mRNA expression suggests that IRS-1 is a potent inhibitor of 24p3 expression, thus extending the observation of Devireddy *et al.* (43) that 24p3 expression is inhibited by IGF-I. Interestingly, 24p3 is also induced by SV40 T antigen (66).

In conclusion, we have examined in this paper some of the signaling aspects of the A and B isoforms in 32D cells, a murine hemopoietic cell line, which is IL-3-dependent and undergoes apoptosis upon IL-3 withdrawal. The IR in these cells requires expression of IRS-1 to send a survival signal. The B isoform sends a stronger differentiation signal than the A isoform. Finally, the A form can translocate IRS-1 to the nucleus while the B form seems to be less effective in this regard, especially with IGF-II.

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