

## Identification of the Mitogen-Activated Protein Kinase Phosphorylation Sites on Human Sos1 That Regulate Interaction with Grb2

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**The Son of sevenless proteins (Sos) are guanine nucleotide exchange factors involved in the activation of Ras by cytoplasmic and receptor tyrosine kinases. Growth factor stimulation rapidly induces the phosphorylation of Sos on multiple serine and threonine sites. Previous studies have demonstrated that growth factor-induced Sos phosphorylation occurs at the C-terminal region of the protein and is mediated, in part, by mitogen-activated protein (MAP) kinase. In this report, we describe the identification of five MAP kinase sites (S-1137, S-1167, S-1178, S-1193, and S-1197) on hSos1. We demonstrate that four of these sites, S-1132, S-1167, S-1178, and S-1193, become phosphorylated following growth factor stimulation. The MAP kinase phosphorylation sites are clustered within a region encompassing three proline-rich SH3-binding sites in the C-terminal domain of hSos1. Replacing the MAP kinase phosphorylation sites with alanine residues results in an increase in the binding affinity of Grb2 to hSos1. Interestingly, hSos2 contains only one MAP kinase phosphorylation site and, as demonstrated previously, has an increased affinity toward Grb2 compared with hSos1. These results suggest a role for MAP kinase in the regulation of Grb2-Sos interactions. Since the binding of Grb2 is important for Sos function, the phosphorylation-dependent modulation of Grb2-Sos association may provide a means of controlling Ras activation.**

The activation of Ras proteins by receptor tyrosine kinases constitutes a critical step in linking extracellular signals to intracellular effector molecules. It is now well established that the coupling of receptor tyrosine kinases to Ras is mediated by the recruitment of the guanine nucleotide exchange factors Son of sevenless (Sos) proteins to the plasma membrane via adaptor molecules such as Grb2 and Shc (2, 5, 8, 15, 20, 22, 26, 27, 30, 32). The molecular basis for this recruitment mechanism seems to involve two types of intermolecular interactions: the ligand-dependent association of the Src homology (SH2) domain of Grb2 with specific phosphotyrosine residues, and the binding of the SH3 domains of Grb2 to proline-rich motifs in the C-terminal domain of Sos proteins (22, 23). Recently, it has been demonstrated that the Pleckstrin and Dbl homology domains in the N-terminal region of Sos also participate in the regulation of Sos function (19, 24, 34).

Growth factor stimulation induces the rapid phosphorylation of Sos on serine and threonine residues (11, 20, 26). Analysis of the phosphorylation of *Drosophila* Sos or human hSos1 expressed in COS-1 cells and mSos1 expressed in HER14 cells has revealed that all these proteins are substrates for mitogen-activated protein MAP kinase both in vivo and in vitro (11, 25, 28). Furthermore, it has been shown that the MAP kinase-mediated phosphorylation occurs within the C-terminal domain of Sos (13, 25, 28). Although little is known about the physiological consequences of Sos phosphorylation, several studies have implicated the phosphorylation of Sos in regulating its interaction with signaling molecules. For example, it has been shown that phosphorylation of Sos is accompanied by its dissociation from Grb2 (10, 13, 36). In addition,

it has been demonstrated that phosphorylation of Sos promotes the dissociation of Sos-Shc and Sos-epidermal growth factor receptor complexes (25, 28) and correlates with inhibition of the binding of Sos-Grb2 complexes with tyrosine-phosphorylated sequences (6). On the basis of these findings, it has been postulated that Sos phosphorylation might constitute a negative-feedback mechanism to control Ras activity. This hypothesis is supported by the findings that prevention of insulin-stimulated Sos phosphorylation and subsequent disassociation of the Grb2-Sos complex by a specific inhibitor of MAP kinase kinase activity results in a prolonged activation of Ras activation (35). However, studies based on the use of cyclic AMP, which inhibits MAP kinase activation at the level between Ras and Raf (7), have indicated that the uncoupling of the Sos-Grb2 complex following Sos phosphorylation has no effect on the duration of insulin-induced Ras activation (13).

In an effort to determine the functional significance of Sos phosphorylation, we have identified all the MAP kinase phosphorylation sites in the C-terminal domain of hSos1 and have studied their role in hSos1-Grb2 interactions. All the growth factor-induced MAP kinase phosphorylation sites were mapped to serine residues located within the C-terminal region of hSos1 containing the first three proline-rich motifs. Mutations of these sites reveal that their phosphorylation by MAP kinase negatively modulates the interaction of hSos1 with Grb2. These findings provide evidence that growth factor-induced phosphorylation of hSos1 by MAP kinase may directly influence complex formation with Grb2.

### MATERIALS AND METHODS

**hSos derivatives.** The construction of expression plasmids encoding hemagglutinin (HA) epitope-tagged hSos1 and hSos2 has been described previously (38). Glutathione-S-transferase (GST)-hSos1C, corresponding to amino acid residues 1018 to 1334 of hSos1 fused in frame to pGEX3, was a gift from J. Schlessinger, New York University. Point mutations were introduced into HA-hSos1 by PCR as described by Zhao et al. (39). The two flanking primers used

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were 5'-AGACCAGTACCATGAGGCAT-3' and 5'-GATAGATCTTCAGGAAGAATGGGC-3'. The following four primers were used to make Ala-for-Ser substitutions: 5'-GATACAGAAGCAGCCCTTGG-3' (S1132A), 5'-GATGGTGCAGATTCTGCTG-3' (S1167A), 5'-TGGGGGAGCGTCCAAATGCTT-3' (S1178A), and 5'-GAATATCGTGGTGCATAGGC-3' (S1193A). The positions of the intended mutation sites are underlined, and the resulting constructs are denoted by the position of the amino acid substituted. The deletion mutant HA-hSos1ΔC was obtained by mutating residue 1071 of hSos1 to a stop codon with the oligonucleotide 5'-GTAGGATCCCTGAGAAACAGAAAGTA-3'.

**Cell culture and transfection.** COS-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum. Transfections of COS-1 cells were performed with calcium phosphate as described by Wigler et al. (37). Briefly, DNA was diluted in Tris-EDTA buffer, and 2 M CaCl<sub>2</sub> was added to a final concentration of 125 mM. The mixture was then diluted in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) Na<sub>2</sub>HPO<sub>4</sub> buffer and allowed to stand at room temperature for 30 min before being added to the culture medium. 12 h after transfection, the medium was aspirated and the cultures were fed with 10 ml of DMEM supplemented with 5% fetal calf serum. The murine myogenic cell line L6 was maintained in DMEM supplemented with 10% fetal calf serum. Both L6 and COS-1 cells were brought to quiescence by incubation for 36 to 48 h in DMEM containing 0.1% FCS and subsequently stimulated with 20% FCS in DMEM at 37°C for 10 to 15 min.

**Antibodies.** The anti-Grb2 mouse monoclonal antibody G16720 (Signal Transduction Laboratories) was used for immunoblotting. The anti-HA mouse monoclonal antibody 12CA5 (Babco) was used for immunoblotting and immunoprecipitation. The anti-Sos antibody 257 was a rabbit polyclonal antibody raised against the synthetic peptide spanning residues 738 to 757 of hSos1 and was a gift from J. Schlessinger (21).

**Cell labeling and immunoprecipitation.** Cells were rinsed with phosphate-free DMEM (Gibco-BRL) and incubated in 4 ml of phosphate-free DMEM supplemented with 1 mCi of <sup>32</sup>P (Du Pont-NEN) per ml for 3 to 4 h before being harvested. At the end of the labeling period, the cells were washed three times with ice-cold phosphate-buffered saline (PBS) and lysed in 1 ml of cold RIPA buffer (150 mM NaCl, 10 mM Tris-HCl [pH 7.4], 1% Triton X-100, 1% deoxycholic acid, 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mg of pepstatin per ml, 50 mM NaF, 1% aprotinin, 10 μg of leupeptin per ml, 1 mM sodium vanadate, 10 mM benzamide, 10 μg of soybean trypsin inhibitor per ml, 1 μM okadaic acid). For coimmunoprecipitation experiments, the cells were washed three times with ice-cold PBS and lysed in 1 ml of cold coimmunoprecipitation buffer (150 mM NaCl, 10 mM Tris-HCl [pH 7.4], 1% Triton X-100, 10% glycerol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mg of pepstatin per ml, 50 mM NaF, 1% aprotinin, 10 μg of leupeptin per ml, 1 mM sodium vanadate, 10 mM benzamide, 10 μg of soybean trypsin inhibitor per ml, 1 μM okadaic acid). The lysates were cleared by centrifugation at 4°C for 15 min at 14,000 × *g*. The clarified lysate was preincubated with protein A-Sepharose (Pharmacia LKB Biotechnology Inc.) for 15 min at 4°C while gently rotating. The immunoprecipitations were performed by incubating the lysates with corresponding antibody for 1.5 to 3 h at 4°C and then with 50 μl of a 50% solution of protein A-Sepharose for 30 min at 4°C. The immune complexes were collected by centrifugation for 15 s at 1,000 × *g*, washed five times with RIPA or coimmunoprecipitation buffer, and eluted with SDS sample buffer. Proteins were resolved by polyacrylamide gel electrophoresis (PAGE) as described by Blattler et al. (3). The gels were transferred to polyvinylidene difluoride (PVDF) membrane and exposed to X-ray film. Bands corresponding to Sos derivatives or GST-hSos1C were excised and digested with *N*-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin (TPCK-trypsin) as described below.

**In vitro phosphorylation by MAP kinase.** The MAP kinase preparation used in this study is a recombinant histidine-tagged MAP kinase provided by M. Cobb, University of Texas Southwestern Medical Center. To obtain an active preparation of the enzyme, the protein was expressed in *Escherichia coli*, purified by affinity chromatography on Ni-nitrilotriacetic acid-agarose and then activated with a *Xenopus* oocyte extract as described previously (31). Before in vitro phosphorylation by MAP kinase, beads were washed four times in ice-cold MAP kinase buffer containing 40 mM HEPES (pH 7.4), 5 mM MgCl<sub>2</sub>, 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), and 0.15 mM CaCl<sub>2</sub>. The kinase reaction was initiated by addition of 50 μM ATP, 200 μCi of [γ-<sup>32</sup>P]ATP (Du Pont-NEN; 6,000 Ci/mmol), and 5 μg of purified activated MAP kinase per ml. The reaction proceeded for 60 min at 30°C and was stopped by the addition of 1 ml of ice-cold kinase buffer followed by the addition of SDS sample buffer.

**Phosphopeptide mapping.** Phosphopeptides were generated by digestion of the protein immobilized on PVDF membrane. Samples were resolved by SDS-PAGE and transferred to PVDF membrane. The radiolabeled bands were excised, and the peptides were digested with TPCK-trypsin (Worthington Biochemical Corp.) and eluted as described previously (4). The peptides were resolved on crystalline cellulose thin-layer chromatography plates by electrophoresis at pH 1.9 (1,000 V for 45 min in a Hunter thin-layer peptide-mapping system) followed by chromatography in butanol-pyridine-acetic acid-H<sub>2</sub>O (15:10:3:12) in the second dimension.

**Phosphopeptide purification.** The phosphorylated tryptic peptides were separated from the bulk of nonphosphorylated peptides on a ferric iminodiacetic

acid column as described (33). The phosphopeptides were resolved by reverse-phase high-performance liquid chromatography (HPLC) with a VYDAC C18 (250 by 2.1 mm) column at a flow rate of 0.15 ml/min with 0.055% trifluoroacetic acid (TFA) modified with 75% acetonitrile–0.05% TFA solution. The phosphopeptides were eluted from the cellulose with 0.1% TFA–30% acetonitrile, and the eluted radioactivity was collected in 0.15-ml fractions and quantified (Cerenkov counts) in a scintillation counter. The identity of the HPLC-purified phosphopeptides was confirmed by comigration with the relevant peptide on two-dimensional phosphopeptide maps. The position of <sup>32</sup>P-phosphorylated residues in purified phosphopeptides was determined with an Applied Biosystems 473 protein sequencer following C-terminal covalent coupling of the peptide to the sample filter as described by Russo et al. (29). Direct protein sequencing of purified phosphopeptides was carried out with an Applied Biosystems 477A sequencer.

**Phosphoamino acid analysis.** To determine the phosphoamino acid content of tryptic peptides, relevant areas of cellulose were scraped from thin-layer plates and placed in microcentrifuge tubes. The peptides were eluted with 0.2 ml of 0.1% TFA in 30% acetonitrile. The cellulose was centrifuged for 5 min in a microcentrifuge; the supernatants were lyophilized and washed three times with water and later hydrolyzed in 50 μl of 6 N HCl for 1 h at 110°C. Samples were dried, and the residual HCl was removed by evacuating twice with water. The samples were dissolved in electrophoresis buffer (pH 1.9) containing phosphoamino acid standards and then spotted onto cellulose thin-layer chromatography plates. Phosphoamino acids were resolved by two-dimensional electrophoresis (pH 1.9 followed by pH 3.5) as described previously by Boyle et al. (4).

## RESULTS

**Characterization of MAP kinase-mediated phosphorylation of hSos1.** It has been shown that upon growth factor stimulation, Sos proteins are phosphorylated by MAP kinase (11, 28). To investigate the physiological role of Sos phosphorylation, we sought to identify the MAP kinase phosphorylation sites on hSos1. We first compared the phosphorylation pattern of hSos1 derived from serum-stimulated COS-1 cells with that of hSos1 phosphorylated by MAP kinase in vitro. For this analysis, an expression plasmid encoding HA-tagged hSos1 (HA-hSos1) was transfected into COS-1 cells. To examine the phosphorylation of hSos1 in vivo, the expressed protein was isolated by immunoprecipitation from serum-stimulated COS-1 cells labeled with <sup>32</sup>P<sub>i</sub>. To examine the phosphorylation of hSos1 in vitro, the expressed protein was immunoprecipitated from serum-starved COS-1 cells and then incubated with purified MAP kinase [(His)<sub>6</sub>-ERK2] in the presence of [γ-<sup>32</sup>P]ATP. The immunoprecipitates were resolved by SDS-PAGE, and the band corresponding to hSos1 was isolated and digested with trypsin. The resulting tryptic phosphopeptides were separated by two-dimensional electrophoresis chromatography. As illustrated in Fig. 1A, the tryptic map obtained from <sup>32</sup>P-HA-hSos1 isolated from serum-stimulated COS-1 cells exhibits five major phosphopeptides (spots 1 to 5) and 2 minor phosphopeptides. The tryptic phosphopeptide map of HA-hSos1 phosphorylated by MAP kinase in vitro exhibits a pattern strikingly similar to that seen with HA-hSos1 phosphorylated in vivo (Fig. 1A and B). An identical pattern was obtained for HA-hSos1, which was treated with potato acid phosphatase prior to the in vitro phosphorylation by MAP kinase (data not shown). Analysis of a mixture of tryptic phosphopeptides derived from hSos1 phosphorylated in vivo or in vitro (Fig. 1C) revealed that the five major phosphopeptides seen following the in vivo phosphorylation of hSos1 (spots 1 to 5) comigrated with phosphopeptides generated from hSos1 phosphorylated in vitro (Fig. 1C). Thus, the phosphorylation of hSos1 in serum-stimulated COS-1 cells is mediated primarily by MAP kinase or by a kinase with identical site specificity. Phosphoamino acid analysis of the five prominent phosphopeptides from <sup>32</sup>P-HA-hSos1 revealed that they each exclusively contain a serine phosphate (Fig. 1D).

To assess the role of MAP kinase in the phosphorylation of endogenous Sos, the two-dimensional tryptic phosphopeptide maps of mSos isolated from resting and serum-stimulated

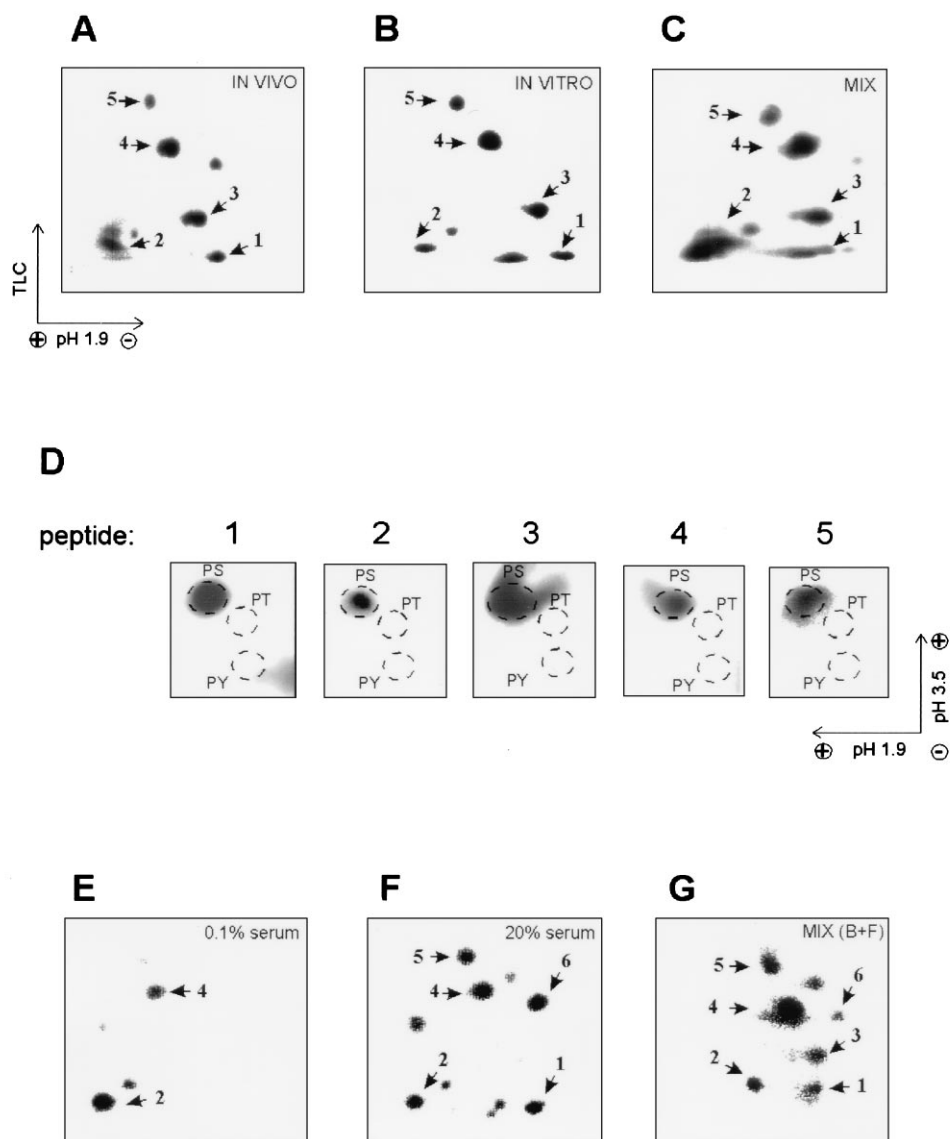


FIG. 1. Characterization of MAP kinase-mediated phosphorylation of hSos1. Tryptic phosphopeptide maps of HA-hSos1 phosphorylated in vivo (A) or in vitro (B) by MAP kinase. (A and B) COS-1 cells were transfected with HA-hSos1, and the transfected protein was immunoprecipitated. (A) HA-hSos1 immunoprecipitate isolated from transfected COS-1 cells labeled with  $^{32}\text{P}_i$  and stimulated with serum. (B) HA-hSos1 immunoprecipitate isolated from unlabeled serum-starved COS-1 cells and then phosphorylated with MAP kinase in vitro. The immunoprecipitates were separated by SDS-PAGE and transferred to PVDF membrane. Sos bands were cut out and digested with trypsin, and the phosphopeptides were analyzed as described in Materials and Methods. (C) Equal counts of the in vivo-labeled sample (A) and the in vitro-labeled sample (B) were mixed and analyzed together. Arrows indicate the five phosphopeptides that exhibit identical migration when derived from HA-hSos1 phosphorylated in vivo or in vitro. (D) Phosphoamino acid content of the tryptic peptides obtained from in vivo labeling. The peptides were eluted from the plates, and their phosphoamino acid content was determined as described in Materials and Methods. Nonradioactive phosphoamino acid standards were detected by ninhydrin staining. (E and F) Sos immunoprecipitates isolated from serum-starved (E) or serum-stimulated (20% fetal calf serum) (F)  $^{32}\text{P}$ -labeled L6 cells. The Sos protein was immunoprecipitated with anti-Sos antibody, and tryptic phosphopeptide mapping was carried out as described above. (G) equal counts of the in vitro-labeled sample (B) and the in vivo-labeled sample (F) were mixed and analyzed together as described above. TLC, thin-layer chromatography.

mouse L6 cells were compared. Serum stimulation induced the appearance of three new phosphopeptides (Fig. 1F, spots 1, 5, and 6). In addition, the phosphorylation of one phosphopeptide present in the unstimulated cells (Fig. 1E, spot 4) was enhanced in response to serum stimulation. Significantly, four of these phosphopeptides (spots 1, 2, 4, and 5) appeared at positions identical to four phosphopeptides generated by MAP kinase phosphorylation in vitro (Fig. 1B, spots 1, 2, 4, and 5). The identities of these species were confirmed in mixing experiments in which phosphopeptides obtained from the in vivo and in vitro phosphorylation were mixed and chromatographed on the same plate (Fig. 1G). These results demon-

strate that MAP kinase contributes significantly to the serum-induced phosphorylation of endogenous Sos. The presence of nonoverlapping phosphopeptides suggests that an additional kinase(s), whose identity is presently unknown, is involved in the phosphorylation of Sos. It is noteworthy that the phosphopeptide maps of endogenous Sos isolated from insulin-stimulated L6 cells exhibit a pattern that is nearly identical to the pattern obtained following serum stimulation (data not shown).

**Identification of the major sites of phosphorylation of hSos1 by MAP kinase.** As reported previously, all the MAP kinase phosphorylation sites in hSos1 reside in the C-terminal domain



of hSos1 (13, 25). We therefore used a GST fusion protein containing all the potential MAP kinase phosphorylation sites (GST-hSos1C; residues 1013 to 1334) to identify the MAP kinase phosphorylation sites. GST-hSos1 was produced in *E. coli* and purified by glutathione affinity chromatography. Following maximal phosphorylation by MAP kinase in the presence of [ $\gamma$ - $^{32}$ P]ATP, the protein was digested with trypsin and the resultant phosphopeptides were isolated on a ferric iminodiacetic acid column and further separated by reversed-phase HPLC on a C<sub>18</sub> column. Five major peaks were detected by HPLC (Fig. 2A), and the eluted phosphopeptides were further purified by thin-layer chromatography. All phosphopeptides contained only phosphoserine, as determined by phosphoamino acid analysis (Fig. 2B, insets). The positions of the phosphorylated serine residues in the phosphopeptides were determined by radiosequencing analysis following the covalent coupling of a small aliquot of each purified phosphopeptide to the sample filter. Significant radioactivity was released from the coupled peptide at cycle 11 of peptide 1, cycle 2 of peptide 2, cycles 4 and 5 of peptide 3, cycle 3 of peptide 4, and cycle 1 of peptide 5 (Fig. 2B). The amino acid sequence surrounding the phosphorylation sites was determined directly by protein sequencing of the phosphopeptides (Fig. 2C). These data, considered together with the cleavage specificities of trypsin (R and K), are consistent with the following assignments for phosphopeptides 1 to 5: 1, S-1167; 2, S-1197; 3, S-1178; 4, S-1193; and 5, S-1132. Intriguingly, while three of these phosphorylation sites (positions 1167, 1178, and 1193) constitute consensus MAP kinase sites, the residues surrounding S-1197 and S-1132 do not form consensus motifs for MAP kinase phosphorylation.

**Expression and characterization of hSos1 mutants lacking the MAP kinase phosphorylation sites.** To confirm the identity of the candidate MAP kinase sites, COS-1 cells were transfected with wild-type hSos1 or with hSos1 mutants in which the serine phosphorylation sites were individually or collectively replaced with alanine residues. We focused on the sites contained within phosphopeptides 1, 3, 4, and 5 because the phosphorylation of these sites is stimulated by serum (Fig. 1A, E, and F). Two days after transfection, the expressed proteins were isolated by immunoprecipitation and phosphorylated with MAP kinase *in vitro*. The levels of expression of all the hSos1 derivatives were nearly identical, as determined by Western blot (immunoblot) analysis (Fig. 3A). Mutation at site 1178 abolished the phosphorylation-dependent mobility shift characteristic of hSos1. The two-dimensional tryptic phosphopeptide maps of the phosphorylated mutants are shown in Fig. 3B. Eliminating S-1167, S-1178, S-1193, and S-1132 individually produced tryptic maps in which phosphopeptides 1, 3, 4, and 5 were missing, respectively, confirming that these sites are the MAP kinase phosphorylation sites in the wild-type protein. Mutations of S-1167, S-1178, S-1193, and S-1132 together led to the disappearance of peptides 1, 3, 4, and 5 and the appearance of a novel peptide. Thus, MAP kinase mediates the serum-induced phosphorylation of four sites in the C-terminal domain of hSos1.

**Effects of MAP kinase-mediated phosphorylation on hSos1-Grb2 interactions.** Previous studies have reported that the phosphorylation of Sos is accompanied by the dissociation of the Grb2-Sos complex (9, 10, 18, 35, 36). This effect is seen *in vivo* in response to insulin-induced phosphorylation of Sos as well as *in vitro* following MAP kinase phosphorylation of the C-terminal domain of hSos1. Furthermore, the serum-induced MAP kinase phosphorylation sites are clustered within a region of 92 amino acids that contain three proline-rich SH3-binding motifs (Fig. 4). To investigate the contribution of MAP

kinase phosphorylation to hSos-Grb2 interaction, we compared the abilities of the wild-type HA-hSos1 (hSos1-WT) and the mutated form of HA-hSos1 (hSos1-4M) to form complexes with Grb2 *in vivo*. hSos1-WT and hSos1-4M were transiently expressed in COS-1 cells. To analyze the phosphorylation of hSos1-4M *in vivo*, serum-starved cells were metabolically labeled with  $^{32}$ P<sub>i</sub> and stimulated with serum. As illustrated in Fig. 5A, the extent of phosphorylation of the hSos1-4M mutant was significantly reduced in comparison with the phosphorylation of hSos1-WT. The *in vivo* phosphorylation sites in hSos1-4M were determined by two-dimensional tryptic phosphopeptide mapping and compared with those obtained from the *in vivo* mapping of hSos1-WT (Fig. 5B and C). The pattern of the map obtained from the hSos1-4M indicates the loss of the four tryptic phosphopeptides corresponding to the serum-induced MAP kinase sites (1, 3, 4, and 5). In addition, the map contains one new peptide, which did not comigrate with any of the phosphopeptides from hSos1-WT phosphorylated *in vivo* (Fig. 5D and E). These observations provide further support that the relevant *in vivo* MAP kinase phosphorylation sites on hSos1 have been identified.

To compare the interactions of hSos1-WT and hSos1-4M with Grb2, transfected cells were lysed and immunoprecipitated with anti-HA antibodies. The immunoprecipitates were then analyzed by immunoblotting with either anti-HA antibodies or anti-Grb2 antibodies. As seen in Fig. 5F, the amount of Grb2 present in the hSos1-4M immunoprecipitates was significantly larger than that present in the hSos1-WT immunoprecipitate. These data suggest that the interaction of hSos1 with Grb2 is negatively regulated by phosphorylation. To test whether hSos1 phosphorylation mediates the serum-induced dissociation of Grb2-Sos complex, we compared the interaction of hSos1-WT and hSos1-4M with Grb2 in serum-starved and serum-stimulated cells. In contrast to hSos1-WT, the interaction of the hSos1-4M mutant with Grb2 remained constant under both serum-starved and serum-stimulated conditions (Fig. 5G). Thus, MAP kinase phosphorylation directly contributes to the serum-dependent uncoupling of the Sos-Grb2 complex. This conclusion is further supported by the observation that the amounts of Grb2 associated with the hSos1-WT and the hSos1-4M mutant were nearly identical under serum-starved conditions (Fig. 5G).

The apparent binding affinity of hSos2 to Grb2 is significantly higher than that of hSos1 (38) (Fig. 6C). To test whether this difference could reflect the differential phosphorylation of hSos1 and hSos2, we examined the *in vivo* and *in vitro* phosphorylation patterns of hSos2. The tryptic map from HA-hSos2 expressed in COS-1 cells and isolated following  $^{32}$ P<sub>i</sub> labeling and serum stimulation shows four major phosphopeptides (Fig. 6A). A similar pattern of phosphorylation was observed for HA-hSos2 isolated from serum-starved cells (data not shown). Only one of these peptides (spot 1) overlaps with the single phosphopeptide detected in the tryptic map obtained from HA-hSos2 phosphorylated by MAP kinase *in vitro* (Fig. 6B). Thus, in contrast to hSos1, the phosphorylation of hSos2 is to a large extent MAP kinase independent. In addition, the amount of Grb2 associated with Sos2 was not altered by exposure to serum (Fig. 6D), indicating that the interaction of Sos2 with Grb2 is not modulated by growth factor stimulation.

## DISCUSSION

The guanine nucleotide exchange factor Sos mediates the coupling of receptor tyrosine kinases to Ras activation. The Sos proteins are rapidly phosphorylated at multiple C-terminal sites in response to growth factor stimulation. Earlier studies

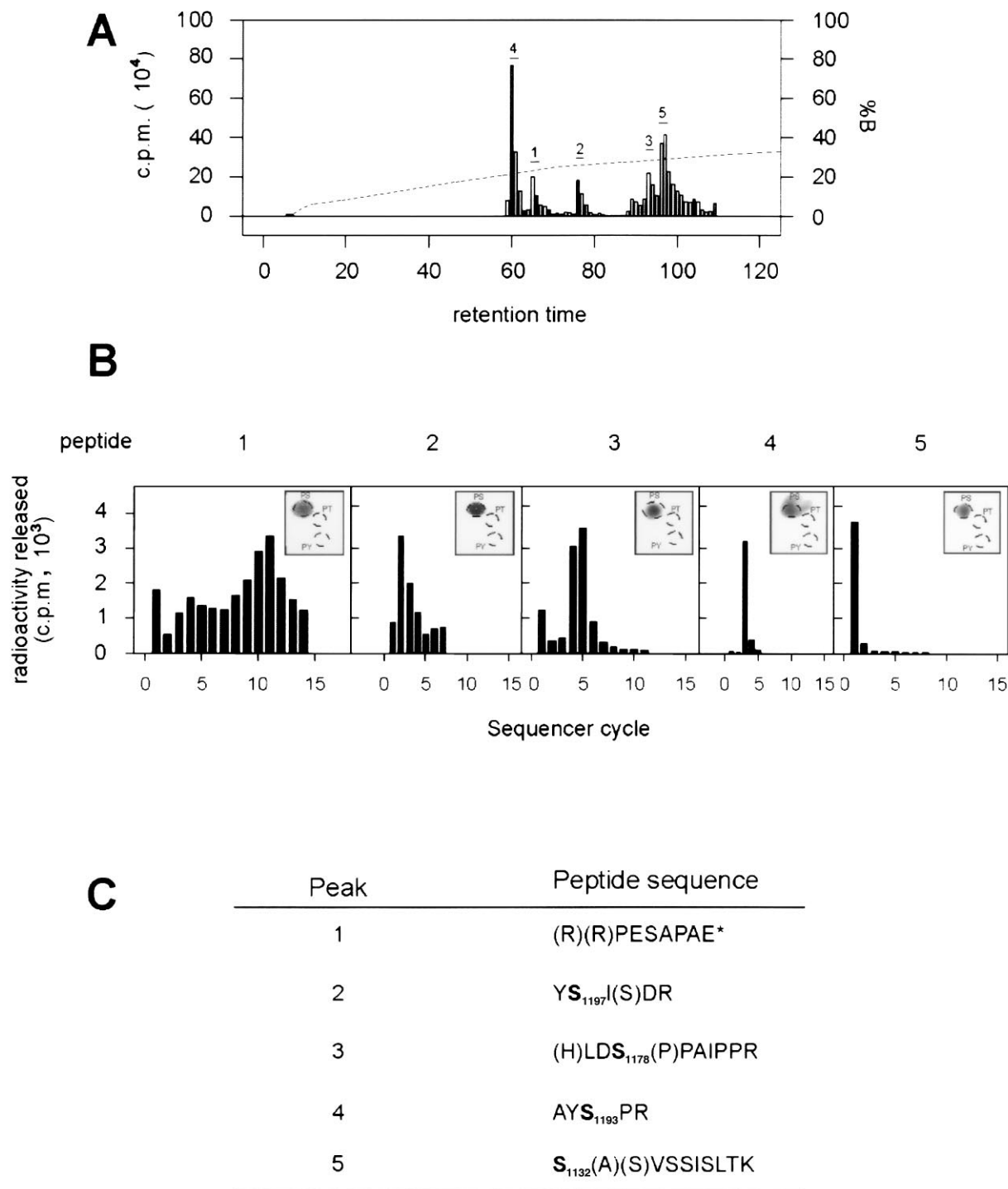


FIG. 2. Phosphopeptide purification and identification of hSos1 phosphorylation sites. (A) GST-hSos1C was phosphorylated in vitro by MAP kinase in the presence of [ $\gamma$ - $^{32}$ P]ATP. Following purification by SDS-PAGE, transfer to PVDF membrane, and trypsin digestion, the resultant phosphopeptides were purified by reversed-phase HPLC on a C<sub>18</sub> column. The sample was applied in an aqueous solution of 0.055% (vol/vol) TFA, and the column was washed with the same buffer. Peptides were then eluted with acetonitrile, using the gradient shown (buffer B contains 0.05% TFA in a mixture of 75% acetonitrile–25% water). The radioactivity in each fraction was determined by Cerenkov counting, and the fractions containing the indicated phosphopeptides were pooled for further purification by TLC as described in Materials and Methods. (B) An aliquot of each partially purified phosphopeptide was covalently coupled to a sequencer sample filter and subjected to protein sequencer analysis. The  $^{32}$ P radioactivity released at each cycle is shown. The phosphoamino acid analysis for each peptide is shown in the insets. (C) The purified phosphopeptides were also subjected to direct protein sequencing. The amino acid identified in each cycle is indicated by the single-letter code. Positions at which unambiguous identification of the amino acid was not possible are in parentheses. Phosphoserine is indicated in boldface type. The asterisk indicates a nonterminated sequence in peptide 1.

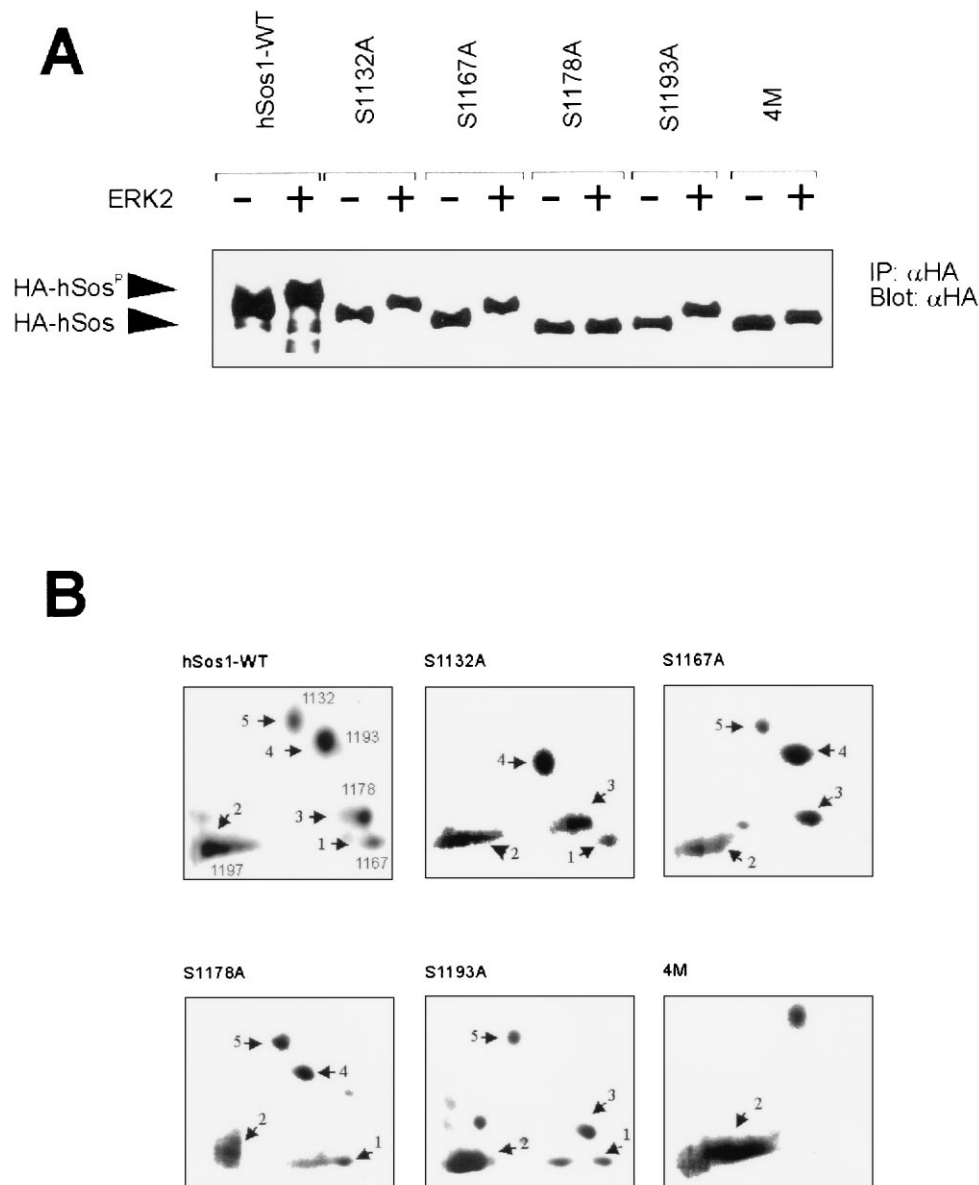


FIG. 3. Site-directed mutagenesis of the MAP kinase sites. (A) Expression of HA-hSos1 mutants lacking the MAP kinase phosphorylation sites. COS-1 cells were transfected with HA-hSos1 or derivatives containing alanine at the indicated residues, and the proteins were immunoprecipitated from unlabeled serum-starved cells and then phosphorylated with MAP kinase in vitro. The immunoprecipitates were separated on an SDS-6% polyacrylamide gel, transferred to nitrocellulose membrane, and analyzed by immunoblotting with anti-HA antibody. (B) Two-dimensional tryptic phosphopeptide maps of MAP kinase-phosphorylated wild-type HA-hSos1 or derivatives containing alanine at the indicated residues. Immunoprecipitates of HA-hSos1 derivatives were separated by SDS-PAGE, transferred to PVDF membrane, and digested with trypsin. Phosphopeptides were analyzed as described in Materials and Methods.

have demonstrated a role for MAP kinase in the phosphorylation of Sos in vivo (9, 11, 25, 28). In this study, we report the identification of the MAP kinase phosphorylation sites on hSos1. Using two-dimensional tryptic mapping of phosphopeptides generated from the hSos1 C-terminal region phosphorylated by purified MAP kinase in vitro in combination with phosphoamino acid analysis and sequence analysis of isolated peptides, we have identified five MAP kinase phosphorylation sites at S-1132, S-1167, S-1178, S-1193, and S-1197. Tryptic analyses of MAP kinase-phosphorylated hSos1 mutants in which the above serine sites were mutated to alanine confirmed the identity of these phosphoacceptor sites.

The ERK1 and ERK2 MAP kinases preferentially phos-

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1126 LPHGPR$ASVSSISLTGKTDEVVPPPPVPPRRRPESAPAESS$PSKIMSK 1174
1175 HLD$PPAIPPRQPTSKAYS$PRYSISDRTSISDPPES$PPLLPPREPVRTP 1223
1224 DVFSS$PLHLQPPPLGKKSDHGNAFFPN$PSFPTPPPPQTPSPHGTRRH 1272
1273 LPSPLTQ$EVDLH$SIAGPPVPPRQ$TSQH$IPKLPKTYKREH$THPSMHRD 1322
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FIG. 4. Sequence of the HA-hSos1 C-terminal region, illustrating the relative positions of the MAP kinase phosphorylation sites (S) and the proline-rich SH3-binding sites (grey boxes).

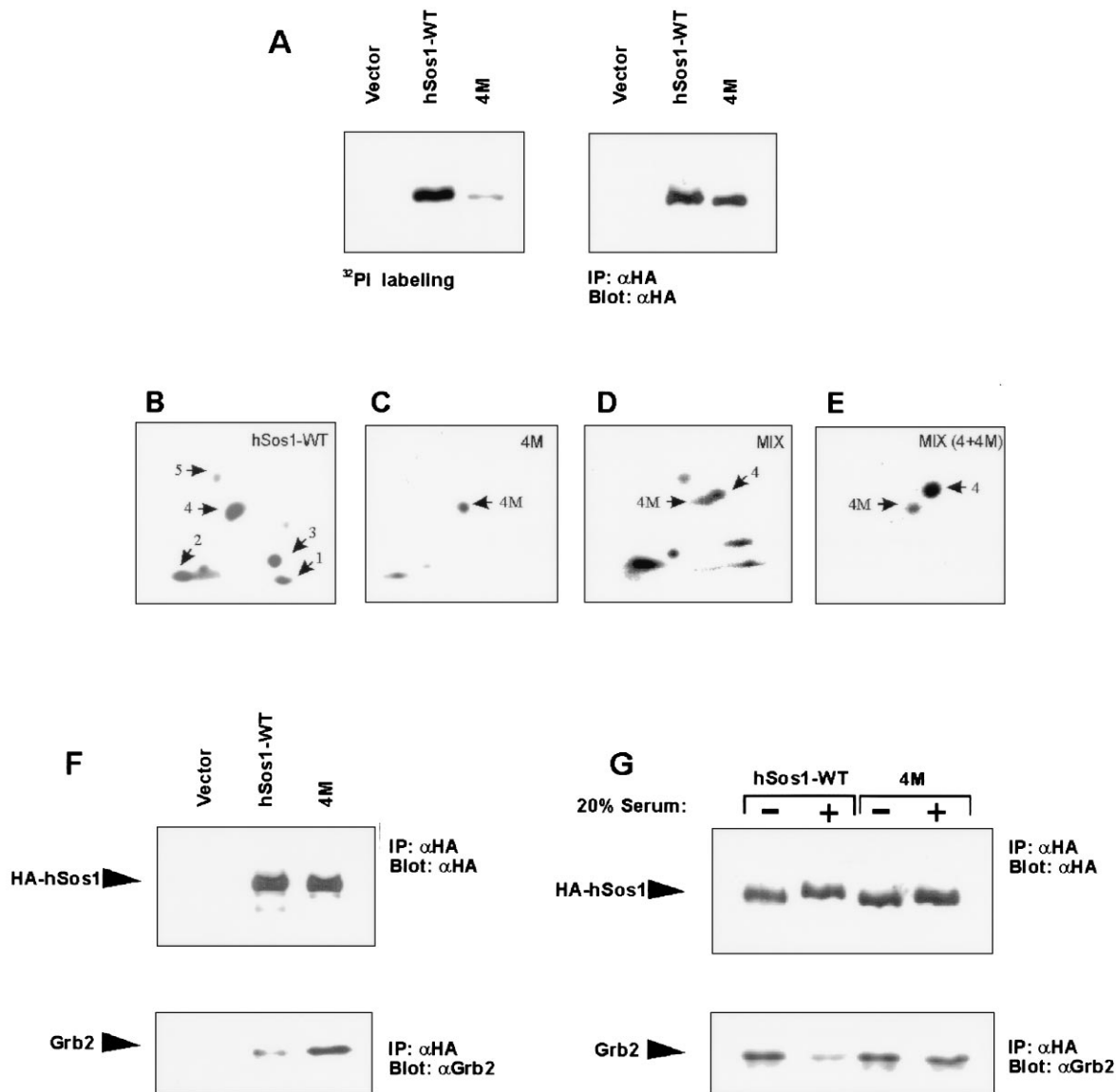


FIG. 5. Effects of MAP kinase phosphorylation on hSos1-Grb2 interaction. (A) COS-1 cells transiently expressing HA-hSos1-WT and HA-hSos1-4M were labeled with  $^{32}\text{P}$ , and stimulated with serum (20%). The immunoprecipitates were resolved on an SDS-6% polyacrylamide gel. Proteins were transferred onto nitrocellulose membrane and visualized by autoradiography (left panel) and by immunoblotting (right panel) with anti-HA antibody. (B and C) Two-dimensional phosphopeptide maps of HA-hSos1-WT (B) and HA-hSos1-4M (C). Phosphopeptide maps were produced as described in Materials and Methods. (D) Phosphopeptides obtained from HA-hSos1-WT and HA-hSos1-4M were mixed and analyzed together. (E) To confirm that phosphopeptide 4M derived from hSos1-4M and phosphopeptide 4 from hSos1-WT represent distinct sites, the corresponding spots were scraped from the thin-layer chromatography plates, eluted with pH 1.9 buffer, pooled, and rerun under the conditions used for previous maps. (F) COS-1 cells were transiently transfected with HA-hSos1-WT or HA-hSos1-4M and immunoprecipitated with anti-HA antibody. The immunoprecipitates were resolved on an SDS-12.5% polyacrylamide gel and immunoblotted with anti-HA antibody (upper panel) or anti-Grb2 antibody (lower panel). (G) COS-1 cells transfected with HA-hSos1-WT or HA-hSos1-4M were serum starved for 24 hours (lanes -) and then stimulated with 20% fetal calf serum for 15 min (lanes +). hSos1 and Grb2 immunoprecipitates were resolved on SDS-6% polyacrylamide and SDS-12.5% polyacrylamide gels, respectively. Immunoblotting analyses were carried out as described for panel F.

phorylate substrates containing PX(S/T)P motifs, where X represents a neutral or basic amino acid (12, 16). However, the first proline is not absolutely required (17). Of the five sites identified within the C-terminal domain of hSos1, three (S-1167, S-1193, and S-1178) are typical MAP kinase sites in that the phosphoacceptor serine is followed by proline. In contrast, the sequences surrounding the two other sites (GPRS-1132AS and PRYS-1197IS) are atypical for MAP kinase phosphorylation sites in that they do not contain proline at the P + 1 position. However, it has been shown that good substrates for

MAP kinase possess noncharged residues at P-3 (16, 17), a feature shared by both the S-1132 and S-1197 sites. In addition, both sites contain a neutral or basic residue at P-1. Thus, it is possible that within a certain sequence context, the P-1 and P-3 residues constitute the critical substrate recognition determinants for MAP kinase.

Comparisons between the tryptic phosphopeptide maps of in vivo-phosphorylated Sos and Sos phosphorylated in vitro by MAP kinase revealed that another Ser/Thr kinase(s) also phosphorylates Sos in vivo. Similar findings have been reported in

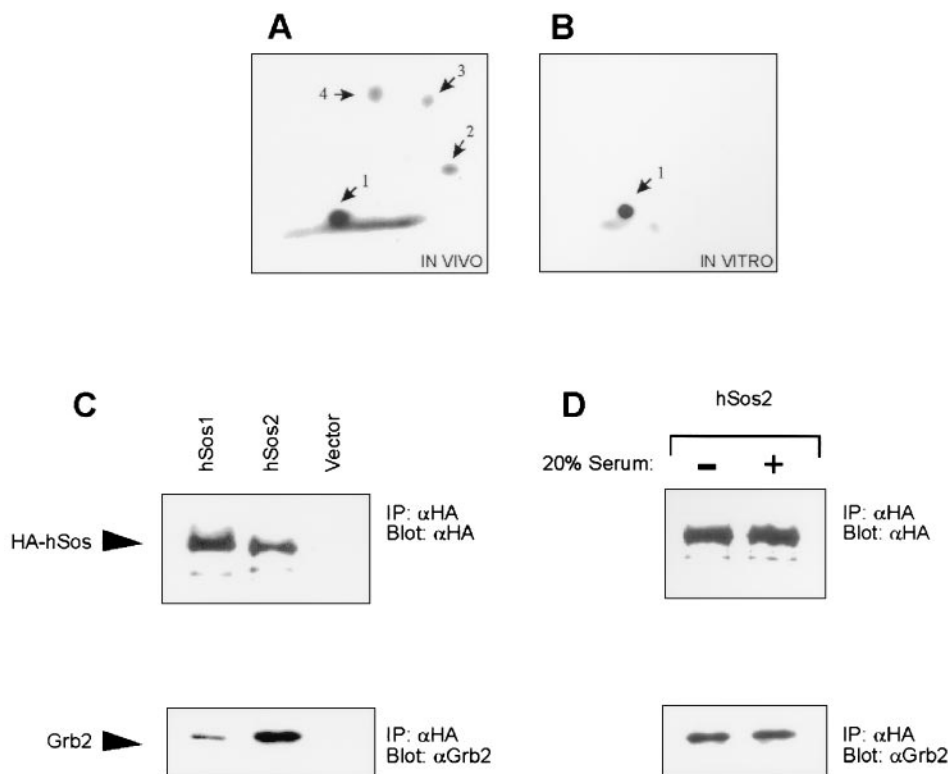


FIG. 6. Characterization of MAP kinase-mediated phosphorylation of hSos2. (A and B) Two-dimensional phosphopeptide maps of HA-hSos2 phosphorylated *in vivo* (A) and *in vitro* (B) by MAP kinase. COS-1 cells were transfected with HA-hSos2, and the transfected protein was immunoprecipitated. (A) an HA-hSos2 immunoprecipitate isolated from transfected COS-1 cells labeled with  $^{32}\text{P}_i$ . (B) An HA-hSos2 immunoprecipitate isolated from unlabeled serum-starved COS-1 cells and then phosphorylated with MAP kinase *in vitro*. (C) COS-1 cells transiently expressing HA-hSos1 or HA-hSos2 were immunoprecipitated with anti-HA antibody. The immunoprecipitates were resolved on an SDS-12.5% polyacrylamide gel and immunoblotted with anti-HA antibody (upper panel) or anti-Grb2 antibody (lower panel). (D) COS-1 cells transiently expressing HA-hSos2 were serum starved for 24 h (lane -) and then stimulated with 20% fetal calf serum for 15 min (lane +). The protein was immunoprecipitated with anti-HA antibody. The immunoprecipitates were analyzed as described for panel C.

earlier studies (11, 25). The identity of this kinase(s) is presently unknown. The C-terminal domain of hSos1 was not phosphorylated by two members of the MAP kinase family, JNK1 and p38 (14), suggesting that these kinases are not involved in the phosphorylation of Sos. In support of this conclusion are the recent observations that activation of the ERK pathway but not the JNK pathway is responsible for Sos phosphorylation (9). In addition, the finding that insulin-stimulated Sos phosphorylation in CHO cells persists under conditions where insulin-induced MAP kinase activation is blocked (18) further indicates the potential involvement of an additional kinase(s) in Sos phosphorylation.

We have previously shown that phosphorylation of the C-terminal domain of hSos1 by MAP kinase *in vitro* reduces its apparent affinity for Grb2 (13). Consistent with this observation, we demonstrate in the present study that conversion of the C-terminal MAP kinase sites in hSos1 to alanine results in increased affinity of hSos1 for Grb2 *in vivo*. Together, these data indicate that phosphorylation at the MAP kinase sites decreases the binding affinity of hSos1 toward Grb2. The molecular basis underlying this effect is not clear. Since the phosphorylation sites are clustered within a small region of the C-terminal domain of hSos1 which contains the first three proline-rich SH3-binding motifs, it is possible that phosphorylation alters the structure of the interacting surfaces critical for SH3 binding. Alternatively, phosphorylation may regulate the ability of the C-terminal region of Sos to interact with other proteins, which in turn might influence the interaction with

Grb2. The identification of the phosphorylation sites should provide a means for analyzing the structural basis for the phosphorylation-dependent regulation of Sos-Grb2 interactions.

Previous studies suggest that the C-terminal domain of Sos has at least two regulatory functions: (i) to mediate the ligand-dependent membrane targeting of Sos through the interaction with Grb2 and Shc adaptor molecules, and (ii) to exert an autoinhibitory effect on Sos activity which presumably can be alleviated by the binding of Grb2 (1, 34). By virtue of its effect on Grb2 binding, the MAP kinase-mediated phosphorylation of Sos could, in principle, modify either one of these functions. However, it is becoming increasingly evident that Sos activity is regulated by additional factors. For example, it has been shown that the Dbl and Pleckstrin domains located in the amino-terminal region of Sos play an important role in controlling Sos activation (19, 24, 34). In addition, we have demonstrated that Sos1 and Sos2 display differential interactions with Grb2 (38), and, as suggested by the present data, this might reflect the difference between the phosphorylation pattern of the two proteins. Thus, the physiological consequences of Sos phosphorylation with respect to receptor-mediated activation of Ras are likely to reflect the relative contributions of multiple regulatory determinants. This might explain why our studies indicate that phosphorylation of Sos is not a prerequisite for the down regulation of receptor-mediated Ras activation in rat L6 cells (13), whereas others have reported that the phosphorylation-dependent dissociation of Sos from Grb2 in mouse 3T3-L1 adipocytes may play an important role in insulin-in-



duced desensitization of Ras. Further characterization of the structural organization and biochemical activity of Sos domains will be necessary to establish how the activity of this critical signaling molecule is regulated.

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