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A Dominant-Negative Mutant of mSOS1 Inhibits Insulin-Induced Ras Activation and Reveals Ras-Dependent and -Independent Insulin Signaling Pathways

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The role of the Grb2-SOS complex in insulin signal transduction was investigated with a deletion mutant of mSOS1 that lacks the guanine nucleotide exchange domain of the wild-type protein. Cells overexpressing either wild-type (CHO-IR/SOS cells) or mutant (CHO-IR/ΔSOS cells) mSOS1 were established by transfection of Chinese hamster ovary cells that express human insulin receptors (CHO-IR cells) with the appropriate expression plasmid. The mutant mSOS1 protein did not contain the guanine nucleotide exchange activity in vitro and associated with Grb2 both in vivo and in vitro. In both CHO-IR and CHO-IR/SOS cells, insulin rapidly stimulated the formation of GTP-bound Ras and the phosphorylation of mitogen-activated protein (MAP) kinase; both these effects of insulin were markedly inhibited in CHO-IR/ΔSOS cells. Insulin-induced glycogen synthase and 70-kDa S6 kinase activities were not affected by expression of either wild-type or mutant mSOS1. These results show that the mutant mSOS1 acts in a dominant-negative manner and suggest that the Grb2-SOS complex mediates, at least in part, insulin-induced activation of Ras in intact cells. The data also indicate that Ras activation is not required for insulin-induced stimulation of glycogen synthase and 70-kDa S6 kinase.

The actions of insulin are initiated by interaction of the hormone with the heterotetrameric insulin receptor, which possesses protein-tyrosine kinase activity (31, 53). Activation of the receptor results in rapid autophosphorylation of receptor tyrosine residues and subsequent tyrosine phosphorylation of cellular substrates, including insulin receptor substrate 1 (IRS-1) (62), Shc (51), and the protein phosphatase PTP-1C/SH–PTP-1 (66). Tyrosine phosphorylation sites serve as specific binding sites for proteins with Src homology 2 (SH2) domains (32). Thus, tyrosine-phosphorylated Shc binds to the SH2 domain-containing protein Grb2 (also called Ash) (60), and tyrosine-phosphorylated IRS-1 binds to Grb2 (60), the 85-kDa subunit of phosphoinositide 3-kinase (PI 3-kinase) (4, 37, 69), Nck (38), and the protein phosphatase Syp/SH–PTP-2/PTP-1D (35) via SH2 domains. We have recently demonstrated that the association of IRS-1 and PI 3-kinase activity is required for insulin-stimulated glucose transport and membrane ruffling but not for insulin-stimulated Ras activation (29, 33).

Grb2, a mammalian homolog of *Caenorhabditis elegans* Sem-5 (18), comprises a central SH2 domain flanked by two SH3 domains (40, 41). Grb2 binds to several proteins, including dynamin (3, 28, 42) and SOS, a guanine nucleotide exchange protein of Ras (10, 11, 21, 26, 39, 54), by interaction of its SH3 domains with proline-rich sequences in the carboxyl-terminal regions of these proteins. Because the SH2 domain of Grb2 binds to tyrosine-phosphorylated Shc (55, 60) as well as IRS-1 (60), insulin stimulation results in the formation of IRS-1–Grb2–SOS and Shc-Grb2-SOS complexes (7, 50, 57, 59). Insulin stimulation also results in a rapid conversion of the GDP-bound form of Ras to the GTP-bound form in intact cells (13, 68). However, there is no direct evidence suggesting that formation of the Grb2-SOS complex is important in insulin-stimulated Ras activation in intact cells.

To evaluate the role of the Grb2-SOS complex in Ras activation by insulin, we have constructed a mutant of mSOS1 that lacks the guanine nucleotide exchange domain but contains the proline-rich sequence and we have expressed this mutant in Chinese hamster ovary cells that also express human insulin receptors (CHO-IR cells). This mutant appeared to act as a dominant-negative protein. With CHO-IR cells overexpressing wild-type or mutant mSOS1, we investigated the role of mSOS1 in Ras activation in the insulin signal transduction pathway and examined Ras-dependent and -independent pathways of insulin signaling.

MATERIALS AND METHODS

Cells and antibodies. CHO-IR cells were established as described previously (2) and maintained in Ham's F-12 medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin (Gibco). Rabbit polyclonal antibodies to mSOS1 (αmSOS1) and 70-kDa S6 kinase (αp70^{S6K}) were generated against synthetic peptides of mouse mSOS1 (residues 1241 to 1260) and rat 70-kDa S6 kinase (residues 2 to 23). Polyclonal antibodies to mitogen-activated protein (MAP) kinase (αY91 and αC92) and monoclonal antibody to rat IRS-1 (1D6) were prepared as described previously (2, 68). Polyclonal antibodies to Grb 2 (αGrb2) and Shc (αShc) were generated against Grb2– and Shc–glutathione *S*-transferase (GST) fusion proteins as previously described (41, 68). Monoclonal antibodies Y13-259 to Ras and 9E10 to the Myc epitope were obtained from the American Type Culture Collection (Rockville, Md.). A specific monoclonal antibody to phosphotyrosine (PY-20) was obtained from ICN Biochemicals (Costa Mesa, Calif.), and a monoclonal antibody to Grb2 was obtained from Transduction Laboratories (Lexington, Ky.).

Construction of expression plasmids. A *Not*I-*Kpn*I fragment of mouse mSOS1 cDNA, encoding full-length mSOS1 (10), was used to prepare an expression

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plasmid, SRαSOS. The *Not*I site of the fragment was filled with Klenow enzyme, and the fragment was then linked with *Kpn*I linker and subcloned into *Kpn*I-digested pcDL-SRα296 (a gift from N. Arai, DNAX, Palo Alto, Calif.) (67). An expression plasmid (SRαΔSOS) encoding a mutant mSOS1 that lacks the guanine nucleotide exchange domain was constructed by removing a *Pst*I-*Pst*I fragment (1.49 kbp) from SRαSOS (see Fig. 1A). Myc-tagged $p42^{mapk}$ expression plasmid (pEXV-Erk2-tag) was obtained from C. J. Marshall (Institute of Cancer Research, London, United Kingdom) (30).

Transfection and establishment of cell lines. CHO-IR cells (5×10^5 cells per dish) were transfected in the presence of Lipofectin (Gibco) with 5 μg of expression vector and 0.5 μg of pSV2 hyg- DNA for 48 h, and then hygromycin B (250 μg/ml) (Wako, Tokyo, Japan) was added to the medium to select for resistant cells. Cells showing a high level of expression of wild-type or mutant mSOS1 were identified by immunoblot analysis with αmSOS1 and then isolated by limiting dilution.

Immunoprecipitation and immunoblot analysis. Dishes of cells were incubated in the absence of serum overnight and then with insulin for the appropriate times. The reaction was stopped by freezing the cells in liquid nitrogen. The cells were lysed in ice-cold buffer A (20 mM Tris-HCl [pH 7.5], 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1% Nonidet P-40) and then centrifuged at 12,000 × *g* for 20 min at 4°C. The supernatant (approximately 100 μg of protein) was subjected to immunoprecipitation with antibodies (1 to 5 μg, as indicated in the figure legends) coupled to protein G-Sepharose (Pharmacia). After being washed three times with ice-cold buffer A, the immune complex was boiled in sodium dodecyl sulfate (SDS) sample buffer, separated on an SDS-polyacrylamide gel, and transferred to a nitrocellulose filter. The filter was probed with the primary antibody and then incubated with horseradish peroxidase-coupled antibodies to mouse or rabbit immunoglobulin G. Immune complexes were detected by enhanced chemiluminescence (ECL; Amersham). We confirmed that αShc or αGrb2 immunoprecipitated the same amount of Shc or Grb2 from three cell lines by immunoblotting under our assay conditions (data not shown).

In vitro guanine nucleotide exchange activity assay. Guanine nucleotide exchange activity was measured in immunoprecipitates prepared from cell lysates (250 μg of protein) with 10 μg of αmSOS1 as described above. After being washed three times with ice-cold buffer A, the immunoprecipitates were washed once with ice-cold buffer B (20 mM Tris-HCl [pH 7.5], 1 mM $MgCl_2$, 0.5 mM EDTA, 100 mM NaCl, 0.01% [wt/vol] bovine serum albumin [BSA], 1 mM dithiothreitol, 0.05% [vol/vol] Triton X-100] and incubated for 30 min at 25°C with 0.2 μCi of [^{35}S]guanosine-5′-*O*-thiotriphosphate ([^{35}S]GTPγS) (final concentration, 1 μM) and 1 pmol of Ha-Ras (a gift from K. Kaibuchi, Kobe University School of Medicine, Kobe, Japan) in 20 μl of buffer B. The reactions were terminated by adding 1 ml of an ice-cold solution containing 20 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 100 mM NaCl, and 1 mM dithiothreitol. The beads were spun down, and 0.9 ml of the supernatant was filtered through a 0.45-μm-pore-size nitrocellulose filter (Schleicher and Schuell, Dassel, Germany) which was then washed three times with 10 ml of the same ice-cold solution. The radioactivity on the filter was measured by liquid scintillation counting. Guanine nucleotide exchange activity was calculated by subtracting the amount of GTPγS bound to Ras in the immunoprecipitates prepared with control rabbit serum from that in the immunoprecipitates prepared with αmSOS1. Under our assay conditions, GTPγS bound to Ha-Ras was linearly increased up to 250 fmol.

In vitro association of mSOS1 and adapter proteins. The plasmid pGEX-Grb2, which encodes a GST-Grb2 fusion protein, was obtained from K. Matsuoka and T. Takenawa (Tokyo Metropolitan Institute of Gerontology and Institute of Medical Science, University of Tokyo, Tokyo, Japan) (41). *Escherichia coli* JM109 cells were transformed with this plasmid, and synthesis of the GST fusion protein was induced by adding 0.5 mM isopropyl-β-D-thiogalactopyranoside to the culture medium. After incubation of the cells for 2 h at 37°C, the fusion protein was purified with glutathione-Sepharose (Pharmacia). CHO-IR cells expressing wild-type or mutant mSOS1 were stimulated with 100 nM insulin for 5 min, lysed in 0.5 ml of ice-cold buffer A, and centrifuged for 20 min at 12,000 × *g* at 4°C. The supernatants (100 μg of protein) were incubated for 2 h at 4°C with GST fusion protein (5 μg) fixed on the resin. In vitro association of proteins with the GST-Grb2 fusion protein was detected by immunoblot analysis with αmSOS1, αShc, or PY-20.

PI 3-kinase activity associated with IRS-1. Serum-starved CHO-IR, CHO-IR/SOS, and CHO-IR/ΔSOS cells cultured in 60-mm-diameter dishes were incubated with or without insulin for 2 min, and the cell lysates were immunoprecipitated with monoclonal antibody to IRS-1 (1D6, 2 μg) as described previously (68, 69). Under the same conditions, the same amount of IRS-1 was immunoprecipitated from three cell lines (data not shown). PI 3-kinase activity in the immunoprecipitates was measured as described previously (23).

Cell labeling and analysis of guanine nucleotides bound to Ras. Cells cultured in 60-mm-diameter dishes were incubated in the absence of serum overnight, labeled for 4 h with $^{32}P_i$ (0.15 mCi/ml) (NEN) in phosphate-free RPMI 1640 (Gibco), and then stimulated with insulin for 2 min. Ras-GTP formation was measured as described previously (45, 68). The cells were lysed in a solution containing 50 mM Tris-HCl (pH 8.0), 20 mM $MgCl_2$, 150 mM NaCl, 1 mM PMSF, aprotinin (20 mg/ml), and 1% Triton X-100. The lysate was centrifuged, and the supernatant was subjected to immunoprecipitation with monoclonal antibody Y13-259 to Ras. After extensive washing of the immunoprecipitate, bound guanine nucleotides were eluted, separated by ascending thin-layer chromatography, and analyzed with a Fuji BAS 2000 image analyzer. The relative molar ratio of GTP and GDP was corrected for the number of phosphates per mole of guanosine, and the amount of Ras-GTP was expressed as a percentage of the total amount of Ras.

Assay of MAP kinase phosphorylation and MAP kinase activity. Cells were incubated in the absence of serum overnight and then treated with various concentrations of insulin for 5 min. The cells were lysed in 300 μl of a solution containing 25 mM Tris-HCl (pH 7.6), 25 mM NaCl, 0.5 mM EGTA (ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid), 10 mM NaF, 10 mM sodium PP_i, 1 mM PMSF, 1 mM sodium orthovanadate, and 10 nM okadaic acid, and the lysate was centrifuged for 30 min at 4°C. The phosphorylation of MAP kinase was examined by the mobility shift of $p42^{mapk}$ on immunoblot analysis with αY91 (47). MAP kinase activity of $p44^{mapk}$ was measured in immunoprecipitates prepared from 100 μg of cell lysate protein with αC92 (5 μg). The immunoprecipitates were incubated for 10 min at 30°C with 1 μCi of [γ-^{32}P]ATP in a solution containing 25 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM EGTA, 40 μM ATP, and myelin basic protein (MBP) (1 mg/ml) as a substrate (27). Under these conditions, the phosphorylation of MBP proceeded linearly for at least 20 min. The reactions were terminated by spotting 15 μl of reaction mixture onto P-81 phosphocellulose paper (Whatman) which was then washed five times with 0.5% phosphoric acid and once with acetone. The papers were dried, and ^{32}P was quantitated by Cerenkov counting.

Assay of 42-kDa MAP kinase activity using a transient expression system. CHO-IR and CHO-IR/ΔSOS cells cultured in 10-cm-diameter dishes were transfected with Myc-tagged $p42^{mapk}$ expression plasmid (1 μg of pEXV-Erk2-tag) in combination with 9 μg of SRα or SRαSOS by calcium phosphate coprecipitation method for 6 to 12 h. Twenty-four hours after transfection, the cells were replated into three 6-cm-diameter culture dishes, one of which was used for immunoblotting. Seventy-two hours after transfection, serum-starved cells were incubated with 100 nM insulin for 5 min, and expressed Myc-tagged $p42^{mapk}$ was immunoprecipitated with 1 μg of antibody 9E10. MAP kinase activity in the immunoprecipitate was measured as described above. After separation of the samples with an SDS–15% polyacrylamide gel, the radioactivity of the MBP was analyzed with a Fuji BAS 2000 image analyzer. The amount of immunoprecipitated Myc-tagged MAP kinase was determined by immunoblotting with polyclonal antibody to MAP kinase (αY91). The MAP kinase activity of each transfectant was normalized by the level of immunoprecipitated Myc-tagged $p42^{mapk}$, and MAP kinase activity was quantified relative to that observed in CHO-IR cells transfected with SRα and Myc-tagged MAP kinase plasmids.

Assay of DNA synthesis and cell growth. For insulin-stimulated DNA synthesis, the cells were grown in 24-well culture plates and serum starved for 2 days in F-12 medium containing 0.1% BSA. After stimulation of the cells with various concentration of insulin for 16 h, DNA synthesis was measured as described previously (2). For calculating cell growth rate, the cells were seeded in six-well culture plates at a density of 5×10^4 per well and were cultured in a medium containing 10% fetal bovine serum. The culture medium was changed every day, and the number of cells was counted every 24 h.

Assay of glycogen synthase activity. Cells cultured in 60-mm-diameter dishes were incubated with various concentrations of insulin for 30 min at 37°C. The cells were then frozen; thawed in 120 μl of a solution containing 50 mM Tris-HCl (pH 7.6), 100 mM KF, 10 mM EDTA, 1 mM PMSF, and 30% (vol/vol) glycerol; and centrifuged at 12,000 × *g* for 30 min at 4°C. The supernatants (30 μl) were assayed for glycogen synthase activity as described previously (65) under linear assay conditions. The ratio of glycogen synthase activity measured in the absence of glucose 6-phosphate to that measured in the presence of 10 mM glucose 6-phosphate was calculated.

Assay of S6 kinase activity in immunoprecipitates. p70 S6 kinase activity was measured as described previously (25), under linear growth conditions. Briefly, dishes (60-mm diameter) of cells were stimulated with insulin for 10 min at 37°C. Cells were lysed in 500 μl of an ice-cold solution containing 50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 20 mM NaF, 1 mM benzamidine, 1 mM EDTA, 5 mM EGTA, 10 mM sodium PP_i, 30 mM *p*-nitrophenylphosphate, 0.1 mM PMSF, and 1% Nonidet P-40, and the lysate was centrifuged at 4°C for 15 min at 12,000 × *g*. The supernatant was subjected to immunoprecipitation with $\alpha p70^{S6K}$ (5 μg) coupled to protein G-Sepharose. After extensive washing of the precipitate, an in vitro kinase assay was performed with reaction buffer containing 1 mM S6 synthetic peptide (KRRRLSSLRASTSKSESSQK; amino acids 230 to 249) and 1 μCi of [γ-^{32}P]ATP at 37°C for 30 min. The reactions were terminated by spotting 15 μl of reaction mixture onto P-81 phosphocellulose paper, and the papers were washed five times with 0.5% phosphoric acid and once with acetone. The papers were dried, and ^{32}P was quantitated by Cerenkov counting.

RESULTS

Expression of mutant mSOS1 in CHO-IR cells. A cDNA encoding a mutant mSOS1 that lacks the guanine nucleotide exchange domain (amino acids 618 to 1036) of the wild-type protein was constructed by removing a *Pst*I-*Pst*I fragment from

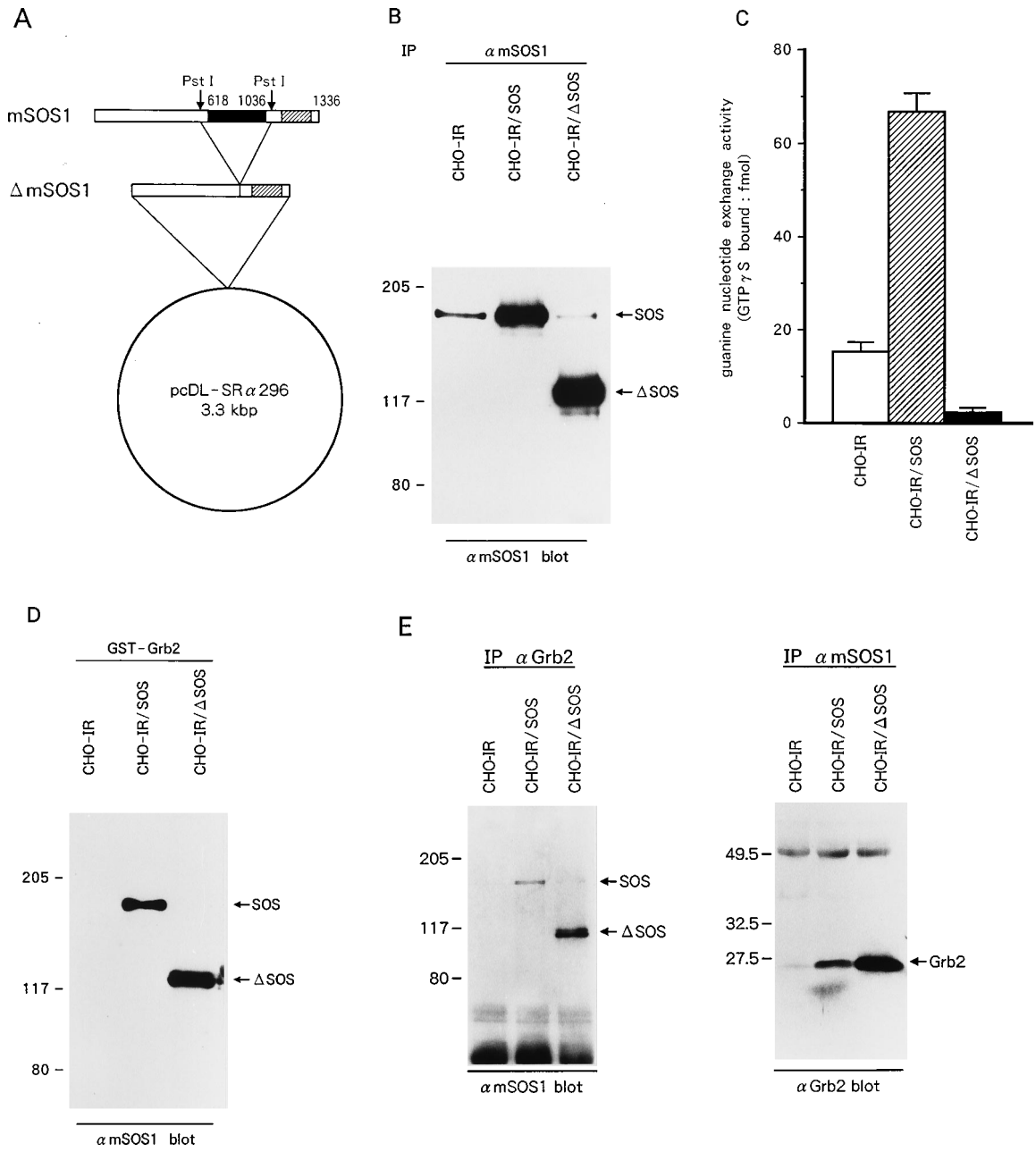


FIG. 1. (A) Construction of the expression plasmids SRαSOS and SRαΔSOS. mSOS1 cDNA (10) was subcloned into *Kpn*I-digested pcDL-SRα296 to generate SRαSOS. The mutant mSOS1 (ΔmSOS1), which lacks the guanine nucleotide exchange domain (amino acids 618 to 1036), was encoded by SRαΔSOS, which was generated by removing a *Pst*I-*Pst*I fragment from SRαSOS. The guanine nucleotide exchange domain (solid box) and the proline-rich domain (hatched box) are indicated. (B) Expression of wild-type and mutant mSOS1 in CHO-IR cells. Lysates were prepared from cell lines expressing wild-type or mutant mSOS1 and were subjected to immunoprecipitation (IP) and immunoblot analysis with αmSOS1. (C) In vitro guanine nucleotide exchange activities of wild-type mSOS1 and mutant mSOS1. The immunoprecipitates prepared from CHO-IR, CHO-IR/SOS, and CHO-IR/ΔSOS cells with αmSOS1 were incubated with [^{35}S]GTPγS and Ras proteins. The amount of GTPγS bound to Ras was measured. Data are the means ± the standard errors of the mean of three separate experiments. (D) In vitro association of Grb2 and mSOS1. Bacterially expressed GST-Grb2 was incubated with lysates of CHO-IR, CHO-IR/SOS, and CHO-IR/ΔSOS cells. Association of mSOS1 with GST-Grb2 was detected by immunoblot analysis with αmSOS1. (E) In vivo association of Grb2 and mSOS1. Lysates from each cell line were subjected to immunoprecipitation with αGrb2 (5 μg) or αmSOS1 (5 μg) and immunoblot analysis with αmSOS1 or αGrb2, respectively. The positions of molecular size standards (in kilodaltons) are indicated to the left of each blot, and the positions of wild-type (SOS) and mutant (ΔSOS) proteins and Grb2 are indicated by arrows (B, D, and E).

the mSOS1 cDNA (10) (Fig. 1A). CHO-IR cells were transfected with the mutant and wild-type mSOS1 expression plasmids, and clones resistant to hygromycin B were selected. As growth of the cells transfected with SRαΔSOS was slow, colonies were small compared with colonies of CHO-IR cells transfected with SRαSOS (data not shown). By screening 30 colonies from each transfectant, 12 independent cell lines expressing the mutant mSOS1(CHO-IR/ΔSOS) and 18 cell lines expressing wild-type mSOS1(CHO-IR/SOS) were isolated. Six CHO-IR/ΔSOS cell lines and three CHO-IR/SOS cell lines

were further purified by the limiting-dilution method, and one line of each type was used for the following experiments. The data described were reproduced with at least two clones of each cell line, which were established from separate colonies of independent origin.

An ~150-kDa protein was detected after immunoprecipitation and immunoblot analysis of CHO-IR/SOS cells with αmSOS1 (Fig. 1B). In contrast, a diffuse band of ~110 kDa was detected with CHO-IR/ΔSOS cells, a size that is consistent with the predicted molecular mass of mutant mSOS1. Wild-type mSOS1 was overexpressed ~15-fold, and mutant mSOS1 was overexpressed ~25-fold, compared with endogenous mSOS1 in CHO-IR cells, as estimated by mSOS immunoblotting with cell lysates of each cell. In vitro guanine nucleotide exchange activity toward Ras in these immunoprecipitates was measured. The immunoprecipitates prepared from CHO-IR/SOS cells with αmSOS1 contained higher levels of guanine nucleotide exchange activity than CHO-IR cell immunoprecipitates (Fig. 1C). In contrast, the immunoprecipitates from CHO-IR/ΔSOS cells contained lower levels of activity than CHO-IR cell immunoprecipitates (Fig. 1C), indicating that the mutant mSOS1 protein does not contain the guanine nucleotide exchange activity toward Ras.

Because mSOS1 has been shown to form a constitutive complex with Grb2 (11, 21, 26, 39, 54), complex formation of mutant mSOS1 and Grb2 was examined by in vitro association experiments with a bacterially expressed GST-Grb2 fusion protein. Lysates prepared from CHO-IR/SOS cells or CHO-IR/ΔSOS cells were incubated with GST-Grb2, and complex formation was detected by immunoblot analysis with αmSOS1. Both wild-type mSOS1 and mutant mSOS1 bound to Grb2 (Fig. 1D), indicating that deletion of the guanine nucleotide exchange domain of mSOS1 did not affect the ability of the protein to bind Grb2. The difference in the amounts of wild-type mSOS1 and mutant mSOS1 associated with GST-Grb2 is consistent with the relative levels of expression of the two proteins in each cell line.

The effects of overexpression of the wild-type or mutant mSOS1 in association with Grb2 in vivo were also examined. Lysates prepared from each cell line were immunoprecipitated with αmSOS1 or αGrb2, and association was detected by immunoblot analysis. Although mSOS1 and Grb2 were immunoprecipitated from CHO-IR cells by αGrb2 and αmSOS1, respectively, markedly increased amounts of these two protein were detected in immunoprecipitates from CHO-IR/SOS and CHO-IR/ΔSOS cells (Fig. 1E), indicating that overexpression of either wild-type mSOS1 or mutant mSOS1 increased with the amount of Grb2-mSOS1 complex. Both a small amount of wild-type mSOS1 protein and a large amount of mutant mSOS1 protein were immunoprecipitated from CHO-IR/ΔSOS cells by αGrb2; densitometric analysis revealed that more than 95% of the SOS associated with Grb2 was the mutant (Fig. 1E). Although two different sizes of mutant mSOS1 in CHO-IR/ΔSOS cells were observed (Fig. 1E), the smaller is probably a proteolytic product of the larger.

Effect of mutant mSOS1 on insulin-induced Ras activation. Given that mutant mSOS1 lacks a guanine nucleotide exchange domain but can interact with Grb2 both in vitro and in vivo, we examined whether signaling from the insulin receptor to Ras was affected in CHO-IR/ΔSOS cells. Cells were labeled with $^{32}P_i$, and the guanine nucleotides bound to Ras were analyzed. We have previously shown that maximal stimulation of Ras is achieved after exposure of CHO-IR cells to insulin for 2 to 5 min (68); we therefore stimulated cells with various concentrations of insulin for 2 min at 37°C. Stimulation of CHO-IR cells with insulin for 2 min increased the percentage of the GTP-bound form of Ras in a dose-dependent manner from 5.6 to 26.0% (Fig. 2). Overexpression of wild-type mSOS1 increased the percentage of Ras-GTP in cells incubated in the absence (7.3%) or presence (maximum, 31.0%) of insulin. In contrast, insulin had only a minor effect on the percentage of Ras-GTP in CHO-IR/ΔSOS cells (4.7 to 7.9%). Thus, insulin-induced Ras activation was inhibited in CHO-IR cells expressing mutant mSOS1, which appears to act as a dominant-negative analog of mSOS1.

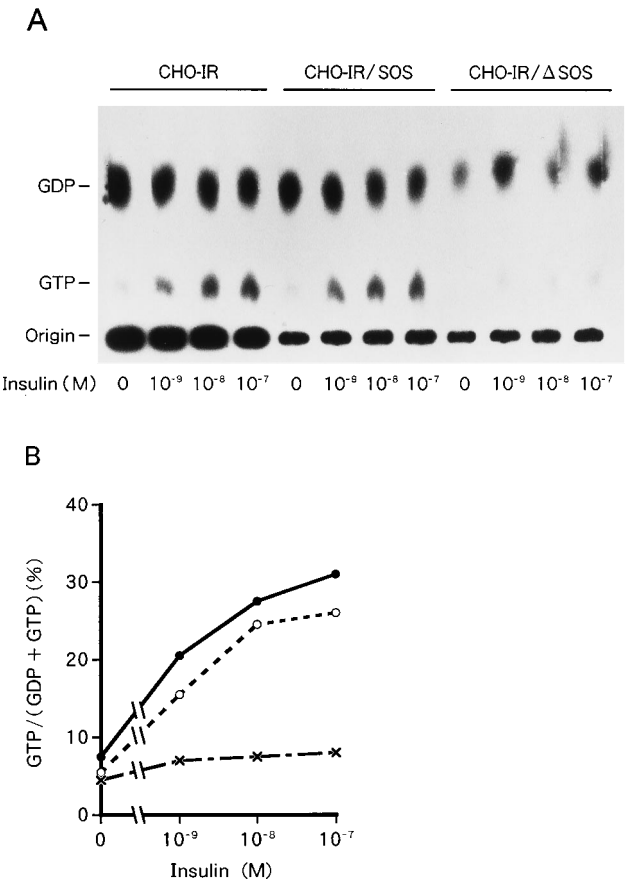


FIG. 2. Effect of expression of wild-type and mutant mSOS1 on insulin-induced Ras activation. Serum-deprived cells were labeled with $^{32}P_i$ and stimulated with various concentrations of insulin for 2 min. Cell lysates were prepared and subjected to immunoprecipitation with Y13-259, and guanine nucleotides precipitated with Ras were separated by thin-layer chromatography. (A) Autoradiogram of the thin-layer plate. The positions of GDP and GTP are indicated by lines. (B) Quantitative analysis of the accumulation of Ras-GTP in CHO-IR (○), CHO-IR/SOS (●), and CHO-IR/ΔSOS (×) cells. The amount of Ras-GTP was expressed as a percentage of the amount of Ras-GDP plus Ras-GTP. The data are the means of the two independent experiments.

Tyrosine phosphorylation of insulin receptor, IRS-1, and Shc in CHO-IR/ΔSOS cells. The effects of overexpression of wild-type or mutant mSOS1 on insulin-induced tyrosine phosphorylation of the insulin receptor, IRS-1, and Shc, and on the association of the tyrosine-phosphorylated proteins with Grb2, were examined. Tyrosine phosphorylation of the insulin receptor, IRS-1 (Fig. 3A), and Shc (Fig. 3B) stimulated by 100 nM insulin for 5 min was increased in CHO-IR/SOS and CHO-IR/ΔSOS cells compared with that in CHO-IR cells. Immunoprecipitation of cell lysates with αGrb2 revealed that Grb2 associated with IRS-1 (Fig. 3C) and Shc (Fig. 3D) in CHO-IR/SOS and CHO-IR/ΔSOS cells to approximately the same extent as in CHO-IR cells.

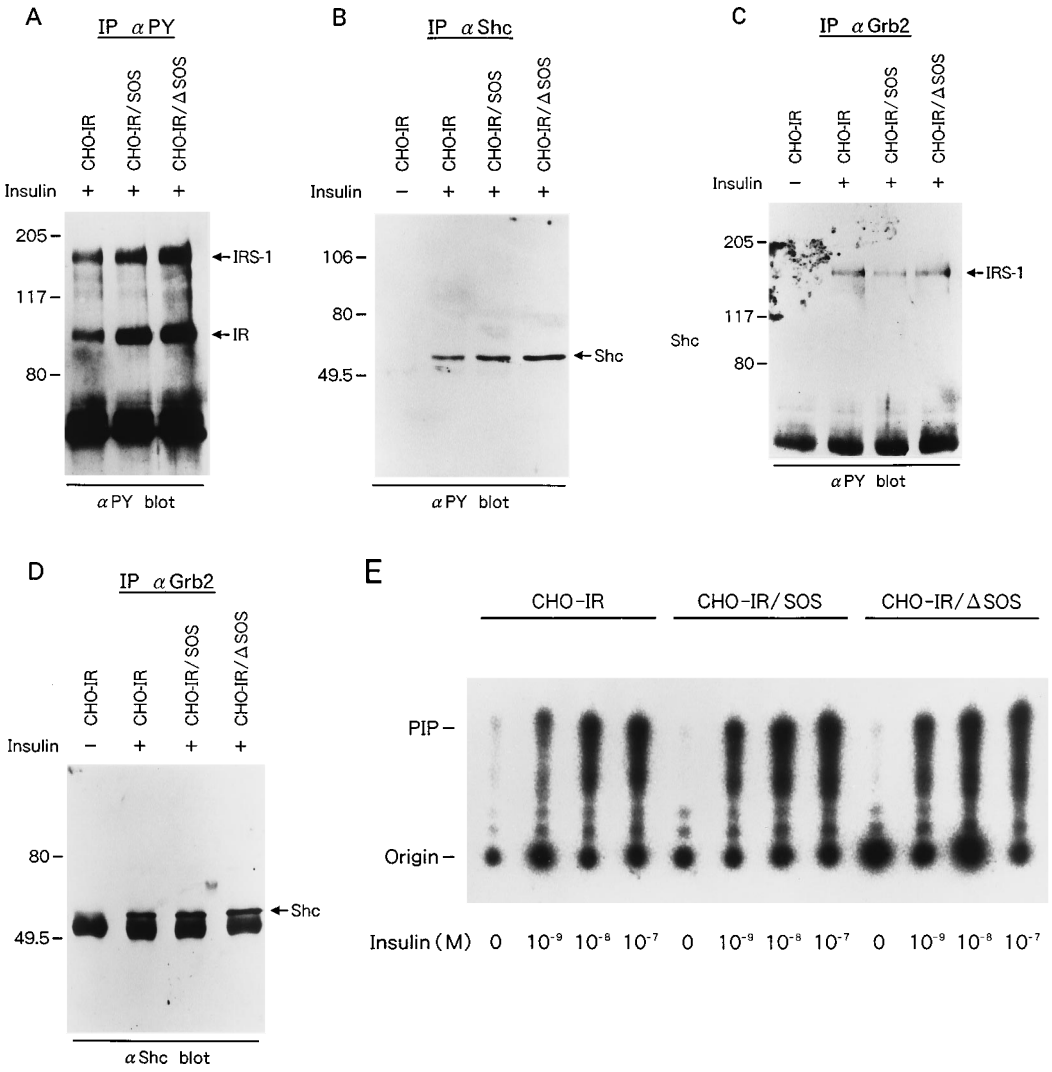
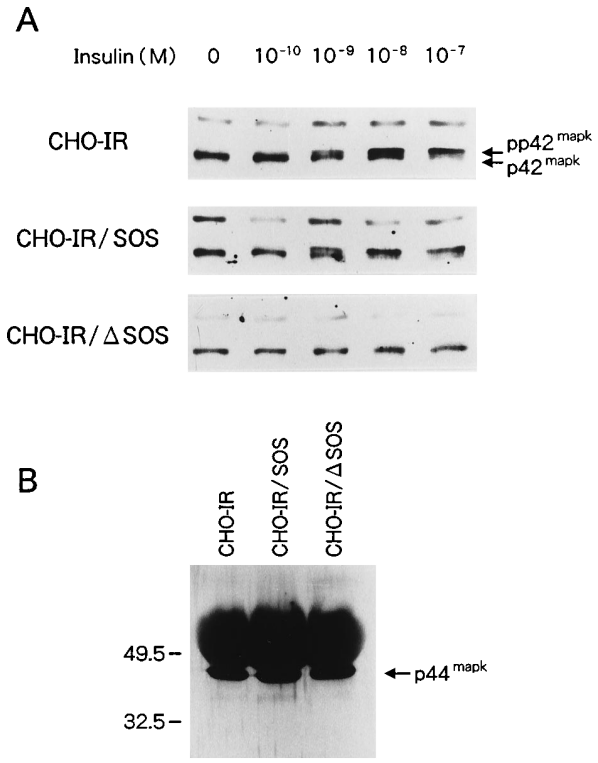


FIG. 3. Effects of wild-type and mutant mSOS1 on insulin-induced tyrosine phosphorylation of insulin receptor, IRS-1 (A), and Shc (B) and on the in vivo association of Grb2 with IRS-1 (C) and Shc (D) and of PI 3-kinase activity with IRS-1 (E). CHO-IR, CHO-IR/SOS, and CHO-IR/ΔSOS cells were stimulated with 100 nM insulin for 5 min. Cell lysates were subjected to immunoprecipitation (IP) with PY-20 (2 μg) (A) or αShc (2 μg) (B), and tyrosine-phosphorylated proteins were detected by immunoblot analysis with PY-20 (A and B). Cell lysates were subjected to immunoprecipitation with αGrb2 (5 μg) and immunoblot analysis with PY-20 (C) or αShc (D). (E) After incubation of cells with insulin for 2 min, the cell lysates were immunoprecipitated with the antibody against IRS-1 (1D6). PI 3-kinase activity in the immunoprecipitate was measured by in vitro kinase assay with phosphatidylinositol as a substrate. Phosphorylated phosphatidylinositol (PIP) was separated by thin-layer chromatography. The relevant proteins are indicated by arrows (A to D) or lines (E), and the positions of molecular size standards (in kilodaltons) are indicated to the left of blots A to D.

PI 3-kinase activity is associated with tyrosine-phosphorylated IRS-1 via the SH2 domains. Stimulation of these three CHO-IR cell lines with insulin for 2 min resulted in similar increases of PI 3-kinase activity in immunoprecipitates with anti-IRS-1 antibodies (Fig. 3E).

Effect of mutant mSOS1 on insulin-induced MAP kinase activation. Phosphorylation of MAP kinase results in a decreased mobility of $p42^{mapk}$ in SDS-polyacrylamide gels (47). Insulin stimulation of CHO-IR cells for 5 min decreased the mobility of $p42^{mapk}$ in a dose-dependent manner (Fig. 4A). Exposure of CHO-IR/SOS cells to insulin also resulted in reduced mobility of $p42^{mapk}$, but the dose-response relation was slightly shifted to the left compared with that of CHO-IR cells. In contrast, the mobility of only a small portion of $p42^{mapk}$ in CHO-IR/ΔSOS cells was decreased even after the incubation of cells with 100 nM insulin for 5 min. To confirm the results from the mobility shift assay, we measured the phosphorylation of MBP after incubation with immunoprecipitates prepared from cells with αC92, which recognizes $p44^{mapk}$. The amounts of $p44^{mapk}$ immunoprecipitated from each of the three cell lines were similar (Fig. 4B). Insulin treatment of CHO-IR and CHO-IR/SOS cells increased MAP kinase activity in the immunoprecipitates in a dose-dependent manner; the dose-response curve was shifted to the left in CHO-IR/SOS cells (Fig. 4C). In contrast, expression of mutant mSOS1 in CHO-IR cells markedly inhibited the stimulation of MAP kinase activity by insulin.

Effects of mSOS1 on insulin-induced MAP kinase activation in CHO-IR/ΔSOS cells. Given that stable expression of the mutant mSOS1 in CHO-IR cells (CHO-IR/ΔSOS cells) markedly inhibited insulin-dependent MAP kinase activation, we examined whether transient expression of mSOS1 could rescue



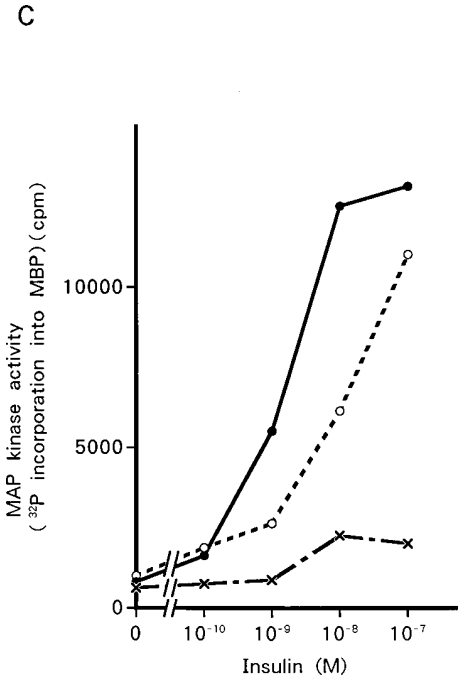


FIG. 4. Effects of wild-type and mutant mSOS1 on insulin-induced activation of MAP kinase. (A) Activation of MAP kinase measured as a phosphorylation-induced decrease in mobility in SDS-polyacrylamide gels. Cells were stimulated with various concentrations of insulin for 5 min. The mobility shift was detected by immunoblot analysis with αY91. The positions of $p42^{mapk}$ and $pp42^{mapk}$ are indicated by arrows. Similar results were obtained in three other experiments. (B) Abundance of $p44^{mapk}$ in immunoprecipitations with αC92. Almost the same amount of $p44^{mapk}$ was immunoprecipitated from each cell line. Molecular standards (in kilodaltons) are indicated on the left). (C) Activation of 44-kDa MAP kinase measured by in vitro kinase assay with MBP as the substrate. The assay was performed with immunoprecipitates from insulin-stimulated CHO-IR (○), CHO-IR/SOS (●), and CHO-IR/ΔSOS (×) cells. Similar results were obtained in two additional experiments.

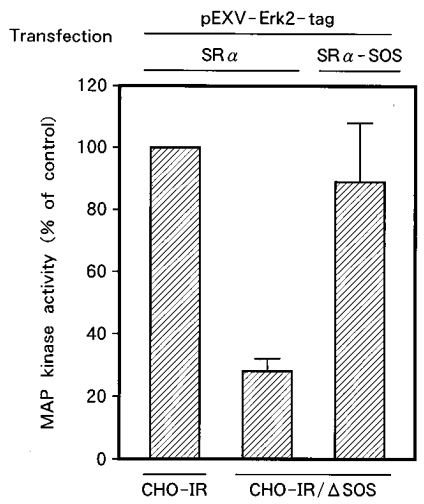


FIG. 5. Effect of wild-type mSOS1 on insulin-induced MAP kinase activation in CHO-IR/ΔSOS cells. CHO-IR/ΔSOS cells were transfected with SRα or SRαSOS together with Myc-tagged $p42^{mapk}$ expression plasmid (pEXV-Erk2-tag) and, after 72 h, were stimulated with 100 nM insulin for 5 min. Activation of $p42^{mapk}$ was measured by in vitro kinase assay with MBP as the substrate. The assay was performed on immunoprecipitates with antibody to the Myc epitope. MAP kinase activity was normalized by the amount of Myc-tagged MAP kinase immunoprecipitated with antibody to the Myc epitope and compared with that of CHO-IR cells cotransfected with SRα and pEXV-Erk2-tag. The data are the means ± standard errors of the mean of three separate experiments.

the inhibition of insulin-dependent MAP kinase activation. CHO-IR/ΔSOS cells were transfected with SRα or SRαSOS together with Myc-tagged $p42^{mapk}$ expression plasmid (pEXV-Erk2-tag) and after 72 h were stimulated with 100 nM insulin for 5 min. We measured the phosphorylation of MBP after incubation with immunoprecipitates prepared from cells with antibody to the Myc epitope, and the level of phosphorylation was normalized by the amount of Myc-tagged $p42^{mapk}$. Transfection of SRαSOS, but not SRα, into CHO-IR/ΔSOS cells restored the insulin-dependent activation of MAP kinase to 89% of that in CHO-IR cells (Fig. 5).

Effects of mutant mSOS1 on cell growth and DNA synthesis. The cell growth rate of each cell line was examined in F-12 medium containing 10% fetal bovine serum. CHO-IR/ΔSOS cells grew slowly (at approximately one-third the rate of CHO-IR cells). CHO-IR/SOS cells grew rather rapidly; however, there was no statistical difference between the growth rates of CHO-IR cells and CHO-IR/SOS cells (Fig. 6A). The cell growth rates of all cell lines with 1% fetal bovine serum were similar (data not shown).

DNA synthesis in each cell line was measured under basal and insulin-stimulated conditions. DNA synthesis without insulin stimulation in CHO-IR/ΔSOS was decreased to approximately one-fourth the rate of CHO-IR cells (9,030 ± 450 dpm/10^6 cells versus 37,200 ± 3,200 dpm/10^6 cells; $n = 4$). However, insulin-dependent increases of DNA synthesis were observed for all three cell lines, with similar dose-response curves (Fig. 6B).

Effects of mutant mSOS1 on insulin-stimulated glycogen synthase and p70 S6 kinase activities. Cells were incubated with various concentrations of insulin for 30 min, and glycogen synthase activity was measured. Insulin stimulated glycogen synthase activity in a dose-dependent manner up to ~2.5- to 3-fold in CHO-IR cells (ratios of 0.23 ± 0.03 to 0.56 ± 0.03), CHO-IR/SOS cells (ratios of 0.17 ± 0.02 to 0.50 ± 0.05), and CHO-IR/ΔSOS cells (ratios of 0.19 ± 0.03 to 0.57 ± 0.01) (Fig.

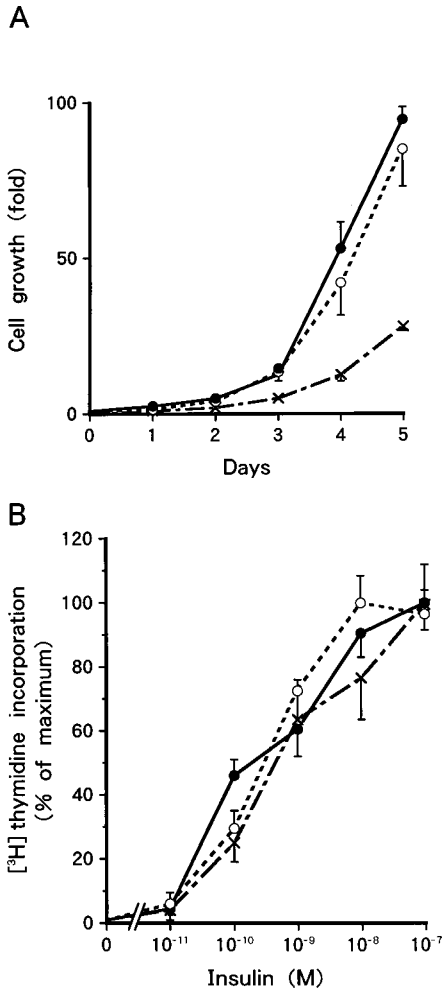


FIG. 6. Cell growth curve and effects of wild-type and mutant mSOS1 on insulin-induced DNA synthesis. (A) Cell growth rate of each cell line cultured in six-well plates measured in F-12 medium containing 10% fetal bovine serum. The cells were counted every 24 h, and the results were expressed as the fold increase in the number of cells. The data are the means ± standard errors of the mean (SEM) of four independent samples. (B) DNA synthesis in each cell line measured in the presence of various concentrations of insulin. The results were expressed as the percent maximum radioactivity incorporated into DNA. The data are the means ± SEM of four independent experiments. ○, CHO-IR; ●, CHO-IR/SOS; ×, CHO-IR/ΔSOS.

7A). The effect of insulin on p70 S6 kinase was also determined in CHO-IR, CHO-IR/SOS, and CHO-IR/ΔSOS cells. The basal activities of p70 S6 kinase were similar in all three cell lines (data not shown), as were the dose-dependent increases in p70 S6 kinase activity in response to insulin (Fig. 7B).

DISCUSSION

The Grb2-SOS complex forms by the interaction of the SH3 domains of Grb2 with the proline-rich sequence in the carboxyl-terminal region of SOS (11, 21, 26, 39, 54). To clarify the role of the Grb2-SOS complex in insulin signaling, we established stable CHO-IR cell lines that overexpress a mutant mSOS1 protein that lacks the guanine nucleotide exchange domain (CHO-IR/ΔSOS cells). The mutant mSOS1 protein did not contain guanine nucleotide exchange activity toward Ras in vitro. As a result of an ~25-fold overexpression of mutant mSOS1, more than 95% of the mSOS1 binding sites on Grb2 were occupied by the mutant protein in vivo.

Insulin stimulates the phosphorylation of several cytoplasmic proteins on tyrosine residues. The SH2 domain of Grb2 recognizes and binds to specific motifs containing phosphorylated tyrosine residues in IRS-1 and Shc (60). Therefore, insulin induces the formation of IRS-1–Grb2–SOS and Shc-Grb2-SOS complexes (7, 50, 60). However, the significance of the formation of these complexes in insulin-induced Ras activation in intact cells has not been clear. We have now shown that insulin-induced Ras activation is markedly impaired in CHO-IR/ΔSOS cells. Insulin-induced tyrosine phosphorylation of the insulin receptor, IRS-1, and Shc was not inhibited in these cells. Furthermore, the association of the Grb2-SOS complex with tyrosine-phosphorylated IRS-1 or Shc and the association of PI 3-kinase activity with IRS-1 were not impaired in CHO-IR/ΔSOS cells. These results suggest that the mutant mSOS1 acts as a dominant-negative protein and specifically inhibits signaling from the insulin receptor to Ras by occupying mSOS1 binding sites on Grb2. Furthermore, overexpression of wild-type mSOS1 increased the amount of Ras-GTP in cells incubated in the absence or presence of insulin. Taken together, our data suggest that the Grb2-SOS complex may mediate, at least in part, insulin-induced activation of Ras in intact cells. Insulin-induced phosphorylation and activation of MAP kinase were markedly impaired in CHO-IR/ΔSOS cells, and the insulin-induced activation of MAP kinase was rescued by the transient expression of wild-type mSOS1 in CHO-IR/ΔSOS cells, suggesting that the mutant mSOS1 acts as a dominant-negative protein and that the observed effects on MAP kinase are due to the primary effect of the mutant mSOS1.

Our study also revealed Ras-dependent and Ras-independent insulin signaling pathways. Insulin-induced phosphorylation and activation of MAP kinase were markedly impaired in CHO-IR/ΔSOS cells, suggesting that Ras-GTP formation is required for the insulin-induced activation of MAP kinase. This conclusion is consistent with previous observations showing that insulin stimulates the phosphorylation of MAP kinase via the activation of Ras in the fibroblast cell lines Rat-1, NIH 3T3, and Swiss 3T3 (12, 20). In contrast, insulin-induced activation of glycogen synthase and p70 S6 kinase was not impaired in CHO-IR/ΔSOS cells, suggesting that Ras-GTP formation is not required for activation of these enzymes by insulin.

The activation of glycogen synthase by insulin has been shown to be mediated by enzyme dephosphorylation (19). Since the MAP kinases are upstream activators of the 90-kDa S6 kinase II (61), the observation that protein phosphatase 1, which dephosphorylates glycogen synthase, is activated in response to phosphorylation by insulin-stimulated protein kinase 1 (19), the homolog of the 90-kDa S6 kinase II in muscle (63), suggested that activation of the Ras-MAP kinase cascade was required for activation of glycogen synthase. However, we have now shown that insulin activated glycogen synthase in CHO-IR/ΔSOS cells, in which insulin-induced Ras and MAP kinase activation was greatly impaired, to an extent similar to that in CHO-IR and CHO-IR/SOS cells. Although we cannot exclude the possibility that glycogen synthase is regulated differently in muscle cells (19) and other types of cells (CHO-IR cells) (14), the activation of MAP kinase by epidermal growth factor did not result in stimulation of glycogen synthase in 3T3-L1 adipocytes (52). These observations suggest that MAP kinase activation is not sufficient for the activation of glycogen synthase. Recently, both p90 S6 kinase (RSK-2) and p70 S6 kinase were shown to inactivate the β isoform of glycogen synthase

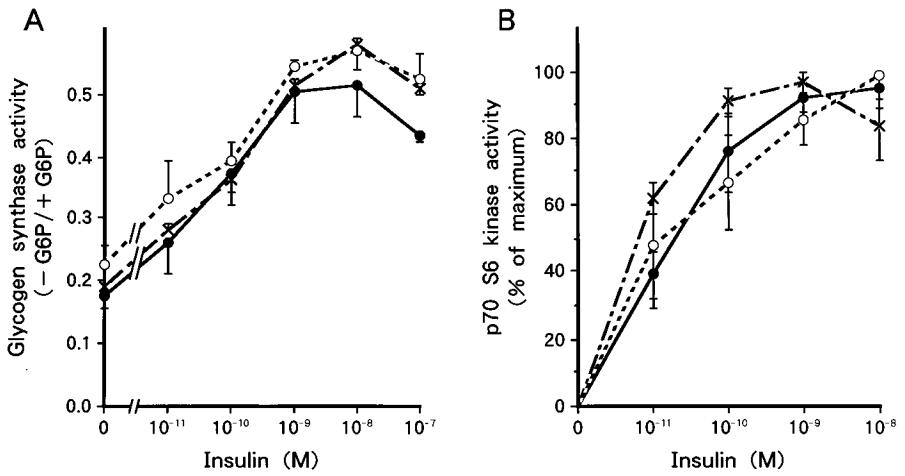


FIG. 7. Effects of expression of wild-type and mutant mSOS1 on insulin stimulation of glycogen synthase (A) and 70-kDa S6 kinase (B) activities. (A) Lysates were prepared from CHO-IR (○), CHO-IR/SOS (●), and CHO-IR/ΔSOS (×) cells after incubation with the indicated concentrations of insulin for 30 min. Glycogen synthase activity was expressed as the ratio of activity measured in the absence of glucose 6-phosphate (−G6P) to that measured in the presence of 10 mM glucose 6-phosphate (+G6P). The data are the means ± standard errors of the mean (SEM) of three separate experiments. (B) Cells were incubated with the indicated concentrations of insulin for 10 min, lysed, and subjected to immunoprecipitation with $\alpha 70^{S6K}$. The immunoprecipitates from CHO-IR (○), CHO-IR/SOS (●), and CHO-IR/ΔSOS (×) cells were assayed for S6 kinase activity with an S6 synthetic peptide as the substrate. The data are the means ± SEM of three separate experiments.

kinase 3, originally identified as an inactivator of glycogen synthase in skeletal muscle (22), in a cell-free system by phosphorylating a serine residue located 9 amino acids from the amino terminus (64). Because insulin-induced activation of p70 S6 kinase was normal in CHO-IR/ΔSOS cells, it is possible that insulin activated glycogen synthase via activation of p70 S6 kinase and subsequent inactivation of glycogen synthase kinase 3β. Taken together, the data indicate that glycogen synthase activity is regulated by insulin in intact cells, at least in part, via a Ras-independent mechanism.

Two major types of serine/threonine kinase participate in the phosphorylation of ribosomal protein S6 (24): the $pp90^{rsk}$ family (1) and p70 S6 kinase (8, 34). The *Xenopus* S6 kinase II, or $pp90^{rsk}$, was first shown to be directly stimulated by insulin-activated MAP kinase in vitro (61), suggesting that MAP kinases are dominant upstream activators of $pp90^{rsk}$. Although p70 S6 kinase is also activated by insulin (49), p70 S6 kinase and MAP kinase lie on distinct signaling pathways (6, 17). The observation that the activation of p70 S6 kinase, but not $pp90^{rsk}$, by insulin was blocked by the immunosuppressant rapamycin (48) allowed us to dissociate insulin signaling via the p70 S6 kinase pathway from that via the MAP kinase and $pp90^{rsk}$ pathway. These data may be consistent with the notion that Ras-dependent signaling does not regulate p70 S6 kinase activity. On the other hand, Ras-transformed cells show increased levels of S6 phosphorylation (9) and p70 S6 kinase activity (5), suggesting that Ras is an upstream activator of p70 S6 kinase. Our observation that p70 S6 kinase was stimulated by insulin in CHO-IR/ΔSOS cells, in which Ras activation was markedly inhibited, suggests that Ras activation is not required for the signaling pathway that results in activation of 70-kDa S6 kinase. Recently, the same conclusion was drawn by a different approach (43). There are conflicting reports of whether PI 3-kinase is the upstream regulator of p70 S6 kinase (15, 16) or not (43). However, our present data did not distinguish these two possibilities.

Although Ras-MAP kinase activation has been tightly linked to the regulation of cell proliferation, we obtained several CHO-IR/ΔSOS cell lines in which insulin-dependent activation of Ras and MAP kinase was inhibited. Skolnik et al. also established L6 cells overexpressing the mutant Grb2(R86K) in which insulin-dependent activation of MAP kinase was inhibited (59). In CHO-IR/ΔSOS cells, insulin-dependent increases of DNA synthesis were observed; however, DNA synthesis without insulin stimulation and cell growth rate with 10% fetal bovine serum were decreased. These data suggest that Ras-MAP kinase pathway is not the only signaling pathway regulating cell proliferation. A similar conclusion was expressed in two recent papers (44, 58). Considering the importance of p70 S6 kinase in the mitogenic response (36) and full activation of this enzyme by insulin in CHO-IR/ΔSOS cells, it is possible that p70 S6 kinase has a role in the insulin-dependent increase of DNA synthesis in CHO-IR/ΔSOS cells.

It is difficult to exclude the possibility that the overexpressed mutant mSOS1 interacts, via its proline-rich sequence, with the SH3 domain containing proteins other than Grb2, such as Nck and Crk, and thereby inhibits insulin-induced Ras activation. However, the following observation suggests that this possibility is unlikely. That is, overexpression of wild-type mSOS1 did not inhibit insulin-induced Ras activation, suggesting that inhibition of insulin-induced Ras activation by exogenously expressed mSOS1 occurs only when the guanine nucleotide-exchanging activity of the protein is abolished. It is possible that insulin-induced activation of glycogen synthase and p70 S6 kinase, which may normally be Ras dependent, was rescued by overexpression of mutant mSOS1 in CHO-IR/ΔSOS cells. However, this possibility is also unlikely, given that insulin-induced activation of glycogen synthase and p70 S6 kinase was normal in CHO-IR-C/S cells (56), in which insulin-induced Ras activation was also markedly impaired by overexpression of a dominant-negative SH–PTP-2 mutant (46).

In conclusion, we have shown that the Grb2-SOS complex mediates, at least in part, insulin-induced activation of Ras in intact cells and that activation of Ras is not required for insulin-induced activation of glycogen synthase and p70 S6 kinase.

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