Phosphorylation of the Ras Nucleotide Exchange Factor Son of Sevenless by Mitogen-activated Protein Kinase*

(Received for publication, November 4, 1993, and in revised form, December 3, 1993)

Andrew D. Cherniack§, Jes K. Klarlund‡, and Michael P. Czech

From the Program in Molecular Medicine and the Department of Biochemistry and Molecular Biology, University of Massachusetts Medical Center, Worcester, Massachusetts 01605

Son of sevenless-1 and -2 (Sos-1 and -2) are guanosine nucleotide exchange factors implicated in the activation of Ras by both the insulin and epidermal growth factor signal transduction pathways. Ras appears to function by initiating the nucleotide exchange of cellular protein kinases including mitogen-activated protein (MAP) kinases. Sos proteins contain numerous sequences in their carboxy-terminal regions which correspond to consensus sites for MAP kinase phosphorylation. To examine whether these sites are substrates for MAP kinases, the cDNA encoding Drosophila Sos (dSos) was tagged with sequences encoding the major antigenic epitope of the influenza virus hemagglutinin (HA) to create a dSosHA fusion construct. dSosHA was transiently expressed in COS-1 cells and immunoprecipitated with anti-HA antibodies. When immune complexes were incubated with purified MAP kinase and [γ-32P]ATP, a phosphorylated band of 180 kDa was observed when analyzed by SDS-polyacrylamide gel electrophoresis. This band was not present in immunoprecipitations from cells transfected with vector alone. No phosphorylation of the 180 kDa band was seen when immunoprecipitates were incubated with [γ-32P]ATP in the absence of MAP kinase. Two dimensional analysis of tryptic peptides from dSosHA phosphorylated by MAP kinase in vitro revealed two major phosphorylated species that were also found in dSosHA isolated from COS-1 cells labeled with [32P]. These results are consistent with the hypothesis that a feedback loop exists wherein growth factor-activated MAP kinases phosphorylate and regulate Sos proteins.

The Ras protein is believed to play a key role in signal transduction pathways initiated by a number of tyrosine kinase receptors such as the receptors for insulin, epidermal growth factor (EGF),* and nerve growth factor (Gibbs et al., 1990; Satoh et al., 1990; Burginger et al., 1991; Medema et al., 1991; Thomas et al., 1992; Wood et al., 1992). Stimulation of the tyrosine kinase activities of the receptors results in the rapid release of Ras-bound guanosine diphosphate, which is catalyzed by a class of proteins, the guanosine nucleotide exchange factors. Formation of biologically active Ras occurs by the subsequent binding of GTP. The first guanosine nucleotide exchange factor identified was the Saccharomyces cerevisiae CDC25 protein (Broek et al., 1987). In higher eukaryotes, two different types of proteins with homology to CDC25 have been found. One includes the p140 Ras GRF-like proteins, which in human, rat, and mouse are found primarily if not exclusively in brain tissue (Martegani et al., 1992; Shou et al., 1992; Wei et al., 1992). These exchange factors have not yet been implicated in any tyrosine kinase receptor signaling pathway. The second group includes the Son of sevenless proteins (Sos). Sos was first identified in Drosophila melangaster, in which genetic studies showed that Sos is downstream of both the Sevenless and EGF receptor tyrosine kinases (Rogge et al., 1991). One human and two murine homologues of Sos have been cloned (Bowtell et al., 1992; Chardin et al., 1993), and unlike the GRF exchange factor, Sos is ubiquitously expressed in mouse and human tissues.

A great deal of recent data have implicated Sos as part of the EGF receptor signaling complex (Buday and Downward, 1993; Egan et al., 1993; Rozakis-Adcock et al., 1993; Li et al., 1993; Gale et al., 1993). Stimulation of the EGF receptor tyrosine kinase results in the formation of a complex between EGF receptor, Sos, and the adapter protein Grb2. The Grb2 protein contains an SH2 domain, which is believed to bind to the EGF receptor autophosphorylation site Y1068, flanked by two SH3 domains, which bind to proline-rich sequences at the COOH terminus of Sos (Rozakis-Adcock et al., 1993; Li et al., 1993). The formation of this complex does not measurably increase the guanosine nucleotide exchange activity that Sos has for Ras in vitro (Buday and Downward, 1993). However, EGF stimulation results in the translocation of Sos from cytoplasmic to particulate fractions. This suggests that EGF stimulation results in a change in the intracellular location of Sos, bringing it in contact with membrane-associated Ras (Buday and Downward, 1993). In contrast to EGF signaling, activation of the insulin receptor tyrosine kinase results in association of Sos-GRB2 complexes with tyrosine-phosphorylated IRS-1 (insulin receptor substrate-1) and Shc (Baltensperger et al., 1993; Skolnik et al., 1993). Thus in the insulin signaling pathway, Sos guanosine nucleotide exchange activity may be regulated through the translocation of IRS-1 and Shc proteins.

We have recently shown that COS-1 cells cotransfected with Drosophila Sos (dSos) cDNA and human H-Ras cDNA contain 10 times the amount of GTP-bound Ras than cells transfected with H-Ras alone. Furthermore, dSos binds to IRS-1 only when an active insulin receptor tyrosine kinase is present (Baltensperger et al., 1993). The dSos cDNA was also found to transform Rat-1 cells (Egan et al., 1993). Taken together these results indicate that dSos protein is functional in mammalian cells.

Phosphoamino acid analysis showed that the dSos protein is phosphorylated on serine and threonine but not tyrosine residues in COS-1 cells (Baltensperger et al., 1993). This indicates that Sos is not a substrate of the insulin receptor itself, but it may be a substrate of serine-threonine kinases downstream of Ras. One group of serine-threonine kinases that are activated by tyrosine kinase signaling pathways through Ras are MAP

* This research was supported by Grant DK30648 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ The two first authors contributed equally to this work.

The abbreviations used are: EGF, epidermal growth factor; GRF, guanine nucleotide-releasing factor; IRS-1, insulin receptor substrate-1; MAP, mitogen-activated protein; PAGE, polyacrylamide gel electrophoresis; GDS, guanine nucleotide dissociation stimulation.
kineses (de Vries-Smits et al., 1992; Medema et al., 1991; Thomas et al., 1992; Wood et al., 1992). The consensus sequence for MAP kinase phosphorylation (Clark-Lewis et al., 1991; Gonzalez et al., 1991) exists seven to nine times in the COOH-terminal domains of all SOS proteins. This study addresses whether dSos is a substrate of MAP kinase. The results reported here demonstrate that dSos is in fact phosphorylated by MAP kinase in vitro and suggest this reaction also occurs in intact cells.

EXPERIMENTAL PROCEDURES

Construction of pCMV5-dSosHA—A cDNA clone of the D. melanogaster SOS gene (dSos) (Bonfini et al., 1992) in BlueScript was a gift of Utpal Banerjee. Sequences encoding the 9-amino acid peptide sequence of the major antigenic epitope of influenza virus hemagglutinin (YPYDVPDYA) were added to the 3' end of the dSos coding sequence by the polymerase chain reaction (Saiki et al., 1988). This was conducted with dSos digested with HindIII and the following two primers: 5'-TC- TAGAGACCTGATCAAGCTTGACATGATCCTGTTTCTACTTG and 5'-GAAGCAATGCTGCGTGCTGTCGTCG. A 196-base pair fragment was generated as described with BamHI and BglII. The resulting 130-base pair BglI-BamHI fragment was ligated to a 5.5-kilobase pair HindIII-BglI fragment of Sos, which contains the 5' end of the dSos gene, and to pCMV5 (Andersson et al., 1989) cut with HindIII and BamHI. The resulting construct is denoted pCMV5-dSosHA. To verify whether dSos is a substrate of MAP kinase, dSos was partially purified by immunoprecipitation and used as a substrate for purified MAP kinase in vitro. In order to immunoprecipitate dSos protein, construct PCMV5-dSosHA was engineered by fusing dSos cDNA to sequences of MAP kinase, dSos was partially purified by immunoprecipitation and used as a substrate for purified MAP kinase in vitro. In order to immunoprecipitate dSos protein, construct PCMV5-dSosHA was engineered by fusing dSos cDNA to sequences encoding the major influenza virus hemagglutinin antigenic epitope. This was subcloned into mammalian expression vector PCMV5 (kindly provided by Roger J. Davis) and 250 μCi of γ-32P-ATP (3000 Ci/mmol) for an additional 30 min at room temperature. The samples were then washed three times with 1 ml of Hepes buffer containing 10 mM nitrophenyl phosphate and 10 mM MgCl₂. The samples were then incubated with purified human p19 isoform of MAP kinase (kindly provided by Roger J. Davis) and 250 μCi of γ-32P-ATP (3000 Ci/mmol) for an additional 30 min at room temperature. To stop the reactions, the samples were washed once with 1 ml of Hepes buffer and SDS-PAGE sample buffer was added.

To test the efficacy of dephosphorylation, two control and two transfectant plates were labeled for 4 h with 2 ml of 32P and immunoprecipitated, and treated with phosphatase as described above.

RESULTS

The consensus sequence for phosphorylation by MAP kinase has been determined to be Pro-X,-Ser/Thr-Pro where X is a neutral or basic amino acid and n = 1 or 2 (Clark-Lewis et al., 1991; Gonzalez et al., 1991). There are seven potential MAP kinase phosphorylation sites in the primary sequence of the dSos protein. To test whether dSos protein is phosphorylated by MAP kinase, dSos was partially purified by immunoprecipitation and used as a substrate for purified MAP kinase in vitro. In order to immunoprecipitate dSos protein, construct PCMV5-dSosHA was engineered by fusing dSos cDNA to sequences encoding the major influenza virus hemagglutinin antigenic epitope. This was subcloned into mammalian expression vector PCMV5 (kindly provided by Roger J. Davis) to create construct PCMV5-dSosHA. COS-1 cells were transfected with PCMV5-dSosHA, and dSosHA protein was immunoprecipitated with an anti-HA monoclonal antibody. The immunoprecipitates were incubated with purified MAP kinase and γ-32P-ATP, and the reaction mixture was analyzed by SDS-PAGE. Fig. 1A shows a prominent 180-kDa band is labeled in this reaction. This band is not present in immunoprecipitates from cells transfected with PCMV5 alone, nor is it present in dSosHA immunoprecipitates incubated only with γ-32P-ATP. These data strongly suggest that the 180-kDa band phosphorylated by MAP kinase is dSosHA.

Since some sites in proteins isolated from cells may already be phosphorylated, the precipitated dSosHA was treated in some experiments with alkaline phosphatase prior to phosphorylation with MAP kinase. As seen in the last two lanes of Fig. 1A, treatment of immunoprecipitates with phosphatase prior to the kinase reaction resulted in an increased phosphorylation of dSosHA by MAP kinase. The efficacy of the phosphatase treatment was verified by examining its effect on dSosHA immunoprecipitated from cells that had been labeled with 32P (Fig. 1B). Phosphatase treatment completely removed the radioactivity that had been incorporated into dSosHA from intact 32P-labeled cells.

Phosphoamino acid analysis was conducted on the 180-kDa band phosphorylated by MAP kinase. As seen in Fig. 1C, MAP kinase phosphorylated dSosHA on both serines and threonines. However, treatment of dSosHA with phosphatase prior to in-
MAP-2 Kinase Phosphorylation of Ras Activator

FIG. 1. Phosphorylation by purified MAP kinase of isolated

dSosHA. A, COS-1 cells were transfected with PCMV5-dSosHA or with

the vector alone as indicated. The expressed protein was immuno-

precipitated, and the indicated samples were treated with calf intestine

alkaline phosphatase. After a 30-min incubation, the phosphatase

was washed from the beads and dSosHA was phosphorylated by MAP kinase

in the presence of nitrophenyl phosphate, a phosphatase inhibitor. After

a 30-min incubation, the beads were washed once and the bound
dSosHA was analyzed by SDS-PAGE and subsequent autoradiography.

B, PCMV5-dSosHA transfected in COS-1 cells was labeled in intact cells

with 32P, and immunoprecipitated. In the indicated samples, the pre-

cipitates were treated with alkaline phosphatase. C, dSosHA was par-

tially hydrolyzed with HCl and analyzed by thin-layer electrophoresis

at pH 3.5 as described under "Experimental Procedures." The positions

of the non-radioactive phosphoamino acids are indicated.

cubation with MAP kinase slightly increased the overall amount of serine phosphorylation incorporated into dSosHA.

To further characterize dSosHA phosphorylation sites, the 180-kDa protein phosphorylated by MAP kinase in immuno-

precipitats was isolated and digested with trypsin. Tryptic peptides were first separated by electrophoresis and then by

chromatography in the second dimension. Two-dimensional maps of tryptic peptides isolated from dSosHA with and without treatment of phosphatase prior phosphorylation are shown in Fig. 2 (A and B). Comparison of peptides derived from both conditions revealed several species appearing at identical positions (Fig. 2, A and B). However, some differences in the pattern of peptides are evident; in particular, peptide 1 (Fig. 2B) was absent in tryptic maps prepared from dSosHA that were not treated with phosphatase (Fig. 2A). This species probably represents a site that is completely phosphorylated in intact cells, and so no further phosphorylation can occur when incubated with MAP kinase. The identities of species were confirmed in mixing experiments in which peptides obtained from phosphatase treated and untreated dSosHA were applied to the same plate and analyzed by two-dimensional separation (data not shown).

The phosphorylation pattern of dSosHA in intact cells was also examined by labeling PCMV5-dSosHA-transfected COS-1 cells with 32P, and isolating dSosHA by immunoprecipitation. Tryptic maps exhibited four major phosphorylated species and several minor species. Two of the major species (peptides 1 and 2 in Fig. 2C) appeared at positions similar to two phosphopeptides that were seen after MAP kinase phosphorylation of phosphatase-treated dSosHA. To further substantiate this, equal amounts of radioactivity from phosphopeptides obtained from both in vitro and intact cell labelings were mixed and spotted on a thin-layer plate. As seen in Fig. 2D, this analysis revealed that the migrations of peptides 1 and 2 obtained by both sources are identical, which strongly suggests that they represent the same phosphorylation sites.

The phosphoamino acid content of the more prominent phosphopeptide species from 32P-labeled dSosHA was determined. Phosphopeptides were eluted from thin-layer plates, partially hydrolyzed with HCl, and analyzed by thin-layer electrophoresis. As illustrated in Fig. 3A, peptides 1 and 2, derived from either dSosHA labeled in intact cells or phosphorylated with

FIG. 2. Tryptic phosphopeptide map of dSosHA. A and B, COS-1 cells were transfected with PCMV5-dSosHA and the expressed protein was immunoprecipitated. In B, the precipitated dSosHA was pretreated with alkaline phosphatase, and in both A and B, immunoprecipitates were then phosphorylated with MAP kinase. Immunoprecipitates were separated by SDS-PAGE, and dSosHA was treated with trypsin overnight. The released peptides were separated by electrophoresis (horizontal) and chromatography (vertical), and the plates were subsequently subjected to autoradiography. The sites of application are indicated by the crosses. C, transfected COS-1 cells were labeled with 32P, for 4 h, and the labeled dSosHA was immunoprecipitated and analyzed by tryptic mapping. D, 982 cpm of the material shown in B and 1041 cpm of the material shown in C were mixed and separated as before. The two species (indicated by 1 and 2) exhibit identical migration whether they are derived from dSos labeled in intact cells or from dSos phosphorylated by MAP kinase in immunoprecipitates.

MAP kinase in immunoprecipitates, contained phosphoserine but no phosphothreonine. The slower migrating labeled species represent partial hydrolysis products. As expected, this pattern generally differs for each phosphorylation site in a given protein (Cooper et al., 1983; Martensen, 1984). Interestingly, the patterns of partial hydrolysis products determined for species 1 from both methods of labeling are identical. This is also true for species 2. These data confirm that phosphopeptide species 1 and 2 derived from dSosHA labeled in intact cells represent the same sites that are phosphorylated by purified MAP kinase in dSosHA immunoprecipitates.

One prominent labeled phosphopeptide species labeled in immunoprecipitates contained only threonine (Fig. 3, B and D). Since only one consensus site for MAP kinase phosphorylation in dSosHA contains threonine (Thr-1481), it is likely that this species represents that site. This species did not appear in material derived from dSosHA labeled in intact cells, and its labeling did not require dephosphorylation prior to phosphorylation by MAP kinase. This site, therefore, does not seem to be phosphorylated in intact cells. A summary of the phosphoamino acid content of these and other labeled peptides is given in Fig. 3B.
DISCUSSION

The Son of sevenless proteins are believed to be involved in the activation of Ras by tyrosine kinase receptors (Baltensperger et al., 1993; Buday and Downward, 1993; Li et al., 1993; Rozakis-Adcock et al., 1993; Skolnik et al., 1993). In this communication, we have shown that the Drosophila Son of sevenless protein is a substrate for MAP kinase in vitro. Some of the same sites that are phosphorylated by MAP kinase in vitro are apparently also phosphorylated in intact cells. This is unambiguously demonstrated for phosphopeptide 1 (Fig. 2) because phosphorylation of this peptide in vitro only occurs after dephosphorylation of its in vivo phosphorylated form. Son of sevenless has been shown to be an adapter protein in different cellular compartments (Benis, 1993; Davis, 1993). Proteins such as c-Myc (Seth et al., 1992), p90rsk (Sturgill et al., 1988), and threonine residues in response to EGF transfection of insulin receptor cDNA into COS-1 cells and subsequent activation of Ras by tyrosine kinase receptors (Baltensperger et al., 1993; Skolnik et al., 1993) have been reported to have high basal levels of GRF nor ralGDS contain long COOH-terminal proline-rich extensions which the potential MAP kinase sites are located.

Phosphorylation may also affect the association between Son and GRB2 or other unidentified proteins. In this case, phosphorylation may change the conformation of Son so that it changes its affinity for binding proteins. A change in phosphorylation state could then affect the intracellular location of Son, which would then alter its ability to activate Ras. A similar mechanism may explain the activation of Son. This phosphorylation occurs concomitantly with partial relocation of CDC 25 to the cytoplasm, which reduces its accessibility to membrane-bound Ras. In this case it has been suggested that a downstream element, cAMP-dependent protein kinase, down-regulates CDC 25 by phosphorylation (Gross et al., 1992). Further studies should determine whether MAP kinase effects Son regulation in a similar manner.

Acknowledgments—We thank Judy Kula for excellent assistance in preparing the manuscript and Dr. Utpal Banerjee for providing the dSoS cDNA and for helpful discussion.

REFERENCES

Albright, C. E., Giddings, B. W., Liu, J., Vito, M., and Weinberg, R. A. (1990) EMBO J. 9, 1233-1247
Anderson, N. G., Li, P., Marsden, L. A., Williams, N., Robert, T. M., and Sturgill, T. W. (1991) Biochem. J. 277, 573-579
Andersson, S., Davis, D. N., Dahlback, H., Jorovav, H., and Russell, D. W. (1989) J. Biol. Chem. 264, 9222-9229
Baltensperger, K., Kozma, L. M., Cherniack, A. D., Klarlund, J. E., Chawla, A., and Czech, M. P. (1993) Science 260, 1801-1802
Blenis, J. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5889-5892
Bonfini, L., Kariovich, C. A., Dasgupta, C., and Banerjee, U. (1992) Science 255, 6034-6038
Bowtell, D., Fu, P., Simon, M., and Senier, P. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6511-6515
Broek, D., Toda, T., Michaeli, T., Levin, R. M., Cobb, M., and SeRon, B. M. (1991) J. Biol. Chem. 266, 15180-15184
Cooper, J. K., Sefton, B. M., and Hunter, T. (1983) Methods Enzymol. 99, 387-402
Davis, R. J. (1993) J. Biol. Chem. 268, 14553-14555
de Vries-Smith, A. M. M., Burgering, B. M. Th., Leesons, S. J., Marshall, C. J., and Bos, J. L. (1992) Nature 357, 55-64
Egan, S. E., Giddings, B. W., Brooks, M. W., Suday, S., Sizeland, A. M., and Weinberg, R. A. (1990) Nature 343, 45-51
Gale, N. W., Kaplan, S., Lowenstein, E. J., Schlesinger, J., and Banerji, D. S. (1990) Nature 343, 88-92
Gartner, A., Nasmyth, K., and Ammerman, G. (1992) Genes Dev. 6, 1200-1202
Gibbs, J. B., Marshall, M. S., Sculnick, E. M., Dox, R. F., and Vogel, V. S. (1990) J. Biol. Chem. 265, 20437-20442
Gonzalez, F. A., Raden, D. L., and Davis, R. J. (1991) J. Biol. Chem. 266, 22159-22163
Gross, E., Goldberg, D., and Levitzki, A. (1992) Nature 356, 765-767
Hunter, T., and Sefton, B. M. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 1311-1315
Karns, I., and Sefton, B. M. (1980) J. Biol. Chem. 255, 5778-5787
Lee, R. M., Cobb, M. H., and Blackshear, P. J. (1992) J. Biol. Chem. 267, 1088-1092
Li, N., Batzer, A., Daly, R., Yajnik, V., Skolnik, E., Chardin, P., Bar-Sagi, D., and Mitchison, T. (1992a) Nature 353, 85-88
Li, N., Wartmann, M., Lin, A. Y., Knopf, L. H., and Davis, R. J. (1993) Cell 72, 269-278
Magari, N., Vannini, M., Ziggeli, R., Cecotti, P., Brambilla, R., Ferrari, C., Stocchi, E., and Alberghina, L. (1992) EMBO J. 11, 2151-2157
Martensson, T. D. (1984) Methods Enzymol. 107, 5-25
Matsuda, S., Gotah, Y., and Nishida, E. (1993) J. Biol. Chem. 268, 3277-3281
Matsuda, R. H., Wulbeita, R., and Sizeland, A. M. (1991) Mol. Cell. Biol. 11, 5963-5976
Northwood, I. C., Gonzalez, F. A., Wartmann, M., Raden, D. L., and Davis, R. J. (1990) J. Biol. Chem. 265, 15296-15300
Rogge, D. R., Karovich, C. A., and Banerjee, U. (1991) Cell 64, 39-48
Rozakis-Adcock, M., Forse, R., Wade, J., Pawson, T., and Bowtell, D. (1993) Nature 363, 83-85
Sakai, R. K., Gelfand, D. H., Stooff, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. (1988) Science 239, 497-491
Sanger, F., Nocken, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463-5467
Sato, T., Endo, M., Nakafuku, M., Akiyama, T., Yamamoto, T., and Katoz, Y. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 7926-7930
Seth, A., Gonzalez, F. A., Ozanne, D. L., and Davis, R. J. (1992) J. Biol. Chem. 267, 24796-24804
Shou, C., Farnsworth, C. L., Neel, B. G., and Pei, L. (1993) Nature 365, 351-354
Shibuki, E., Bhatia, A., Li, P., Lee, H., Lowenstein, E., Mohammadi, M., Margolis, B., and Schlessinger, J. (1993) Science 260, 1953-1955
Sturgill, T. W., Ray, L. B., Erikson, E., and Maller, J. L. (1988) Nature 334, 715-718
Takishima, K., Graywolf, Pratiner, I., Ingebritsen, T., and Rosen, M. R. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 2520-2524
Thomas, S. M., DeMarco, M., D’Arcangelo, G., Lageousa, S., and Brugge, J. S. (1993) Cell 73, 691-701
Wei, W., Mestler, R. D., Sanyal, P., Gonzalez, F. E., McKinney, D., Dasgupta, C., Li, P., Liu, X., and Brock, D. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 7100-7104
Wilson, A. D., Niman, H. L., Loughead, A. M., Chernov, M. L., Connolly, M. L., and Lerner, R. A. (1984) Cell 37, 767-778
Wolffman, A., and Macara, I. G. (1990) Science 248, 67-69
Woot, K. W., Sarnecki, C., Roberts, T. M., and Blenis, J. (1992) Cell 68, 1041-1050

2 A. D. Chorniak and M. D. Czech, unpublished results.
3 J. K. Klarlund and M. P. Czech, unpublished results.