The Ras/Rac Guanine Nucleotide Exchange Factor Mammalian Son-of-sevenless Interacts with PACSIN 1/Syndapin I, a Regulator of Endocytosis and the Actin Cytoskeleton

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Mammalian Son-of-sevenless (mSos) functions as a guanine nucleotide exchange factor for Ras and Rac, thus regulating signaling to mitogen-activated protein kinases and actin dynamics. In the current study, we have identified a new mSos-binding protein of 50 kDa (p50) that interacts with the mSos1 proline-rich domain. Mass spectrometry analysis and immunodepletion studies reveal p50 as PACSIN 1/syndapin I, a Src homology 3 domain-containing protein functioning in endocytosis and regulation of actin dynamics. In addition to PACSIN 1, which is neuron-specific, mSos also interacts with PACSIN 2, which is expressed in neuronal and nonneuronal tissues. PACSIN 2 shows enhanced binding to the mSos proline-rich domain in pull-down assays from brain extracts as compared with lung extracts, suggesting a tissue-specific regulation of the interaction. Proline to leucine mutations within the Src homology 3 domains of PACSIN 1 and 2 abolish their binding to mSos, demonstrating the specificity of the interactions. In situ, PACSIN 1 and mSos1 are co-expressed in growth cones and actin-rich filopodia in hippocampal and dorsal root ganglion neurons, and the two proteins co-immunoprecipitate from brain extracts. Moreover, epidermal growth factor treatment of COS-7 cells causes co-localization of PACSIN 1 and mSos1 in actin-rich membrane ruffles, and their interaction is regulated through epidermal growth factor-stimulated mSos1 phosphorylation. These data suggest that PACSINS may function with mSos1 in regulation of actin dynamics.

Ras functions as a molecular switch in the transduction of a wide variety of growth and differentiation signals induced by extracellular ligands (1). Activation of Ras is mediated by several Ras-specific guanine nucleotide exchange factors (GEFs) that convert GDP-Ras into GTP-Ras (1). Prominent among these is mammalian Son-of-sevenless (mSos). mSos interacts through a C-terminal proline-rich domain (PRD) with the Src homology 3 (SH3) domains of Grb2, an adaptor protein that targets mSos to activated growth factor receptors (2–7). Additionally, mSos interacts through the PRD with the endocytic adaptor proteins amphiphysin II (8) and intersectin (9, 10). These interactions may function to target mSos to Ras activation on the endocytic pathway (11–13).

In addition to the PRD, mSos contains a CDC25 homology domain, encoding Ras GEF activity (3–5, 14), and a Dbl homology domain, endowed with GEF activity for Rac, a member of the Rho superfamily of GTases (15, 16). In fact, mSos is the prototype member of a family of bifunctional GEFs, including Ras-GRF1 and Ras GRF-2, having dual specificity for Ras and Rac (16). Through its Dbl homology domain, mSos binds directly to Rac (17). However, the GEF activity of mSos toward Rac appears to be unique relative to other Rho family GEFs in that it catalyzes guanine nucleotide exchange as part of a macromolecular complex with Eps8 and E3b1, two proteins functioning in growth factor signaling (18, 19). Rac activation has multiple effects in cells, the most prominent being alterations in the actin cytoskeleton leading to membrane ruffling and lamellipodia formation (20). Thus mSos, through its ability to activate Rac, is thought to play a functional role in growth factor-mediated regulation of actin dynamics (18, 21).

To screen for novel mSos binding partners, we performed overlay assays of brain extracts with fusion proteins encoding the PRD of mSos1. A major mSos1-binding protein of 50 kDa was detected and purified by affinity chromatography. Mass spectrometry analysis identified the protein as PACSIN 1/syndapin I. PACSIN 1 was originally identified based on its differential expression in intact and lesioned mouse brain (22), and syndapin I was independently identified through its SH3 domain-dependent interaction with dynamin 1 (23). We will

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1 The abbreviations used are: GEF, guanine nucleotide exchange factor; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propane-sulfonic acid; CT, C-terminal; EGF, epidermal growth factor; GST, glutathione S-transferase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; mSos, mammalian son-of-sevenless; NT, N-terminal; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PRD, proline-rich domain; SH, Src homology.
use the name PACSIN throughout to collectively refer to PACSIN and syndapin. Whereas PACSIN 1 is neuron-specific, its closely related homologue PACSIN 2 is expressed in brain and several nonneuronal tissues (24, 25). Interestingly, the PACSINs appear to be involved in regulation of endocytosis and the actin cytoskeleton. Through a C-terminal SH3 domain, the PACSIN isoforms interact with the endocytic regulatory enzymes dynamin 1 and dynamin 1, as well as with N-WASP, a stimulator of Arp2/3-mediated actin nucleation and assembly [23, 25, 26]. Overexpression of full-length PACSIN stimulates cortical actin assembly, leading to filopodia formation, and the PACSINs localize to sites of high actin turnover, such as filopodia and lamellipodia (25).

After identifying PACSIN 1 as a mSos1 binding partner, we confirmed the interaction in vitro and used co-immunoprecipitation analysis to demonstrate the interaction in vivo. Interestingly, mSos1 co-distributes with PACSIN 1 in the growth cones and filopodia of cultured hippocampal neurons, and both proteins co-localize with actin in the filopodia from growth cones of dorsal root ganglia neurons in culture. Further, PACSIN 1 and mSos1 are co-localized in growth factor-induced membrane ruffles in COS-7 cells, and their interaction is regulated by mSos1 phosphorylation. Together, these data provide further evidence for a role of mSos in regulation of the actin cytoskeleton.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Affinity-purified antibodies against PACSIN 2 (26) and amphiphysin 1 and II (27) were described previously. Polyclonal anti-serum 2704 against rat syndapin 1 was a generous gift of Dr. Regis Kelly (University of California, San Francisco) (23). Monoclonal antibodies against α-tubulin and FLAG epitope (Sigma), tetra-His epitope (Qiagen), dynamin 1 (Hudy-1) (Upstate Biotechnology Inc.), and a polyclonal antibody C23 against mSos1 (Santa Cruz Biotechnology) were obtained commercially.

**DNA Constructs and Recombinant Proteins**—His-tagged rat syndapin I (25) and Flag-tagged mouse Sos1 (28) in mammalian expression vectors were generous gifts of Dr. Regis Kelly (University of California, San Francisco) and Dr. Jeffrey Pessin (University of Iowa), respectively. A protein construct encoding the SH3 domain of rat syndapin I (residues 376–441) was generated by polymerase chain reaction with Vent (San Francisco) and Dr. Jeffrey Pessin (University of Iowa), respectively. pGEX-2T. GST fusion proteins encoding full-length mouse PACSIN 1 and mSos1 are co-localized in growth factor-induced membrane ruffles in COS-7 cells, and their interaction is regulated by mSos1 phosphorylation. Together, these data provide further evidence for a role of mSos in regulation of the actin cytoskeleton.
Figure 1. Overlay analysis of mSos1-binding proteins. A, proteins of cytosolic fractions from rat brain were separated by SDS-PAGE, transferred to nitrocellulose, and overlaid with GST fused to amino acids 1111–1228 (GST-NT) or amino acids 1223–1341 (GST-CT) of the PRD of mouse Sos1 or with GST alone. The migratory positions of three major bands that bind to GST-NT and that of the molecular weight standards are indicated on the right and left, respectively. B, a crude Triton X-100-soluble rat brain extract was incubated with GST, GST-NT, or GST-CT conjugated to glutathione-Sepharose beads. Proteins specifically bound to the beads (B) along with aliquots of the brain extract (starting material (SM)) and equal amounts of the unbound material (void (V)) were processed for Western blot with an antibody that recognizes both amphiphysin I (amph I) and amphiphysin II (amph II). The molecular masses of amphiphysin I and II are indicated on the left.

RESULTS AND DISCUSSION

We previously demonstrated that mSos interacts through its PRD with the endocytic protein intersectin, suggesting that intersectin may target mSos to Ras on the endocytic pathway (9, 10, 13). As the PRD of mSos contains multiple SH3 domain-binding consensus sites, we sought to identify additional mSos binding partners. Overlay of adult rat brain extracts with a GST fusion protein encoding the N-terminal half of the mouse Sos1 PRD (GST-NT) (amino acids 1111–1228) identified three proteins of 120 (p120), 90 (p90), and 50 (p50) kDa (Fig. 1A). None of the bands were detected with a GST fusion protein encoding the mouse Sos1 PRD C-terminal half (GST-CT) (amino acids 1223–1341) or with GST alone (Fig. 1A). Previously, Leprince et al. (8) identified amphiphysin II as a mSos1 binding partner. To determine whether p120 and p90 correspond to amphiphysin I (120 kDa) and amphiphysin II (90 kDa), respectively, we used GST-NT, GST-CT, or GST alone in pull-down assays with soluble rat brain extracts. Western blots of the pull-downs demonstrated that both amphiphysin I and II bind specifically to GST-NT (Fig. 1B), suggesting that they represent p120 and p90. Intersectin also bound selectively to GST-NT, whereas Grb2 bound equally well to GST-NT and GST-CT (data not shown). Surprisingly, neither Grb2 nor intersectin was detected on the overlay assays, possibly due to lower levels of expression in brain extracts than the amphiphysins or p50. The abundant SH3 domain-containing protein, endophilin 1, which is readily detected on overlays with the PRDs of synaptojanin 1 (33) and dynamin 1 (34), was not seen on the overlays with the PRD of mSos1, further demonstrating the specificity of the interactions detected.

To characterize p50, we performed overlays with GST-NT on tissue extracts. p50 was detected in brain but not in a variety of nonneuronal tissues (Fig. 2A). This is consistent with the distribution of mSos1 that is expressed at higher levels in brain than in other tissues (9). Within brain, subcellular fractionation revealed p50 in both soluble and particulate fractions (Fig. 2B). The greatest enrichment was seen in the second lysed supernatant fraction (Fig. 2B, L5), which contained soluble proteins generated from the lysis of crude synaptosomes. This distribution is similar to that previously described for mSos1 (9), as well as that of the presynaptically enriched endocytic regulatory enzymes dynamin 1 and synaptojanin 1 (29).

To identify p50, we used the mSos1 GST fusion proteins to affinity purify mSos1-binding proteins from a soluble rat brain extract. As determined by Coomassie Blue staining, a 50-kDa band that bound to GST-NT but not to GST-CT or to GST alone was the major affinity-selected protein (Fig. 3). Minor bands at 120, 90, and 70 kDa were also weakly detected. The 50-kDa band was excised from the gel and subjected to trypsin digestion, and the fragments were analyzed by matrix assisted laser desorption ionization mass spectrometry at the W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University. A ProFound search of the peptide masses provided a tentative identification for p50 as PACSIN 1/syndapin I. PACSIN 1 was identified based on its up-regulation during neuronal differentiation in mouse (22), whereas its rat orthologue syndapin I was identified through its SH3 domain-dependent interaction with dynamin 1 (23). PACSIN 1, which contains an SH3 domain at its C terminus, is a neuron-specific protein with a predicted molecular mass of 50 kDa, consistent with its identification as p50. To support this identification, we performed overlay assays with GST-NT or GST alone on cells transfected with a cDNA encoding His6-tagged full-length PACSIN 1. GST-NT specifically interacted with a protein species that perfectly co-migrated with PACSIN 1, as detected with an anti-His6 Western blot (Fig. 4A), demonstrating that
PACSIN 1 directly interacts with mSos1. The identity of p50 as PACSIN 1 was confirmed with the demonstration that immunodepletion of PACSIN 1 from brain extracts using an anti-PACSIN 1 antibody completely depletes p50, as determined by GST-NT overlay (Fig. 4B).

In addition to PACSIN 1, a second member of the PACSIN family, referred to as PACSIN 2, has been recently described (24, 25). In contrast to PACSIN 1, which is expressed exclusively in neurons, PACSIN 2 is expressed in brain and nonneuronal tissues (24, 25) (Fig. 5A). Because mSos1 is enriched in brain but is also expressed in nonneuronal tissues (9), we hypothesized that mSos1-PACSIN 2 interactions may occur both within and outside the nervous system. To explore this question, we performed pull-down assays from Triton X-100-solubilized extracts prepared from brain, as well as from lung, which expresses high level of PACSIN 2 (Fig. 5A) (26). As expected, we detected binding of PACSIN 2 from both tissues to the mSos1 PRD (Fig. 5B). In fact, PACSIN 2 is likely to represent the 70-kDa band that was weakly detectable on the Coomassie blue stained pull-downs from brain extracts using mSos1 GST-NT (Fig. 5). Surprisingly, the level of PACSIN 2 recovered on the mSos1-PRD fusion protein was consistently greater when using brain versus lung extracts, even though PACSIN 2 was more abundant in the extracts from lung (Fig. 5, A and B). Moreover, a second, slightly smaller band that reacted with the PACSIN 2 antibody was detected in the GST-NT pull-downs from brain extracts but not from lung extracts (Fig. 5B). This protein may represent the short splice variant of PACSIN 2 previously described in rat tissues (25).

The differential binding of the long form of PACSIN 2 to the mSos1 PRD in brain compared with lung reveals a tissue-specific regulation of the interaction. The reason for this observation is currently unknown. However, it is possible that a tissue-specific posttranslational modification of PACSIN 2 alters its affinity for the mSos PRD.

To address whether the observed interactions are dependent on the classical SH3 domain binding interface, we generated point mutations in the SH3 domains of PACSIN 1 and 2 that converted proline to leucine (P434L for PACSIN 1; P478L for PACSIN 2). Comparable mutations in the Caenorhabditis elegans Grb2 homologue sem-5 cause a lethal phenotype by preventing sem-5 interactions with its PRD-containing binding partners (35). Using wild-type and mutated PACSIN 1 and 2 expressed as GST fusion proteins, we performed pull-down assays from brain extracts (Fig. 5C). mSos1 was found to interact with both wild-type fusion proteins, whereas the proline to leucine mutations abolished mSos1 binding to both PACSINs, demonstrating that the interactions are specifically mediated through the PACSIN SH3 domains.

To explore the potential interaction between PACSIN 1 and mSos1 in situ, we performed co-immunoprecipitation experiments from rat brain extracts. Immunoprecipitation of PACSIN 1 led to co-immunoprecipitation of mSos1 (Fig. 6). The interaction was specific, as no mSos1 precipitated in the presence of normal rabbit serum, and the abundant brain protein tubulin was not detected in the anti-PACSIN 1 immunoprecipitates (Fig. 6). Only a limited percentage of the total mSos1 in the brain extract co-immunoprecipitated with PACSIN 1. This is not surprising given that PACSIN 1 interacts through its SH3 domain with multiple binding partners, including the abundant brain proteins dynamin 1 and synaptojanin 1 (23).
fact, dynamin 1 was found to strongly co-immunoprecipitate with PACSIN 1 (Fig. 6). As the interactions between PACSIN 1 and its various SH3 domain-binding partners are likely to be competitive, the PACSIN-mSos interaction may be restricted to specific subcellular domains that are enriched for mSos1 relative to other PACSIN binding partners. Alternatively, the PACSINs may be at the core of large protein complexes in which they simultaneously interact with multiple binding partners. Consistent with the later possibility, it has been recently demonstrated that the PACSINs can self-associate to form homo- and hetero-oligomers (26).

To further examine the potential for interactions between PACSIN 1 and mSos1 in situ, we sought to determine whether the proteins were co-distributed in neurons. Immunofluorescence analysis of hippocampal neurons at 2 days in vitro with polyclonal antibodies against each protein revealed strong staining in the neuronal cell bodies, with fluorescent punctae observed along the length of the neurites and in growth cones (Fig. 7, A and C). Staining for both proteins extended into filopodia emanating from the growth cones (Fig. 7, B and D). To examine the localization within growth cones in more detail, we performed immunofluorescence analysis of primary rat dorsal root ganglia neurons maintained in culture for 1 day. Similar to hippocampal neurons, both PACSIN 1 and mSos1 were detected in dorsal root ganglia growth cones and were seen to extend into filopodia (Fig. 8). Interestingly, co-staining with phalloidin, which reveals filamentous actin, demonstrated that both mSos1 (Fig. 8A) and PACSIN 1 (C and D) were strongly co-localized with actin filaments at the plasma membrane and throughout the length of the filopodia. PACSIN has been demonstrated to localize at sites of high actin turnover, including filopodia and filopodial tips in nonneuronal cells (25). Further, overexpression of full-length PACSIN causes filopodia formation in an N-WASP-dependent manner, although the mechanism of this activation is unknown (25). The co-localization of mSos1 with PACSIN 1 in filopodia suggests that mSos1 may cooperate with PACSIN 1 in filopodia formation or function. This role may be particularly relevant during neuronal development, as actin-dependent filopodial dynamics are critical in the response of neuronal growth cones to extracellular guidance cues (26). Filopodia formation is dependent on N-WASP (37), which is activated by interactions with SH3 domains (38), as well as by binding to phosphatidylinositol bisphosphate (39).
Thus, PACSIN may cause filopodia formation via SH3 domain-dependent stimulation of N-WASP. As activated Rac binds to phosphatidylinositol 4-phosphate 5-kinase, leading to phosphatidylinositol (4,5)bisphosphate production (41, 42), mSos could contribute to N-WASP stimulation via activation of Rac (17–19, 21).

We next investigated the possibility of direct co-localization of PACSIN 1 and mSos1 in actin-rich structures. Fibroblasts are well established to form membrane ruffles upon treatment with growth factors (43, 44). We therefore co-transfected COS-7 cells with FLAG-tagged mSos1 and His6-tagged PACSIN 1 and examined their distribution before and after EGF treatment. Interestingly, both PACSIN 1 and mSos1 relocalized from a predominantly cytoplasmic distribution to become concentrated and co-localized at membrane ruffles following EGF treatment (Fig. 9A). Co-staining of PACSIN 1 transfected cells with anti-PACSIN 1 antibody and fluorescent phalloidin confirmed that the structures at which PACSIN 1 and mSos1 were co-localized were actin-rich membrane ruffles (Fig. 9B). Growth factor-induced ruffle formation is mediated by Ras-dependent Rac activation (43). Recent data suggest that mSos plays an important dual role in coupling Ras to Rac (18). Through its CDC25 homology domain, mSos activates Ras, which in turn activates phosphatidylinositol 3-phosphate kinase (45). The products of phosphatidylinositol 3-phosphate kinase catalytic activity stimulate the GEF activity of mSos toward Rac, causing Rac activation (17, 18). Indeed, mSos has been demonstrated to function directly in growth factor-induced membrane ruffle formation (18, 21). Our observation that full-length mSos1 targets to sites of Rac activation in response to EGF stimulation is consistent with its role in Rac-dependent actin reorganization. The co-localization of PACSIN 1 with mSos1 suggests that through direct protein interactions, PACSIN may regulate mSos function in ruffle formation.

**FIG. 8.** Co-localization of mSos1 and PACSIN 1 with actin in growth cones and filopodia of dorsal root ganglia neurons in culture. Growth cones of E16 dorsal root ganglia neurons from an explant culture stained with an antibody against mSos1 (A) or PACSIN 1 (B) and co-stained with phalloidin-Alexa 488 (phalloidin) to demonstrate F-actin. Arrows point to filopodia demonstrating co-localization of mSos1 or PACSIN with actin. Scale bar, 5 μm in low power (top three panels of A and B) and 2 μm in high power (bottom three panels in A and B).

**FIG. 9.** EGF treatment stimulates PACSIN 1/mSos1 co-localization in membrane ruffles. COS-7 cells, co-transfected with His6-tagged PACSIN 1 and FLAG-tagged mSos1 (A) or transfected with PACSIN 1 alone (B), were serum-starved and left untreated (−EGF) or were treated with 100 ng/ml EGF for 2 min (+EGF). PACSIN 1 was detected with an anti-PACSIN 1 rabbit antibody, and mSos1 was detected with anti-FLAG monoclonal antibody. F-actin was detected using phalloidin-tetramethylrhodamine isothiocyanate (phalloidin). Magnification, ×630.

**FIG. 10.** Regulation of the PACSIN 1-mSos1 interaction by phosphorylation. Serum-starved COS-7 cells were treated with 100 ng/ml of EGF for 5 min at 37 °C (EGF +). When indicated, prior to the EGF treatment, cells were preincubated with PD-098059 (PD +). Lysates from cells were incubated with GST-PACSIN 1 SH3 coupled to glutathione-Sepharose beads, and material specifically bound to the beads was processed for SDS-PAGE, along with an aliquot of cell lysate (starting material (SM)). The migratory position of mSos1 and the upwardly shifted phosphorylated mSos1 (phospho-mSos1) and that of GST-PACSIN 1 SH3 domain are indicated on the right.
formation. In fact, PACSIN 1 interacts with mSos1 within the same region that mediates mSos1 interactions with Eps8 and E3b1 (18). The binding of these proteins to mSos stimulates mSos GEF activity toward Rac (18). Thus, PACSIN interactions with mSos may play a comparable modulatory role.

We next sought to determine whether the interaction between PACSIN 1 and mSos1 was subject to regulation in response to growth factor stimulation. Several laboratories have demonstrated that MEK-dependent feedback phosphorylation of mSos leads to Ras desensitization by inducing the dissociation of mSos from Grb2 and Shc (10, 28, 46–49). We thus examined mSos1-PACSIN 1 interactions following MEK-induced mSos1 phosphorylation. Treatment of COS-7 cells with EGF led to a small but highly reproducible upward shift in mSos1 mobility (Fig. 10). This shift is characteristic of MEK-induced mSos1 phosphorylation (28, 49) and was blocked by preincubation with the MEK inhibitor PD-098059 (Fig. 10). Interestingly, phosphorylated mSos1 showed less binding to the SH3 domain of PACSIN 1 than did nonphosphorylated mSos1 (Fig. 10; representative of three separate experiments). In contrast, the binding of N-WASP to the PACSIN 1 SH3 domain was identical under the various treatment conditions (data not shown). Thus, phosphorylation of mSos1 negatively regulates its interaction with PACSIN 1. Interactions between SH3 domain-containing proteins and their proline-rich binding partners are often regulated by phosphorylation. For example, phosphorylation of dynamin 1 and synaptojanin 1 reduces their interactions with Eps8 and synaptojanin 1. Therefore, it appears that phosphorylation of mSos regulates its interaction with PACSIN 1. Interactions between PACSIN 1 and mSos1 may play a comparable modulatory role. It occurs rapidly following EGF treatment of COS-7 cells (both the mSos-Grb2 complex is detectable only after several minutes of feedback phosphorylation of mSos and the dissociation of the mSos interaction.

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