Nuclear Translocation of Insulin Receptor Substrate-1 by the Simian Virus 40 T Antigen and the Activated Type 1 Insulin-like Growth Factor Receptor*

32D cells are a murine hemopoietic cell line that undergoes apoptosis upon withdrawal of interleukin-3 (IL-3) from the medium. 32D cells have low levels of the type 1 insulin-like growth factor (IGF-I) receptor and do not express insulin receptor substrate-1 (IRS-1) or IRS-2. Ectopic expression of IRS-1 delays apoptosis but cannot rescue 32D cells from IL-3 dependence. In 32D/IRS-1 cells, IRS-1 is detectable, as expected, in the cytosol/membrane compartment. The SV40 T antigen is a nuclear protein that, by itself, also fails to protect 32D cells from apoptosis. Co-expression of IRS-1 with the SV40 T antigen in 32D cells results in nuclear translocation of IRS-1 and survival after IL-3 withdrawal. Expression of a human IGF-I receptor in 32D/IRS-1 cells also results in nuclear translocation of IRS-1 and IL-3 independence. The phosphotyrosine-binding domain, but not the pleckstrin domain, is necessary for IRS-1 nuclear translocation. Nuclear translocation of IRS-1 was confirmed in mouse embryo fibroblasts. These results suggest possible new roles for nuclear IRS-1 in IGF-I-mediated growth and anti-apoptotic signaling.

32D cells are a murine hemopoietic cell line that is interleukin (IL)-3-dependent for growth (1). In the absence of IL-3, 32D cells undergo apoptosis (2–4). Parental 32D cells have low levels of IGF-IR, about 2 × 10^5 receptors/cell (5), and do not express insulin receptor substrate-1 (IRS-1) and IRS-2 (6, 7). The IRS proteins are major substrates of the IGF-I and insulin receptors and play an important role in the signaling from both receptors (reviewed in Refs. 8 and 9). Expression of a human IGF-IR in 32D cells to about 17 × 10^5 receptors/cell (32D IGF-IR cells) prevents apoptosis caused by IL-3 withdrawal (4, 5, 10). However, after 48 h of exponential growth, the cells begin to differentiate along the granulocytic pathway (7). Ectopic expression of IRS-1 in 32D IGF-IR cells (32D IGF-IR/IRS-1 cells) inhibits differentiation. The cells become IL-3-independent and form tumors in mice (11). In parental 32D cells, expression of only IRS-1 does not prevent apoptosis, although it delays it slightly (3, 12).

The interactions of the IGF axis with the SV40 T antigen (hitherto abbreviated as T antigen) are complex. The SV40 T antigen interacts closely with IRS-1, as demonstrated by reciprocal co-immunoprecipitation. This is true in both MEF (13) and 32D cells (3). Truncation of the 250 amino-terminal residues of the T antigen abrogates both its ability to co-transform cells in combination with IRS-1 and its ability to co-precipitate IRS-1 (13). 32D cells expressing T antigen are not protected from apoptosis induced by IL-3 withdrawal, and they actually die even faster than parental 32D cells (3). Thus, neither IRS-1 nor T antigen singly can protect parental 32D cells from apoptosis. However, a combination of the two results in the survival of 32D cells after IL-3 withdrawal (3). IRS-1 is known to interact also with nucleolin (14). Both T antigen and nucleolin are predominantly nuclear proteins, although minor fractions of either protein can be detected in the cytosol (15, 16), indeed even on the cell surface in the case of nucleolin (17). It has been therefore tacitly assumed that IRS-1, anchored to the receptors, was interacting with the minor cytosolic fractions of either T antigen or nucleolin. A recent report, however, has indicated that under certain conditions IRS-1 can be translocated to the nuclei (18).

The situation is further complicated by the fact that T antigen induces 24p3 (19). 24p3 is a lipocalin that Devireddy et al. (20) reported to be responsible for the apoptosis caused by IL-3 withdrawal in several types of hemopoietic cell lines, including 32D cells. According to these authors, IGF-I inhibits the transcription of 24p3. A reasonable hypothesis at this point would be that T antigen cannot transform 32D cells because it does not inhibit 24p3 transcription, in fact it superinduces it. Ectopic expression of IRS-1, activated by IGF-I, could inhibit 24p3 transcription and thus rescue 32D/T cells from apoptosis.

The purpose of this investigation was to determine the subcellular localization of IRS-1 in 32D-derived cells that survive and grow versus cells that do not survive and undergo apoptosis. In addition, we have tested the hypothesis that survival may depend on the inhibition of 24p3 transcription. Using immunohistochemistry and confocal microscopy, we show here that IRS-1 is localized to the cytosol in 32D IRS-1 cells, which undergo apoptosis upon IL-3 withdrawal (3, 7, 21). However, in 32D IRS-1/T cells and in 32D IGF-IR/IRS-1 cells, which survive (and grow) in the absence of IL-3 (3, 7, 11), IRS-1 is found also in the nuclei. We have demonstrated the nuclear localization of IRS-1 by a variety of procedures and showed that it also occurs in MEF, where we have confirmed it by subcellular fractionation. In 32D IGF-IR cells, we show that a mutant IRS-1 with a deleted phosphotyrosine-binding (PTB) domain will not translocate to the nuclei, whereas the pleckstrin homology (PH)
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Experimental Procedures

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Cell Lines—32D, 32D/T, 32D IRS-1, and 32D IRS-1/T cells have been described by Zhou-Li et al. (3). 32D IRS-1/IGF-IR cells express both the human wild type IGF-IR and mouse IRS-1 (7), which are IL-3 independent, and form tumors in nude mice (11). 32D IGF-IR cells expressing the mutant IRS-1 proteins have been described previously (22, 23). They are 32D IGF-IR cells stably transfected with plasmids expressing mutant IRS-1 proteins. The mutants used in these experiments include an IRS-1 with a deletion of the PH domain, a mutant with a deletion of the PTB domain, and another plasmid expressing only the PH/PTB domain. The expression levels of human IGF-IR and endogenous IRS-1 (26). In some experiments, parental 32D and 32D/T cells were transiently transfected with the pIRS-1 Flag plasmid (see below), using the FuGENE 6 transfection reagent (Roche Molecular Biochemicals), following the manufacturer’s instructions. After 48 h, the transfected cells were examined by confocal microscopy. Only transfected cells were positive for the FLAG antibody.

Plasmids—pIRS/FLAG was generated from pGR159 MSCV puc retroviral vector by fusing in-frame the wild type mouse IRS-1 sequence with the FLAG sequence (Kodak) at the 3' end. The IRS-1 sequence fused in-frame with the FLAG epitope at the 3' end was produced by PCR. The detailed methodology for the construction of the retroviral vector has been described previously (26). 24p3 cDNA was obtained by reverse transcription-PCR from 32D cells, 4 h after IL-3 withdrawal. 32D cells were washed three times with Hanks’ buffer and seeded at 5 × 10^5 cells/ml in RPMI medium supplemented with 10% FBS for 4 h. The cells were collected and washed with cold PBS, and RNA was extracted using RNeasy kit from Qiagen. Reverse transcription-PCR was performed with C. hydrogenoforms thermosetable polymerase according to the manufacturer’s instructions (one-step reverse transcription-PCR system; Roche Molecular Biochemicals). The primers used were: forward 5’-AGACCTAGTAGCTGTGGAAACC-3’ and reverse 5’-GGGGGCGATATTCATTACG-3’. The annealing temperature used was 56 °C. The 24p3 cDNA was subcloned in pCR 2.1 vector using the Topo TA cloning kit (Invitrogen) according to the manufacturer’s instructions. The sequence of 24p3 cDNA was monitored using T7 and M13 reverse primers and by comparison with the sequence in the BLAST program.

Confocal Microscopy—32D and 32D-derived cells (see above) were washed three times with Hanks’ buffer and seeded 2 × 10^5 cells/ml in RPMI medium supplemented with 10% FBS or IGF-1 50 ng/ml on poly-l-lysine coverslips for16 h. The cells were fixed with 3.5% formaldehyde solution for 30 min., washed three times with PBS, permeabilized with 0.3% Triton X-100 solution in PBS for 2 min, and washed three times with PBS. The blocking was done for 30 min using 10% normal donkey serum (Santa Cruz Biotechnology) diluted in PBS, and the slides were incubated for 45 min with the appropriate antibodies. Confocal analysis was performed on a Bio-Rad MRC-600 Ar/Kr laser scanning confocal microscope interfaced to a Zeiss Axiovert 100 microscope with Zeiss Plan-Apo 63× oil immersion objective and a Zeiss 40× objective. The samples were analyzed with simultaneous excitation at 488 and 568 nm with proper filters to visualize fluorescein and rhodamine-lissamine signals.

Immunohistochemistry—32D and 32D-derived cells were washed three times with Hanks’ buffer and seeded at a density of 5 × 10^5/ml of RPMI supplemented with 10% FBS or IGF-1 50 ng/ml. The cells were harvested after 16 h, and cytosins were prepared. The fibroblasts were then fixed on coverslips (5 × 10^5 cells/ml in Dulbecco’s modified Eagle’s medium plus 10% FBS. After the cells had attached to the coverslip, they were shifted to serum-free medium for 24 h and then stimulated with IGF-1 (50 ng/ml) for 6 h. The cells were harvested with cold PBS, collected by scraping, and washed twice in a 15-ml Falcon tube with cold PBS. The cells were resuspended in buffer A (50 mm Hepes, pH 7.4, 1 mM EDTA, protease and phosphatase inhibitors from Sigma, 100 mM dithiothreitol, and 0.5% Triton X-100), kept on ice for 10 min, and then homogenized with a tight fitting Dounce and examined under microscope to confirm that the cells were lysed. After centrifugation at 4 °C, 500 × g for 10 min, the supernatant was collected, centrifuged again at 12,000 × g for 10 min, and collected as the cytoplasmic fraction. The pellet of the first centrifugation was washed at least three times with buffer B (50 mm NaCl, 10 mm Hepes, pH 8, 25% glycerol, 0.1 mM EDTA, protease and phosphatase inhibitors from Sigma, and 100 mM dithiothreitol), resuspended in buffer C (350 mm NaCl, 10 mm Hepes, pH 8, 25% glycerol, 0.1 mM EDTA, protease and phosphatase inhibitors from Sigma, and 100 mM dithiothreitol), and kept rocking for 30 min at 4 °C. After centrifugation at 12,000 × g for 10 min, the supernatant collected represented the nuclear fraction. 20 µg of cytoplasmic and nuclear fractions were separated on a 4–15% gradient gel (Bio-Rad) and transferred to a nitrocellulose membrane.

Antibodies—The primary antibodies used were: nucleolin monoclonal antibody, SV40 T antigen monoclonal antibody, Id2 polyclonal antibody, IRS-1(C20) polyclonal antibody with the epitope mapping at the carboxyl terminus of IRS-1, and IRS-1(C19) with the epitope mapping at the amino terminus of IRS-1 from Santa Cruz Biotechnology. Anti-FLAG fluorescein isothiocyanate-conjugated antibody and another anti-IRS-1 polyclonal antibody were from Upstate Biotechnology, Inc. Clathrin polyclonal antibody was a kind gift of Dr. J. H. Keen (Thomas Jefferson University). All of these antibodies were used at a concentration of 5 µg/ml. After washing three times with PBS, the samples were incubated with appropriate secondary antibodies conjugated to rhodamine or fluorescein (Santa Cruz Biotechnology and Jackson ImmunoResearch Laboratories). In some experiments the nuclei were stained with propidium iodide from Molecular Probes. For Western blots, the antibodies used were anti-IRS-1(C20), anti-e-Jun (Santa Cruz Biotechnology), and anti-glyceraldehyde-3-phosphate dehydrogenase (Research Diagnostics, Inc.).

Northern Blots—Northern blots were carried out using standard techniques using the full-length 24p3 mouse cDNA described above.

Results

32D-derived Cell Lines—Parental 32D cells die quickly after IL-3 withdrawal, with most of the cells dead by 24 h (1, 2). Cell death is easily demonstrated by simply counting the number of cells (10, 21). The evidence that the mode of cell death is apoptosis has been repeatedly documented in previous papers from this and other laboratories. In our first paper (3), we used fluorescence-activated cell sorter analysis to show that the same cell lines used in the present experiments underwent apoptosis after IL-3 withdrawal. We confirmed apoptosis by the TUNEL method in a subsequent paper (4). We have monitored the extent of apoptosis in the present experiments, and they exactly reproduced the data of Zhou-Li et al. (3). Those data had shown that a sizable fraction of 32D/T cells are already dead at 16 h after IL-3 withdrawal, whereas death of 32D IRS-1 cells was delayed and less prominent. The number of apoptotic cells was negligible in 32D IRS-1/T cells or in 32D IGF-IR/IRS-1 cells (3, 4, 7). All 32D-derived cells grow normally if incubated in IL-3 (not shown, but repeatedly shown in previous PLoS).

Subcellular Localization of IRS-1 in 32D-derived Cells—IRS-1 is known to interact directly with both the insulin and the IGF-1 receptors, and the domains required for their interaction have been identified (27). Because of its direct interaction with the receptors, its size, and its downstream signaling, it has been generally assumed that IRS-1 is an exclusively cytosolic (or plasma membrane) protein (for the most recent references, see Refs. 28 and 29). However, as already men-
tioned, IRS-1 is known to interact with the SV40 T antigen (3, 13) and nucleolin (14). These two proteins are predominantly nuclear proteins. In addition, a recent report (18) has shown that IRS-1 can translocate to the nuclei in medulloblastoma cells and cell lines. In an attempt to explain the co-operative effect of SV40 T antigen and IRS-1, we have investigated their localization in 32D cells.

Fig. 1 shows confocal and regular microscopy pictures of 32D-derived cells, stained with the appropriate antibodies (see “Experimental Procedures”). 32D cells (and derivatives) growing in IL-3 have the appearance of blast cells, with large nuclei, large and often diffuse nucleoli, and a variable rim of cytoplasm around the large nuclei (7). Fig. 1A shows that 32D/T cells are completely negative for IRS-1 staining, as expected from the fact that parental 32D cells do not express IRS-1 (6, 7). In 32D IRS-1 cells (3), IRS-1 is detectable (Santa Cruz antibody) in cells growing in complete medium plus IL-3 (Fig. 1B). The nucleus was stained with hematoxylin, and IRS-1 clearly appears localized in the cytoplasm of these cells. The amount of IRS-1 in the cytosol of individual cells varies, which can be explained by the fact that this is a mixed population. When the amount of IRS-1 is substantial, the color of the nucleus is darker, as one would expect, in immunohistochemistry, from cells in which the nucleus is covered by a thin layer of cytosol. The average amount of IRS-1 in these cells, as detectable by Western blots, is high (7). These results were confirmed by confocal microscopy. Fig. 1C shows that the T antigen is a nuclear protein in 32D/T cells, the amount detectable in the cytosol (stained for clathrin) being negligible. Parental 32D cells do not stain at all with an antibody to the T antigen (not shown). Fig. 1D shows a 32D/IRS-1 cell in which IRS-1 (green) is limited to the cytoplasmic rim, whereas the antibody to nucleolin (red) stains the nucleus diffusely. Fig. 1E shows another example of a 32D/IRS-1 cell, but with the colors reversed (IRS-1 is now stained red and nucleolin green). These experiments were repeated several times, with reproducible results.

**IRS-1 Co-localizes with the SV40 T Antigen in the Nucleus of 32D IRS-1/T Cells**—In contrast, when the same antibodies are used to stain 32D IRS-1/T cells, IRS-1 is now seen predominantly in the nucleus, co-localizing with the T antigen (Fig. 2). A halo of IRS-1 can still be seen in the cytosol, but a substantial proportion of IRS-1 is now in the nucleus. The images of A and B of Fig. 2 were obtained using two different antibodies for IRS-1, from different commercial sources (see above). To point the localization, we show in Fig. 2 isolated cells, but the great majority of 32D IRS-1/T cells showed nuclear co-localization of the two proteins (see also below). The results are consistent with nuclear localization of IRS-1 in 32D cells expressing T antigen. With the results of Fig. 1, we can also state that IRS-1 is not required for the localization in the nuclei of T antigen, whereas T antigen is needed for the nuclear localization of IRS-1.

**Subcellular Localization of IRS-1 in 32D IGF-IR/IRS-1 Cells**—However, the nuclear localization of IRS-1 is not limited to T antigen expressing cells. 32D IRS-1/IGF-IR cells are transformed by any criteria, because they form tumors in nude mice (11). Confocal microscopy (Fig. 3) shows that IRS-1 in these cells co-localized with nucleolin, predominantly in the nucleus. In these cells, nucleolin gives a diffuse nuclear staining, which is at variance with MEF, where the anti-nucleolin antibodies give a discrete nucleolar-shaped staining. A weak halo of IRS-1 is also detectable in the cytosol. Therefore, ectopically expressed IRS-1 is cytosolic in 32D/IRS-1 cells but mostly nuclear when the cells also express either the T antigen or increased levels of IGF-IR.
IRS-1 co-localizes with nucleolin in the cell nuclei. The antibodies used are described under “Experimental Procedures.” The merged picture shows that most of the IRS-1 co-localizes with nucleolin in the cell nuclei.

Confirmation of the Nuclear Localization of IRS-1—Because of often justified doubts on the specificity of commercial antibodies (especially in immunohistochemistry), we have attempted to confirm our results by using a FLAG-tagged IRS-1. This is a plasmid in which mouse IRS-1 (30) is tagged with a FLAG epitope at its 3’ end (see “Experimental Procedures”). This plasmid was transiently transfected into 32D/T cells, and the cells were examined 48 h later. Because the efficiency of transfection of parental and derived 32D cells is fairly low, this transient expression experiment offered the advantage of using the untransfected cells as controls for the FLAG epitope. Untransfected cells are negative for the FLAG epitope and stain only with the Id2 antibody (Id2 is a nuclear protein). Fig. 4 shows different experiments in which 32D/T cells were transfected with the IRS-1/FLAG plasmid and stained with antibodies to Id2 and FLAG. The merged pictures show that there is FLAG staining in the cytoplasm, but a substantial part is in the nucleus, co-localizing with the Id2 protein. Although the few cells positive for the FLAG epitope (transfected) show nuclear localization of IRS-1/FLAG, the untransfected cells are only positive for Id2. As an additional control, IRS-1/FLAG was transfected into parental 32D cells. The results are shown in Fig. 5. Again, only a fraction of cells were transfected and stained positive for FLAG (central panel). The nuclei were stained red with propidium iodide, and the merged picture clearly shows that in parental 32D cells, IRS-1/FLAG is cytosolic. The untransfected cells again serve as controls that parental 32D cells do not stain for FLAG.

Subcellular Localization of Mutant IRS-1 Proteins—Another indirect way of confirming the ability of IRS-1 to translocate to the nuclei is to use mutant IRS-1 proteins. In addition, such an experiment can give clues on the mechanism of IRS-1 translocation. The IRS-1 mutants used have been described in previous papers from our laboratory (22, 23, 31) and are the same used by Yenush et al. (27) to study the insulin receptor. These IRS-1 mutants were expressed in 32D IGF-IR cells (23), so that they can be compared directly with the 32D IGF-IR/IRS-1 cells described above in Fig. 3. The results are shown in Fig. 6. As the negative control, we show 32D IGF-IR cells (first two upper panels), which do not express IRS-1 and are completely negative, whether in serum or in serum plus IGF-I. A positive control is 32D IGF-IR/IRS-1 cells in IGF-I. Confirming the results by confocal microscopy of Fig. 3, IRS-1 can be detected in the nuclei of cells stimulated with IGF-I (right upper panel), although a significant amount can also be detected in the cytosol. There is nuclear translocation in 32D IGF-IR cells expressing the δPH IRS-1 mutant, whereas the δPTB mutant

![Image 308x212 to 555x390](file)

![Image 53x601 to 293x729](file)

![Image 552x728](file)

![Image 552x728](file)
densed. As already mentioned, when the amount of IRS-1 is abundant (or the nucleus very small), the cytoplasmic layer slightly modifies the color of the nuclei, but the difference between the upper right panel and the central lower panel is obvious. The right lower panel shows 32D cells stably expressing the PH/PTB plasmid (24, 27). These cells had to be stained with an antibody to the amino terminus of IRS-1 (see "Experimental Procedures"), and the panel shows that this mutant does translocate to the nucleus. However, a substantial fraction of this mutant IRS-1 is also detectable in the cytoplasm. These results have been confirmed by confocal microscopy (Fig. 7).

Nuclear Localization of IRS-1 in MEF—At this point, we tested nuclear translocation of IRS-1 in MEF for two reasons. The first was to confirm in another cell line that IRS-1 can translocate to the nuclei. The second reason was that subcellular fractionation is easier in MEF than in 32D cells, which grow in suspension. The MEF we have chosen are R/H11002/GR15 cells (26). These are R/H11002/derived cells expressing endogenous IRS-1 in significant amounts. The results of subcellular fractionation (see "Experimental Procedures") are shown in Fig. 8. IRS-1 is detectable in the nuclear fraction of R/HGR15 cells in amounts slightly higher than in the cytosol. The antibody to glyceraldehyde-3-phosphate dehydrogenase shows that the nuclear fraction was devoid of detectable contamination by cytosolic proteins. The antibody to c-Jun was used to monitor the purity of the cytosolic fraction.

Time Course of 24p3 mRNA Expression in 32D-derived Cells—It has been reported by Devireddy et al. (20) that IGF-I represses the transcription of 24p3 induced by IL-3 withdrawal. We wanted to determine whether the presence of IRS-1 affected the expression of 24p3. The expression of 24p3 was
determined by Northern blots at the times after IL-3 withdrawal indicated in Fig. 9. 24p3 mRNA is detectable in parental 32D cells as quickly as 4 h after IL-3 withdrawal (Fig. 9A). In 32D/T cells, the appearance of 24p3 RNA is also rapid (Fig. 9B). In 32D IRS1 and 32D IRS-1/T cells, 24p3 mRNA is not detectable at least up to 48 h after IL-3 withdrawal (Fig. 9B). The expression of 24p3 mRNA is also inhibited in 32D IRS-1/IGF-IR. These experiments therefore confirm the results of Devireddy et al. (20) that 24p3 is induced by IL-3 withdrawal and that its induction is inhibited by an activated IGF-IR. However, 24p3 induction is also inhibited in 32D/IRS-1 cells, which do not survive IL-3 withdrawal. This is an important observation, because it indicates that IRS-1 is sending a signal even in 32D cells not expressing either the T antigen or the human IGF-IR. The signal is sufficient for inhibition of 24p3 but not sufficient to ensure survival.

**Discussion**

Our findings can be summarized as follows: 1) The combined expression of IRS-1 and the SV40 T antigen results in IRS-1 translocation to the nucleus (this paper) and survival in the absence of IL-3 (3). 2) Ectopic expression of IRS-1 in 32D/IRS-1 cells shows that IRS-1 is localized to the cytosol, as expected. The cells do not survive IL-3 withdrawal, although apoptosis is delayed (3, 12). 3) IRS-1 also translocates to the nuclei in 32D IRS-1/IGF-IR cells, which are transformed and form tumors in mice (11). 4) Nuclear translocation of IRS-1 has been confirmed using a FLAG-tagged IRS-1 transiently expressed in 32D/T cells (using as controls parental 32D cells). 5) Mutant IRS-1 proteins indicate that the PTB domain is necessary for nuclear translocation. Deletion of the PH domain does not inhibit the nuclear translocation of IRS-1. A truncated IRS-1, comprising only the PH and PTB domain is partially translocated to the nucleus. 6) IRS-1 can also translocate to the nucleus of MEF and is more prominent after IGF-1 stimulation. In these cells, the presence of IRS-1 in the nucleus has been confirmed by subcellular fractionation. 7) The experiments with 24p3 mRNA confirm the results of Devireddy et al. (20) for IL-3 withdrawal and those of Hraba-Reveney (19) for the SV40 T antigen. However, at least in this model, the role of 24p3 in apoptosis is not as clear cut as in the experiments of Devireddy et al. (20). IRS-1, by itself, inhibits the induction of 24p3 but does not protect parental 32D cells from apoptosis.

The evidence for nuclear localization of IRS-1 must rest first of all on the proper identification of the protein. The following findings support the identification of the nuclear protein as IRS-1: 1) By confocal microscopy and immunohistochemistry, two antibodies against IRS-1 from different commercial sources gave the same results. Both antibodies showed a cytosolic localization of IRS-1 in 32D IRS-1 cells and a nuclear localization in cells that also expressed either T antigen or the human IGF-IR. We tried a third antibody, with its competing peptide, on another cell line, and it confirmed that our antibodies specifically recognize IRS-1, and only IRS-1. 2) Introduction of IRS-1 with a FLAG epitope shows nuclear localization of the FLAG-tagged protein in 32D/T cells and cytosolic localization in parental 32D cells. The FLAG antibody does not recognize untransfected cells. Incidentally, our preliminary data on FLAG-tagged IRS-1 indicate that the tag does not seem to affect the subcellular localization nor the mitogenic function of IRS-1. 3) The mutant IRS-1 protein that in the same cells is found only in the cytosol (ΔPTB) is an indirect confirmation that the antibody correctly recognizes IRS-1. 4) In MEF, we have been able to confirm the nuclear localization of IRS-1 by immunohistochemistry and subcellular fractionation. The latter procedure unfortunately was not found to be sufficiently reliable in 32D-derived cells. Perhaps, subcellular fractionation in 32D-derived cells is difficult because our 32D cells expressing IRS-1 have a tendency to attach strongly to the plate, from which sometimes they can be removed only by trypsinization. On the basis of our findings, we conclude that the evidence is convincing that under certain conditions, IRS-1 can translocate to the nuclei of 32D-derived cells and MEF.

There are some differences in the amount of cytosolic IRS-1 when the cells are examined by confocal microscopy or by immunohistochemistry (compare for instance Figs. 6 and 7). This is probably due to the fact that in confocal microscopy, the cell section is so thin that low amounts of IRS-1 in the cytosol go undetected or almost undetected. This explanation is supported by the appearance of the nuclei, which are covered by a layer of cytosol in immunohistochemistry but not in confocal microscopy. The cytosol layer in immunohistochemistry modifies slightly the color of the nuclei (in comparison with IRS-1-negative cells), although not nearly as much as when the IRS-1 is translocated into the nucleus. There is also variability on the amounts of IRS-1 in individual cells, which is to be expected in mixed populations. We should add that a limitation in detecting IRS-1 by immunohistochemistry is the dilution of the antibody. At a dilution below 1:200, false positive cells begin to appear in cell lines known not to express IRS-1.

The results with the mutant IRS-1 proteins are also informative. The fact that the ΔPTB mutant does not translocate in response to IGF-1 stimulation is not surprising because this mutant is not completely inactive but is strongly impaired in its function (23, 27). It indirectly confirms, however, that the wild type IRS-1 found in the nucleus is really IRS-1, because the same antibody was used as for the wild type IRS-1. More
interesting is the ability of the δPH mutant to translocate to the nucleus (see below).

A number of reports have appeared indicating nuclear translocation of signaling molecules. Among the signaling molecules that are known to translocate to the nucleus are: MAPK (32), STAT proteins (33), p70S6K/TOR (34, 35), Akt (36), β-catenin (37), the epidermal growth factor receptor (38), a cleaved ErbB-4 receptor (39), phosphatases (40), and IRS-3 (41). Translocation of IRS-1 has just been reported by Lassak et al. (18) in medulloblastoma cells and cell lines.

Indeed, Jans and Hassan (42) have summarized in a review the evidence rapidly accumulating that growth factors and their receptors can accumulate in the nuclei of cells (see also Ref. 43). Although the evidence is rigorous in certain cases and less rigorous in others, a picture is emerging that nuclear translocation of ligands, receptors, and signaling molecules is more common than generally believed. Carmo-Fonseca (44) has proposed that gene regulation may in part occur by nucleocytoplasmic shuttling.

As to the mechanism of translocation, we can only speculate at this point. Jans and Hassan (42) give a long list of ligands and their membrane receptors that have been reported to localize also in the nucleus. For most of them, they also provide the putative sequence for a NLS. The sequences are very different from each other, the only common feature being several basic amino acids residues (see Table I in Ref. 42). Using these rather loose criteria, there are putative NLS in IRS-1. One sequence goes from residues 14 to 28 and reads RKVGYLRK-PSMKHR, where 8 of 15 amino acids are basic. The sequence is perfectly conserved in mouse, human, and rat. In IRS-2 (which can also translocate to the nucleus3), the sequence reads RKCGYLRKPSMKHR, which is a lot of homology, especially considering that the sequences around it are widely divergent. (The basic amino acids are also highly conserved in GAB-1 and even chico, the Drosophila IRS; in fact, in the last one, it looks even more like a NLS.) There is another sequence that was selected by Keller et al. (30) in their original report on the isolation of mouse IRS-1 as a possible NLS. This latter sequence begins at residue 51 and reads KKWRHK. Both sequences are in the PH domain. Unfortunately, deletion of this domain does not prevent nuclear translocation of IRS-1 in 32D IGF-IR cells. The ability of δPH IRS-1 to translocate to the nucleus also seems to rule out nucleolin as a possible chaperone for nuclear translocation. The PH domain has been reported by Burks et al. (14) as necessary for the interaction of IRS-1 with nucleolin.

If IRS-1 has to be chaperoned to the nucleus, its presence in the nuclei of cells expressing the T antigen (largely a nuclear protein) is easy to explain, because the two proteins interact with each other (3, 13). We can rule out IRS-1 translocating T antigen, because 32D/T cells (no IRS-1) have a nuclear T antigen (Fig. 1C). As for 32D IGF-IR/IRS-1 cells, an obvious candidate for the protein that may chaperone IRS-1 into the nucleus would have been nucleolin. Nucleolin, besides interacting with IRS-1 (14), is dephosphorylated by the activation of the IGF-IR (45). The results with δPH mutant suggest that other possible candidates, like importins, have to be considered.

Another question deals with the biological significance of nuclear translocation and its mechanism of action. It is not clear yet what is the functional significance of the nuclear translocation, i.e. whether it simply accompanies signaling events or produces totally different effects. Lin et al. (38) reported that the epidermal growth factor receptor translocated into the nucleus functions as a transcription factor. As to IRS-1, its interaction with nucleolin (14) strongly suggests a role in rRNA processing, which is modulated by nucleolin (46). Indeed, we know from previous experiments and from the literature that IRS-1 and its downstream signaling molecules regulate the size of the cells. A role for IGF-IR/IRS-1 signaling in determining cell size is supported by the finding that IRS-1- and p70S6K knock-out mice are somewhat smaller than their wild type littermates (47–49). But the importance of IRS-1 and p70S6K in cell size regulation was rigorously demonstrated by the observations that homologues of either IRS-1 (50) or the S6 kinase (51) or Akt (52) regulate cell size in Drosophila. IRS-1 controls cell size in 32D-derived cells (11). In turn, cell size is known to depend on the amounts of protein and rRNA/cell (53, 54). It would be tempting to correlate IRS-1 translocation to the nucleus with transformation, because both 32D/IRS-1/T cells and 32D IRS-1/IGF-IR cells are IL-3-independent. Interestingly, 32D IGF-IR cells expressing the PH domain deletion mutant are also IL-3-independent (22). For the moment, we cannot say that IL-3 independence requires IRS-1 translocation. Obviously, alternative pathways for growth must be available, otherwise 32D cells would not be growing in IL-3. However, the importance of the IRS-1 pathway in regulating cell size in vivo (see above) indicates that the role of IRS-1 in cell growth is not trivial.

Our 24p3 data were partially disappointing. It is true that 24p3 mRNA increases after IL-3 withdrawal, but the only interesting finding was that its increase is inhibited by the expression of IRS-1. It confirms that IGF-1 can inhibit the expression of 24p3 mRNA (20), but, more importantly, it shows that IRS-1 is sending a signal even in 32D IRS-1 cells, a signal that inhibits 24p3 transcription but is not sufficient for prolonged survival (3). The role of 24p3 in apoptosis in this model is still unclear.

In conclusion, we present the following hypothesis for the generation of IL-3 independence in 32D cells by the IGF-IR. Our hypothesis is that there are three separate signals from the IGF-IR that must converge to transform 32D cells. The first is an anti-apoptotic signal, originating from the activated IGF-IR and increased by IRS-1 (4, 7). In this step, the role of inhibition of 24p3 transcription (20) is possible but needs confirmation. The second signal is an inhibition of the differentiation program through the increase in Id2 protein expression (24). Overexpression of Id2 protein inhibits differentiation but does not increase survival of 32D cells (55, 56). The third signal is the sustained proliferation signal proceeding from IRS-1 through the phosphatidylinositol 3-kinase/p70S6K pathway (11). The SV40 T antigen could replace in part the IGF-IR in this function, although the IGF-IR is still needed, even at low levels. In this scheme, the IRS-1 plays an important role, especially in the last two processes. The question to be addressed in future experiments is whether the nuclear translocation of IRS-1 results in new signals, different from those of the membrane-located IRS-1. An obvious possibility is the half-life of IRS-1, because molecules in the nucleus (for instance) are less exposed to degradation than when located in the cytosol (57, 58). Finally, it is unlikely that the nuclear translocation of IRS-1 may be simply an artifact of tissue cultures. Evidence of nuclear IRS-1 has already been reported in biopsies of at least two human tumors (18, 59).

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