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Overexpression of Human Insulin Receptor Substrate 1 Induces Cellular Transformation with Activation of Mitogen-Activated Protein Kinases

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The insulin receptor substrate 1 protein (IRS-1) is a specific substrate for insulin receptor tyrosine kinase. Expression and tyrosyl phosphorylation of IRS-1 play an important role during normal hepatocyte growth, and the gene is overexpressed in hepatocellular carcinoma tissue. We determined if IRS-1 overexpression directly contributes to cellular transformation. The human IRS-1 gene was subcloned into a mammalian expression vector driven by the cytomegalovirus early promoter. NIH 3T3 cells transiently transfected with this vector subsequently developed transformed foci. Several stably transfected cell lines were established, and they grew efficiently under low-serum conditions and formed colonies when plated in soft agar. Cell lines overexpressing IRS-1 displayed increased tyrosyl phosphorylation of IRS-1 and association with Grb2 but not with the p85 subunit of phosphatidylinositol 3′-kinase. Since Grb2 is a component of the son-of-sevenless–*Ras* pathway and upstream in the mitogen-activated protein kinase (MAPK) cascade, enzymatic activities of the major components of this cascade, such as MAPK kinase and MAPK, were evaluated and found to be substantially increased in three independent cell lines with IRS-1 protein overexpression. Such cells, when injected into nude mice, were highly tumorigenic, and there may be a correlation between the degree of MAPK activation and tumor growth rate. This report describes the generation of a transformed phenotype by overexpression of a molecule without a catalytic domain far upstream in the signal transduction cascade and suggests that prolonged activation of MAPKs by this mechanism may be one of the molecular events related to hepatocellular transformation.

Insulin is a pleiotrophic factor with both mitogenic and metabolic effects on cells. Insulin receptor substrate 1 (IRS-1) protein is the principal intracellular substrate of insulin receptor tyrosine kinase activity and is the most upstream molecule in the signal transduction cascade mediated by insulin, interleukin 4, and insulin-like growth factor I stimulation (26, 41). However, IRS-1 appears not to have a catalytic domain (26, 41). Previously, we reported the cloning of the human IRS-1 (hIRS-1) gene and demonstrated overexpression in human hepatocellular carcinoma (HCC) tissue compared with adjacent, noninvolved, healthy liver tissue (28). We have also presented evidence that expression and phosphorylation of IRS-1 are important during normal liver growth induced by partial hepatectomy as a presumed "docking" protein involved both in transmission of the insulin signal and possible interaction with other molecules essential for regulation of hepatocyte growth (30, 31). It has been amply demonstrated that IRS-1 has multiple tyrosine phosphorylation residues that allow it to associate with other signal molecules containing *src* homology 2 (SH2) domains, such as the p85 subunit of phosphatidylinositol 3′-kinase (PI3-K) (2, 25); growth factor receptor-bound protein 2 (Grb2) (3, 32), which interacts with son of sevenless (SOS) and induces *Ras* activation (18, 42); and SH-PTP2 or Syp (19). These protein-protein interactions play essential roles in the intracellular signaling process (34, 35). More important, the insulin signal transduction pathway activates the mitogen-activated protein kinase (MAPK, or extracellular signal-regulated kinase [Erk]) cascade (32, 37). This MAPK cascade is composed of MAPKs, MAPK kinase (MAPKK) {Erk kinase (MAPK-Erk kinase [MEK])}, and Raf or MEK kinase (9, 10, 20, 27). In addition, Ras functions as an activator of Raf and leads to activation of the cascade (43). It has also been established that Ras-dependent signal transduction also converges to activate the MAPK cascade, and this event appears to be necessary for cell proliferation to occur (1, 4).

It has been proposed that IRS-1 is essential for insulin-induced mitogenic effects (29, 40). Thus, the components of this cascade, including IRS-1, may be required for hepatocyte proliferation and even contribute to hepatic carcinogenesis. Therefore, we tested the hypothesis that constitutive overexpression of this molecule may result in generation of the malignant phenotype. In the present investigation, we determined if upregulation of the IRS-1 gene activates the downstream MAPK cascade and is associated with malignant transformation of NIH 3T3 cells.

MATERIALS AND METHODS

Antibodies. Anti-hIRS-1 polyclonal antibodies were prepared in rabbits as previously described (28). Anti-Erk1 and anti-Erk2 rabbit polyclonal antibodies and anti-Grb2 rabbit polyclonal antibody were purchased from Santa Cruz Biotechnology, Inc. The anti-p85 subunit of PI3-K rabbit polyclonal antibody was obtained from Upstate Biotechnology Inc., Lake Placid, N.Y. Antiphosphotyrosine mouse monoclonal antibody was kindly provided by Brian Drucker (Dana-Farber Cancer Institute, Boston, Mass.).

Expression plasmids. Mammalian expression vector pBK-CMV, driven by the cytomegalovirus immediate-early promoter and containing the neomycin resistance gene, was purchased from Stratagene Cloning Systems, La Jolla, Calif. The entire open reading frame of hIRS-1 (*Nsi*I-*Eco*RI fragment) was subcloned in the proper orientation into pBK-CMV at the *Nhe*I and *Sal*I sites, and this construct was designated pBK-CMV-hIRS-1. Plasmid construct pLNCX-UP1,

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containing the v-*src* and neomycin resistance genes, was used as a positive control (24).

Cell culture and plasmid transfections. For transfection experiments with the expression vectors mentioned above, NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Mediatech, Washington, D.C.) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma Chemical Co., St. Louis, Mo.) without insulin supplementation. Insulin was not added to cell culture medium in these experiments. The calcium phosphate (5 Prime-3 Prime, Inc., Boulder, Colo.) or liposome-mediated transfection method using Lipofectin (GIBCO BRL, Life Technologies, Inc., Gaithersburg, Md.) was carried out in accordance with manufacturer instructions. Subconfluent NIH 3T3 cells (3×10^5 cells per 6-cm-diameter dish) were transfected with pBK-CMV (negative control), pBK-CMV-hIRS-1, or pLNCX-UP1 (positive control).

To confirm the efficiency of transfection of NIH 3T3 fibroblasts, NIH 3T3 cells were transfected with several amounts of the vector DNAs indicated in Results. After 48 h, the cells were split and cultured in DMEM supplemented with 10% FBS and 400 μg of Geneticin (G418; GIBCO BRL, Life Technologies, Inc.) per ml. After 2 weeks, the surviving colonies were counted.

Analysis of transformed foci. An assay was performed to examine transformed foci following transfection by the methods cited above. NIH 3T3 cells were split among 12 dishes at 48 h after transfection with the three plasmids. Six dishes were used for determination of the number of transformed foci. The remaining six were cultured in Geneticin- and 10% FBS-containing DMEM and used for evaluation of transfection efficiency. Cells were grown until confluent for 14 days with a complete medium change every 3 days, and the transformed foci were counted. G418-resistant colonies were counted 2 weeks after transfection. Results are expressed as the number of transformed foci per microgram of plasmid DNA and the number of G418-resistant colonies per microgram of plasmid DNA.

Isolation of IRS-1 stable transfectants. Growth in Geneticin-containing medium was used to establish stably transfected cell lines. DMEM containing 10% FBS and 400 μg of Geneticin per ml was added as the culture medium following plasmid transfection. Populations of NIH 3T3 cells grew and were selected as cell lines which acquired the Geneticin resistance phenotype. In Geneticin-containing medium, each cell line was cloned by limiting dilution of cells derived from the pooled population.

Cell growth under low-serum conditions. Cell growth rates in low serum concentrations were also measured. Clonal cell lines selected in the presence of Geneticin were seeded at a density of 10^4 cells in six-well dishes containing DMEM with 10% FBS. Replicate dishes were counted 24 h later to confirm that all of the plates contained approximately the same initial cell number, and they were designated day 0 in the time course curve. The medium was removed and replaced with culture medium containing 1% FBS. The culture medium was changed every 2 days, and cell counts were performed.

Anchorage-independent cell growth assays. NIH 3T3 cells stably transfected with pBK-CMV, pBK-CMV-hIRS-1, or pLNCX-UP1 were assessed for altered growth characteristics by colony formation in soft agar. For this procedure, 10^4 cells of each stably transfected pool of cells or established cell lines which had been previously determined to express IRS-1 at a higher level than mock-transfected control cells were seeded in DMEM containing 10% FBS and 0.4% soft agar (SeaPlaque GTG Agarose; FMC Bioproducts, Rockland, Maine) and placed over a bottom agar containing medium consisting of DMEM containing 10% FBS and 0.53% agar. Cells were fed once a week with DMEM containing 10% FBS and 0.2% agar. Two weeks after seeding, colonies larger than 0.1 mm in diameter were scored as positive for growth.

Immunoprecipitation and Western blot (immunoblot) analysis. Cell lysates from stable IRS-1-transfected cell lines were prepared in Triton lysis buffer [50 mM Tris-HCl (pH 7.5), 1% Triton X-100, 2 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N,N′*-tetraacetic acid (EGTA), 10 mM EDTA, 100 mM NaF, 1 mM $Na_4P_2O_7$, 2 mM $NaVO_4$, 1 mM phenylmethylsulfonyl fluoride, 25 mg of aprotinin per ml, 3.5 mg of pepstatin A per ml, 25 mg of leupeptin per ml]. These samples were subjected to either immunoprecipitation or Western blot analysis as previously described (28, 30, 31). Following sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, proteins were transferred onto Immobilon P membranes (Millipore Corp., Bedford, Mass.). Antibodies bound to proteins or identifying phosphotyrosine residues on proteins were detected with the ECL system (Amersham). Signals on the films were quantitated by densitometry as previously described (31).

MAPKK activity. Endogenous MAPKK activity was evaluated by measuring ^{32}P incorporation into full-length human recombinant MAPK-Erk1 and myelin basic protein (MBP; Sigma Chemical Co.). A recombinant enzyme-agarose conjugate was obtained from Upstate Biotechnology Inc. This assay was performed as described in a previous report (20), with some modification. Briefly, 25 μg of protein from each whole-cell lysate in 50 mM β-glycerophosphate (pH 7.3)–1.5 mM Na-EGTA–100 μM $NaVO_4$–400 μM phenylmethylsulfonyl fluoride–25 mg of aprotinin per ml–3.5 mg of pepstatin A per ml–25 mg of leupeptin per ml–1 mM dithiothreitol was applied for detection of MAPKK activity. A 10-μl portion of each sample was mixed with 6 μl of 20 mM *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid (HEPES; pH 7.4)–1 mM EGTA–1 mM dithiothreitol–0.4 mg of bovine serum albumin per ml containing 50 μg of recombinant Erk1–glutathione *S*-transferase protein conjugated to agarose beads plus 4 μl of 50 mM $MgCl_2$ and 0.5 mM [γ-^{32}P]ATP. Samples were incubated for 30 min at 30°C and washed three times with lysis buffer, three times with 0.5 M LiCl–100 mM Tris-HCl (pH 7.6), and then twice with 150 mM Tris-HCl (pH 7.4)–3 mM dithiothreitol–30 mM $MgCl_2$. Next, 10 μl of the reaction mixture (150 mM Tris-HCl [pH 7.4], 3 mM dithiothreitol, 30 mM $MgCl_2$, 150 μM [γ-^{32}P]ATP, 0.75 mg of MBP per ml) was added to the pellets. After 15 min of incubation at 30°C, samples were quenched with loading buffer (60 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 0.025% bromophenol blue, 5% 2-mercaptoethanol) and analyzed by SDS-polyacrylamide gel electrophoresis. Erk1–glutathione *S*-transferase and MBP bands were excised from the dried and stained gels and counted with a liquid scintillation counter (Beckman). As a control, all procedures were performed with only lysis buffer to exclude the activity of recombinant enzyme induced through autophosphorylation. All experiments were repeated at least three times with similar results.

Kinase assay using MBP. Enzymatic activity of MAPKs was measured by a previously described method (13, 37). In brief, cell lysates were electrophoresed on an SDS-polyacrylamide gel containing 0.5 mg of MBP per ml. Next, SDS was removed from the gel by washing with 20% 2-propanol in 50 mM Tris-HCl (pH 8.0) for 1 h and then with 50 mM Tris-HCl (pH 8.0) containing 5 mM 2-mercaptoethanol for an additional 1 h at 20°C. The enzyme in the gels was denatured by treatment with 6 M guanidine HCl and 50 mM Tris-HCl (pH 8.0) for 1 h at 20°C and renatured in 50 mM Tris-HCl (pH 8.0) containing 0.04% Tween 40 and 5 mM 2-mercaptoethanol. After renaturation, the gel was preincubated in kinase buffer (40 mM HEPES [pH 8.0], 2 mM dithiothreitol, 0.1 mM EGTA, 20 mM $MgCl_2$) for 1 h at 25°C. Phosphorylation of MBP was carried out by incubation of the gel with kinase buffer containing 25 μCi of [γ-^{32}P]ATP (Du Pont) for 1 h at 25°C. After incubation, the gel was washed in 5% (wt/vol) trichloroacetic acid solution containing 1% sodium PP_i, dried, and then subjected to autoradiography at −80°C. The locations corresponding to MAPK-phosphorylated MBP were determined by using the autoradiographs, and relevant areas were cut out and counted with a liquid scintillation counter (Beckman). All experiments were repeated at least three times with similar results.

Tumor formation in nude mice. To investigate tumorigenicity in nude mice, stably transfected cells were resuspended at a concentration of 10^7/0.1 ml of 0.9% NaCl and 100 μl was injected subcutaneously into the back of each nude mouse. Nude mice were observed for 1 month, and solid tumor formation and growth rate were determined.

RESULTS

To confirm expression of IRS-1 protein in neomycin-resistant NIH 3T3 cells following transfection, pooled transfectants were prepared. As shown in Fig. 1A, IRS-1 expression was detected at higher levels in a pooled population of NIH 3T3 cells transfected with the hIRS-1 gene than were endogenous IRS-1 concentrations present in NIH 3T3 cells transfected with an empty vector alone. FOCUS HCC cells were used as a positive control since they have high endogenous levels of hIRS-1 protein compared with normal human liver (28). Parental and clonal cell lines were maintained with 10% FBS–DMEM. As shown in Fig. 1B and C, IRS-1 expression in these three cell lines was four- to sixfold higher than endogenous IRS-1 levels present in NIH 3T3 Neo^r control cells transfected with the empty vector. There was a severalfold increase in tyrosyl phosphorylation of IRS-1 in all three cloned cell lines, as well as in the pooled parental cells, compared with that in mock-transfected NIH 3T3 control cell line; these cells were grown in 10% FBS without insulin addition to the culture medium (Fig. 1D and E).

The transforming potential of the hIRS-1 gene was studied and compared to the activity exhibited by the v-*src* gene in pLNCX-UP1 (positive control) and pBK-CMV (negative control). As shown in Table 1, cells transfected with the hIRS-1 gene formed four to five times more transformed foci per microgram of DNA than did those transfected with the empty vector; v-*src*-transfected NIH 3T3 cells, however, exhibited the highest number of transformed foci. This experiment also demonstrated transfection efficiencies to be about 0.1 to 0.3% for NIH 3T3 cells under these conditions (Table 1). The appearance of a confluent culture of empty-vector-transfected cells is shown in Fig. 2A. Typical transformed foci induced by the hIRS-1 gene are shown in Fig. 2B and appeared similar to those of v-*src*-transfected cells, as shown in Fig. 2C. There was

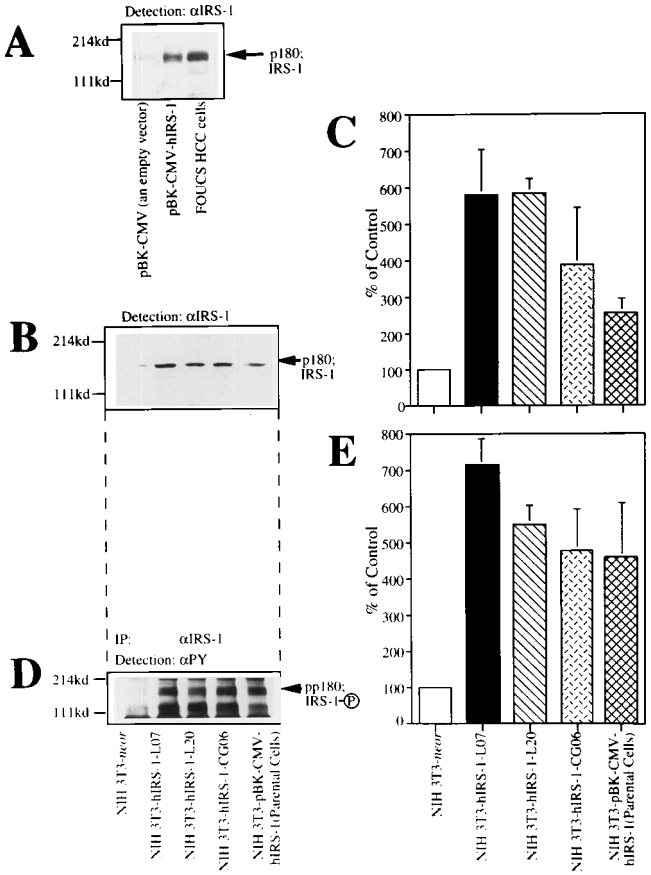

FIG. 1. Expression and tyrosyl phosphorylation of IRS-1 in stably transfected NIH 3T3 cell lines. (A) NIH 3T3 cells were transfected with the pBK-CMV or pBK-CMV-hIRS-1 construct. Whole-cell lysates were prepared and blotted after SDS-polyacrylamide gel electrophoresis. Immunodetection was performed with a rabbit anti-hIRS-1 (αIRS-1) polyclonal antibody. NIH 3T3 cells transfected with pBK-CMV, NIH 3T3 cells stably transfected with pBK-CMV-hIRS-1, and FOCUS HCC cells (positive control) were used in this experiment. (B and D) Lysates of three cloned cell lines stably transfected with IRS-1, the pooled parental cells, and control cell lines were analyzed by Western blot analysis of IRS-1 protein levels, as well as tyrosine phosphorylation on IRS-1. The levels of IRS-1 expression (C) and tyrosine phosphorylation (E) were assessed by three independent blots and quantitated by densitometry. Error bars indicate the standard error of the mean. kd, kilodaltons.

TABLE 1. Growth characteristics of NIH 3T3 cells transiently transfected with the human IRS-1 gene

Transfection vector	Mean no. of transformed foci/μg of DNA ± SD[a]	Mean no. of G418-resistant colonies/μg of DNA ± SD[a]
Expt 1[b]		
pBK-CMV	14.2 ± 3.4	31.7 ± 12.2
pBK-CMV-hIRS-1	66.7 ± 12.3	20.0 ± 9.8
pLNCX-UP1 (v-*src*)	196.0 ± 35.0	16.7 ± 2.36
Expt 2[c]		
pBK-CMV	15.0 ± 7.6	83.3 ± 18.5
pBK-CMV-hIRS-1	53.3 ± 3.6	80.0 ± 17.3
pLNCX-UP1 (v-*src*)	250.0 ± 10.0	83.3 ± 27.3

[a] Values are means and standard deviations of six determinations from three independent transfections.
[b] Calcium phosphate precipitation method done with 20 μg of DNA of each construct.
[c] Liposome-mediated transfection method done with 10 μg of DNA of each construct.

no difference in size between the transformed foci exhibited by hIRS-1- and v-*src*-transfected NIH 3T3 cells.

Following transfection of NIH 3T3 cells, stable cell lines were obtained and grown in DMEM containing 10% FBS and 400 μg of Geneticin per ml without insulin addition. Transfection efficiencies for all three plasmids (pBK-CMV, pBK-CMV-hIRS-1, and pLNCX-UP1 [v-*src*]) were evaluated by counting Geneticin-resistant colonies. To determine if stably transfected IRS-1 cell lines acquire the property of anchorage-independent cell growth, a mixture of NIH 3T3 cells overexpressing IRS-1 which had obtained the neomycin resistance phenotype was employed in this experiment. These cells were placed in medium containing soft agar, and colony formation was assessed at 2 weeks. A few small colonies were observed with NIH 3T3 cells transfected with pBK-CMV (negative controls), as shown in Fig. 2D, and nontransfected NIH 3T3 cells (data not shown). On the other hand, NIH 3T3 cells overexpressing IRS-1 displayed typical colony formation in soft agar (Fig. 2E) similar to that observed with v-*src*-transformed cells (Fig. 2F).

Next, several cell lines were established by dilutional cloning from the pooled population overexpressing IRS-1 protein. To determine the growth characteristics of each cell line with respect to serum requirements, we made a comparison of the growth rates of four cell lines isolated from the neomycin-resistant pooled population overexpressing IRS-1 with those of two stable cell lines transfected with pBK-CMV containing the neomycin resistance gene and normal NIH 3T3 cells (negative controls). The results are presented in Fig. 3. The NIH 3T3-hIRS-1-L07 cell line displayed a substantially increased growth rate in 1% FBS. In addition, two other cell lines overexpressing IRS-1, namely, NIH 3T3-hIRS-1-L20 and NIH 3T3-hIRS-1-CG06, also grew at approximately the same rate as NIH 3T3-hIRS-1-L07 cells in 1% FBS. The other cell lines, NIH 3T3-hIRS-1-C20, NIH 3T3-Neo^r-1, and NIH 3T3-Neo^r-2, were unable to proliferate in culture medium containing 1% FBS, and their growth was similar to that of nontransfected parental NIH 3T3 cells (Fig. 3A). The three cell lines that grew in 1% FBS expressed high levels of IRS-1. In contrast, NIH 3T3-hIRS-1-C20 cells had IRS-1 levels similar to those of the two NIH 3T3-Neo^r cell lines (Fig. 3B). Finally, tyrosine phosphorylation of IRS-1 was prominant in L07, L20, and CG06 cells but not in C20, NIH 3T3-Neo^r-1, and NIH 3T3-Neo^r-2 cells and normal NIH 3T3 cells (Fig. 3C).

To further determine if the three cloned cell lines overexpressing IRS-1 acquire the property of anchorage-independent cell growth, cells were cultured in soft agar and colony formation was assessed at 2 weeks. Table 2 presents the quantitative assessment of these experiments and demonstrates that all of the stably transfected cells overexpressing IRS-1 were capable of anchorage-independent growth in soft agar. Colony formation exhibited by cells with IRS-1 overexpression was less than that observed with v-*src*-transformed cells. These studies indicate that overexpression and tyrosyl phosphorylation of IRS-1 led to anchorage-independent cell growth. From these results, cell lines NIH 3T3-hIRS-1-L07, NIH 3T3-hIRS-1-L20, and NIH 3T3-hIRS-1-CG06 were used for further analysis of IRS-1-induced transforming activity.

To evaluate signal transduction molecules downstream of IRS-1 that may be involved in the transformation process, binding of the p85 subunit of PI3-K and Grb2 to IRS-1 was studied. Among the three cell lines overexpressing IRS-1 and the NIH 3T3-Neo^r control cell line assessed under standard culture conditions (see Materials and Methods), there were no

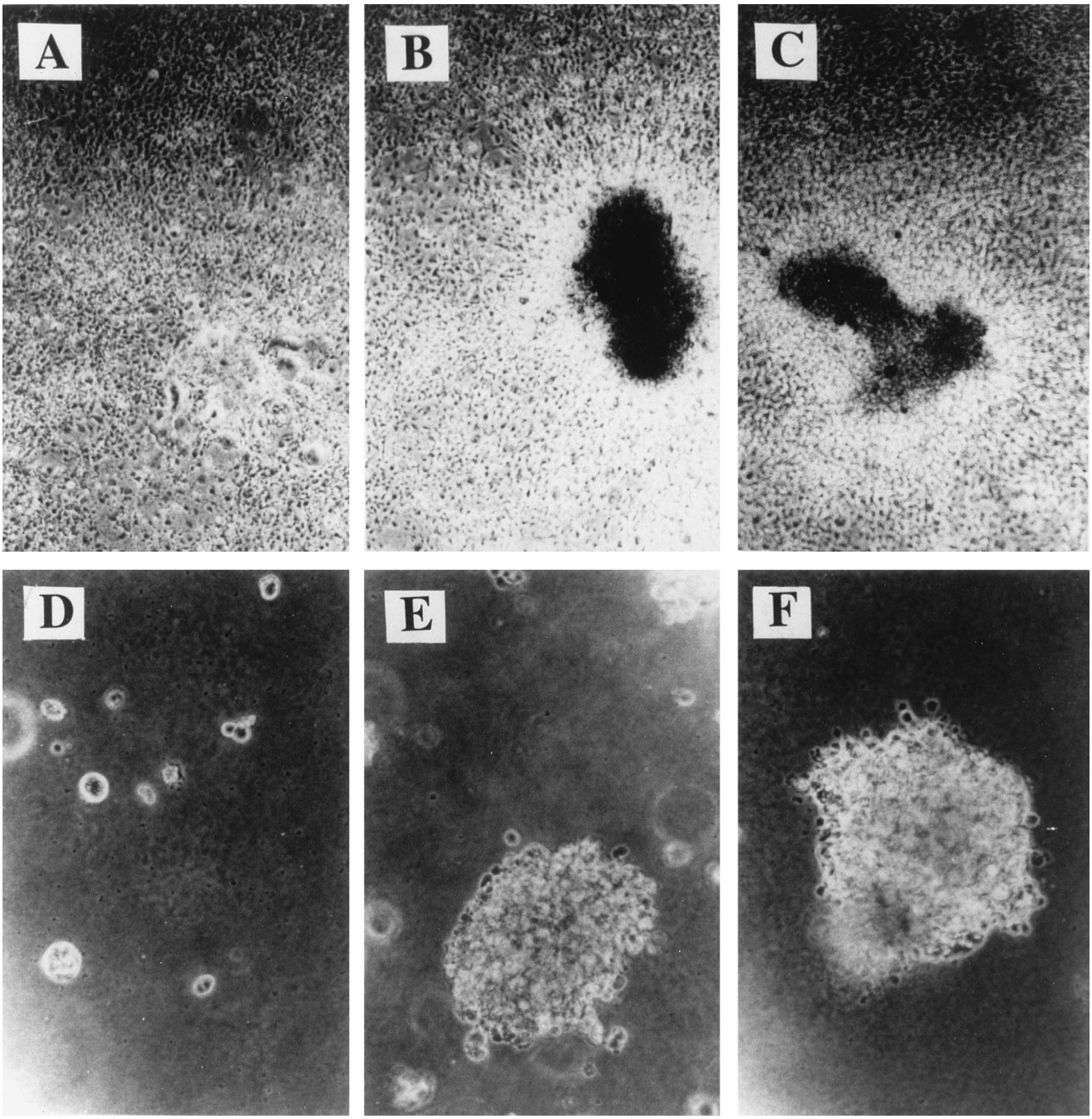

FIG. 2. Appearance of transformed foci and colony formation in soft agar of NIH 3T3 cells stably transfected with the hIRS-1 gene. Photomicrograph of the appearance of confluent NIH 3T3 cell cultures 14 days after transient transfection with a pBK-CMV (A), pBK-CMV-hIRS-1 (B), or pLNCX-UP1 (C) construct. Photographs of typical colonies in soft agar cultures taken after 14 days of growth show NIH 3T3 cells transfected with pBK-CMV (negative control) (D), NIH 3T3 cells transfected with pBK-CMV-hIRS-1 (E), and v-*src*-transformed NIH 3T3 cells transfected with the pLNCX-UP1 construct (positive control) (F). This experiment was repeated three times with essentially identical results.

significant differences in p85 protein levels, as illustrated by Fig. 4A. Figure 4E depicts the levels of Grb2 expression in these four cell lines. Figure 4B and F presents the combined results of densitometric studies from three different experiments indicating the levels of PI3-K and Grb2 protein expression in such cell lines, respectively. One cell line that overexpressed hIRS-1 (NIH 3T3-hIRS-1-L20) was found to have endogenous Grb2 protein levels twice as high as those of the other three. Next, the association of IRS-1 with these two signal transduction molecules was determined. As shown in Fig. 4C and D, there was a small increase in the association of IRS-1 with the p85 subunit of PI3-K in NIH 3T3-hIRS-1-L07, NIH 3T3-hIRS-1-L20, and NIH 3T3-hIRS-1-CG06 cells compared with NIH 3T3-Neo^r cells. In contrast, as shown in Fig. 4G and H, the association of IRS-1 with Grb2 was increased two- to fourfold in the three cell lines overexpressing IRS-1

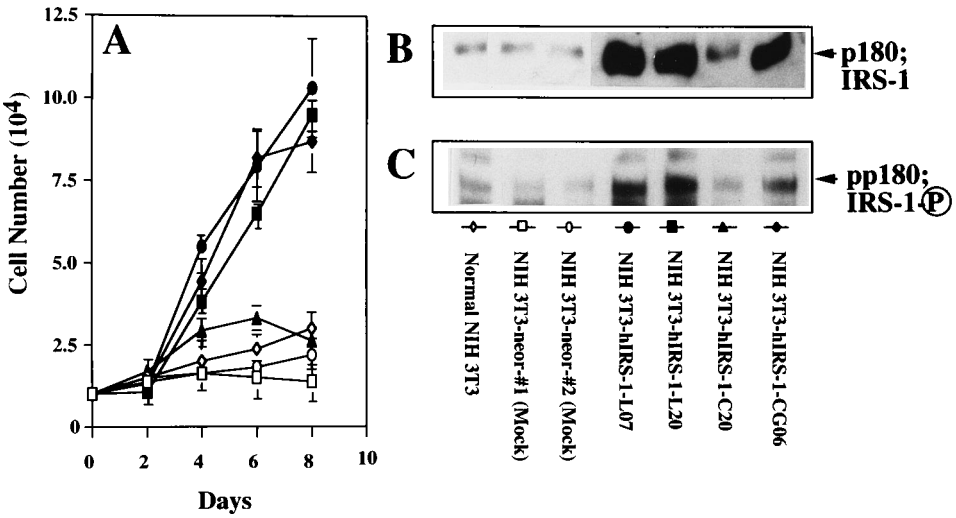

FIG. 3. Growth of NIH 3T3 cell lines overexpressing IRS-1 in low serum concentrations. The cell lines indicated (C) were grown in 1% serum (A). Whole-cell lysates prepared under standard culture conditions were used for detection of IRS-1 expression (B) and tyrosine phosphorylation of IRS-1 (C). Results are expressed as the mean and standard deviation determined from cell counts of six wells derived from each cell line. Three independent experiments were performed, and the results were identical.

compared with that of a Neo^r gene-transfected control cell line ($P < 0.05$).

Accordingly, the major components in the MAPK cascade, namely, MAPKK and MAPKs, were assessed. As shown in Fig. 5A, phosphorylation of recombinant human Erk1 as a substrate of MAPKK was increased two- to fourfold in three cell lines, NIH 3T3-hIRS-1-L07, NIH 3T3-hIRS-1-L20, and NIH 3T3-hIRS-1-CG06, that overexpressed IRS-1 compared with that in NIH 3T3-Neo^r cells ($P < 0.05$, $P < 0.001$, and $P < 0.01$ versus control, respectively). This finding suggests that enzymatic activation of MAPKK was induced in all three cell lines overexpressing IRS-1. Recombinant Erk1 as an MBP kinase was activated following phosphorylation by endogenous MAPKK, as shown in Fig. 5B ($P < 0.05$, $P < 0.01$, and $P < 0.01$ for the NIH 3T3-hIRS-1-L07, NIH 3T3-hIRS-1-L20, and NIH 3T3-hIRS-1-CG06 cell lines versus the control cell line, respectively). However, there were no statistically significant differences among these three cell lines overexpressing IRS-1 with respect to MAPKK activity. Thus, MAPKK was activated and led to activation of MAPK enzymatic activity as downstream events of the Grb2-SOS-*Ras* signal transduction pathway.

TABLE 2. Anchorage-independent growth of NIH 3T3 cell lines stably transfected with the human IRS-1 gene

Cell line	Avg efficiency of colony formation in soft agar (%) ± SD[a]	
	Expt 1[b]	Expt 2[b]
NIH 3T3-Neo^r-1[c]	0.014 ± 0.03	0.036 ± 0.02
NIH 3T3-Neo^r-2[c]	0.018 ± 0.01	0.024 ± 0.01
NIH 3T3-IRS-1-L07	3.2 ± 1.1	3.5 ± 0.5
NIH 3T3-IRS-1-L20	2.9 ± 1.1	2.6 ± 0.4
NIH 3T3-IRS-1-CG06	2.6 ± 0.7	1.6 ± 0.3
NIH 3T3-IRS-1-C20	0.042 ± 0.02	0.060 ± 0.3
NIH 3T3-v-*src*[d]	5.1 ± 0.5	8.3 ± 1.1

[a] Values for growth in soft agar are normalized to the total cell number in each well. These experiments were repeated three times with similar results.
[b] Done with 10% FBS.
[c] Cell line stably transfected with pBK-CMV.
[d] Cell line stably transfected with pLNCX-UP1.

Next, protein levels and enzymatic activities of MAPK were assessed in these three cell lines; the results are summarized in Fig. 6. Concentrations of the MAPK, Erk1 and Erk2 proteins were approximately the same in all of the cell lines overexpressing IRS-1 (NIH 3T3-hIRS-1-CG06, NIH 3T3-hIRS-1-L20, and NIH 3T3-hIRS-1-L07); similar levels were also observed in the NIH 3T3-Neo^r negative control cell line (Fig. 6A, B, C, and D). However, as shown in Fig. 6E and F, cell lines overexpressing IRS-1 demonstrated enzymatic activation of MAPKs at a substantially higher level than that found in NIH 3T3 cells transfected with the Neo^r-encoding gene. The highest level of kinase activity was observed in the NIH 3T3-hIRS-1-L07 cell line (fourfold; $P < 0.001$), followed by NIH 3T3-hIRS-1-L20 ($P < 0.01$) and NIH 3T3-hIRS-1-CG06 ($P < 0.05$) at two- to threefold compared with the control. These results suggest that activation of a MAPK cascade involving enzymatic activation of both MAPKK and MAPKs is consistently associated with IRS-1 overexpression in all three of the independent cell lines studied and that activation occurs principally through the Grb2-SOS-*Ras* signal transduction pathway.

Finally, to further investigate the phenotype of the three cell lines overexpressing IRS-1 and associated activation of downstream MAPKs, the ability of such cell lines to form solid tumors in nude mice was assessed (Table 3). Following subcutaneous injection of 10^7 cells into nude mice, there was progressive tumor formation at the injection site and tumors reached a mean size of 0.5 cm within 14 to 20 days and 14 to 28 days with the NIH 3T3-hIRS-1-L07 and NIH 3T3-hIRS-1-CG06 cell lines, respectively. It is noteworthy that cells overexpressing IRS-1 produced tumors in most mice, in contrast to the NIH 3T3-Neo^r control cell line transfected with an empty pBK-CMV vector, which was not tumorigenic. However, v-*src*-transfected NIH 3T3 cells were the most tumorigenic and produced 0.5-cm tumors within 7 to 10 days. It was of interest that the measured MAPK enzymatic activity in NIH-3T3-hIRS-1-L07 cells was 1.5- to 2-fold higher than that found in NIH 3T3-hIRS-1-CG06 cells. NIH 3T3-hIRS-1-L07 cells, when injected into nude mice, subsequently formed larger and more frequent tumors than did the NIH 3T3-hIRS-1-CG06 cell line. Thus, there may be a correlation between tumor

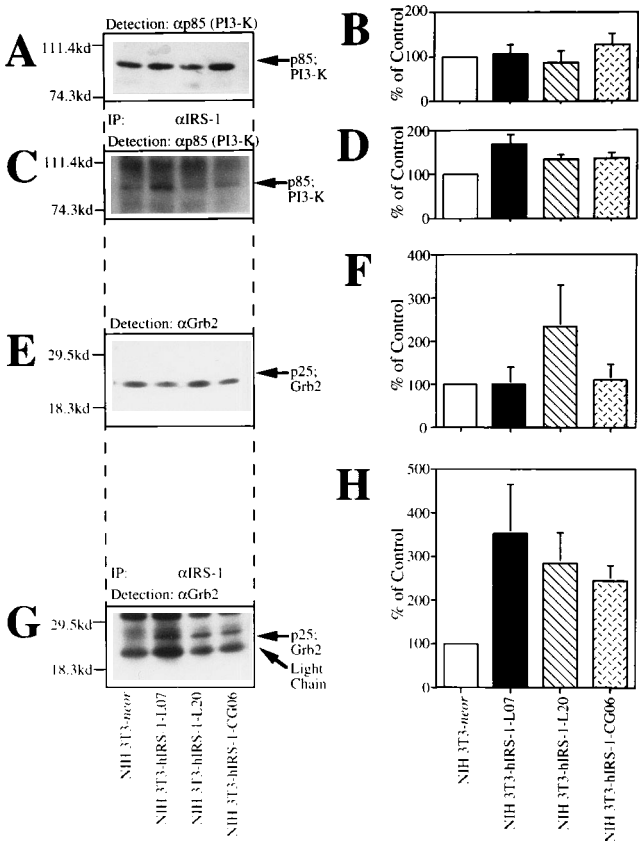

FIG. 4. Expression and association of the p85 subunit of PI3-K and Grb2 with IRS-1 in three cell lines overexpressing IRS-1. Expression of p85 (A and B) and its association with IRS-1 in these cell lines (C and D) were detected with a rabbit anti-p85 antibody and with the same antibody following immunoprecipitation with an anti-IRS-1 antibody (αIRS-1), respectively. Grb2 expression was detected with a rabbit anti-Grb2 antibody (αGrb2) in cell lines overexpressing IRS-1 and in the control (E and F). Immunoprecipitates with αIRS-1 were electrophoresed, blotted, and detected with αGrb2 (G and H). The level of expression of each protein and its association with IRS-1 were assessed by three independent blots and quantitated by densitometry. Error bars indicate the standard error of the mean. Note that all three cell lines overexpressing IRS-1 exhibited a higher level of association of IRS-1 with Grb2 (G and H). kd, kilodaltons.

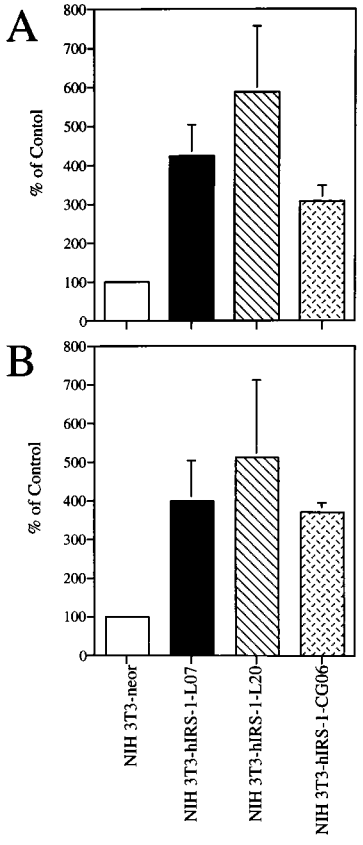

FIG. 5. MAPKK activation in IRS-1-transformed NIH 3T3 cell lines. Whole-cell lysates were prepared from NIH 3T3-hIRS-1-CG06, NIH 3T3-hIRS-1-L20, NIH 3T3-hIRS-1-L07, and NIH 3T3-Neo^r cells. Enzymatic activation of MAPKK was determined by phosphorylation of a full-length Erk1–glutathione *S*-transferase fusion protein (A). Following phosphorylation by endogenous MAPKK, enzymatic activation of recombinant Erk1 as an MBP kinase was measured by ^{32}P incorporation into MBP (B). Following this reaction, samples were subjected to SDS-polyacrylamide gel electrophoresis. Erk1–glutathione *S*-transferase and MBP bands were excised from the dried and stained gels, and the extent of phosphorylation of these proteins was determined as described in Materials and Methods. Error bars indicate the standard error of the mean.

growth rate and degree of MAPK activation in these hIRS-1-transfected NIH 3T3 cell lines.

DISCUSSION

Previous studies have demonstrated that insulin is a mitogenic factor (5, 6, 33) for the liver. Upon insulin binding to the insulin receptor, the β subunit becomes autophosphorylated on tyrosine residues and this is followed by activation of receptor tyrosine kinase activity towards endogenous intracellular substrates. A specific substrate for insulin receptor β subunit kinase activity has been found to be IRS-1 (26, 41). IRS-1, upon tyrosyl phosphorylation, associates with proteins containing SH2 domains through its multiple binding sites. Several functional domains of the IRS-1 molecule have been described. There is a pleckstrin homology domain in the amino-terminal region of the molecule that can bind to the G protein βγ subunit and phosphatidylinositol-4,5-bisphosphate (14, 38). The other functional domains include a YVNI motif that binds Grb2, several YMXM motifs that bind the p85 subunits of PI3-K, a YIDL motif that binds the Syp tyrosine phosphatase 1D protein, and a motif that binds the Nck oncogene product but is not detected in hIRS-1 (26, 41).

One of the recent advances in understanding the transmission of growth signals from the cell surface to the nucleus has been the description of how receptors activate the *Ras* pathway (4) and how signal transduction molecules such as Raf converge to activate the MAPK cascade (1, 9, 10, 20, 27). Key to understanding these pathways was the recognition that proteins containing SH2 domains have binding sites for specific tyrosine-phosphorylated proteins. There are specific protein-rich motifs recognized by SH3 domains that allow such proteins to interact with a number of different down- and upstream activators. Other important discoveries were the identification of adaptor proteins, such as Grb2, that connect other signal transduction molecules through SH2 and SH3 domains and the recent recognition that the mammalian homolog of *Drosophila* SOS links *Ras* to the epidermal growth factor and insulin receptors on the membrane through Grb2, thus linking growth factor receptor kinases with the *Ras* signal transduction pathway (3, 4, 18, 32, 42). Finally, it has been recently reported that *Ras* activation takes place in the context of Raf and 14-3-3 proteins (11, 12, 16).

Evidence suggests that insulin and IRS-1 are important to

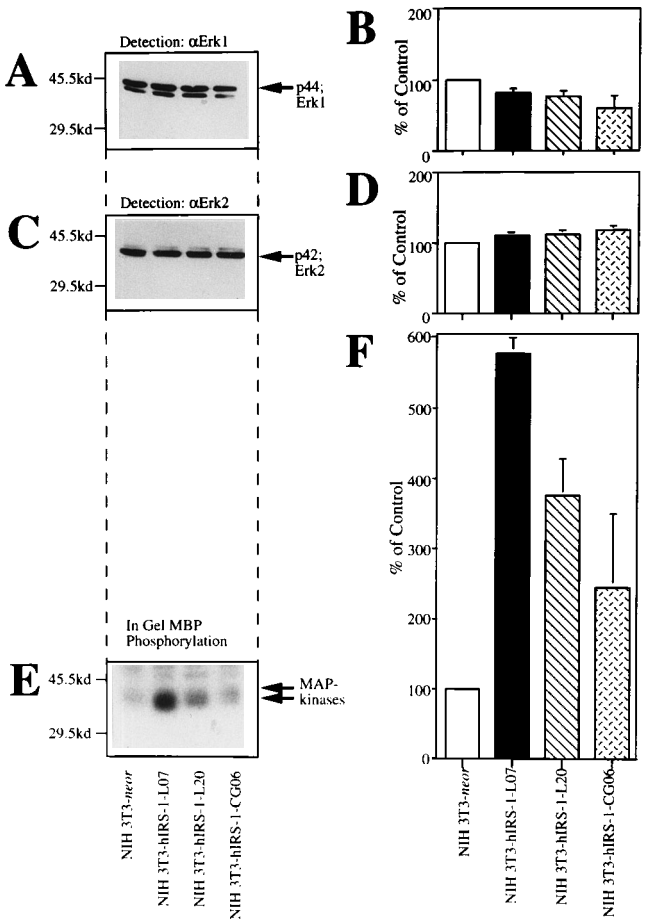

FIG. 6. Expression and enzymatic activation of MAPK proteins (Erk1 and Erk2) in cell lines overexpressing IRS-1. Protein lysates were prepared from NIH 3T3-hIRS-1-CG06, NIH 3T3-hIRS-1-L20, NIH 3T3-hIRS-1-L07, and NIH 3T3-Neo^r cells. Immunodetection of various proteins was performed with the indicated antibodies (A and C). Cellular proteins were subjected to electrophoresis on an SDS-polyacrylamide gel containing MBP, and enzymatic activation of MAPKs was determined. Expression levels of Erk1 and Erk2 were determined by densitometry (B and D), and MAPK enzymatic activity (E) was estimated by measuring the incorporation of ^{32}P into MBP (F). Error bars indicate the standard error of the mean. kd, kilodaltons.

hepatocyte proliferation (30, 31). IRS-1 expression and tyrosyl phosphorylation have been examined during normal liver cell growth induced by two-thirds hepatectomy. IRS-1 protein and RNA levels increased two- to threefold from 0.5 to 18 h after partial hepatectomy. More important, tyrosyl phosphorylation of IRS-1 was enhanced 9- to 18-fold over the initial level 8 to 12 h after partial hepatectomy and prior to the major wave of DNA synthesis at 24 h. These observations suggest that IRS-1 plays a role in hepatocyte growth by linking the insulin receptor at the cell surface to intracellular signal-transducing molecules important in cell proliferation. Finally, the hIRS-1 gene was found to be overexpressed three- to fourfold in 85% of human HCC compared with adjacent, uninvolved liver cells, and this finding suggests a possible link to malignant transformation as well (28).

IRS-1 lacks a catalytic domain, and no mutations in the molecule in human tumors have been described to indicate that it acts as a dominant oncogene (7). However, given the facts that tyrosyl phosphorylation of IRS-1 is a major event during liver regeneration and the protein has been found to be

TABLE 3. Tumorigenicity of NIH 3T3 cell lines stably transfected with the human IRS-1 gene

Cell line	No. of tumors/no. of mice injected[a]	No. of days for tumors to reach 0.5 cm
NIH 3T3-Neo^r[b]	0/10	
NIH 3T3-IRS-1-L07	9/9	14–21
NIH 3T3-IRS-1-CG06	7/10	14–28
NIH 3T3-v-*src*[c]	10/10	7–10

[a] These experiments were repeated three times with similar results.
[b] Cell line stably transfected with pBK-CMV.
[c] Cell line stably transfected with pLNCX-UP1.

overexpressed three- to fourfold in HCC tumors, the question of whether simple upregulation of the IRS-1 gene severalfold is sufficient to induce cellular transformation was raised. To evaluate this hypothesis, transient transfection experiments were performed with NIH 3T3 cells. These studies demonstrated that cells transfected with the IRS-1 gene overexpressed the protein and led to the formation of transformed foci. Stably transfected cell lines overexpressing IRS-1 were established and found to form colonies in soft agar, grow in low-serum-containing medium, and produce large tumors in nude mice. Thus, there is evidence to support the role of IRS-1 as a transforming protein under these experimental conditions. We presume that overexpressed IRS-1 must have appropriate tyrosyl phosphorylation from intact receptor tyrosine kinases for cellular transformation to occur. In support of this hypothesis, recent findings by Zhou-Li et al. (44) demonstrated that transfected IRS-1 was not transforming in mutant fibroblasts negative for insulin-like growth factor 1 (IGF-1) receptors but IRS-1 was transforming in the presence of large T antigen in such cell lines. We have found that addition of 10 and 100 ng of IGF-1 per ml to 10% FBS further enhanced the number of colonies that grew in soft agar to about twofold in IRS-1-overexpressing NIH 3T3 cell lines (35a). It is possible but unlikely that constitutive phosphorylation of IRS-1 was responsible for the transforming activity because of the absence of a critical phosphatase enzyme in these transformed cell lines, and further studies are necessary to examine that possibility.

There are several molecular mechanisms that may induce cellular transformation upon overexpression of IRS-1. Two recent studies are provocative in this regard and have demonstrated that transformation of mammalian cells may be initiated by constitutively activating MAPKK by using gain-of-function deletion mutants (8, 23). Expression of these mutants in mammalian cells activated MAPKs and increased AP-1-regulated transcription. Such cells formed transformed foci, grew efficiently in soft agar, and were highly tumorigenic in nude mice. These studies indicate that constitutive activation of MAPKK is sufficient to promote cellular transformation. The present study also indicates that cells overexpressing IRS-1 develop the transforming phenotype with activation of MAPKK and MAPK. Although the levels of MAPKK and MAPK enzymatic activation were lower in our study than in other investigations (20, 23), it is possible that prolonged activation of the MAPK cascade to any degree is sufficient to transform mammalian cells both in vitro and in vivo. Taken together, these findings indicate that an absolute intracellular increase in IRS-1 and tyrosine-phosphorylated IRS-1 may lead to cellular transformation in association with prolonged activation of the Grb2-SOS-*Ras* and MAPK cascades. Interestingly, there may be a correlation between the enzymatic activity and the transformed characteristics of the cells, since cells

with the highest MAPK activities formed more frequent and larger tumors in nude mice.

Constitutive activation of components in signal transduction pathways involved in cell proliferation and differentiation may often lead to cellular transformation. Signals generated from growth receptors on the cell surface converge on the MAPK cascade (9, 10, 27). Cellular oncogenes such as *Ras*, Src, and Raf have been shown to be upstream molecules in this cascade, and they strongly induce cellular transformation in their activated form. Indeed, it has been suggested that the Raf signaling pathway which phosphorylates and activates MAPKK (20) may be essential for induction of cellular transformation by most oncogenes that initiate their effects through activation of the MAPK cascade (1, 39). On the other hand, association of IRS-1 and Grb2 induces *Ras* activation (18, 42). Nck and Syp, which are known to be proteins that associate with IRS-1, bind directly to SOS and Grb2-SOS, respectively (15, 22), and these SH2-containing molecules function downstream of IRS-1 and converge on the *Ras* signaling pathway to subsequently activate the MAPK cascade. In this regard, our study has shown that overexpression of IRS-1 in three independent NIH 3T3 cell lines increased the association of IRS-1 with Grb2 (Fig. 4G and H), was associated with downstream enzymatic activation of MAPKK and MAPKs (Fig. 5 and 6), and induced cellular transformation. Preliminary experiments suggest that this downstream activation occurs in conjunction with an enhanced *Ras*-bound GTP/GDP ratio (17). It is concluded that overexpression of IRS-1 and increases in its phosphorylation can induce cellular transformation with activation of potent oncogenic signal transduction pathways, such as the Grb2-SOS-*Ras* and MAPK cascades. Although this study has shown the possibility that the Grb2-SOS-*Ras* and MAPK cascades are involved in IRS-1-induced cellular transformation, it is still unknown if IRS-1 may be constitutively phosphorylated and what types of protein kinases or protein phosphatases are involved in the transformation processes.

It was of interest that no significant difference in association of IRS-1 with the 85-kDa subunit of PI3-K was observed among the three cell lines overexpressing IRS-1 compared to the Neo^r gene-transfected control cell line. It is still possible that the PI3-K pathway contributes to cellular transformation, and this question needs to be addressed in studies with site-directed mutant IRS-1 proteins. In this regard, Tanaka et al. have recently shown in a preliminary study that a double mutation (Y F) in the Grb2- and Syp-binding motifs of IRS-1 completely abolished the transforming activity of the molecule in stably transfected NIH 3T3 cells (36). We believe, therefore, that downstream activation of MAPKs through the Grb2-SOS-*Ras* pathway, as a consequence of IRS-1 overexpression, may lead or contribute to the oncogenic properties of this protein in human HCC.

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