Regulation of Epidermal Growth Factor Receptor Signaling by Phosphorylation of the Ras Exchange Factor hSOS1*

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In response to stimulation with epidermal growth factor (EGF), the guanine nucleotide exchange factor human SOS1 (hSOS1) promotes the activation of Ras by forming a complex with Grb2 and the human EGF receptor (hEGFR). hSOS1 was phosphorylated in cells stimulated with EGF or phorbol 12-myristate 13-acetate or following co-transfection with activated Ras or Raf. Co-transfection with dominant negative Ras resulted in a decrease of EGF-induced hSOS1 phosphorylation. The mitogen-activated protein kinase (MAPK) phosphorylation of hSOS1 in vitro within the carboxyl-terminal proline-rich domain. The same region of hSOS1 was phosphorylated in vivo, in cells stimulated with EGF. Tryptic phosphopeptide mapping showed that MAPK phosphorylated hSOS1 in vitro on sites which were also phosphorylated in vivo. Phosphorylation by MAPK did not affect hSOS1 binding to Grb2 in vitro. However, reconstitution of the hSOS1-Grb2-hEGFR complex showed that phosphorylation by MAPK markedly reduced the ability of hSOS1 to associate with the hEGFR through Grb2. Similarly, phosphorylated hSOS1 was unable to form a complex with Shc through Grb2. Thus phosphorylation of hSOS1, by affecting its interaction with the hEGFR or Shc, down-regulates signal transduction from the hEGFR to the Ras pathway.

The guanine nucleotide exchange factor human SOS1 (hSOS1)1, the homologue of Drosophila Son of sevenless, promotes the activation of Ras following stimulation with epidermal growth factor (EGF) or with other growth factors acting through receptor tyrosine kinases (2). The molecular mechanism of Ras activation by EGF involves the interaction of hSOS1 with the activated human EGF receptor (hEGFR) through the adapter protein Grb2 (3). Grb2 links the hEGFR to hSOS1 by binding phosphotyrosine 1068 on the cytosolic tail of the hEGFR with its SH2 domain, and the carboxyl-terminal proline-rich region of hSOS1 with its SH3 domains (4, 5). It has been suggested, that upon formation of the hSOS1-Grb2-hEGFR complex, hSOS1 is recruited to the plasma membrane where it activates Ras by promoting GDP release and GTP binding (6, 7).

Stimulation with EGF also triggers the interaction of the hEGFR with the Shc proteins. The mammalian Shc gene encodes three proteins of 46, 52, and 62 kDa, and upon treatment with EGF Shc binds the activated hEGFR and undergoes tyrosine phosphorylation (8). Phosphorylation of Shc on tyrosine residue 317 creates a binding site for the SH2 domain of Grb2 and promotes the formation of the complex hSOS1-Grb2-Shc which participates in the activation of Ras (9), (10). Active, GTP-bound Ras binds and causes the translocation of the serine/threonine kinase Raf to the plasma membrane where Raf is activated by an event as yet unidentified (11, 12). Active Raf phosphorylates Mek which phosphorylates and activates the mitogen-activated protein kinases (MAPK) Erk1 and Erk2 (13). MAPK phosphorylates a number of cytosolic kinases and nuclear transcription factors which contribute to elicit the cellular responses following growth factor stimulation (14). EGFR induced activation of the Ras signaling pathway is often short lived, Ras-GTP loading and MAPK activation reach a maximum within 2–5 min and return to a basal level in 1 h (15). Several mechanisms which cooperate to the down-regulation of Ras signaling have been described. Among these mechanisms phosphorylation by MAPK or by other serine/threonine kinases acting downstream of Ras results in desensitization of the hEGFR (16) and in down-regulation of Raf and Mek (17). In cells treated with EGF or insulin hSOS1 as well as its murine counterpart, mSOS1 (18), undergoes serine/threonine phosphorylation (19, 20). These observations, and the fact that MAPK phosphorylates Drosophila SOS in vitro on sites which are also phosphorylated in vivo (21), have suggested that MAPK phosphorylates hSOS1 in response to growth factors activating receptor tyrosine kinases. It has been speculated that phosphorylation of hSOS1 by MAPK constitutes a negative feedback mechanism participating in the down-regulation of Ras signaling (22).

Here we show that active MAPK phosphorylates hSOS1 in vitro within the carboxyl-terminal proline-rich domain, on sites which are also phosphorylated in cells stimulated with EGF. Phosphorylation by MAPK does not affect hSOS1 interaction with Grb2 in vitro, nevertheless, phosphorylated hSOS1 demonstrates a markedly decreased ability to form a complex with the hEGFR or Shc through Grb2. Although other serine/threonine kinases cooperate with MAPK in hSOS1 phosphorylation in vivo, our data suggest that hSOS1 phosphorylation participates in the down-regulation of EGF induced activation of Ras.

MATERIALS AND METHODS

COS1 Cell Transfection—The cDNAs of interest (10 μg each), cloned into the pEXV3 expression vector, were transfected into COS1 cells by electroporation (23). COS1 cells, grown to 75% confluence, were harvested and washed twice in HEBS buffer (20 mM HEPES pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na2HPO4, 6 mM glucose). 2.5 × 10⁶ cells were resuspended in 240 μl of HEBS and placed into a 0.4-cm Bio-Rad electroporation cuvette containing 100 μg of sonicated salmon testes DNA (10 μg) (Sigma) and the plasmid DNA, to make a total volume of 260 μl. Cells were electroporated at 250 V/125 microfarads giving a time constant of 5 to 6 ms. Following electroporation the cells were seeded

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1 The abbreviations used are: hSOS1, guanine nucleotide exchange factor human SOS1; hEGFR, human EGF receptor; EGF, epidermal growth factor; PMA, phorbol 12-myristate 13-acetate; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase(s).
Immuno precipitation and Immunoblotting—An identical protocol was used for COS1 and HER14 cells. Cells were serum starved for 16 h, treated with EGF and/or phorbol 12-myristate 13-acetate (PMA), and lysed in 1 ml/dish of ice-cold 20 mM Tris-HCl, pH 8, 135 mM NaCl, 1.5 mM MgCl2, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM Pefabloc, 20 mM NaF, 1 mM NaVO4. Lysates were cleared of the insoluble material, normalized for protein concentration, and immunoprecipitated at 4°C for 2 h using the required antibody. Protein A-Sepharose beads were then added for a further hour to recover the immunoprecipitates. Immunoprecipitates were washed 3 times with 1 ml of lysis buffer, resolved by SDS-PAGE (6% gels, Novex, San Diego, CA) and, when required, electrophoretically transferred to a poly(vinylidene difluoride) membrane (Millipore, Bedford, MA). Membranes were incubated with the required antibody and immunoreactive proteins were visualized using enhanced chemiluminescence following incubation with a secondary antibody conjugated to horseradish peroxidase. To strip the membranes of the prebound antibody, membranes were incubated in 200 mM glycine, pH 2.2, 0.1% SDS, 1% Tween 20, for 1 h at room temperature. For metabolic labeling, COS1 cells, transfected with the required plasmids, were serum starved for 16 h and incubated for a further 4 h in phosphate-free Eagle’s medium (2 ml/60 mm plate) containing 0.5 mCi/ml of 32Pi. Cells were lysed and the proteins of interest were immunoprecipitated, resolved on SDS-PAGE, and visualized by autoradiography.

In vitro Phosphorylation of hSOS1—Bacterially produced GST-Erk1 was incubated with active Raf and recombinant Mek for 30 min at 30°C in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl2, 1 mM dithiothreitol, 50 μM ATP (24). Under these conditions active Raf, which was purified from SF9 cells co-infected with cr-f, r-raf, and v-src baculorusses, phosphorylated Mek which in turn phosphorylated and activated GST-Erk1 (24). An aliquot of the Raf-Mek-GST-Erk1 kinase reaction was then mixed with the required amount of baculovirus produced, epitope (EYMMPE, Glu-Glu)-tagged hSOS1 (25) and incubated at 30°C for a further 30 min in the same reaction buffer. When required (γ-32P]ATP (6000 Ci/mmol, 10 mCi/ml) was added to the kinase reaction at a final concentration of 0.5 μCi/ml. Typically 3 μg of recombinant hSOS1 which was pre-activated by incubation with 0.5 μg of Raf and 1 μg of Mek, was used to phosphorylate 10 μg of recombinant hSOS1 in a 100 μl reaction.

Two-dimensional Tryptic Peptide Mapping—COS1 cells, transiently expressing epitope-tagged (Glu-Glu)-tagged hSOS1, were metabolically labeled with [35S] and hSOS1 was immunoprecipitated using an anti-Glu-Glu mAb. At the same time 10 μg of recombinant hSOS1 were phosphorylated in vitro using activated GST-Erk and [γ-32P]ATP. hSOS1 was resolved by SDS-PAGE and electrophoretically transferred onto a poly(vinylidene difluoride) membrane. The hSOS1 band was visualized by autoradiography, cut out, and incubated for 12 h with 15 μg of trypsin in 70 μl of 50 mM ammonium bicarbonate, pH 8, at room temperature. Following the first 12 h of incubation a further 30 min of trypsin were added and the incubation was continued for another 6 h. The tryptic peptides were lyophilized twice in water and once in pH 1.9 electrophoresis buffer (formic acid/acetic acid/water, 1/3/66, v/v). Lyophilized peptides were resuspended in 12 μl of electrophoresis buffer and 10,000 cpm, typically corresponding to 2–6 μl, were loaded to 20 × 20-cm glass-backed cellulose plates. Peptides were separated in two dimensions by electrophoresis at 1 kV for 1.5 h using pH 1.9 electrophoresis buffer followed by chromatography (2-butanol/pyridine/acetic acid/water, 6.4/5/1/44) and autoradiographed (27).

Reconstitution of the hSOS1-GST-Grb2 Complex—Recombinant hSOS1 was phosphorylated in vitro using activated GST-Erk and, when required, [γ-32P]ATP. Bacterially produced GST-Grb2 (10 pmol) was mixed with [35S] labeled phosphorylated hSOS1 (6.5 pmol) and the required amount of unmodified or phosphorylated hSOS1 in 200 μl of lysis buffer. Tubes were rotated at 4°C for 15 min then the GST-Grb2-hSOS1 complexes were recovered using glutathione-agarose beads. Beads were washed 3 times, complexes were resolved on SDS-PAGE, and bands bound to GST-Garb2 was quantified using an AMBIS β scanner. Under these conditions the amount of [35S] labeled phosphorylated hSOS1 remaining bound to GST-Grb2 estimated the ability of the unmodified hSOS1 and of phosphorylated hSOS1 to form a complex with GST-Grb2.

Reconstitution of the hSOS1-GST-Grb2-HEGFR Complex—HEGFR was immunoprecipitated using an anti-HEGFR mAb (EGFR1) (28) from HER14 cells (29), which were serum starved for 16 h and treated with EGF or PMA as required. Immunoprecipitates were immobilized onto Protein A-Sepharose and washed 3 times with lysis buffer. hSOS1 Phosphorylation in COS1 Cells—We studied hSOS1 phosphorylation in COS1 cells transiently expressing epitope (Glu-Glu)-tagged hSOS1. Stimulation of the cells with EGF or PMA resulted in hSOS1 phosphorylation, as indicated by its decreased electrophoretic mobility (Fig. 1A). A similar mobility shift was observed in mSOS1 immunoprecipitated from EGF-treated fibroblasts and was reversed by treatment with phosphatases (19). The electrophoretic mobility of hSOS1 was also decreased following co-expression with oncogenic Ras (H-Ras G12V) or with a membrane-targeted, constitutively active ver-
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Fig. 2. A, hSOS1 deletion mutants. Dark shaded areas, CDC25 homology domain; light shaded areas, proline-rich region. B, GST-Erk1 phosphorylates hSOS1 within the carboxyl-terminal proline-rich region. Epitope (Glu-Glu)-tagged hSOS1 deletion mutants were transiently expressed in COS1 cells and, following overnight serum deprivation, immunoprecipitated using an anti-Glu-Glu mAb prebound to protein G-Sepharose. Immunoprecipitates were phosphorylated in vitro using [γ-32P]ATP and GST-Erk1 which had been activated in vitro using a reconstituted Raf-Mek-MAPK signaling pathway. Immunoprecipitates were resolved by SDS-PAGE and phosphorylated proteins were detected by autoradiography. C, hSOS1 is phosphorylated within the proline-rich region in EGF-treated COS1 cells. COS1 cells, transiently expressing epitope (Glu-Glu)-tagged hSOS1 deletion mutants, were metabolically labeled with 32P, in phosphate-free minimum essential Eagle's medium (0.5 mCi/ml, 2 ml/6 cm dish). At the end of the incubation with the radiolabel, cells were stimulated with 10 nM EGF for 5 min. hSOS1 deletion mutants were immunoprecipitated using an anti-Glu-Glu mAb. Immunoprecipitates were resolved on SDS-PAGE and phosphorylated proteins were detected by autoradiography.

Erk1 was able to phosphorylate its substrate myelin basic protein. GST-Erk1 efficiently phosphorylated hSOS1 which demonstrated that hSOS1 was phosphorylated in vivo within the region containing amino acids 1066 and 1333, thus within the same region that was phosphorylated in vitro by GST-Erk1 (Fig. 1C). A low amount of hSOS1 phosphorylation was also detected in unstimulated cells (Fig. 2C): this could be due to the high basal level of MAPK activity which has been described in COS1 cells (21). We also studied which region of hSOS1 was phosphorylated in response to stimulation with PMA. These experiments showed that, as with EGF-stimulated cells, treatment with PMA resulted in phosphorylation of hSOS1 only within the proline-rich domain (not shown).

Tryptic Peptide Mapping of Phosphorylated hSOS1—To further characterize the involvement of MAPK in hSOS1 phosphorylation, we carried out two-dimensional tryptic phosphopeptide analysis of hSOS1 phosphorylated in vivo, in EGF-stimulated COS1 cells labeled with 32P, and in vitro, using GST-Erk1 and [γ-32P]ATP. The study of the tryptic maps showed that 11 phosphopeptides were generated using in vitro phosphorylated hSOS1 (Fig. 3A) and 13 phosphopeptides were generated using in vivo phosphorylated hSOS1 (Fig. 3B). Analysis of a mixture of radiolabeled phosphopeptides prepared from both conditions showed that 7 of them were generated using either in vitro or in vivo phosphorylated hSOS1 (Fig. 3, A and B, peptides 1 to 7), strongly suggesting that MAPK phosphorylated hSOS1 in vivo, on sites situated within these peptides. A further 6 phosphopeptides were detected only when analyzing in vivo phosphorylated hSOS1, implying that, besides MAPK, other serine/threonine kinases also phosphorylated hSOS1 in vivo.
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Phosphorylation by MAPK Decreases hSOS1 Ability to Form a Complex with the hEGFR and GST-Grb2—Proline-rich regions are extended and flexible domains often participating in protein-protein interactions (32). We speculated that phosphorylation of the hSOS1 carboxyl-terminal proline-rich domain regulated, although indirectly, the interaction of hSOS1 with the activated hEGFR. To test this hypothesis, we reconstituted the hSOS1-Grb2-EGFR complex in vitro. We immunoprecipitated the hEGFR from HER14 fibroblasts and incubated the immunoprecipitates with epitope (Glu-Glu)-tagged, recombinant hSOS1 and GST-Grb2. At the end of the 1-h incubation, formation of the hSOS1-Grb2-EGFR complex was detected by resolving the immunoprecipitates on SDS-PAGE followed by Western immunoblotting using an anti-Glu-Glu mAb. The association of hSOS1 with the hEGFR was entirely dependent on the addition of GST-Grb2 to the reconstitution assay (not shown) and on the activation of the hEGFR, which was achieved by stimulating the HER14 cells with EGF. Furthermore, the amount of hSOS1 that we found associated with the hEGFR immunoprecipitates increased proportionally with the concentration of EGF we used to stimulate the HER14 cells for a longer time or when we used a lower concentration of EGF.

Phosphorylation by MAPK Does Not Affect hSOS1 Binding to Grb2 in Vitro—The hSOS1 carboxyl-terminal proline-rich region contains the binding sites for the adapter Grb2 (4). We studied if phosphorylation by GST-Erk1 modified the affinity of hSOS1 for GST-Grb2 in vitro. We used either unmodified or in vitro phosphorylated hSOS1 to compete with the binding of 32P-labeled phosphorylated hSOS1 to GST-Grb2. A the end of a 15-min incubation the hSOS1-GST-Grb2 complexes were captured, resolved on SDS-PAGE, and the amount of 32P-labeled phosphorylated hSOS1 remaining bound to GST-Grb2 was quantified. Fig. 4 shows that unmodified or phosphorylated hSOS1 demonstrated a nearly identical capacity to compete with 32P-labeled phosphorylated hSOS1 for binding GST-Grb2 suggesting that the affinity of hSOS1 for Grb2 was not significantly modified by GST-Erk1 phosphorylation.

Phosphorylation by MAPK Does Not Affect hSOS1 by MAPK. Bacterially produced GST-Grb2 (10 pmol) was mixed with 32P-labeled phosphorylated hSOS1 (6.5 pmol) and the indicated amounts of unmodified hSOS1 (●) or cold-phosphorylated hSOS1 (○). Tubes were rotated at 4°C for 15 min then the GST-Grb2-hSOS1 complexes were captured using glutathione-agarose beads and resolved on SDS-PAGE. The amount of 32P-labeled phosphorylated hSOS1 bound to GST-Grb2 was quantified using an AMBIS β scanner (100% = 1,000 cpm). Under the conditions of this assay, 32P-labeled phosphorylated hSOS1 saturated only 50% of GST-Grb2 binding capacity. The averages (± S.D.) of three independent experiments are shown.

Fig. 3. Tryptic phosphopeptide mapping of phosphorylated hSOS1. A, recombinant hSOS1 was phosphorylated in vitro using activated GST-Erk1 and [γ-32P]ATP. Phosphorylated hSOS1 was resolved by SDS-PAGE and transferred onto a poly(vinylidene difluoride) membrane. The band corresponding to hSOS1 was identified by autoradiography, cut out, and digested with trypsin. Tryptic peptides, corresponding to 10,000 cpm, were applied on cellulose plates, separated by electrophoresis (horizontal) followed by chromatography (vertical), and visualized by autoradiography. The site of application is situated near the bottom right corner of the panel. B, COS1 cells transiently expressing Glu-Glu-tagged hSOS1 were metabolically labeled with 32Pi and stimulated with 10 nM EGF for 2 min. hSOS1 was immunoprecipitated with an anti-Glu-Glu mAb, resolved by SDS-PAGE and analyzed as described in A. A + B, tryptic phosphopeptides from A and B (corresponding to 7,000 cpm from each sample) were mixed and analyzed as described in A. Seven phosphopeptide species (phosphopeptides 1 to 7) showed identical migration whether they were generated from hSOS1 phosphorylated in vitro or in vivo.
hEGFR Complex—Serine/threonine phosphorylation of the hEGFR by MAPK and by other kinases such as CaM kinase 2 or protein kinase C also contributes to the acute desensitization of EGF signaling (16, 33). To study if serine/threonine phosphorylation of hEGFR affects its capacity to associate with GST-Grb2 and hSOS1, we reconstituted the hSOS1-GST-Grb2-hEGFR complex using hEGFR immunoprecipitated from HER14 fibroblasts which were treated with PMA prior to stimulation with EGF. Treatment with PMA causes phosphorylation of hEGFR on serine and threonine residues which are also phosphorylated in response to prolonged stimulation with EGF and which are important for receptor desensitization (34). Al-

though with reduced capacity, unmodified hSOS1 was still able to form a complex with GST-Grb2 and hEGFR immunoprecipitated from PMA-treated cells, whereas in vitro phosphorylated hSOS1 failed to bind through GST-Grb2 to the same hEGFR preparation (Fig. 5B). Thus, serine/threonine phosphorylation of the hEGFR alone was not sufficient to prevent its interaction with hSOS1, whereas phosphorylation of both hSOS1 and hEGFR entirely blocked the assembly and/or impaired the stability of the hSOS1-GST-Grb2-hEGFR complex.

Phosphorylation of the hSOS1 Proline-rich Region Affects Its Interaction with GST-Grb2 and the hEGFR—We used the hSOS1 deletion mutant SOSΔ4, which comprised amino acid residues 1066 to 1333, to study if phosphorylation of the hSOS1 proline-rich region was sufficient to prevent the formation of the hSOS1-GST-Grb2-hEGFR complex. Fig. 5C shows that in vitro phosphorylation by GST-Erk1 decreased the capacity of SOSΔ4 to bind the hEGFR through GST-Grb2.

PMA Treatment Prevents the Interaction of mSOS1 with the hEGFR in Vivo—We used HER14 cells to study the effects of phosphorylation of mSOS1 and hEGFR in vivo. In these cells, a brief treatment with EGF alone induced co-immunoprecipitation of the hEGFR and the endogenous mSOS1, which is 98% identical to hSOS1 (1) (Fig. 5D). However, we failed to co-immunoprecipitate mSOS1 and the hEGFR from cells in which serine/threonine phosphorylation of hSOS1 and the hEGFR was induced with PMA prior to EGF stimulation (Fig. 5D). Treatment with PMA inhibits the tyrosine kinase activity of hEGFR and reduces its ability to autophosphorylate on tyrosine residues (34). Nevertheless, our failure to co-immunoprecipitate mSOS1 and hEGFR from PMA-treated cells could be attributed only in part to the diminished tyrosine phosphorylation of the hEGFR, as hEGFR, immunoprecipitated from cells pretreated with PMA and stimulated with EGF, was still able to associate with unmodified hSOS1 in vitro (Fig. 5B).

Phosphorylation of hSOS1 Impairs the Formation of the hSOS1-GST-Grb2-Shc Complex—Another route leading to activation of Ras in response to stimulation with EGF involves the formation of a complex of hSOS1 with tyrosine-phosphorylated Shc and Grb2. We studied if the assembly of the hSOS1-GST-Grb2-Shc complex was also impaired by hSOS1 phosphorylation. To reconstitute the hSOS1-Grub2-Shc complex in vitro, we immunoprecipitated Shc from COS1 cells transfected with Shc cDNA (8). Immunoprecipitates were incubated with GST-Grb2 and with the S100 fraction of a lysate of COS1 cells expressing Glu-Glu-tagged hSOS1, which were either untreated or stimulated with PMA to induce hSOS1 phosphorylation. The formation of the hSOS1-GST-Grb2-Shc complex was detected by resolving the immunoprecipitates on SDS-PAGE followed by Western immunoblotting using an anti-Glu-Glu mAb. Under these conditions hSOS1 formed a complex with GST-Grb2 and Shc and the assembly of such complex was entirely dependent on the addition of GST-Grb2 to the reconstitution assay (Fig. 6A). In contrast, phosphorylated hSOS1 was unable to form a complex with Shc and GST-Grb2 (Fig. 6A).

To establish if phosphorylation of mSOS1 affected its interaction with Shc in vivo, we attempted to co-immunoprecipitate mSOS1 and Shc from HER14 fibroblasts. Although we were able to co-immunoprecipitate Shc and mSOS1 from cells stimulated with EGF, we failed to co-immunoprecipitate Shc and mSOS1 from cells which were pretreated with PMA and stimulated with EGF (Fig. 6B). Treatment of HER14 cells with PMA caused a decrease of Shc tyrosine phosphorylation which could account for our failure of co-immunoprecipitating mSOS1 and Shc. Nevertheless, a decrease of Shc tyrosine phosphorylation was not sufficient to prevent the formation of the hSOS1-GST-Grb2-Shc complex in vitro, as Shc immunoprecipitated...
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Phosphorylation of hSOS1 occurred within the region containing the binding sites for the SH3 domains of Grb2. Nevertheless, we did not detect any significant difference between the ability of either unmodified or in vitro phosphorylated hSOS1 to bind GST-Grb2. Different from our data, it has recently been suggested that stimulation with insulin of 3T3-L1 adipocytes or Chinese hamster ovary cells induced phosphorylation of mSOS1 and disassociation of the mSOS1-Grb2 complex (20). Stimulation with insulin results in the activation of the Ras pathway and of other insulin-specific signaling systems which operate distinct negative feedback mechanisms (35). Therefore it is possible that in response to stimulation with insulin, mSOS1 is phosphorylated by kinases different from those activated by EGF, or that mSOS1-Grb2 interacts with yet unidentified proteins, this resulting in disassociation of the mSOS1-Grb2 complex.

Recombinant hSOS1, phosphorylated using GST-Erk1, showed a decreased capacity to form a complex with GST-Grb2 and the activated hEGFR in vitro. Similarly phosphorylated hSOS1 failed to form a complex with GST-Grb2 and tyrosine-phosphorylated Shc in vitro. These data suggest that one effect of MAPK phosphorylation of hSOS1 was to impair its participation in signaling complexes with the hEGFR or Shc, thus uncoupling signal transduction from the hEGFR to Ras. Down-regulation of EGF signaling also occurs through the serine/threonine phosphorylation of the hEGFR which results in inhibition of the tyrosine kinase activity of the receptor and in the decrease of its affinity for EGF (16). In addition, serine/threonine phosphorylation of the hEGFR cooperated with hSOS1 phosphorylation in blocking the assembly of the hSOS1-GST-Grb2-hEGFR complex in vitro.

The mechanism by which phosphorylation of hSOS1 by MAPK interferes with the participation of hSOS1 in signaling complexes is not clear. It is possible that phosphorylation by MAPK decreases the stability and induces the disassembly of the hSOS1-GST-Grb2-hEGFR complex. It is also possible that, binding of Grb2 to the hyperphosphorylated carboxyl terminus of hSOS1 results in alteration of Grb2 structure and loss of its affinity for binding tyrosine-phosphorylated proteins. The study of the crystal structure of Grb2 suggests that a conformational change affecting the SH3 domains of Grb2 would be unlikely to alter the SH2 domain (36). Nevertheless, binding of the SH2 domain of Grb2 to tyrosine-phosphorylated Shc positively regulates the interaction of the SH3 domains with the proline-rich region of mSOS1 (37). Thus, conformational changes appear to be transmitted through Grb2 and these changes are involved in regulating Grb2 interaction with target proteins.

MAPK is not the only kinase that phosphorylates hSOS1 in vivo, therefore, to fully understand the biochemical function of hSOS1 phosphorylation, it will be necessary to identify the other serine/threonine kinases as well as the sites that are phosphorylated. It is plausible that phosphorylation of hSOS1 in vivo, by analogy with the effects of phosphorylation by MAPK in vitro, also impairs the participation of hSOS1 in complexes with hEGFR or Shc. Indeed, EGF failed to induce co-immunoprecipitation of mSOS1 and hEGFR or Shc in cells which were pretreated with PMA. In conclusion our data suggest that hSOS1 phosphorylation, by limiting the access of hSOS1 to Ras, contributes to regulate the duration or the amplification of signals originating from activated tyrosine kinases. In agreement with this view, prolonged Ras-GTP loading has been detected in insulin-stimulated cells in which phosphorylation of mSOS1 was inhibited using a specific Mek inhibitor (38). It has been proposed that the duration of the activation of the Ras pathway (sustained versus transient) is critical to enacting...
specific cellular responses (39). According to this model, hSOS1 phosphorylation contributes to secure the execution of the appropriate cellular programs in response to growth factor stimulation.

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