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Insulin Receptor Substrate-1, p70^{S6K}, and Cell Size in Transformation and Differentiation of Hemopoietic Cells*

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After an initial burst of cell proliferation, the type 1 insulin-like growth factor receptor (IGF-IR) induces granulocytic differentiation of 32D IGF-IR cells, an interleukin-3-dependent murine hemopoietic cell line devoid of insulin receptor substrate-1 (IRS-1). The combined expression of the IGF-IR and IRS-1 (32D IGF-IR/IRS-1 cells) inhibits IGF-I-mediated differentiation, and causes malignant transformation of 32D cells. Because of the role of IRS-1 in changing the fate of 32D IGF-IR cells from differentiation (and subsequent cell death) to malignant transformation, we have looked for differences in IGF-IR signaling between 32D IGF-IR and 32D IGF-IR/IRS-1 cells. In this report, we have focused on p70^{S6K}, which is activated by the IRS-1 pathway. We find that the ectopic expression of IRS-1 and the inhibition of differentiation correlated with a sustained activation of p70^{S6K} and an increase in cell size. Phosphorylation *in vivo* of threonine 389 and, to a lesser extent, of threonine 421/serine 424 of p70^{S6K} seemed to be a requirement for inhibition of differentiation. A role of IRS-1 and p70^{S6K} in the alternative between transformation or differentiation of 32D IGF-IR cells was confirmed by findings that inhibition of p70^{S6K} activation or IRS-1 signaling, by rapamycin or okadaic acid, induced differentiation of 32D IGF-IR/IRS-1 cells. We have also found that the expression of myeloperoxidase mRNA (a marker of differentiation, which sharply increases in 32D IGF-IR cells), does not increase in 32D IGF-IR/IRS-1 cells, suggesting that the expression of IRS-1 in 32D IGF-IR cells causes the extinction of the differentiation program initiated by the IGF-IR, while leaving intact its proliferation program.

The type 1 insulin-like growth factor receptor (IGF-IR),[1] activated by its ligands plays an important role in the growth of cells in at least four different ways. It is mitogenic, both *in vivo* and *in vitro*, it is quasi-obligatory for transformation, it can protect cells from a variety of apoptotic injuries, and it can also induce differentiation in certain types of cells (1). The fact that the IGF-IR can induce cell differentiation (that eventually results in inhibition of growth and cell death) is in clear contradiction to its other properties, which are more of a growth-promoting type. The mechanism by which the IGF-IR switches from one signaling to another is in itself of considerable interest.

We have recently shown that, in the case of the IGF-IR, the choice between stimulation of cell proliferation or differentiation depends on the availability of the immediate substrates of the IGF-IR (2). In those experiments, we used as a model 32D cells (3), which are diploid murine hemopoietic cells. 32D cells have an absolute requirement for interleukin-3 (IL-3) for growth, and undergo apoptosis when IL-3 is withdrawn (4–6). IGF-I (7, 8) or overexpression of the IGF-IR (9–11) prevent apoptosis caused by IL-3 withdrawal. Furthermore, IGF-I can induce in these cells a differentiation program along the granulocytic pathway (2). A characteristic of 32D cells is that they are devoid of insulin receptor substrate-1 (IRS-1) and IRS-2 (12–14), whereas Shc proteins are strongly expressed. This unbalance between the two major substrates of the IGF-IR suggested that the absence of IRS-1 could play a role in the induction of differentiation by IGF-I in 32D IGF-IR cells. Indeed, re-introduction of IRS-1 in these cells resulted in inhibition of IGF-I-mediated differentiation, which was also inhibited by a dominant negative mutant of Shc (2). It seems therefore that proliferation or differentiation of 32D IGF-IR cells depends on the availability of substrates, with IRS-1 favoring proliferation and Shc proteins favoring differentiation. An important aspect of those experiments was that the IGF-IR, by itself, causes 32D cells to grow very rapidly for the first 48 h. During this 48-h period, there is no appreciable difference in the rate of growth between 32D IGF-IR cells and 32D IGF-IR/IRS-1 cells (Refs. 2 and 11, and this paper). This is in agreement with the established fact that differentiation of cells, especially hemopoietic cells, requires a short period of cell proliferation (5, 15). A typical example is the granulocyte colony-stimulating factor, which induces both the proliferative and the differentiating programs, with the latter eventually prevailing.

In previous papers (2, 10), we reported that IRS-1 greatly increases PI 3-kinase and Akt activation in 32D cells. Since this pathway connects with the activation of p70^{S6K} (16), we have focused, in the present investigation, on p70^{S6K} (17). We have found that sustained activation of p70^{S6K} (*in vivo* phosphorylation of threonine 389 and threonine 421/serine 424) correlates with the inhibition of differentiation (and therefore

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[1] The abbreviations used are: IGF-IR, insulin-like growth factor I receptor; IGF-I, insulin-like growth factor I; OKA, okadaic acid; MPO, myeloperoxidase; PAGE, polyacrylamide gel electrophoresis; IRS, insulin receptor substrate; PI, phosphoinositol; IL, interleukin; FACS, fluorescence-activated cell sorting.

with the transformed phenotype). In addition, rapamycin and okadaic acid (OKA), both of which inhibit p70^{S6K} activation, induce differentiation of 32D IGF-IR/IRS1 cells. Inhibition of differentiation is accompanied by an increase in size of the cells, which is in agreement with the effect of homologues of IRS-1 (18) and p70^{S6K} (19) on cell size in *Drosophila*. By determining the expression of myeloperoxidase (MPO) mRNA, we also find that the differentiation program is already activated in 32D IGF-IR cells while they are still actively proliferating. MPO mRNA, however, does not increase in 32D IGF-IR/IRS1 cells. These results suggest that IRS-1 may indeed extinguish the differentiation program of 32D IGF-IR cells (without affecting its proliferative program), and that this effect may be mediated by the sustained activation of p70^{S6K}.

Finally, we report here that 32D IGF-IR/IRS1 cells, in addition to be able to grow in the absence of IL-3 without differentiating, can also form tumors in mice. These latter experiments confirm and extend previous reports that overexpression of IRS-1 results in transformation (20, 21).

EXPERIMENTAL PROCEDURES

Cell Lines—32D IGF-IR cells were obtained from the murine hematopoietic cell line 32D clone 3 (3) by stable transfection with the human wild type IGF-I receptor. 32D IGF-IR/IRS-1 are 32D IGF-IR overexpressing the wild type IRS-1 and 32D IGF-IR/vector are 32D IGF-IR cells stably transfected with the empty vector used to deliver IRS-1. These three cell lines are mixed populations obtained by retroviral infection and have already been described by Valentinis *et al.* (2) and Peruzzi *et al.* (11). Cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Inc.), 10% WEHI cell conditioned medium as a source of IL-3 and the required antibiotics to maintain the selective pressure (250 μg/ml G418, from Life Technologies, Inc., or 250 μg/ml hygromycin, from Calbiochem). For brevity, the WEHI cell conditioned medium will be referred to as IL-3.

Growth and Differentiation Analysis—Cells exponentially growing were collected, washed three times, and seeded in IL-3-free medium (RPMI 1640 medium containing only 10% heat-inactivated fetal bovine serum) supplemented with 50 ng/ml IGF-I. In some experiments, inhibitors (10 nM or 20 nM okadaic acid (Sigma), or 2.5 or 10 ng/ml rapamycin (Sigma)) were added. Cells were seeded at a density of 5×10^4 cells/ml. After 4 and 6 days, viable cells were counted by trypan blue exclusion (Life Technologies, Inc.) and cytospins were performed for morphological analysis. To establish the ability for IL-3-independent growth, cells from the same experiments at day four were re-plated in fresh IL-3-free medium with IGF-I (50 ng/ml) at a density of 5×10^4 cells/ml. Cells were counted by trypan blue exclusion after 4 additional days (for a total of 8 days of culture). To evaluate the degree of granulocytic differentiation, cytospins were stained with Wright-Giemsa and the cells in the different stages of differentiation counted at the microscope. Differentiation was expressed as percentage of bands and polymorphonuclear cells in the total number of scored cells.

Immunoblots—For the detection of phosphorylated proteins, exponentially growing cells were washed three times and incubated in serum-free medium (RPMI 1640 medium supplemented with 0.1% bovine serum albumin (Sigma)) for 4 h before stimulation with 20 ng/ml IGF-I (Life Technologies, Inc.). When inhibitors were used, cells were pre-incubated with the inhibitors for 1 h (okadaic acid) or 15 min (rapamycin) before stimulation with IGF-I. At the desired time point, cells were collected, washed with cold phosphate-buffered saline, and resuspended in lysis buffer in ice for 30 min. Cell lysates were clarified by centrifugation at 13,000 rpm for 15 min, and equal amounts of proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose filter. For immunoblotting, membranes were blocked with 5% nonfat dry milk in buffer consisting of 10 mM Tris (pH 8.0), 150 mM NaCl, and 0.1% Tween 20 overnight at 4 °C and probed with the indicated primary antibodies, followed by incubation with horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin (Oncogene Science). Blots were developed with the enhanced chemiluminescence system according to the manufacturer's instruction (Amersham Pharmacia Biotech)

Antibodies—The phosphorylation of specific amino acids in p70^{S6K} (phospho-Thr^{389}, phospho-Thr^{421}/Ser^{424}, and phospho-Ser^{411}) was detected with antibodies purchased from New England Biolabs. The total amount of p70^{S6K} loaded was monitored after stripping of the filters by immunoblotting with an anti p70^{S6K} antibody (C-18, Santa Cruz). IRS-1 was detected with an antibody to its carboxyl-terminal (Upstate Biotechnology, Inc., Lake Placid, NY).

Determination of Cell Size—Exponentially growing cells were prepared and seeded under the same conditions used for growth analysis. After 24 and 48 h, cells were collected and immediately analyzed for size and cell cycle distribution by flow cytometry. Cell size was determined by forward scattering (FS). Cell cycle distribution was analyzed by staining the cells with 10 μg/ml Hoechst 33342 (Molecular Probes) at 37 °C for 30 min. The cell suspension was analyzed using an Elite ESP-EPICS flow cytometer (Coulter Corp., Hialeah, FL). Software was Elite version 4.5.

Northern Blots—For the detection of the MPO mRNA level, exponentially growing cells were prepared and seeded under the same conditions used for growth analysis. At the indicated time points, cells were collected and total RNA was extracted with the Tri-Reagent solution (Sigma) following the manufacturer's instructions. Ten μg of total RNA for each sample were run on a 1% agarose formaldehyde gel, blotted onto a nitrocellulose membrane, and hybridized with a 1.45-kilobase myeloperoxidase cDNA fragment obtained from the pUC19-MMPO6 plasmid (a kind gift of Dr. Mauro Valtieri). The cDNA probe was labeled with [α-^{32}P]dCTP by the Random Primed DNA labeling kit (Roche Molecular Biochemicals) and purified using the QuickSpinn G-50 Sephadex columns (Roche Molecular Biochemicals). Hybridization was performed in 5× SSC, 50% formamide, 0.1% SDS, 100 μg/ml salmon sperm DNA at 42 °C for 16 h.

Tumor Formation in Nude Mice—Cells exponentially growing were collected, washed with phosphate-buffered saline and injected intraperitoneally into NIH Swiss nude mice. Each animal was injected with 10×10^6 cells and sacrificed after 6 weeks, and its organs were isolated, weighed, and fixed in 10% buffered formalin. Histological sections of the organs were stained with hematoxylin and eosin and examined for the presence of infiltrating 32D cells.

RESULTS

The parental 32D cells cl. 3 (5) have an absolute requirement for IL-3 for growth, and undergo apoptosis within 24 h after IL-3 withdrawal (11, 13, 22). 32D IGF-IR cells and 32D IGF-IR/IRS1 cells have been described previously (2, 11). Both 32D IGF-IR cells and 32D IGF-IR/IRS-1 cells grow at similar rates in IGF-I-supplemented medium for the first 48 h and do not show any morphological sign of differentiation (2, 11). We have monitored the deoxybromouridine labeling of these cells, growing in IGF-I, between IL-3 withdrawal (0 time) and 24 h, and between 24 and 48 h. With both cell lines, and for both time intervals, the labeling with deoxybromouridine was 100% (data not shown). Thus, in the first 48 h, the two cell lines are essentially indistinguishable, although 32D IGF-IR cells eventually differentiate and decrease in number.

In a previous paper (2), we had shown that tyrosyl phosphorylation of Shc was essentially similar in the 2 cell lines, and that IRS-1 was tyrosyl-phosphorylated when 32D IGF-IR/IRS1 cells were stimulated with IGF-I. Soon *et al.* (10) have shown that, in the absence of IRS-1, PI 3-kinase activity is undetectable in 32D IGF-IR cells, which survive and grow (for at least 48 h) in the presence of PI 3-kinase inhibitors (11). Ectopic expression of IRS-1 causes a dramatic increase in PI 3-kinase activity, and these findings of Soon *et al.* (10) have been confirmed (data not shown). Akt/PKB is strongly activated by IGF-I in 32D IGF-IR/IRS-1 cells, but only weakly activated in 32D IGF-IR cells (2). This is not surprising since Akt activation is markedly increased by PI 3-kinase (23–26), whose activity, in turn, is strongly increased by IRS-1 (27). In this report, we have focused our attention on p70^{S6K}, whose activity is strongly dependent on the activation of PI 3-kinase (16) and the Akt/PKB pathway (28).

Activation of p70^{S6K}—The analysis of the specific phosphorylated amino acid was performed on samples run on a 4–15% gradient gel. We used one membrane for each blot of p70^{S6K} phospho-amino acid to detect a clear, specific signal without interference from the stripping of the membrane. The time of observation ranged from 0 to 60 min. To show the mobility shift

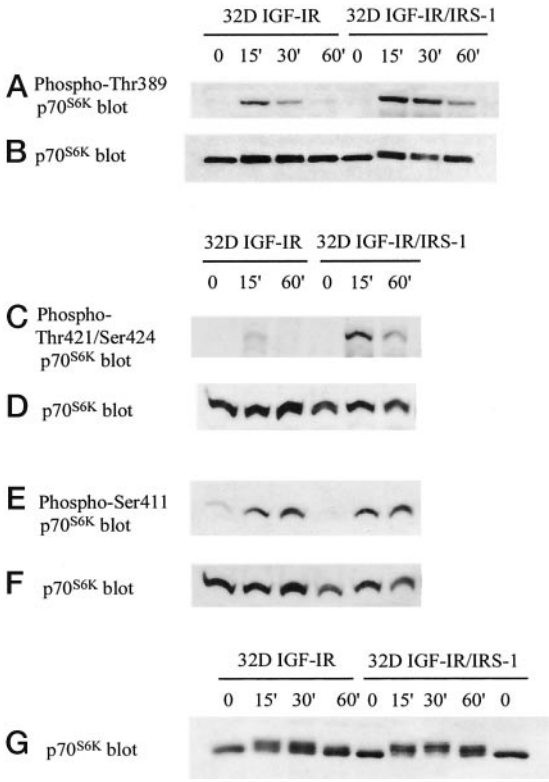

FIG. 1. **Activation of p70^{S6K} in 32D IGF-IR and 32D IGF-IR/IRS-1 cells.** Cells exponentially growing were seeded in serum-free medium for 4 h before stimulation with 20 ng/ml IGF-I (see "Experimental Procedures"). Lysates were prepared from cells at the times indicated (in min) after IGF-I stimulation. From the same experiments, equal amounts of proteins were subjected to three separate 4–15% gradient gel SDS-PAGE and transferred to nitrocellulose membranes, and each filter was blotted with a different phospho-p70^{S6K} antibody: phospho-Thr^{389} (*panel A*), phospho-Thr^{421}/Ser^{424} (*panel C*), and phospho-Ser^{411} (*panel E*). The membranes were stripped and re-blotted with an anti-p70^{S6K} antibody to assess total level of protein (*panels B*, *D*, and *F*). The Western blot shown in *panel G* was performed on a 7.5% gel that allows a better separation of the different phosphorylated forms of p70^{S6K} (mobility shift). A representative experiment is shown. Identical results were obtained using lysates from three different experiments.

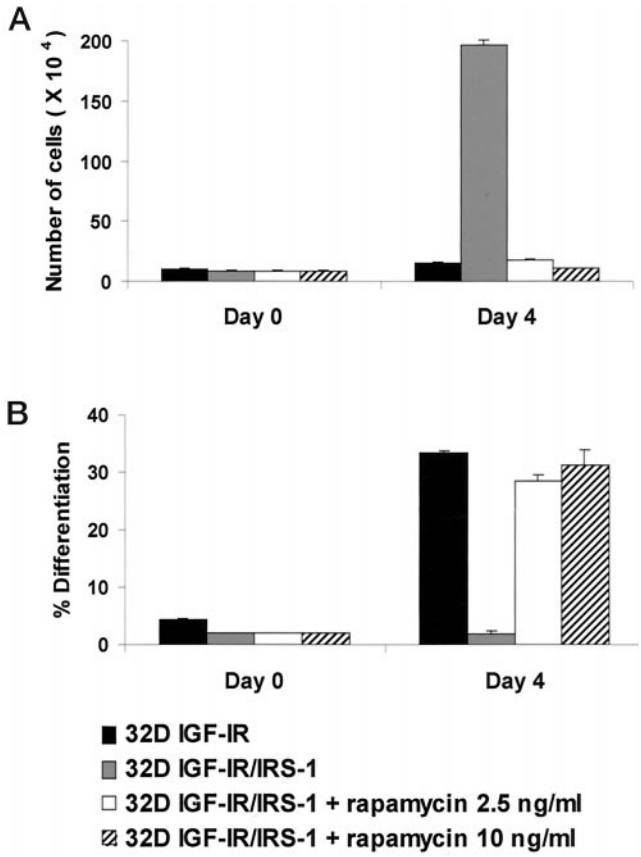

FIG. 2. **Effect of rapamycin on the proliferation and differentiation of 32D-derived cells.** 32D IGF-IR (*closed bars*) and 32D IGF-IR/IRS-1 (*gray bars*) were seeded in IL-3-free medium supplemented with 50 ng/ml IGF-I at a density of 1×10^5 cells in 2 ml total volume. 32D IGF-IR/IRS-1 were also incubated with 50 ng/ml IGF-I and 2.5 ng/ml rapamycin (*open bars*) or 10 ng/ml rapamycin (*striped bars*). Cells were analyzed for growth and differentiation after 4 days of culture. *Panel A*, cell proliferation was evaluated by counting the total number of viable cells. *Panel B*, Wright-Giemsa-stained cells were evaluated for the presence of morphological characteristics of differentiation (percentage of bands and polymorphonuclear cells on the total number of cells scored). A representative experiment (duplicate with range) is shown.

of p70^{S6K}, that is generally accepted as an indicator of p70^{S6K} activity (16, 29), samples were run on a 7.5% gel.

Using the antibody to phosphothreonine 421/phosphoserine 424 (30), p70^{S6K} phosphorylation is clearly detectable, after IGF-I stimulation, in 32D IGF-IR/IRS1 cells, but not in 32D IGF-IR cells (Fig. 1, *panel C*). A difference was also detected using an antibody to phospho-threonine 389 (Fig. 1, *panel A*), which is the residue whose dephosphorylation most closely parallels loss of kinase activity (28). The response to IGF-I stimulation was stronger and more prolonged in 32D IGF-IR/IRS-1 cells than in 32D IGF-IR cells. The antibody to phosphoserine 411 of p70^{S6K} could not detect clearly reproducible differences between the two cell lines, after stimulation with IGF-I (Fig. 1, *panel E*). *Panels B*, *D*, and *F* show the total amounts of p70^{S6K} in each lane. A mobility shift blot is shown in Fig. 1 (*panel G*); a shift is detectable in both cell lines, but it is more accentuated, especially at 30 min, and more sustained in 32D IGF-IR/IRS-1 cells. However, it is evident that the IGF-IR can activate p70^{S6K} even in the absence of IRS-1.

Effect of Rapamycin on 32D IGF-IR/IRS1 Cells—Threonine 389 belongs to the group of serine/threonines of p70^{S6K} that are rapamycin-sensitive. In fact, they were originally considered to be the only residues that were rapamycin-sensitive, with the residues in the C terminus (like threonine 421 and serine 424) considered insensitive (29, 31, 32). More recent reports have questioned this difference (33–35). Although rapamycin actually targets mTOR, its effect is most crucial on the phosphorylation of threonine 389 (28). We reasoned that, if rapamycin-sensitive residues are less activated in 32D IGF-IR cells, then rapamycin, at appropriate concentrations, ought to induce differentiation of 32D/IGF-IR/IRS1 cells.

For this purpose, 32D IGF-IR/IRS-1 cells were treated with rapamycin in the presence of IGF-I. As shown in Fig. 2 (*panel A*), the proliferation of 32D IGF-IR/IRS-1 cells is inhibited by rapamycin (at concentrations of 2.5 and 10 ng/ml). After 4 days, the cell number of rapamycin-treated 32D IGF-IR/IRS-1 cells is essentially the same as in untreated 32 IGF-IR cells. At the same time, differentiation resumes under rapamycin treatment reaching the same percentage observed for 32D IGF-IR cells (*panel B*).

The effect of rapamycin on p70^{S6K} activation is summarized in Fig. 3. In the presence of rapamycin, IGF-I fails to phosphorylate threonine 389 (*panel A*) and also, to a lesser extent, serine 411 (*panel E*). Despite some difficulty in identifying the phospho-p70^{S6K} in *panel C*, one can say that rapamycin also inhibits phosphorylation of threonine 421/serine 424. This well known inhibition by rapamycin (see "Discussion") was confirmed by mobility shift (Fig. 3, *panel G*). The amounts of p70^{S6K} protein in each lane are shown in Fig. 3 (*panels B*, *D*,

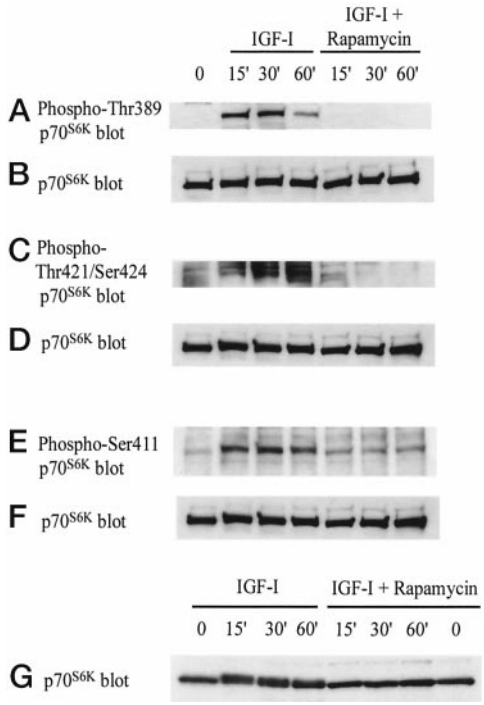

FIG. 3. **Effect of rapamycin on p70^{S6K} activation in 32D IGF-IR/IRS-1 cells.** 32D IGF-IR/IRS1 cells were incubated in serum-free medium for 4 h. Samples treated with rapamycin were pre-incubated with 10 ng/ml of rapamycin for 15 min before stimulation with IGF-I 20 ng/ml. The incubations were stopped at the times indicated (in min), and the lysates obtained were run on four separate gel-SDS-PAGE. The membranes were blotted with the three different anti-phospho-p70^{S6K} antibodies described in *panels A*, *C*, and *E*. After stripping, the filters were re-blotted with an anti-p70^{S6K} antibody to monitor the total levels of protein (*panels B*, *D*, and *F*). The samples in *panel G* were run on a 7.5% gel to enhance the mobility shift of the total p70^{S6K} (see previous figures).

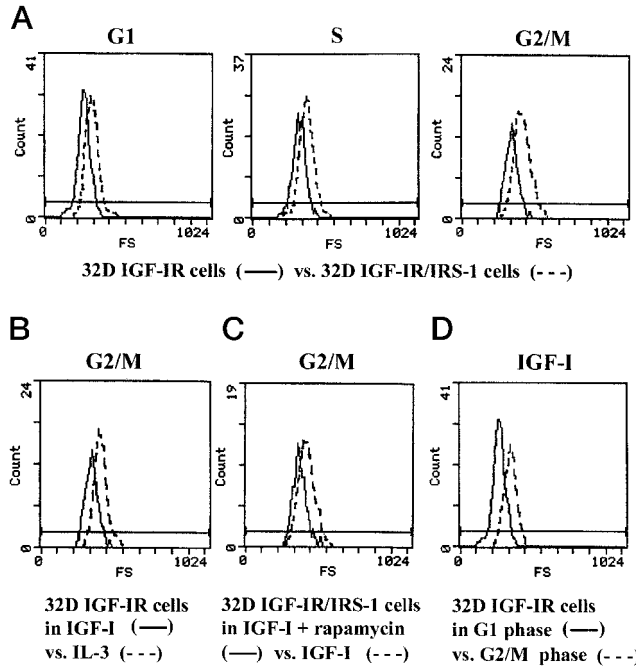

FIG. 4. **Cell size analysis of 32D-derived cells.** Cell size was analyzed by FS as described under "Experimental Procedures." The differences in size are presented as the superimposed histograms generated by FACS analysis. *Panel A* gives a comparison between 32D IGF-IR and 32D IGF-IR/IRS-1 cells in three different phases of the cell cycle (48 h after IL-3 withdrawal and supplementation with IGF-I). The *lower panels* (*B–D*) give three controls. *Panel B*, FS for the G_2/M subpopulation of 32D IGF-IR cells in IL-3 or in IGF-I (48 h). *Panel C*, FS for the G_2/M subpopulation of 32D IGF-IR/IRS1 cells in IGF-I or in IGF-I plus rapamycin (48 h). *Panel D* is a comparison of FS for the 32D IGF-IR cells in the G_1 or G_2/M phases of the cell cycle (48 h in IGF-I). These experiments were repeated three times.

and *F*). These experiments confirm that the p70^{S6K} pathway plays a major role in determining the fate of 32D IGF-IR cells (differentiation *versus* transformation).

Cell Size—It has recently been reported that, in *Drosophila*, both the IRS homologue CHICO (18) and the S6 kinase homologue (19) regulate cell size. We compared the cell size of 32D IGF-IR and 32D IGF-IR/IRS1 cells, by forward scatter analysis. Since the cell cycle distribution of a cell population affects cell size, we also stained the cells with Hoechst 33342 and determined the cell cycle distribution. There was no difference between the two cell lines (data not shown). We then compared cell size of subpopulations in different phases of the cell cycle (see "Experimental Procedures"). Fig. 4 (*panel A*) compares the cell size distribution generated by FACS analysis for subpopulations of 32D IGF-IR and 32D IGF-IR/IRS1 cells in G_1, S, and G_2/M phases of the cell cycle 48 h after IL-3 withdrawal and supplementation with IGF-I. In all three phases of the cell cycle, the mean of the forward scatter of 32D IGF-IR/IRS1 cells was 15–20% higher than the mean of 32D IGF-IR cells. This difference was highly reproducible in repeated experiments. Similar results, but somewhat less pronounced, were detected at 24 h after IL-3 withdrawal and IGF-I supplementation. Fig. 4 (*panels B–D*), shows three necessary controls. In *panel B*, the subpopulation of 32D IGF-IR cells in the G_2/M phase was analyzed in either IL-3 or IGF-I. The cells have a larger size in IL-3, indicating that 32D IGF-IR cells are not just smaller than 32D IGF-IR/IRS1 cells. In the experiment shown in *panel C*, we compared 32D IGF-IR/IRS1 cells (again in G_2/M) in either IGF-I or in IGF-I plus rapamycin. Rapamycin decreases the size of the cells, almost to the level of untreated 32D IGF-IR cells. Finally, in *panel D*, we show the size difference of 32D IGF-IR cells in the G_1 and G_2 phases of the cell cycle. Since cells in G_2 are generally twice as big as cells in G_1, this experiment shows that modest differences in scattering revealed by FACS analysis can actually equal almost a doubling in size.

Differentiation Markers—Since 32D IGF-IR cells proliferate for at least 48 h, one can assume that the IGF-IR activates both the proliferation program and the differentiation program, the latter eventually prevailing (see Introduction). We have hypothesized that IRS-1 extinguishes the differentiation program, leaving the proliferation program intact. As a first approach to test this hypothesis, we have examined the appearance of a known differentiation marker, the MPO mRNA in our 2 cell lines. The results of one such experiment are shown in Fig. 5, where we examined MPO mRNA levels in cells in IL-3 or in IGF-I at various intervals after IL-3 withdrawal. MPO mRNA levels remain high in 32D IGF-IR cells 24 h after IL-3 withdrawal and increase at 48 h. MPO mRNA levels are decreased in 32D IGF-IR/IRS1 cells and diminish further by 48 h. Thus, the hypothesis is supported by these findings. 32D IGF-IR/IRS-1 cells do not seem to activate the differentiation program, that is, instead, activated in 32D IGF-IR cells. We have also examined 32D IGF-IR/IRS1 cells treated with rapamycin, which induces differentiation (see above). The MPO mRNA levels now increase even in 32D IGF-IR/IRS1 cells (*last two lanes* of Fig. 5), especially at 48 h.

Effect of Okadaic Acid—We have determined the effect of OKA on the growth and differentiation of 32D IGF-IR and 32D IGF-IR/IRS-1 cells. OKA is a potent inhibitor of serine phosphatases (36), and the serine phosphatase PP2A interacts directly with p70^{S6K} (37). Among the many effects of OKA, it

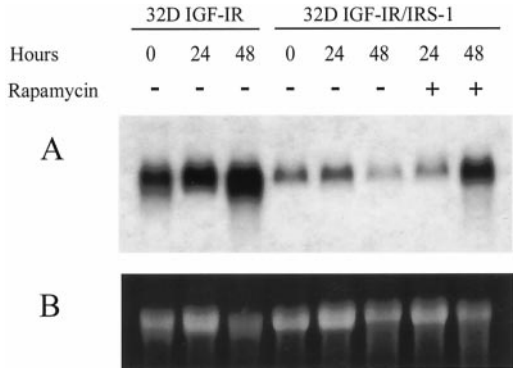

FIG. 5. **Expression of myeloperoxidase mRNA in 32D-derived cells.** RNAs were prepared from cells seeded in IL-3-free medium (0 h) or supplemented with 50 ng/ml IGF-I (24 and 48 h) alone or in combination with rapamycin (10 ng/ml). The RNAs were subjected to Northern blot as described under "Experimental Procedures." The autoradiograph obtained after hybridization with the probe for the myeloperoxidase mRNA is shown in *panel A*. The 28 S ribosomal RNA stained with ethidium bromide is reported to monitor the total amount of RNA present on the filter (*panel B*).

induces apoptosis, and its apoptotic effect is counteracted by the IGF-IR (38). OKA also induces serine phosphorylation of IRS-1 (39), which causes inhibition of IRS-1 signaling (40). We reasoned that OKA ought to inhibit the activation of p70^{S6K}, reduce the growth and induce the differentiation of 32D IGF-IR/IRS1 cells. Fig. 6 (*panel A*) shows that OKA inhibits the proliferation of 32D IGF-IR/IRS1 cells, and that this inhibition is complete at a concentration of only 20 nM.

At the same time, we examined the extent of differentiation of 32D IGF-IR/IRS1 cells in the presence or absence of OKA. At a concentration of 20 nM, OKA induces differentiation of 32D IGF-IR/IRS1 cells (Fig. 6, *panel B*). The effect is only partial, when compared with 32D IGF-IR cells (*closed bars*), but it is nevertheless significant. Increasing the concentration of OKA unfortunately results in massive cell death, which obscures its differentiating effect. We monitored the effect of OKA on IRS-1, by studying its shift in mobility (38, 39), due to the extensive phosphorylation of serine residues. OKA induced a mobility shift of IRS-1 in our cell lines, as expected (data not shown).

We tested the effect of OKA on the phosphorylation and activation of p70^{S6K} in 32D IGF-IR/IRS-1 cells. OKA inhibits the long term phosphorylation of threonine 389 (Fig. 7, *panel A*). *Panel B* shows the amounts of p70^{S6K} in each lane. The samples shown in *panel C* were run on a 7.5% gel (see above). OKA causes a decrease in mobility shift of p70^{S6K}, especially visible at 30 min after IGF-I stimulation (*panel C*). The experiments of Figs. 7 were repeated several times, with similar results.

IL-3 Independence Test—In a previous paper (2), we reported that 32D IGF-IR/IRS1 cells grew, in a medium supplemented with 10% fetal bovine serum and IGF-I, for at least 4 days, whereas 32D IGF-IR cells had stopped growing at 48 h and had begun to differentiate. We have asked whether 32D IGF-IR/IRS1 cells are transformed cells. The first prerequisite of a transformed 32D cells is its ability to grow indefinitely in the absence of IL-3 (41). To determine IL-3 independence, we tested the cell lines after re-plating. For this experiment, we also added as a control 32D IGF-IR cells transduced with the vector used to introduce IRS-1 (empty vector). As described under "Experimental Procedures," the cell lines were grown for 4 days without IL-3 but with IGF-I, and the cell number was determined. Then, the same number of viable cells from each cell line was re-incubated in IL-3-deficient, IGF-I-supplemented medium. Cell number was determined after an additional 4 days in culture. The results are shown in Fig. 8. 32D

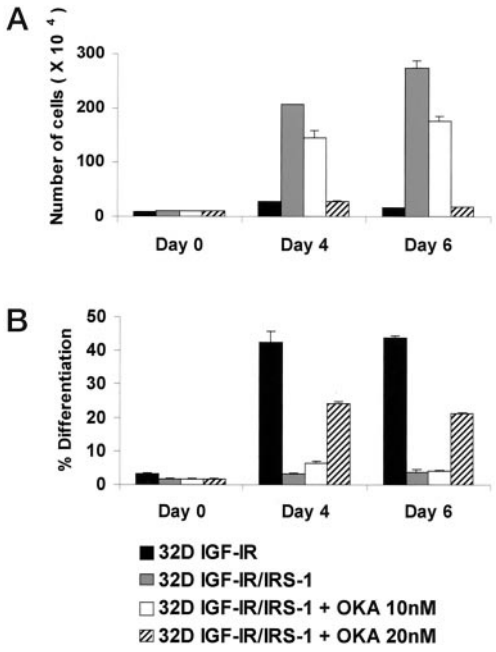

FIG. 6. **Effect of okadaic acid on the proliferation and differentiation of 32D-derived cells.** 32D IGF-IR (*closed bars*) and 32D IGF-IR/IRS-1 (*gray bars*) were seeded in IL-3-free medium supplemented with 50 ng/ml IGF-I at the density of 1×10^5 cells in 2 ml total volume. 32D IGF-IR/IRS-1 were also incubated in 50 ng/ml IGF-I and 10 nM OKA (*open bars*) or 20 nM OKA (*striped bars*). Cells were analyzed for growth and differentiation after 4 and 6 days of culture. *Panel A*, cell proliferation was evaluated by counting the total number of viable cells. *Panel B*, Wright-Giemsa-stained cells were evaluated for the presence of morphological characteristics of differentiation (percentage of bands and polymorphonuclear cells on the total number of cells scored). A representative experiment (duplicate with range) is shown.

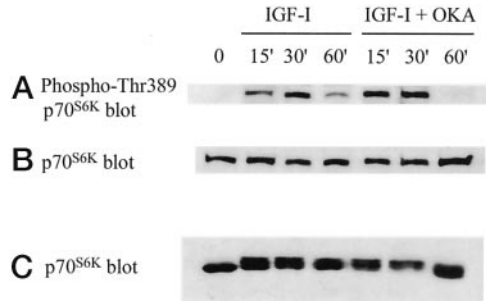

FIG. 7. **Effect of okadaic acid on p70^{S6K} activation in 32D IGF-IR/IRS-1 cells.** 32D IGF-IR/IRS-1 cells were incubated in serum-free medium for 4 h. Samples treated with OKA were pre-incubated with 500 nM OKA for 60 min before stimulation with IGF-I (20 ng/ml). Control cells were incubated only with IGF-I. Lysates obtained at the times indicated (in min) were run on three separate gels (SDS-PAGE) and the membranes blotted with the three different anti-phospho-p70^{S6K} antibodies. For convenience, we show only the results obtained with the anti-phosphothreonine 389 antibody (*panel A*) and the total level of protein (*panel B*). The blot shown in *panel C* was run on a 7.5% gel to enhance the mobility shift of the total p70^{S6K} (see "Experimental Procedures").

IGF-IR and 32D IGF-IR/vector cells grew in the first 4 days after IL-3 withdrawal (albeit with considerable less efficiency than 32D IGF-IR/IRS-1 cells) and showed differentiation (36% and 37%, respectively). After re-plating, only a few cells (61% of them differentiated) were left at the end of the experiment on day 8. In contrast, 32D IGF-IR/IRS1 cells grew again in the absence of IL-3, without any evidence of differentiation. These experiments therefore show that 32D IGF-IR/IRS1 cells have become IL-3-independent and can be passaged in its absence.

Finally, all these cell lines have repeatedly tested negative

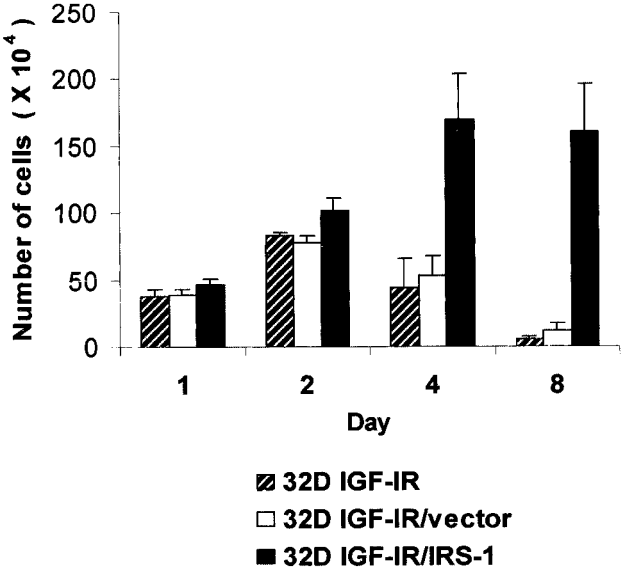

FIG. 8. **Growth of 32D-derived cells in the absence of interleukin-3.** 32D IGF-IR (*striped bars*), 32D IGF-IR/vector (*open bars*), and 32D IGF-IR/IRS-1 cells (*closed bars*) were seeded in IL-3-free medium supplemented with 50 ng/ml IGF-I at the density of 5×10^4 cells/ml in a total volume of 2 ml. At day 4 viable cells were counted by trypan blue exclusion, and 10×10^4 cells for each cell line were re-plated in 2 ml of fresh IL-3-free medium with IGF-I (50 ng/ml). Cells were counted after 4 additional days for a total of 8 days of culture. Data are the average of three separate experiments (with standard deviations).

for mycoplasma. This is important, because Feng *et al.* (42) have recently reported that mycoplasma infection induces malignant transformation of 32D cells.

Tumor Formation in Animals—IL-3 independence has been often taken as a measure of transformation of 32D cells (41). However, a more stringent criterion for transformation is the ability to form tumors in animals. We tested both 32D IGF-IR and 32D IGF-IR/IRS1 cells for their ability to form tumors in nude mice (see "Experimental Procedures"). Because 32D cells are of hemopoietic origin, and because they were injected intraperitoneally, we expected them to localize predominantly in the abdominal organs, especially spleen and liver (which, incidentally, is the major site of IGF-I production in the animal body). The results are shown in Fig. 9, where we compare the pathology of mice injected with either 32D IGF-IR cells and 32D IGF-IR/IRS-1 cells. However, the parental 32D cells behaved, as expected, like the 32D IGF-IR cells (data not shown). 32D IGF-IR/IRS1 cells form tumors in nude mice, causing marked increases in weight of both liver and spleen (Fig. 9, compare *panels A* and *B*). On average, the livers doubled their weight, while the weight of spleen increased 10-fold. Histologically (Fig. 9), the structures of both liver and spleen are obliterated by the infiltration of leukemic cells. Only remnants of hepatocytes can be seen in the liver (compare *panels C* and *D*). In the spleen, the small lymphocytes of the normal spleen (*panel F*) have been replaced by larger leukemic cells (*panel E*). 32D IGF-IR/IRS-1 cells can also form tumors in syngeneic mice, but only when the cells are injected intravenously (data not shown). Therefore, even by the quite stringent criterion of tumor formation in animals, 32D IGF-IR/IRS1 cells are transformed.

DISCUSSION

As previously reported, in the absence of IRS-1, the IGF-IR induces only a burst of cell proliferation (about 48 h), after which, the cells (32D IGF-IR cells) undergo granulocytic differentiation (Ref. 2 and this paper). The novel findings in this report can be summarized as follows. 1) A comparison of signaling between the 2 cell lines shows that in 32D IGF-IR/IRS1 cells, the phosphorylation of threonine 389 and threonine 421/serine 424 of $p70^{S6K}$ is stronger and more sustained, when compared with the 32D IGF-IR cells. 2) In support of a role of $p70^{S6K}$ in the transformation of 32D IGF-IR/IRS1 cells, we show that rapamycin, which inhibits the activation of $p70^{S6K}$ (Refs. 29, 32, and 43, and this paper) causes differentiation of 32D IGF-IR/IRS1 cells. 3) The ectopic expression of IRS-1 and the sustained activation of $p70^{S6K}$ result in an increase in cell size of 32D IGF-IR/IRS-1 cells, increase that is inhibited by rapamycin. 4) Despite the fact that, in the first 48 h, both 32D IGF-IR and 32D IGF-IR/IRS1 cells proliferate very actively (Ref. 11 and this paper), a differentiation marker, MPO mRNA, is already prominent at 24 h in 32D IGF-IR cells. MPO mRNA levels do not increase in 32D IGF-IR/IRS-1 cells, but they do so in the same cells treated with rapamycin. Secondary findings include the following. 5) OKA, which also inhibits the activation of $p70^{S6K}$, causes differentiation of 32D IGF-IR cells. 6) 32D cells expressing both the IGF-IR and IRS-1 (32D IGF-IR/IRS-1 cells) have a transformed phenotype, as evidenced by IL-3 independent growth and the ability to form tumors in animals.

There are several reports in the literature that cells in general, and hemopoietic cells in particular, must undergo one or two rounds of DNA synthesis before they can differentiate (5, 15). Therefore, it is not surprising that 32D IGF-IR cells do grow for 48 h before they begin to differentiate. The question is how IRS-1 can inhibit the differentiation program in these cells, and actually transform them. It is generally assumed that the IGF-IR and the insulin receptor use for mitogenesis 3 main pathways (44–47). The first is through IRS-1 to PI-3 kinase to $p70^{S6K}$ (16, 30); the second is through IRS-1 to Grb2 to Ras and Erk, and the third one is from Shc to Grb2, to Ras again. Both $p70^{S6K}$ and ERK are required for mitogenesis (17, 44, 48). Although IGF-IR signaling is certainly more complicated than this, this simplification gives an operational scheme for any attempt to elucidate pathways involved in two different and contradictory programs. The first exception to this scheme is provided by the observation that the IGF-IR (but not the IR) does not need IRS-1 for mitogenesis and survival in 32D cells (2, 9–11), at least for the first 48 h. It seems that the IGF-IR has pathways for protection from apoptosis, cell proliferation and differentiation that are IRS-1-independent (2, 10) and are not shared with the IR (11).

One reasonable target of IRS-1 is $p70^{S6K}$, which is activated by IRS-1 (16, 49), although it may be also activated by other transducing molecules (50). In a hierarchical model of kinase activation, the activation of $p70^{S6K}$ is apparently dependent on the phosphorylation of a putative auto-inhibitory C terminus, which causes a change in conformation, which, in turn, allows the catalytic domain to be activated (28, 51). Our results show that the presence of IRS-1 causes a sustained phosphorylation of threonine 389, and of threonine 421/serine 424, phosphorylation that is shorter and less intense in 32D IGF-IR cells. This difference is highly reproducible, and results also in a difference in the mobility of the $p70^{S6K}$ protein. A mobility shift of $p70^{S6K}$ has often been taken as an indication of activation (16, 29, 52). In addition, phosphorylation of threonine 389 is largely obliterated when 32D IGF-IR/IRS-1 cells are treated with rapamycin, which induces differentiation. As mentioned above, threonine 389 belongs to the group of serine/threonines of $p70^{S6K}$ that are rapamycin-sensitive. Indeed, they were originally considered to be the only residues that were rapamycin-sensitive, while the residues in the C terminus (like threonine 421 and serine 424) were considered insensitive (29, 31, 32). More recent reports have questioned this difference (33–35).

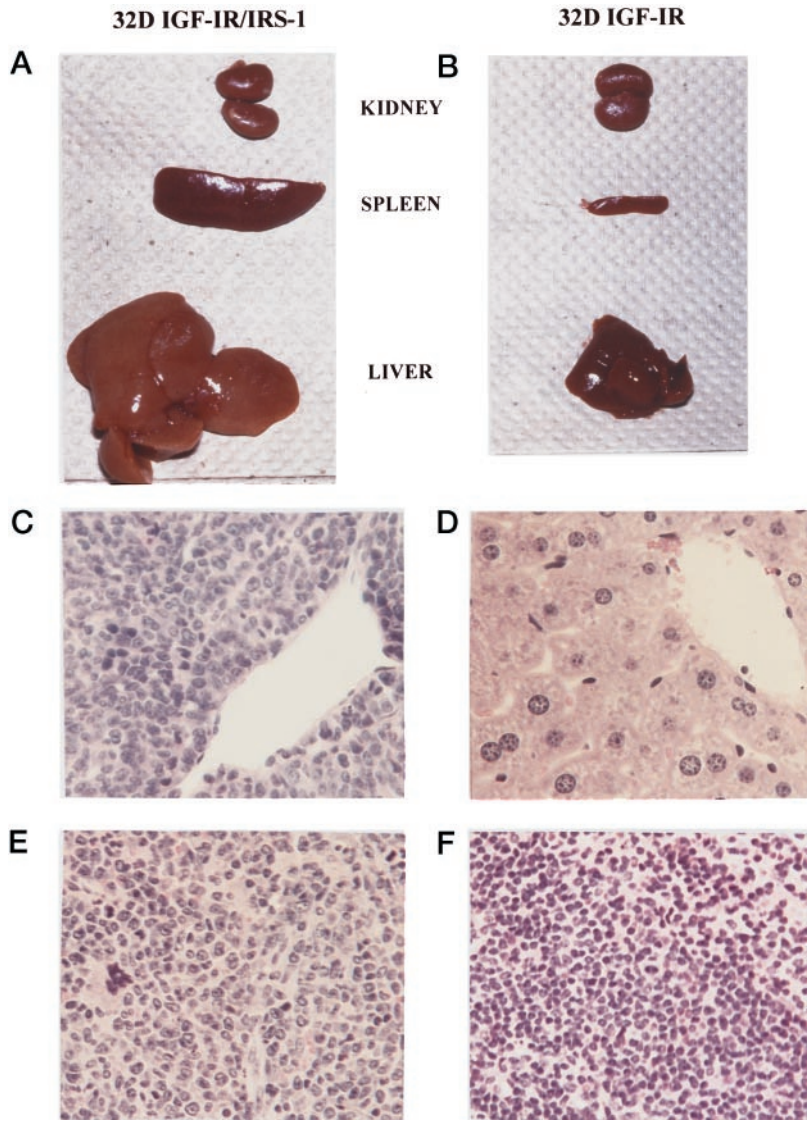

FIG. 9. **Pathology of liver and spleen in Mice injected with 32D IGF-IR/IRS-1 cells.** The animals and the cell lines are described under "Experimental Procedures". *Panel A*, a representative picture of liver, spleen, and kidneys from nude mice injected with 32D IGF-IR/IRS-1 cells. *Panel B*, same for mice injected with 32D IGF-IR cells. *Panel C*, histology of liver in mice injected with 32D IGF-IR/IRS-1 cells. *Panel D*, liver section of animals injected with 32D IGF-IR cells. *Panels E* and *F*, spleen sections of mice injected with 32D IGF-IR/IRS1 or 32D IGF-IR cells, respectively. All magnifications for histology are ×400. The only tissues markedly increased in weight in mice injected with the 32D IGF-IR/IRS-1 cells were the liver and spleen.

Incidentally, the induction of differentiation in 32D IGF-IR/IRS-1 cells by rapamycin, a specific inhibitor (through mTOR) of $p70^{S6K}$ (28), at concentrations that do not cause apoptosis, provides evidence for a role of $p70^{S6K}$ in this model that goes beyond a simple correlative finding.

In no way should our findings be construed as indicating that $p70^{S6K}$ activation is not needed for proliferation of 32D cells. The abundant literature on the subject shows that $p70^{S6K}$ is required for the G_1 to S transition (17), and indeed we show that $p70^{S6K}$ is activated in both 32D IGF-IR and 32D IGF-IR/IRS1 cells, that proliferate for the first 48 h. The difference between our two cell lines is in the sustained stimulation of $p70^{S6K}$, a difference confirmed when the 32D IGF-IR/IRS1 cells are induced to differentiate by rapamycin (or OKA). One corollary of these findings is that the IGF-IR can activate $p70^{S6K}$ in the absence of IRS-1. The human insulin receptor fails to activate $p70^{S6K}$ in the absence of IRS-1 (52). These studies also emphasize the need to examine phosphorylation of $p70^{S6K}$ with different phosphospecific antibodies and at different intervals after stimulation. Weng *et al.* (34) carried out an extensive study of $p70^{S6K}$ phosphorylation and activation, using several phosphospecific antibodies. Their time courses (their Fig. 2) extended to 30 min after insulin stimulation, with no suggestion of a decrease at that time (unless rapamycin or wortmannin were added). The cells used by Weng *et al.* (34) were CHO-IR cells, which have IRS-1 and overexpress the insulin receptor. Han *et al.* (29) also looked at $p70^{S6K}$ activity at times up to 1 h after stimulation, and showed that the inhibitor SQ20006 caused inhibition. Our results seem to indicate that if we had examined $p70^{S6K}$ phosphorylation only at 15 min after IGF-I stimulation, the difference between the two cell lines would have been much less convincing. It is only at later times that differences emerge, and these differences are corroborated by changes in mobility shifts.

As to the mechanism(s) by which IRS-1 and $p70^{S6K}$ bring about the transformed phenotype, we have explored in this paper two possibilities. The first possibility is an effect on cell size. An effect of IGF-IR signaling on cell size was suggested by the experiments of Surmacz *et al.* (53), who showed that IGF-I could activate the ribosomal DNA promoter, and further supported by the finding that $p70^{S6K}$ knock-out mice are somewhat smaller than their wild type littermates (54). But the importance of IRS-1 and $p70^{S6K}$ in cell size regulation was demonstrated more rigorously by the recent reports that homologues of both IRS-1 and the S6 kinase regulate cell size in *Drosophila* (18, 19). We show here that cell size is increased in 32D IGF-IR/IRS-1 cells, but not when the cells are treated with rapamycin. The increase in the mean of the forward scatter is small but highly reproducible, and, for a proper assessment, one should compare our data with Fig. 2 of Bohni *et al.* (18). In this figure,

Bohni *et al.* (18) used, like us, FACS analysis to estimate the effect of CHICO on cell size in *Drosophila*. Their difference (also measured by forward scattering) was 10–14%, the same difference we have noticed in our cells. Furthermore, our comparison of the G_1 and G_2 subpopulations of the same cell line (Fig. 4, *panel D*) shows that the small difference revealed by forward scattering reflects a considerable difference in actual size.

The second possibility (the two possibilities are not mutually exclusive) is based on a simple hypothesis. This hypothesis calls for the IGF-IR to send signals for two separate programs: differentiation and mitogenesis (see above). In this scenario, the main function of IRS-1 would be to extinguish the differentiation program. It has been reported that certain markers of differentiation in hemopoietic cells can be detected very early, while the cells are still proliferating (55). If our hypothesis is correct, a marker of differentiation would be visible at early times in 32D IGF-IR cells, but not in 32D IGF-IR/IRS1 cells. Our results with an early differentiation marker, the MPO mRNA, are compatible with this hypothesis. The levels of MPO mRNA are high in 32D IGF-IR cells at 24 h after IL-3 withdrawal (with the cells rapidly proliferating), while they are much lower in 32D IGF-IR/IRS-1 cells. In further support of our hypothesis is the finding that MPO mRNA is also increased in 32D IGF-IR/IRS-1 cells treated with rapamycin. Incidentally, 32D IGF-IR/IRS1 cells have not lost the capacity to undergo granulocytic differentiation. They are induced to do so not only by OKA and rapamycin, but also by granulocyte colony-stimulating factor (data not shown).

Since PI 3-kinase plays an important role in activation of $p70^{S6K}$ (see above), it is reasonable to ask whether inhibitors of PI 3-kinase have any effect on the growth of these cells. Addition of PI 3-kinase inhibitors has little effect on the growth of 32D IGF-IR cells (10, 11). It seems therefore that the IGF-IR can stimulate growth by a mechanism by passing PI 3-kinase. However, Akt is weakly but reproducibly activated by IGF-I in 32D IGF-IR cells (2). An obvious next step is to determine whether the activation of $p70^{S6K}$ by the IGF-IR, in the absence of IRS-1, may directly involve PDK1 (28).

An intriguing conclusion of this paper is that IRS-1 can make the difference between terminal differentiation (and death) and malignant transformation in 32D cells expressing the IGF-IR. Although 32D IGF-IR cells are stimulated to proliferate by IGF-I for the first 48 h (Ref. 11 and this paper), only 32D IGF-IR/IRS1 cells can form tumors in nude and syngeneic mice (or grow continuously in the absence of IL-3). There is often a discrepancy between decreased growth factor requirements and ability to form tumors in experimental animals (56). For this reason, we wish to document here that the 32D IGF-IR/IRS1 cells not only have lost the IL-3 requirement, but are also capable of forming tumors in mice.

Finally, there are two other considerations that should be discussed at this point. The first is the use of a dominant negative mutants of $p70^{S6K}$. It would have confirmed the data obtained with rapamycin, but the problem with dominant negative mutants of $p70^{S6K}$ is the existence of a similar kinase, S6K2, that can, at least partially, replace the $p70^{S6K}$ function (28, 54). A second legitimate concern is the connection between an effect seen at 60 min (sustained activation of $p70^{S6K}$) and an event (differentiation) that materializes only after 48 h or even later. This is a problem with all signal transduction events when one wishes to correlate them to later processes, such as proliferation or transformation. However, Rose *et al.* (57), in an elegant experiment, have shown that some transducing signals are required throughout the cell cycle, while other are required only for the first 15 min after growth factor stimulation. We may also add that the extinction of the differentiation program (see Fig. 5) must occur early after IGF-I stimulation.

In conclusion, our experiments offer a valuable model for the study of transformation *versus* differentiation in murine hemopoietic cells expressing the IGF-IR. The presence or absence of IRS-1 determines whether the cells will transform or differentiate, respectively. The mechanism requires the sustained activation of the $p70^{S6K}$ pathway. We would like to speculate (see above) that the main function of IRS-1 in this model is to inhibit the activation of the differentiation program, resulting in continuous cell proliferation. Our results with the MPO mRNA suggest that this hypothesis may be correct. It is therefore possible that the differences reported in this paper between 32D IGF-IR cells and 32D IGF-IR/IRS-1 cells may already be programmed in the early stages of IGF-I stimulation, before the fate of the cells actually diverges. The next step would be to investigate how the various transcription factors that are involved in myeloid differentiation are affected by IRS-1 in the various conditions described in this paper.

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Insulin Receptor Substrate-1, p70^{S6K}, and Cell Size in Transformation and Differentiation of Hemopoietic Cells

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