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Involvement of Shc in Insulin- and Epidermal Growth Factor-Induced Activation of p21^{ras}

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Shc proteins are phosphorylated on tyrosine residues and associate with growth factor receptor-bound protein 2 (Grb2) upon treatment of cells with epidermal growth factor (EGF) or insulin. We have studied the role of Shc in insulin- and EGF-induced activation of p21^{ras} in NIH 3T3 cells overexpressing human insulin receptors (A14 cells). A14 cells are equally responsive to insulin and EGF with respect to activation of p21^{ras}. Analysis of Shc immunoprecipitates revealed that (i) both insulin and EGF treatment resulted in Shc tyrosine phosphorylation and (ii) Shc antibodies coimmunoprecipitated both Grb2 and mSOS after insulin and EGF treatment. The induction of tyrosine phosphorylation of Shc and the presence of Grb2 and mSOS in Shc immunoprecipitates followed similar time courses, with somewhat higher levels after EGF treatment. In mSOS immunoprecipitates, Shc could be detected as well. Furthermore, Shc immune complexes contained guanine nucleotide exchange activity toward p21^{ras} in vitro. From these results, we conclude that after insulin and EGF treatment, Shc associates with both Grb2 and mSOS and therefore may mediate, at least in part, insulin- and EGF-induced activation of p21^{ras}. In addition, we investigated whether the Grb2-mSOS complex associates with the insulin receptor or with insulin receptor substrate 1 (IRS1). Although we observed association of Grb2 with IRS1, we did not detect complex formation between mSOS and IRS1 in experiments in which the association of mSOS with Shc was readily detectable. Furthermore, whereas EGF treatment resulted in the association of mSOS with the EGF receptor, insulin treatment did not result in the association of mSOS with the insulin receptor. These results indicate that the association of Grb2-mSOS with Shc may be an important event in insulin-induced, mSOS-mediated activation of p21^{ras}.

After stimulation, most if not all growth factor receptor tyrosine kinases activate p21^{ras}. This activation of p21^{ras} appears to be regulated predominantly through activation of guanine nucleotide exchange on p21^{ras} (7, 17, 23). Recently, several proteins have been implicated to function in this activation.

First, there are the guanine nucleotide exchange factors mSOS 1 and 2 (5). These proteins were isolated as the murine homologs of the *Drosophila melanogaster* Son of Sevenless protein, a putative guanine nucleotide exchange factor that mediates signaling from the Sevenless receptor tyrosine kinase to Ras1 in regulatory processes in eye development (35).

The second implicated protein is the 23-kDa growth factor receptor-bound protein 2 (Grb2; also called Ash) (19, 21). Grb2 consists of a Src homology2 (SH2) domain flanked by two SH3 domains. Whereas SH2 domains are involved in the binding of tyrosine-phosphorylated protein domains (1, 15, 27, 40, 43), SH3 domains appear to bind to proline-rich sequences (30). The involvement of Grb2 in the regulation of p21^{ras} was first revealed by its homolog in *Caenorhabditis elegans*, Sem-5, which functions between a receptor tyrosine kinase (Let-23) and a p21^{ras} homolog (Let-60) in vulval development (11, 26). Furthermore, the *D. melanogaster* Grb2 homolog Drk binds in vitro to both the Sevenless receptor and SOS; i.e., the SH2 domain of Drk binds to the tyrosine-phosphorylated Sevenless receptor, and the SH3 domains of Drk bind to the proline-rich sequences present in SOS (24, 36). In mammalian cells, Grb2 and mSOS form a stable complex as well. Upon epidermal growth factor (EGF) treatment, this complex associates with the tyrosine-phosphorylated EGF receptor. The current hypothesis is that this association triggers the activation of p21^{ras} (6, 12, 14, 18, 33).

A third protein that has been implicated in the regulation of p21^{ras} is Shc. Shc has been isolated as a protein with a region homologous to the SH2 domain of c-Fes, and it contains a single SH2 domain and a glycine/proline-rich region (28). Antisera against Shc immunoprecipitate three different Shc proteins of 46, 52, and 66 kDa (p46^{shc}, p52^{shc}, and p66^{shc}, respectively). p46^{shc} and p52^{shc} are both encoded by a 3.4-kb mRNA, and p66^{shc} is translated from a distinct transcript. Shc proteins are phosphorylated upon activation of the EGF receptor, the insulin receptor, and the platelet-derived growth factor receptor (28, 29). Upon EGF receptor activation, Shc associates with the EGF receptor through its SH2 domain (28, 34). The involvement of Shc in p21^{ras} activation has been demonstrated by the observation that overexpression of Shc leads to p21^{ras}-dependent neurite outgrowth in PC12 cells (34). Moreover, in v-*src*-transformed cells, Shc is found in a complex with Grb2 (22, 33), and a small fraction of Shc was detected in mSOS immunoprecipitates (12). After EGF receptor stimulation, a complex between Shc and Grb2 is also formed (22, 28, 34).

We were interested in the role of Shc in insulin receptor signaling. Previously, we reported that after insulin treatment, Shc is phosphorylated on tyrosine residues and associates with Grb2 (29), a result independently obtained by others (16, 38,

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42). Grb2 does not associate directly with the insulin receptor (38, 42), which may imply that insulin-induced activation of $p21^{ras}$ is mediated by Shc, a possibility which is also not excluded for EGF-induced activation of $p21^{ras}$. However, adding to the complexity, association between Grb2 and insulin receptor substrate 1 (IRS1) has also been reported (38). IRS1, a 180-kDa substrate of the insulin receptor, has several putative tyrosine phosphorylation sites that may serve as docking sites for SH2 domain-containing proteins (32, 41). For instance, IRS1 binds to the 85-kDa subunit of phosphatidylinositol 3-kinase (2, 41) and to the 70-kDa Syp phosphotyrosine phosphatase (13).

In this report, we demonstrate that upon insulin treatment of A14 fibroblasts, a complex is formed between Shc and mSOS which exhibits guanine nucleotide exchange activity on $p21^{ras}$ in vitro. Although we did find association of Grb2 with IRS1, we were unable to detect a complex between IRS1 and mSOS. We conclude that the association between Shc and mSOS may be an important event in insulin-induced, mSOS-mediated activation of $p21^{ras}$.

MATERIALS AND METHODS

Cells and cell culture. The A14 cells used in this study are NIH 3T3 cells expressing 7×10^5 human insulin receptors per cell (9). A14 cells were cultured in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal calf serum (GIBCO). For insulin and EGF treatment, cells were placed in serum-free Dulbecco's modified Eagle's medium for 18 h and subsequently treated with insulin (5 μg/ml) or EGF (20 ng/ml) for the indicated times.

Fusion proteins and generation of antisera. Bacteria expressing the glutathione *S*-transferase (GST)–Shc fusion protein were generously provided by Tony Pawson (University of Toronto) (28). Upon induction with 0.1 mM isopropyl-β-D-thiogalactopyranoside, these bacteria express a fusion protein of GST and the SH2 domain of Shc. The GST-Shc protein was purified from bacterial cell lysates by using a glutathione-agarose column (39). The purified GST-Shc was injected in rabbits to generate antisera against Shc. The antiserum from one of the immunoresponsive rabbits was affinity purified on a GST-Shc column. Murine *grb2* cDNA was isolated from a mouse brain library by using oligonucleotides of human *grb2* sequences and PCR. The integrity of the cloned murine *grb2* was confirmed by sequence analysis. By using oligonucleotides, full-length *grb2* was cloned in frame with the C-terminal part of the GST-coding sequence in a modified pGEX2T expression vector (a kind gift of R. Plasterk, The Netherlands Cancer Institute, Amsterdam, The Netherlands). GST-Grb2-expressing bacteria were isolated by standard procedures. The GST-Grb2 protein was purified, and antisera were raised as described above for GST-Shc. Polyclonal anti-Grb2 used for immunoblotting was from Upstate Biotechnology, Inc. Antiphosphotyrosine antibody PY20 was from ICN, and antiphosphotyrosine antibody FB2 was a generous gift from R. Clark (Onyx Pharmaceuticals, Richmond, Calif.). Polyclonal anti-IRS1 serum was obtained by immunizing rabbits with a fusion protein between GST and part of the IRS1 protein (25). Monoclonal anti-IRS1 was from Upstate Biotechnology. Polyclonal anti-mSOS serum was raised by immunizing rabbits with an mSOS peptide (S3) and affinity purified on an S3 affinity column (6).

Immunoprecipitation and immunoblotting. Cells were lysed in lysis buffer (30 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 0.1 μM aprotinin, 1 μM leupeptin, 1 mM sodium orthovanadate, 10 mM sodium fluoride), and lysates were cleared by centrifugation. For immunoprecipitations, affinity-purified anti-Shc (2 μg), affinity-purified anti-mSOS (5 μg), polyclonal anti-IRS1 (20 μl), and polyclonal anti-Grb2 (20 μl) sera were coupled to 10 μl of protein A-Sepharose. Antibody-loaded beads were washed three times with lysis buffer and then incubated in cell lysates for 3 h at 4°C. Immunoprecipitates were washed four times with lysis buffer and once with 10 mM Tris-HCl (pH 7.0)–1 mM EDTA, after which proteins were eluted and denatured in sample buffer (10% glycerol, 60 mM Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate [SDS], 0.3 M β-mercaptoethanol) at 95°C for 5 min. Eluted proteins were separated on SDS-polyacrylamide gels and electroblotted to nitrocellulose. Immunoblotting was performed after blocking of the nitrocellulose filters in 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) containing 0.1% Tween 20 (TPBS) for 2 h at 4°C. The filters were incubated with the indicated primary antibodies in proper dilutions in TPBS–0.1% BSA for 3 h at 4°C. Filters were washed three times for 10 min in TPBS and subsequently incubated with the proper secondary antibodies or protein A-coupled with horseradish peroxidase (HRP) in TPBS–0.1% BSA for 2 h at 4°C. After three 10-min washes in TPBS, bound HRP was made visible by enhanced chemiluminescence (Amersham).

In vitro exchange activity assay. Shc immunoprecipitations from serum-starved A14 cells treated with insulin or EGF were performed as described above. Control immunoprecipitations were performed with 10 μg of nonspecific rabbit immunoglobulin G (IgG). After being washed three times with lysis buffer, the pellets were washed once with exchange buffer (50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.5], 1 mM $MgCl_2$, 1 mM dithiothreitol, 100 mM KCl, 0.1 μg of BSA per ml, 0.05% Triton X-100). To the final pellets, 10 μl of exchange buffer containing 10 ng of $p21^{c\text{-}Ha\text{-}ras}$ and 2 μCi of [α-^{32}P]GTP (final concentration, 1 μM) was added. The samples were shaken at 21°C for the indicated times. The reactions were stopped by the addition of 1 ml of ice-cold buffer (50 mM HEPES [pH 7.5], 5 mM $MgCl_2$, 1 mM dithiothreitol, 10 μg of BSA per ml, 0.1 mM GTP). The beads were centrifuged, and 900 μl of the supernatant was collected on nitrocellulose filters, which were subsequently washed three times with 10 ml of cold PBS plus 5 mM $MgCl_2$. The filters were counted for Cerenkov radiation.

RESULTS

mSOS associates with Shc after insulin and EGF treatment. NIH 3T3 cells expressing high levels of human insulin receptors (A14 cells) respond equally well to insulin and EGF treatment with respect to $p21^{ras}$ activation (8). Therefore, this cell line provides a good model system with which to study the effects of the two growth factors on intermediates in the signaling process from receptor to $p21^{ras}$. We previously reported that insulin treatment of A14 cells induced tyrosine phosphorylation of Shc (29). First, we compared the time course and level of insulin-induced tyrosine phosphorylation of Shc with those observed after EGF treatment. A14 cells were treated for different periods with either insulin or EGF, and Shc proteins were immunoprecipitated and analyzed for the level of tyrosine phosphorylation. Both insulin and EGF treatment induced Shc tyrosine phosphorylation, with maximum phosphorylation around 5 min after treatment (Fig. 1A). In both cases, $p52^{Shc}$ appeared to be the major tyrosine-phosphorylated Shc protein. EGF treatment resulted in

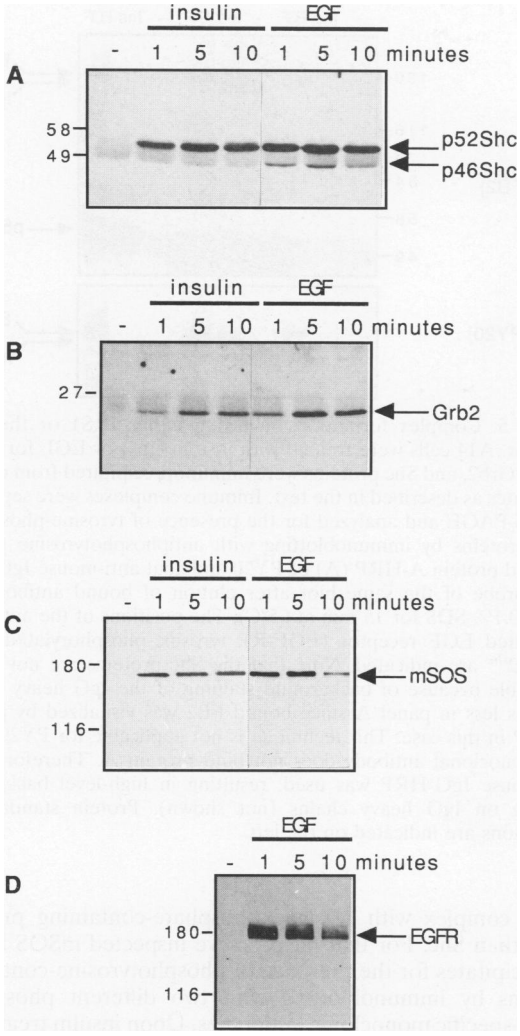

FIG. 1. Insulin- and EGF-induced complex formations involving Shc, Grb2, and mSOS. (A) Insulin- and EGF-induced tyrosine phosphorylation of Shc. A14 cells were stimulated for different periods with insulin or EGF, as indicated. Shc proteins were immunoprecipitated from cleared cell lysates and analyzed for tyrosine phosphorylation levels by immunoblotting with antiphosphotyrosine (PY20) and goat anti-mouse IgG-HRP. The Shc proteins are indicated by arrows. The diffuse band between p46^{shc} and p52^{shc} is present in all lanes and represents the IgG heavy chain. Protein standards in kilodaltons are indicated on the left. (B) Insulin- and EGF-induced complex formation between Shc and Grb2. A14 cells were treated as described above. Coprecipitation of Grb2 with Shc immune complexes was visualized by immunoblotting with polyclonal anti-Grb2 serum (Upstate Biotechnology) and protein A-HRP. The Grb2 protein is indicated. Low-level background staining above Grb2 is due to the presence of IgG light chain at this position. The 27-kDa standard is indicated on the left. (C) Insulin- and EGF-induced complex formation between Shc and mSOS. Shc immune complexes were obtained as described above and analyzed for the presence of mSOS by immunoblotting with affinity-purified anti-mSOS and protein A-HRP. The mSOS protein is indicated on the right; protein standards in kilodaltons are indicated on the left. (D) EGF-induced coprecipitation of the autophosphorylated EGF receptor with Shc proteins. A14 cells were treated with EGF for the times indicated, and Shc proteins were immunoprecipitated from the cell lysates. The autophosphorylated EGF receptor was visualized by immunoblotting with PY20 and goat anti-mouse IgG-HRP. The EGF receptor (EGFR) is indicated on the right; protein standards in kilodaltons are indicated on the left.

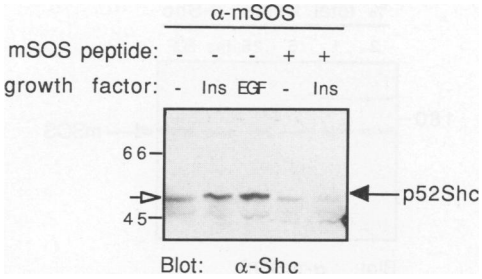

FIG. 2. Specific coimmunoprecipitation of Shc with mSOS immunoprecipitates from insulin- and EGF-treated A14 cells. A14 cells were not treated (−), treated with insulin (Ins) for 1 min, or treated with EGF for 1 min. mSOS immunoprecipitations were performed on the cleared lysates in the absence (−, Ins, and EGF) or presence (− and Ins) of competitor peptide as described earlier (6). Shc proteins were visualized by immunoblotting with affinity-purified anti-Shc (α-Shc) and protein A-HRP. p52^{shc} and a nonspecific band which has a somewhat faster mobility than p52^{shc} (open arrow) are indicated. Protein standards in kilodaltons are indicated on the left.

slightly higher levels of Shc tyrosine phosphorylation than did insulin treatment.

Shc tyrosine phosphorylation triggers association with the 23-kDa Grb2 protein through the SH2 domain of Grb2 and a phosphotyrosine residue of Shc (34). Indeed, both insulin and EGF treatment of A14 cells resulted in coprecipitation of Grb2 with Shc immune complexes, as detected by immunoblotting with a polyclonal anti-Grb2 serum (Fig. 1B). Not surprisingly, coprecipitation of Grb2 with Shc correlated with the time course of Shc tyrosine phosphorylation.

The Grb2 protein interacts through its SH3 domains with proline-rich sequences in the carboxy-terminal part of mSOS, a p21^{ras} guanine nucleotide exchange protein (12, 18, 33). This led us to test whether both insulin- and EGF-induced Shc-Grb2 complex formation would result in the appearance of mSOS in Shc immune complexes. Indeed, probing of Shc immunoprecipitates with an anti-mSOS peptide serum revealed the presence of mSOS after both insulin and EGF treatment (Fig. 1C). mSOS coprecipitation correlated with both Shc tyrosine phosphorylation and Shc-Grb2 complex formation. Finally, after EGF treatment, the tyrosine-phosphorylated EGF receptor also appeared in Shc immunoprecipitates (Fig. 1D), in agreement with previous reports (22, 28). In contrast, after insulin treatment, Shc does not associate with either the insulin receptor or IRS1 (29). To demonstrate the complex formation between Shc and mSOS more conclusively, we analyzed mSOS immunoprecipitates for the presence of Shc. mSOS immunoprecipitates from insulin- and EGF-treated A14 cells both contained considerable amounts of Shc (Fig. 2). Coprecipitation of Shc with mSOS was blocked in the presence of the mSOS peptide (S3) that was used to raise the mSOS polyclonal antiserum. To get an impression of the amount of mSOS involved in complex formation with Shc, A14 cells were stimulated with insulin or EGF for 1 min and Shc complexes were immunoprecipitated. The amount of coprecipitating mSOS was visualized by immunoblotting and compared with the amount of mSOS present in whole cell lysates (Fig. 3). Since our Shc antiserum precipitates more than 90% of the Shc present in cell lysates (not shown), we estimate that only a few percent of total mSOS is involved in insulin- and EGF-induced complex formation with Shc.

Shc immunoprecipitates contain guanine nucleotide exchange activity. mSOS has been shown to contain guanine nucleotide exchange activity toward p21^{ras} in vitro (6). To

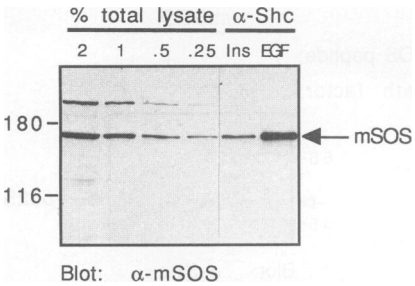

FIG. 3. Quantification of the amount of mSOS involved in complex formation with Shc. A14 cells were stimulated for 1 min with insulin (Ins) or EGF, and Shc was immunoprecipitated from the cell lysates. The amount of coprecipitating mSOS was visualized by immunoblotting as described in the text and compared with the amount of mSOS that is present in different quantities of total cell lysates (2, 1, 0.5, and 0.25% of the amount of cell lysate used in the immunoprecipitations). mSOS is indicated by the arrow, and protein standards in kilodaltons are indicated on the left. The protein of about 200 kDa present in total cell lysates is most likely not related to mSOS, since this protein is not detected in mSOS immunoprecipitates that were probed with anti-mSOS (α-mSOS), and it does not bind to a GST-Grb2 fusion protein (not shown).

investigate whether the observed Shc-mSOS complexes exhibit a similar activity, we analyzed Shc immune complexes for the presence of guanine nucleotide exchange activity toward $p21^{ras}$. Shc immunoprecipitates were mixed with [α-^{32}P]GTP and $p21^{c\text{-}Ha\text{-}ras}$ in exchange buffer, and guanine nucleotide binding to $p21^{ras}$ was measured. As depicted graphically in Fig. 4, Shc immunoprecipitates from insulin- and EGF-treated cells displayed guanine nucleotide exchange activity toward $p21^{ras}$.

mSOS does not form a stable complex with the insulin receptor or IRS1. Next we addressed whether mSOS forms a

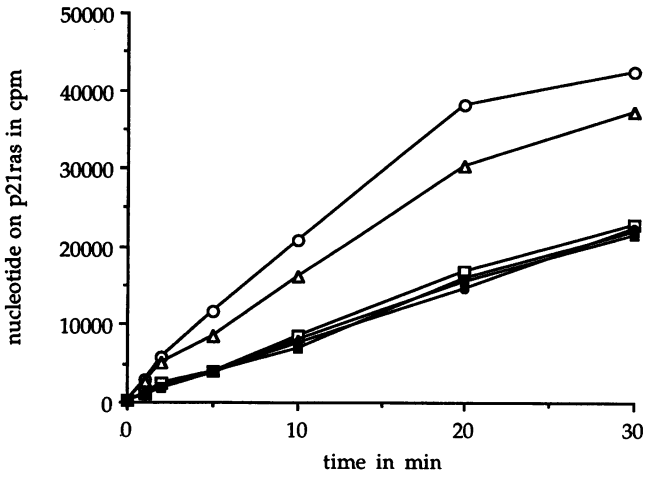

FIG. 4. Shc immune complexes contain $p21^{ras}$ guanine nucleotide exchange activity. Shc immunoprecipitations from lysates of untreated A14 cells (□) or cells that were treated for 1 min with insulin (△) or EGF (○) were performed as described in the text. Immunoprecipitations with nonspecific rabbit IgG from untreated (■), insulin-treated (▲), and EGF-treated (●) A14 cells were performed as controls. After washing, the immune complexes were incubated with pure bacterially expressed $p21^{c\text{-}Ha\text{-}ras}$ and [α-^{32}P]GTP for the indicated times. Labeled nucleotide bound to $p21^{ras}$ was determined by binding to nitrocellulose filters and Cerenkov counting.

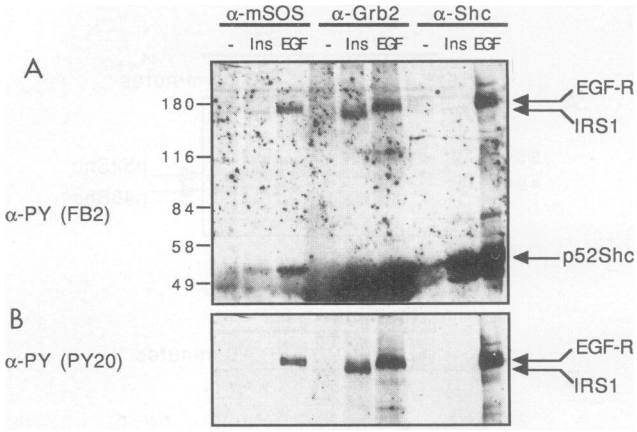

FIG. 5. Complex formations involving either IRS1 or the EGF receptor. A14 cells were treated with insulin (Ins) or EGF for 1 min. mSOS, Grb2, and Shc proteins were immunoprecipitated from cleared cell lysates as described in the text. Immune complexes were separated by SDS-PAGE and analyzed for the presence of tyrosine-phosphorylated proteins by immunoblotting with antiphosphotyrosine (α-PY) FB2 and protein A-HRP (A) or PY20 and goat anti-mouse IgG-HRP (B; reprobe of the same blot after elution of bound antibodies in TPBS–0.1% SDS for 15 min at 65°C). The positions of the autophosphorylated EGF receptor (EGF-R), tyrosine-phosphorylated IRS1, and $p52^{shc}$ are indicated. Note that the Shc proteins are not clearly detectable because of background staining of the IgG heavy chains, which is less in panel A since bound FB2 was visualized by protein A-HRP in this case. This technique is not applicable for PY20, since this monoclonal antibody does not bind protein A. Therefore, goat anti-mouse IgG-HRP was used, resulting in high-level background staining on IgG heavy chains (not shown). Protein standards in kilodaltons are indicated on the left.

stable complex with tyrosine phosphate-containing proteins other than Shc. For that purpose, we inspected mSOS immunoprecipitates for the presence of phosphotyrosine-containing proteins by immunoblotting with two different phosphotyrosine-specific monoclonal antibodies. Upon insulin treatment, we observed only $p52^{shc}$ in mSOS immunoprecipitates (Fig. 5A); no evidence could be obtained for the presence of the 95-kDa insulin receptor β subunit or the 180-kDa IRS1 protein (Fig. 5A and B). In contrast, after EGF treatment, both $p52^{shc}$ (Fig. 5A) and the 185-kDa autophosphorylated EGF receptor were readily detected in mSOS immunoprecipitates, as previously reported (6, 12, 33). Similarly, the EGF receptor was detectable in Grb2 immunoprecipitates as well as Shc immunoprecipitates. In the case of insulin treatment, we did not observe the insulin receptor β subunit or IRS1 in Shc immunoprecipitates (Fig. 5), nor did we detect Shc in insulin receptor and IRS1 immunoprecipitates from insulin-treated cells (not shown) (29, 38, 42). The insulin receptor β subunit was also not observed in Grb2 immunoprecipitates. However, we did observe a 180-kDa phosphoprotein in Grb2 immunoprecipitates, which is most likely IRS1. These results may indicate that upon insulin treatment, a complex between IRS1-Grb2 and mSOS is formed, but the mSOS antiserum is unable to recognize this complex. To exclude this possibility, we immunoprecipitated IRS1 complexes from insulin-treated cells and analyzed these complexes for the presence of mSOS (Fig. 6). As shown before, mSOS was easily detectable in Shc immunoprecipitates upon insulin treatment; however, mSOS was not detected in the IRS1 immunoprecipitates. Immunoblotting with antiphosphotyrosine revealed that the majority of IRS1 was immunoprecipitated by the polyclonal anti-IRS1

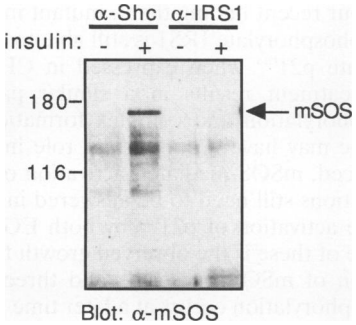

FIG. 6. mSOS is not detectable in IRS1 immunoprecipitates. A14 cells were not treated (−) or treated with insulin (+) for 1 min. Shc and IRS1 were precipitated from the cell lysates, after which the immune complexes were analyzed for the presence of mSOS as described in the text. mSOS is indicated by the arrow, and protein standards in kilodaltons are shown on the left.

serum (not shown). Taken together, these results indicate that in insulin-treated A14 cells, the amount of mSOS associated with IRS1 is considerably smaller than the amount associated with Shc.

To investigate whether the observed association between IRS1 and Grb2 is induced by insulin treatment, we analyzed Grb2 immunoprecipitates for the presence of IRS1. As shown in Fig. 7, there was some IRS1 present in Grb2 immune complexes from serum-starved A14 cells that were not treated with insulin. The amount of Grb2-associated IRS1 was somewhat increased upon insulin treatment. Furthermore, IRS1 present in Grb2 immunoprecipitates from insulin-treated A14 cells appeared to be phosphorylated on tyrosine residues, since it had a lower mobility in SDS-polyacrylamide gel electrophoresis (PAGE), and it was recognized by antiphosphotyrosine. In IRS1 immunoprecipitates, we were not able to conclusively show the presence of Grb2 because of high background staining in the 25-kDa region (not shown). Similar results were obtained with Chinese hamster ovary (CHO) cells expressing high levels of human insulin receptors (25a). The presence of IRS1-Grb2 complexes in quiescent cells coupled with the fact that we did not observe complex formation between IRS1 and mSOS suggests that in A14 cells, IRS1 may not be the most important intermediary protein in insulin-induced, mSOS-mediated activation of guanine nucleotide exchange on p21^{ras}.

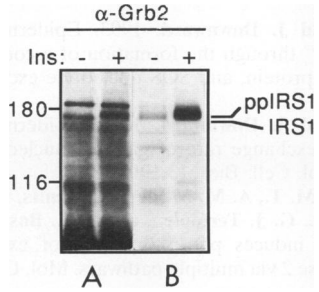

FIG. 7. IRS1-Grb2 complexes are precipitated from both untreated (−) and insulin-treated (+) A14 cell lysates. Grb2 was immunoprecipitated from cell lysates of untreated and insulin-treated cells (1 min). Immune complexes were separated by SDS-PAGE and analyzed for the presence of IRS1 by immunoblotting with monoclonal anti-IRS1 antibody and goat anti-mouse IgG-HRP (A) or PY20 and goat anti-mouse IgG-HRP (B). IRS1 and phosphorylated IRS1 (ppIRS1) are indicated on the right; protein standards in kilodaltons are indicated on the left.

DISCUSSION

Shc proteins were identified as SH2 domain-containing proteins of 46, 52, and 66 kDa (28). These proteins are phosphorylated on tyrosine residues upon activation of a variety of receptor tyrosine kinases and are phosphorylated constitutively in v-*src*-transformed cells (12, 22, 28, 29). Furthermore, after EGF treatment, Shc binds to the autophosphorylated EGF receptor (28, 34). The involvement of Shc in the activation of p21^{ras} has been suggested by the observation that overexpression of Shc leads to p21^{ras}-dependent neurite outgrowth in PC12 cells (34). Moreover, Shc associates with Grb2 after the induction of Shc tyrosine phosphorylation (22, 34). Grb2 is a protein that consists of an SH2 domain flanked by two SH3 domains. The SH2 domain presumably mediates the binding to Shc as well as to the EGF receptor (40). The SH3 domains recognize and bind to proline-rich sequences, several of which are present in mSOS, a p21^{ras} guanine nucleotide exchange protein (5, 12, 18, 33). On the basis of genetic evidence in both *C. elegans* and *D. melanogaster* and biochemical evidence related to EGF receptor signaling, the current model is that the Grb2-mSOS complex mediates receptor tyrosine kinase-induced activation of p21^{ras}. However, Grb2 may also bind to other proline-rich sequence-containing proteins and in this way may mediate other signaling pathways. Indications of an additional role for Grb2 came from the *C. elegans* homolog of Grb2, Sem-5. Disruption of the gene for Sem-5 has a more profound effect on sex myoblast migration and vulval development than deletion of the p21^{ras} homolog Let-60 (11). Also, microinjection of Grb2 (Ash) antiserum in fibroblasts has an effect on both cell proliferation and membrane ruffling, whereas inhibition of p21^{ras} affects only cell proliferation (20, 31). Interestingly, the SH3 domains of Grb2 were shown to direct localization of Grb2 to membrane ruffles (4). Therefore, the observed association of Grb2 with both the EGF receptor and Shc may represent different signaling functions of Grb2. To investigate whether the Shc-Grb2 complex is involved in the activation of p21^{ras}, we investigated whether Shc forms an active complex with mSOS after receptor tyrosine kinase activation. Such an experiment is difficult to perform with use of EGF receptor signaling, since both Grb2 and Shc can bind directly to the EGF receptor. However, Shc appears not to bind to the insulin receptor or IRS1, making insulin receptor signaling a good model system for studying the function of Shc in receptor tyrosine kinase-induced activation of p21^{ras}. Therefore, we used the A14 cell line, which is a NIH 3T3 cell line overexpressing the human insulin receptor. A14 cells are equally responsive to insulin and EGF with respect to activation of p21^{ras}, which allows a comparison between the two signaling pathways (8). We observed that after both insulin and EGF treatment, Shc is phosphorylated on tyrosine residues and associates with both Grb2 and mSOS (Fig. 1). Complex formation between Shc and mSOS was demonstrated by the presence of mSOS in Shc immunoprecipitates and by the presence of Shc in mSOS immunoprecipitates (Fig. 2). Only a fraction of the total amount of mSOS could be detected in a complex with Shc (Fig. 3), but this amount was sufficient to induce an increase in guanine nucleotide exchange on p21^{ras} in vitro (Fig. 4). From these results, we conclude that after both EGF and insulin treatment, Shc forms an active complex with mSOS. How this complex formation contributes to the activation of p21^{ras} in vivo is not yet known. For EGF signaling, it has been suggested that binding of mSOS to the membrane-bound

EGF receptor may be the mechanism for p21^{ras} activation (6). In insulin- and EGF-treated A14 cells, the amount of mSOS involved in complex formation with Shc is too little to allow a quantitative analysis of mSOS translocation to the membrane. However, since at least 50% of the total amount of Shc is present in the particulate (p100) fraction of A14 cells (unpublished observations), growth factor-induced Shc tyrosine phosphorylation may be sufficient to target mSOS to the membrane fraction.

After insulin treatment, the association between Shc and mSOS is most likely mediated by Grb2. In the case of EGF receptor signaling, however, part of the Grb2 and mSOS found in Shc immune complexes may also be coprecipitated through direct association between Grb2-mSOS and the EGF receptor. In fact, this may partially account for the observed higher levels of Grb2 and mSOS found in Shc immune complexes after EGF treatment (Fig. 1B and C). The observed higher levels of Shc-mSOS complexes upon EGF treatment are also reflected by the level of Shc-associated p21^{ras} guanine nucleotide exchange activity in the in vitro assay. However, in vivo, we do not observe a difference in the level of p21^{ras} activation upon insulin and EGF treatment (8). Apparently, EGF treatment results in the formation of a surplus of Shc-mSOS complexes. Alternatively, insulin-induced activation of p21^{ras} may be mediated by an additional mechanism, not involving Shc-mSOS complex formation. In this respect, it is noteworthy that the amount of Shc-mSOS complexes present after 60 min of EGF treatment is reduced to the level observed upon insulin treatment, yet the level of p21^{ras}-GTP remains high in both cases. These last observations support the idea of excess Shc-mSOS complex formation in case of EGF treatment.

It has previously been reported that after insulin stimulation, a complex between Grb2 and IRS1 is formed (38, 42). IRS1 is a substrate of the insulin receptor with several putative tyrosine phosphorylation sites. We investigated whether this complex is also present in A14 cells upon insulin treatment and, if so, whether this complex also contains mSOS. Indeed, we detected IRS1-Grb2 complexes; however, we failed to demonstrate the presence of IRS1 in mSOS immunoprecipitates. To get an impression of our detection limit, we inspected mSOS immunoprecipitates for the presence of tyrosine phosphate-containing proteins after both insulin and EGF treatment, using two different phosphotyrosine-specific monoclonal antibodies. Whereas the EGF receptor after EGF treatment and Shc after both EGF and insulin treatment were readily detectable in mSOS immunoprecipitates, no evidence for the presence of IRS1 (or the insulin receptor β subunit) could be obtained. From these results, we conclude that in our cell system, insulin treatment does not induce complex formation between IRS1 and mSOS. Skolnik et al. (38) and Tobe et al. (42) did observe an increase in complex formation between IRS1 and Grb2 (Ash) in CHO cells overexpressing the human insulin receptor. Whether IRS1 and SOS form a complex in these cells has not yet been reported. However, the possibility that complexes between mSOS and IRS1 occur after insulin treatment cannot be excluded. Skolnik et al. (37) reported the formation of a complex between IRS1 and mSOS in L6 myoblasts overexpressing Grb2, and Baltensperger et al. (3) reported the formation of a complex between IRS1 and *D. melanogaster* SOS in COS cells that were transiently transfected with recombinant *D. melanogaster* SOS cDNA. Our results indicate that after insulin treatment, a complex between Shc and mSOS is formed as well. In fact, in A14 cells, this Shc-mSOS complex is much more prominent than the complex between IRS1 and mSOS, suggesting a role for Shc in insulin-induced, mSOS-mediated p21^{ras} activation. This conclusion is supported by our recent finding that a mutant insulin receptor which fails to phosphorylate IRS1 is still able to phosphorylate Shc and activate p21^{ras} when expressed in CHO cells (25). Since EGF treatment results in a similar pattern of Shc tyrosine phosphorylation and complex formation with Grb2 and mSOS, Shc may have an important role in both insulin- and EGF-induced, mSOS-mediated activation of p21^{ras}.

Several questions still need to be answered in order to fully understand the activation of p21^{ras} by both EGF and insulin treatment. One of these is the observed growth factor-induced phosphorylation of mSOS on serine and threonine residues (33). This phosphorylation occurs at a later time point than the formation of the observed complexes and the activation of p21^{ras}. Moreover, this phosphorylation is dependent on p21^{ras}-mediated activation of the Raf1-Erk2 serine/threonine kinase cascade, indicating that this phosphorylation is not involved in the initial activation of p21^{ras} (10). Clearly, receptor tyrosine kinase-induced activation of p21^{ras} still has its secrets.

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