High Affinity Binding of the Pleckstrin Homology Domain of
mSos1 to Phosphatidylinositol (4,5)-Bisphosphate

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mSos1 has been implicated in coupling mammalian tyrosine kinases to the Ras GTPase. Because activation of Ras induced by growth factor stimulation likely requires the localization of mSos1 to the plasma membrane, we have investigated the possibility that the PH domain of mSos1 might mediate an interaction of mSos1 with phospholipid membranes. A glutathione S-transferase fusion protein containing the pleckstrin homology (PH) domain of mSos1 bound specifically and tightly to phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) with a Kd of 1.8 ± 0.4 μM. This interaction was saturable and was competed away with the soluble head group of PI(4,5)P2, inositol 1,4,5-triphosphate. Substitution of Arg452 within the PH domain with Ala had only a slight effect on binding to PI(4,5)P2, whereas substitution of Arg459 severely compromised the ability of the mSos1 PH domain to bind to PI(4,5)P2 containing vesicles. Purified full-length mSos1 and mSos1 complexed with Grb2 were also tested for binding to various phospholipid vesicles, and the association of mSos1 PH domain with phospholipid vesicles containing PI(4,5)P2 and that this interaction is facilitated by the ionic interaction of Arg459 with the negatively charged head group of PI(4,5)P2. The association of the mSos1 PH domain with phospholipid may therefore play a role in regulating the function of this enzyme in vivo.

Guanine nucleotide exchange factors catalyze the conversion of Ras from its inactive GDP state to an active GTP-bound state, leading to the activation of downstream effectors (1). A Ras-guanine nucleotide exchange factor specifically implicated in tyrosine kinase signaling is Sos, which was first identified in Drosophila (2, 3); subsequently two mammalian homologues have been described, mSos1 (murine form of the son-of-sevenless 1) and mSos2 (4). Sos is a large protein (~150 kDa) that consists of an N-terminal region, with a Dbl homology domain and a pleckstrin homology (PH)1 domain, a catalytic region that is responsible for the protein’s guanine nucleotide exchange activity, and a C-terminal proline-rich tail with binding sites for the SH3 domains of the Grb2 adaptor protein.

Because Ras is membrane-associated, activation of Sos may involve a change in Sos subcellular localization from the cytosol to the membrane, where it can gain access to GDP-bound Ras (1, 4, 5). It has been observed that targeting of mSos1 to the plasma membrane is sufficient for the activation of the Ras signaling pathway, consistent with the notion that mSos1 is active when situated at the membrane (6, 7). One possibility is that the association of mSos1 with the SH3 domains of Grb2 may serve to modify mSos1 localization upon growth factor stimulation through interaction of the Grb2 SH2 domain with the tyrosine phosphate of activated growth factor receptors (8–10) or through an intermediate docking protein, such as Shc or IRS-1 (11–13).

Recent evidence has raised the possibility that the interactions of Grb2 SH3 domains with the C-terminal tail of mSos1 proteins may have more complex functions than simple relocalization and conversely has suggested that other regions of mSos1 may be involved in membrane targeting. Deletion of the mSos1 C-terminal region yields an mSos1 variant that transforms Rat1 cells (14). Similarly, Drosophila Sos constructs lacking the proline-rich region are able to support the development of R7 photoreceptor cells in a genetically sensitized background (15) and to stimulate Ras activation when ectopically expressed in COS-1 monkey cells (16). These results are consistent with the possibility that a membrane localization region exists in Sos apart from the C-terminal proline-rich domain.

A logical candidate for this potential membrane binding region is the PH domain of Sos. Pleckstrin contains N-terminal and C-terminal repeats of about 100 amino acids (17) that have been found in a large number of proteins including GTPase-activating proteins, tyrosine or serine/threonine kinases, kinase substrates, structural and regulatory elements of the cytoskeleton, and phospholipases (18). A number of ligands for the PH domains have been reported including βγ subunits of heterotrimeric G proteins (19) and protein kinase C (20). The PH domains of several proteins (pleckstrin, spectrin, and phospholipase C-61) have been shown to interact in vitro with phospholipid vesicles containing PI(4,5)P2 and/or its soluble head group Ins(1,4,5)P3 (21–23), although only the phospho-

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lipase C-81 PH domain has thus far shown a high affinity association with phospholipids.

In this study, we demonstrate that the isolated PH domain of mSos1 binds PI(4,5)P₂ with high affinity and specificity. Our results are consistent with the idea that Sos localization may be facilitated by the interaction of its PH domain with the plasma membrane.

**EXPERIMENTAL PROCEDURES**

**Bacterial Expression and Purification of Proteins**—The PH domain of mSos1 (amino acids 392–552) was generated by polymerase chain reaction and cloned in frame into the Bacillus subtilis expression vector pGEX-4T2 to form the fusion protein GST-PH. The R452A and R459A mutants were generated by polymerase chain reaction using the above pGEX-PH domain of mSos1 vector as template. The R452A/R459A double mutant was generated using the mutated R452A GST-PH domain of mSos1 vector as template.

The *Escherichia coli* BL21(DE3) strain was used in the transformations of expression plasmids containing the PH domain (wild type and mutants). A single, transformed colony was used to inoculate 25 ml of medium containing 20 μg/ml of ampicillin. Cells were grown at 37 °C, induced by the addition of isopropyl-1-thio-β-D-galactopyranoside, harvested by centrifugation at 5000 rpm in a Sorvall SS34 rotor for 20 min at 4 °C, and resuspended in 10 ml of buffer that consisted of 50 mM Hepes (pH 7.5), 1 mM EDTA, 150 mM NaCl, and 10% glycerol. The GST fusion proteins were immobilized onto glutathione-agarose beads, which were subsequently washed three times with ice-cold buffer consisting of 25 mM Hepes (pH 7.5), 0.1 M NaCl, 1 mM EDTA, and 1 mM dithiothreitol (Buffer A). Digestion of the PH-PD domain of mSos1 was carried out overnight at 4°C with 2 μg of thrombin. The purified PH domain of mSos1 (wild type and mutants) were then dialyzed in 2 × 1 liter of fresh Buffer A.

The construction of mSos1 and Grb2 baculovirus transfer vectors, overexpression in insect cells, and methodology for purification of protein have been described elsewhere (24).

**Preparation of Unilamellar Vesicles**—Dinystoyl PC, dipalmityl PS, and PI, were purchased from Sigma. PI(4)P₂ and PI(4,5)P₂ were purchased from Calbiochem. PI(3,4,5)P₃ was a kind gift from Dr. Ching-Shih Chen, of the University of Kentucky (25). Sucrose-loaded vesicles were prepared essentially as described (26, 27) by freeze-thawing the aqueous suspension five times and passing them through a 0.1-μm filter (Millipore, Millex-VV filter unit).

**Binding of Isolated mSos1 PH Domain and Full-length mSos1 to Unilamellar Lipid Vesicles**—Binding of the mSos1 PH domain to sucrose-loaded unilamellar vesicles was measured by mixing 5–10 μg of mSos1 PH domain with 500 μl of buffer containing sucrose-loaded unilamellar vesicles (containing total lipid ranging from 20 to 400 μM) in 250 μl of solution containing Buffer A. The unilamellar vesicles were then sedimented by centrifugation at 100,000 × g for 40 min at 25 °C. The pellets were resuspended in H₂O and were run on a 12% SDS-polyacrylamide gel. Binding of full-length mSos1 and the mSos1Grb2 complex were determined in the same way.

To quantitate the interaction of the PH domain of mSos1 to PI(4)P₂, a concentration series of this interaction with respect to increasing PI(4,5)P₂ levels was carried out as before (27). The amount of [PH domain]₀ was determined after ultracentrifugation by measuring protein concentration of the resuspended vesicle using the Bradford dye reagent (Bio-Rad). Assuming a one-to-one complex between the PH domain of mSos1 and PI(4,5)P₂, the data from the protein assays were fitted by nonlinear regression to the equation

\[
\frac{[\text{PH domain}]_0}{[\text{PI(4,5)P}_2]} = \frac{K_a}{K_d} + \frac{1}{K_d}
\]

where \(K_a\) is the dissociation constant for the interaction between the PH domain of mSos1 and PI(4,5)P₂.

**Analysis of the Phospholipid Binding Properties of GST-mSos1 PH Domain by Right Angle Light Scattering**—The phospholipid binding properties of the PH domain of mSos1 were inferred by right angle scattering (29). Protein samples were centrifuged at 13,000 × g for 5 min to remove any particulate material. Samples were then diluted into the same buffer (filtered, 0.2 μm) to a final concentration of 20 μg/ml in a quartz cuvette containing aliquots of various phospholipid vesicles (final concentration, 20 μg/ml in a final volume of 2 ml). The right angle scattering intensity was monitored continuously over time in a Perkin-Elmer LS50B luminescence spectrophotometer at room temperature, with the excitation and emission monochromators set to 320 nm and the slit widths at 2.5 and 15 nm, respectively. The emission signal was attenuated to 1% full scale. The time course of the scattering intensity was monitored until a stable reading was obtained (<5 min).

**RESULTS**

The observation that the PH domain of Sos has a functional role in vivo (14–16) and may be involved in membrane association led us to test the ability of this domain to interact with PI derivatives in vitro. For this purpose, the PH domain of mSos1 was expressed as a GST fusion protein in bacteria, containing residues 392–552 of mSos1. Previous studies have shown that a positively charged face of PH domains is critical for binding PI(4,5)P₂ (26). To assess whether the mSos1 PH domain has basic residues that might be involved in phospholipid binding, the amino acids spanning β strands 1 and 2 of PH domains of known structure were aligned with the corresponding region of the mSos1 PH domain (18; Fig. 1). Conserved lysine or arginine residues, previously shown to be involved in interacting with the 4- and 5-monoesters of PI(4,5)P₂, were identified in the mSos1 PH domain.

**Specific Binding of the mSos1 PH Domain to PI(4,5)P₂**—To investigate the interaction of the mSos1 PH domain with phospholipids, the GST-mSos1 PH fusion protein was purified and digested with thrombin to release the PH domain. The digestion mixture was then incubated with sucrose-loaded unilamellar vesicles, containing 5% PI(4,5)P₂/95% PC, which were then subjected to a centrifugation assay. Following centrifugation, the supernatant and pellet fractions were analyzed by SDS-polyacrylamide gel electrophoresis, as shown in (Fig. 2A).

When the concentration of PI(4,5)P₂ was held at 20 μM, the majority of the mSos1 PH domain was found in the pellet, indicating an association with the phospholipid vesicles. Consistent with this observation, the supernatant of this sample
showed a corresponding decrease in the amount of the mSos1 PH domain, in comparison with control samples. In contrast, no mSos1 PH domain was pelleted with vesicles composed exclusively of the neutral phospholipid PC or with vesicles containing 5% PS, another negatively charged phospholipid. The GST moiety released by thrombin cleavage, which served as an internal control, remained in the supernatant in every case and showed no binding to any of the phospholipids tested. These results show that the mSos1 PH domain binds specifically to phospholipid vesicles containing a phosphoinositide.

To examine whether the mSos1 PH domain showed preferential binding to specific phosphoinositides, we tested the ability of the intact GST-PH domain fusion protein to bind phospholipid vesicles containing either PI(3,4,5)P3, PI(4)P, or PI. As shown in Fig. 2B, the GST fusion protein was detected in pelleted phospholipid vesicles containing PI(4,5)P2 and to a much smaller degree in association with vesicles containing PI(4)P. No mSos1 PH domain fusion protein was detected in association with vesicles that contained PI(3,4,5)P3, PI, or PS. These data suggest that the isolated mSos1 PH domain shows a specific interaction with PI(4,5)P2 and binds weakly to PI(4)P. Again, GST alone showed no interaction with any of the phospholipid vesicles tested (Fig. 2B).

**Right Angle Light Scattering of GST-PH Bound Unilamellar Vesicles**—To further examine the interaction of the PH domain of mSos1 with phospholipid vesicles, light scattering was used, as has been previously employed for other lipid binding proteins (29). Right angle light scattering is a very sensitive method for measuring protein-lipid interactions and is dependent on the molecular weight of the measured particle. Therefore, the intensity of light scattering should increase upon association of a protein with a phospholipid vesicle. The increments in scattering intensity upon the addition of the GST-PH domain to various phospholipid vesicles are shown in Fig. 3A. The mSos1 PH domain interacted specifically with phospholipid vesicles containing 5% PI(4,5)P2, as demonstrated by an increase in scattering intensity of about 50% upon the addition of the GST-PH fusion protein. Little, if any, association was observed with vesicles containing other phospholipids (Fig. 3A). The fusion protein itself induced an increase of about 5 intensity units (data not shown), which explains the small but consistent increases in intensity observed for other phospholipids, notably vesicles containing 100% PC or 5% PI. A slightly greater increase was observed for vesicles containing 20% PS or 5% PI(3,4,5)P3 or PI(4)P, indicating that negatively charged lipids do exhibit a weak interaction with the PH domain of mSos1. However, this interaction was not nearly as significant as that observed for PI(4,5)P2. GST itself, when titrated with PI(4,5)P2 containing phospholipids, showed no increase in light scattering intensity (Fig. 3A).

**Fig. 3B** shows the effect on scattering intensity of adding the GST-PH fusion protein in increasing concentrations to a fixed amount of PI(4,5)P2-containing phospholipid vesicles. Saturation started to become apparent at about 4 μM, indicative of a binary interaction between the PH domain of mSos1 and PI(4,5)P2. This increase in right angle light scattering could be competed away completely by Ins(1,4,5)P3, at a concentration of 30 μM (Fig. 3B).

Quantitation of Binding of Wild Type and Mutant mSos1 PH Domains to PI(4,5)P2—The ability of the mSos1 PH domain to bind sucrose-loaded unilamellar vesicles containing varying concentrations of PI(4,5)P2 was quantitated using the ultracentrifugation assay. We also assessed the involvement of two basic residues of the mSos1 PH domain, Arg452 and Arg459, in the recognition of PI(4,5)P2 by expressing GST-PH domain fusion proteins in which either or both of these residues were substituted with Ala. The wild type PH domain of mSos1 bound tightly to PI(4,5)P2 containing phospholipid vesicles, with a KD of 1.8 ± 0.4 μM (Table I). These results suggest that the isolated wild type mSos1 PH domain shows a relatively high affinity for PI(4,5)P2, in comparison with other PH domains. Surprisingly, the R452A mutant mSos1 PH domain exhibited only a slight decrease in its affinity to PI(4,5)P2, displaying a KD of 5.1 ± 0.7 μM. However, the R459A mutant displayed a greater than 20-fold loss in binding affinity for PI(4,5)P2, indicating that it has a much reduced affinity for PI(4,5)P2, and the double R452A/R459A mutant showed a further decrease in affinity.

To determine if these substitutions had caused any drastic conformational changes, the folding of the wild type and mutant PH domains was investigated by circular dichroism. CD measurements indicated that the secondary structure contents of the GST-PH domain R452A, R459A, and the R452A/R459A mutants were identical to that of the wild type protein (data not shown). Therefore, these substitutions did not cause any gross structural perturbation, suggesting that the decreased affinity of the R459A mutant PH domain for PI(4,5)P2 is likely due to a specific effect on phospholipid binding rather than a general effect on the overall structure of the PH domain.

**Binding of Full-length mSos1 and a mSos1-Grb2 Complex to PI(4,5)P2**—Full-length mSos1, purified from baculovirus infected Hi5 cells (24), was tested for its ability to bind various phospholipid vesicles. mSos1 demonstrated a specific interaction with PI(4,5)P2-containing vesicles (data not shown), albeit at lower affinity than that exhibited by the GST-PH domain fusion protein (KD 53 ± 7 μM, Table I). Because mSos1 is found constitutively bound to Grb2 in vivo, we tested the ability of a
heterodimeric complex containing full-length mSos1 and Grb2 to bind phospholipids. The purified mSos1-Grb2 complex demonstrated a specific interaction with PI(4,5)P₂-containing vesicles. The addition of a phosphopeptide modeled on the Shc Tyr317 phosphorylation site that binds with high affinity to the Grb2 SH2 domain did not influence binding of the mSos1-Grb2 complex to PI(4,5)P₂.

It has been noted that SH2 domains can interact with phosphoinositides (25). We therefore tested the binding of Grb2 to the various phospholipids used in this study. Neither Grb2 purified from baculovirus-infected insect cells nor a GST-Grb2 fusion protein expressed in E. coli displayed any detectable interaction with the various phospholipids using ultracentrifugation and analysis of phospholipid vesicles.

**DISCUSSION**

The results presented in this study demonstrate that the PH domain of mSos1 binds to PI(4,5)P₂ with high affinity and specificity. To date, a number of PH domains have been shown to interact with phosphatidylinositol derivatives (21, 22, 30) *in vitro*. In most cases the affinities are rather low, raising questions about whether the interactions occur *in vivo*. It is difficult to compare the $K_d$ value obtained for the mSos1 PH domain with those obtained on other pleckstrin domains, because the number of lipid binding sites are dependent on the composition and the size of the phospholipid vesicles. However, the affinity of the mSos1 PH for domain for PI(4,5)P₂ appears to be sufficient to contribute to the localization of the full-length mSos1 to the membrane.
The apparent $K_d$ for binding of the mSos1 PH domain to Pl(4,5)P$_2$ is greater than that reported for the pleckstrin N-terminal PH domain (1.8 versus 30 μM (21), respectively) and of the same order of magnitude as reported for the phospholipase C-61 PH domain (≈1.7 μM; (23, 27)). However, the mSos1 PH domain does not share the high affinity of the phospholipase C-61 PH domain for the soluble inositol phosphate, Ins(1,4,5)P$_3$ (note Fig. 3B). For phospholipase C-61, it has been suggested that interaction of its PH domain with Pl(4,5)P$_2$ at the membrane surface allows the enzyme to hydrolyze its substrate in a processive manner and that the tight interaction of the reaction product Ins(1,4,5)P$_3$ could remove this anchor and reduce catalytic efficiency (23, 27, 31, 32). Apparently, this type of inhibition by Ins(1,4,5)P$_3$ is unlikely to be involved in the dissociation of the mSos1 PH domain from the membrane surface. There have been several examples of PH domains binding to Pl(4,5)P$_2$ and Ins(1,4,5)P$_3$ with different affinities (21, 23, 26, 27, 30), although various protein structures suggest that these domains do not interact directly with the acyl chains within the lipid membranes. Neutron diffraction experiments with Pl(4)P/PC mixed membranes demonstrate that the head group inositol is inclined at an angle to the bilayer, perhaps due to electrostatic interaction of the negatively charged 4-phosphate with the trimethyl ammonium moiety of PC (33). Thus, it may be possible that the phosphorylated inositol head groups bound to acyl chains present in a lipid membrane adopt different orientations than that of the soluble inositol triphosphate. In any case, the binding of Ins(1,4,5)P$_3$ to the mSos1 PH domain has no clear biological function. It may be possible for Ins(1,4,5)P$_3$ to compete with Pl(4,5)P$_2$ for the PH domain, although it seems unlikely that concentrations of Ins(1,4,5)P$_3$ in living cells would reach the levels required to displace mSos1 (27), unless local concentrations of Ins(1,4,5)P$_3$ can reach levels of 10 μM or higher.

A number of recent studies have shown that PH domains have distinct structural characteristics (21, 22, 30), even in the absence of any strong primary sequence homology (18). Indeed, the Shc and IRS-1-phosphotyrosine binding (PTB) domains have recently been demonstrated to share the same fold as PH domains (28, 34). The Pl(4,5)P$_2$ binding site in the N-terminal PH domain of pleckstrin has been identified (21) at the lip of the β-barrel in the N-terminal half of the domain. Two residues found to be important for this interaction were Lys$^{13}$ and Lys$^{22}$ (26). The corresponding amino acids within the PH domain of mSos1 are Arg$^{452}$ and Arg$^{459}$ (18). We have identified Arg$^{459}$ of mSos1 as being critical for the strong interaction of this protein’s PH domain with Pl(4,5)P$_2$ in vitro. This residue is analogous to the Arg$^{26}$ residue of the Btk PH domain (18), whose functional significance has been demonstrated by its mutation to cysteine in XID mice (35, 36). The finding that substituting Arg$^{452}$ with Ala has only a small effect on binding could be interpreted as indicating that this residue does not form an ionic interaction with the negatively charged Ins(1,4,5)P$_3$ or alternatively that a compensatory mechanism occurs upon substitution of Arg$^{452}$ that replaces the lost interaction.

The weaker interactions of the full-length mSos1 and mSos1-Grb2 complex compared with that of the isolated PH domain are interesting. It is tempting to speculate that a cellular factor is required to allow the PH domain of mSos1 to interact efficiently with Pl(4,5)P$_2$, which may thereby regulate mSos localization and function. Unlike some other SH2 domains (25), Grb2 showed no interaction with the phospholipids tested in this study.

The role of these postulated protein-membrane interactions could be, as proposed previously for the Grb2/mSos1 interaction, mainly to localize of the enzyme (mSos1) to its substrate (Ras). However, formation of protein-phospholipid binary complexes also could increase subsequent reaction rates by limiting the dimensions required for translational diffusion. This could effectively increase the frequency of productive collisions between membrane-bound proteins by reducing the possible orientations of the reacting species. Thus, requiring that the complex between mSos1 and Ras forms on the plasma membrane surface could be a means to achieve the rapid amplification of signaling through the mSos1/Ras pathway, as well as providing a mechanism to modulate mSos activity by controlling its subcellular localization.

In addition to phospholipid interaction, it has been suggested that another possible function of PH domains is to bind to Gβγ subunits (19). In vitro studies using human Sos1 failed to show saturable binding to Gβγ subunits (37). However, it is possible that as with βARK PH domain (38), Pl(4,5)P$_2$ interaction with the mSos1 PH domain may mediate or synergize with the interaction of this domain with Gβγ subunits or with some as yet unidentified protein ligand.

In conclusion, we have shown that there is a strong interaction of the PH domain of mSos1 to Pl(4,5)P$_2$ in vitro and that this interaction can be competed away by Ins(1,4,5)P$_3$. The native mSos1-Grb2 complex also bound Pl(4,5)P$_2$, albeit more weakly than the isolated PH domain. Site-directed mutagenesis of conserved basic residues within the PH domain of mSos1 has demonstrated that Arg$^{459}$ plays a critical role in mediating this interaction with Pl(4,5)P$_2$.

### Table I

| PH domain          | $K_d$ ± S.D.* |
|--------------------|--------------|
| Wild type          | 1.8 ± 0.4    |
| R452A              | 5.1 ± 0.7    |
| R459A              | 79 ± 19      |
| R452A/R459A        | 87 ± 22      |
| mSos1              | 53 ± 26      |
| mSos1/Grb2         | 69 ± 19      |

*$^a$ S.D. is standard deviation for determination of the parameter $K_d$, estimated by fitting Equation 1 to the binding data.

$b$ Standard error as calculated from three independent binding experiments using Equation 2.
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