The role of the PH domain in the signal-dependent membrane targeting of Sos

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The pleckstrin homology (PH) domain is a conserved protein module present in diverse signal transducing proteins. To investigate the function of the PH domain of the Ras exchanger Sos, we have generated a recombinant (His)₆-tagged PH domain from human Sos (PH-Sos). Here, we show that PH-Sos binds with high affinity (1.5 μM) to lipid vesicles containing the negatively charged phospholipid phosphatidylinositol 4,5-bisphosphate (PIP₂). When microinjected into serum-deprived rat embryonic fibroblasts or COS cells, PH-Sos displays a homogenous subcellular distribution. However, PH-Sos rapidly accumulates in the plasma membrane following serum stimulation and, under these conditions, is localized preferentially to the leading edge of motile cells. Surprisingly, the membrane localization of PH-Sos is not dependent on its ability to bind PIP₂. Overexpression of the PH domain of Sos has a pronounced dominant-negative effect on serum-induced activation of the Ras signaling pathway. These results suggest that the PH domain of Sos participates in regulating the inducible association of Sos with the membrane, and indicate the presence of specific ligands that interact with this domain to bring about the activation of Ras.

Keywords: leading edge/lipids/PH domains/Ras/Sos

Introduction

Pleckstrin homology (PH) domains are sequence motifs that share homology with an internal repeat in pleckstrin, the major substrate of protein kinase C in platelets (Konishi et al., 1994; Yao et al., 1994). They contain ~120 amino acids, and are present in a large variety of proteins involved in intracellular signaling or cytoskeletal organization (reviewed by Musacchio et al., 1993; Gibson et al., 1994; Shaw, 1995; Lemmon et al., 1996). These include protein kinases such as Akt and Btk; guanine nucleotide exchange factors for small GTPases such as Dbl and Sos; phospholipase C isoforms (PLC-β, -γ and -δ); and cytoskeletal proteins such as β-spectrin. Despite the fact that the sequence identity between PH domains is limited, their tertiary structures appear to be strikingly similar. The three-dimensional structures of the PH domains from β-spectrin, pleckstrin, dynamin and PLCδ₁ reveal that each PH domain consists of a seven stranded β-sandwich of two orthogonal antiparallel β-sheets closed at one end with a C-terminal amphipathic α-helix (Lemmon et al., 1996). Recently, a second class of PH domains, the phosphotyrosine-binding (PTB) or phosphotyrosine interaction (PI) domains from Shc and insulin receptor substrate-1 (IRS-1), have been characterized structurally (Zhou et al., 1995; Eck et al., 1996). These PTB domains share no sequence homology with PH domains, but have a very similar overall architecture.

By analogy with SH2 and SH3 domains, it has been postulated that PH domains are structural modules involved in mediating intermolecular interactions. However, the identity of the physiological ligands of PH domains is, to a large extent, unknown. Several PH domains, specifically those from β-spectrin, pleckstrin, dynamin and PLC-δ₁, have been shown to bind to phosphoinositides and their head groups. The PH domain of PLC-δ₁ binds specifically to both inositol 1,4,5-trisphosphate [Ins(1,4,5)P₃] and phosphatidylinositol 4,5-bisphosphate (PIP₂) with high affinities [Kₐ = 1.7 μM for PIP₂ and Kₐ = 0.21 μM for Ins(1,4,5)P₃] (Lemmon et al., 1995). It has been proposed that this interaction serves to recruit the enzyme to membrane regions enriched with its substrate. In contrast, the PH domains from β-spectrin and pleckstrin bind to phospholipids weakly and with little specificity (Harlan et al., 1994; Hyvonen et al., 1995), and the physiological relevance of these interactions is not known. In addition to lipid binding, the PH domain of β-adrenergic receptor kinase (β-ARK) has been shown to be required for its interaction with βγ subunits of heterotrimeric G proteins (Pitcher et al., 1995), and the PH domain of Akt contributes to the formation of Akt protein complexes (Datta et al., 1995). The ligands for PTB domains are specific protein sequences containing phosphorylated tyrosines (Bork and Margolis, 1995; Zhou et al., 1995). PTB-mediated interactions play a role in the recruitment of signaling molecules to activated receptors (Batzler et al., 1995; Yajnik et al., 1996).

Although little is known about the molecular basis for the function of PH domains, several lines of evidence indicate that these domains are critical for the biological activity of various signaling molecules. First, specific point mutations in the PH domain of Bruton’s tyrosine kinase (Btk) are thought to constitute the primary genetic defect underlying X-linked immunodeficiency in mice and agammaglobulinemia in humans (Rawlings et al., 1993; Thomas et al., 1993; Vihinen et al., 1995). Second, the PH domain of β-ARK is required for its membrane association and activation (Pitcher et al., 1995). Third, the coupling of insulin receptors and IRS-1 depends in part on the PH domain of IRS-1 (Myers et al., 1995; Voliovitch et al., 1995). Fourth, the PH domain of PLC-δ₁ is necessary and sufficient for the interaction of PLC-δ₁ with the plasma membrane (Cifuentes et al., 1994; Paterson et al., 1995). Fifth, the PH domain of the protein kinase Akt has been implicated in the activation of Akt by phosphatidylinositol 3-kinase (PI-3 kinase) (Franke et al., 1995). Finally, the
PH domain of oncogenic Dbl mediates its targeting to the cytoskeletal matrix and is essential for its oncogenic activity (Zheng et al., 1996b).

PH domains are present in the Ras guanine nucleotide exchange factors Sos and p140Ras-GRF and have been implicated in the regulation of their guanine nucleotide exchange activity (Karlovich et al., 1995; McCollam et al., 1995; Wang et al., 1995; Buchsbaum et al., 1996). In this study, we have examined the function of the PH domain of Sos. We report that the isolated PH domain of Sos (PH-Sos) binds to acidic phospholipids with high affinity and is targeted to specific regions within the plasma membrane. Surprisingly, lipid binding does not appear to be required for the membrane targeting of PH-Sos. Rather, the localization of PH-Sos to the membrane is dependent on growth factor stimulation. Our results suggest a role for PH-Sos in the signal-dependent recruitment and activation of Sos.

**Results**

**PH-Sos is localized to the plasma membrane**

To study the function of the PH domain of hSos1 (PH-Sos), we generated a recombinant fusion protein in which a fragment encompassing residues 422–551 of hSos1 was N-terminally fused to six histidines followed by a T7 epitope tag. Using Ni affinity chromatography, the PH-Sos was purified to homogeneity (Figure 1A) and microinjected into subconfluent cells. The distribution of the injected protein was then examined by indirect immunofluorescence staining (Figure 1B). Analysis of the fluorescence staining patterns by confocal microscopy revealed that both in COS cells (panel a) and in rat embryo fibroblasts (panel b) the PH-Sos was present throughout the cytoplasm but was specifically enriched at the cell periphery. These results indicate that the PH-Sos can target itself to the plasma membrane.

**High affinity binding of PH-Sos to phosphatidylinositol-4,5-bisphosphate**

Several PH domains have been shown to bind negatively charged phospholipids (Rebecchi et al., 1992; Harlan et al., 1994; Yagisawa et al., 1994; Hyvönen et al., 1995; Lemmon et al., 1995; Zheng et al., 1996a). We tested whether PH-Sos would interact with phospholipids using a vesicle binding assay (Rebecchi et al., 1992). Different concentrations of purified PH-Sos were mixed with 1 mM lipid vesicles containing or not containing 2% PIP2. The vesicles were pelleted by ultracentrifugation and then analyzed for the presence of the bound PH-Sos by the bicinchoninic acid (BCA) protein assay. In the presence of PIP2, PH-Sos bound to the phospholipid vesicles in a concentration-dependent manner (Figure 2A) with an apparent dissociation constant of 1.5 μM (Figure 2B). This binding affinity is similar to that observed for the binding of the isolated PLC-δ1 PH domain (Kd = 1.7 μM) or PTB domain (Kd = 7.3 μM) to PIP2 in phospholipid vesicles (Lemmon et al., 1995; Zhou et al., 1995). In contrast, the binding of PH-Sos to phospholipid vesicles was relatively insignificant in the absence of PIP2 (Figure 2A).

**Phospholipid binding is not critical for the membrane association of the PH-Sos**

Most proteins that contain PH domains require membrane association for their function. Thus, it has been proposed that the interaction between PH domains and phospholipids may serve as a mechanism to anchor PH domain-containing proteins to the membrane. To investigate whether the lipid-binding activity of the PH-Sos plays a role in its membrane association, we generated a mutant of PH-Sos in which two residues, Lys456 and Arg459, were mutated simultaneously to glutamic acid. Based on an alignment with other PH domains of known structures, these positively charged residues in the PH-Sos are predicted to be located at the N-terminal portion of the β2 strand (Figure 2C), a region shown to be critical for lipid binding (Harlan
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Fig. 2. Binding of PH-Sos to acidic phospholipids and identification of residues which are critical for the lipid binding of PH-Sos. (A) Concentration dependence of the binding of PH-Sos to lipid vesicles. Increasing concentrations of purified PH-Sos protein (WT) were incubated with 1 mM lipid vesicles containing 2% PIP2 (●) or 0% PIP2 (○). PH-Sos mutant, KR→EE (▲), was incubated with 1 mM lipid vesicles containing 2% PIP2 under the same conditions as for PH-Sos WT. PH-Sos bound and PH-Sos free were separated and measured as indicated in Materials and methods. (B) Apparent dissociation constants, 1.5 μM for WT and 73 μM for KR→EE, were calculated by fitting the experimental data to the Scatchard equation: Y/[PH-Sos]f = Kd (1 – Y). (C) Sequence alignment and secondary structure comparison of PH-Sos domain with other PH domains of known structure. Conserved elements of secondary structure (seven β sheets and one α helix) are represented by boxes located under the primary sequence. The main amino acid residues involved in the interaction of PH-PLCδ with phosphoinositides are marked by asterisks. The positions of mutations in PH-Sos that were found to affect lipid binding (see B) are indicated by the arrows. Potential protein kinase C phosphorylation sites are indicated by a square. Potential casein kinase II phosphorylation sites are indicated by a dot.

et al., 1994; Ferguson et al., 1995; Zheng et al., 1996a). The PIP2 dependence of the binding of the wild-type and mutant PH-Sos was compared by the vesicle binding assay. As shown in Figure 2A, PIP2 had no effect on the binding of the double mutant PH-Sos, indicating that this mutant completely lost the PIP2-dependent binding activity. Scatchard analysis reveals that the apparent dissociation constant for the mutant PH-Sos is 73 μM (Figure 2B).

To test whether the impaired lipid binding of the PH-Sos double mutant affected its cellular localization, a purified preparation of the mutant protein was microinjected into COS cells and the subcellular localization of the injected protein was analyzed by immunofluorescence staining and confocal microscopy. Figure 3 illustrates that similarly to the wild-type PH-Sos, the double mutant was able to localize to the plasma membrane. Therefore, the PIP2 binding activity is not essential for the membrane localization of PH-Sos.

**PH-Sos is recruited to the plasma membrane in a serum-dependent manner and is localized preferentially to the leading edges of motile cells**

To gain insights into the mechanisms that regulate the localization of the PH-Sos to the plasma membrane, we tested the effect of growth factor stimulation on the subcellular distribution of PH-Sos. When microinjected into serum-starved COS cells, the PH-Sos displayed a homogenous cytoplasmic distribution (Figure 4A, panel a). However, following serum stimulation, the PH-Sos rapidly accumulated in the plasma membrane (Figure 4A, panel b). We have consistently observed that the membrane staining of PH-Sos is localized preferentially to one side of the cell periphery which is not in contact with other cells. This polarized staining pattern resembles the localization of membrane proteins associated with the leading edges of motile cells. Therefore, we tested whether the PH-Sos is localized preferentially to the leading edge by performing a wound-healing experiment. In cell monolayers, the individual cells that are most adjacent to a newly formed wound rapidly reorient themselves such that their leading edges point towards the wound. Experimentally, this phenomenon can be mimicked by introducing a scratch in a confluent monolayer of cells. Under these conditions, the space generated by the scratch represents the wound. For our experiments, a scratch was introduced in a confluent monolayer of rat embryo fibroblasts and the PH-Sos was microinjected into cells positioned along the wound. Immunofluorescence staining revealed that the PH-Sos was concentrated at the leading edges of the cells (Figure 4B). The preferential increase in the concentration of PH-Sos at the leading edge was also observed by confocal microscopy (not shown). These observations raise the possibility that PH-Sos may be involved in the targeting of Sos to specialized regions within the plasma membrane.
Fig. 3. Comparison between the subcellular localization of PH-Sos (WT) and PH-Sos mutant (KR→EE). Subconfluent COS cells were microinjected with purified preparations (2 mg/ml) of WT or mutant PH-Sos. The distribution of the injected protein was examined by indirect immunofluorescence staining as described in the legend to Figure 1. Fluorescence images were viewed by confocal microscopy.

Fig. 4. Regulation of the subcellular localization of PH-Sos. (A) Serum-dependent membrane localization of PH-Sos. Serum-starved subconfluent COS cells were microinjected with PH-Sos. Two hours after injection, cells were stimulated with (10%) serum for 5 min. Localization of the injected protein in serum-starved (a) and serum-stimulated (b) cells was determined by indirect immunofluorescence staining and confocal microscopy as described in Figure 1. (B) Localization of PH-Sos to the leading edge of motile cells. A scratch was made in a confluent monolayer of REF-52 cells. The cells near one side of the wound were microinjected with PH-Sos (2 mg/ml) and the distribution of the injected protein was analyzed by indirect immunofluorescence staining and conventional fluorescence microscopy. The fluorescence staining image is shown in panel b and the corresponding phase-contrast micrograph is shown in panel a. Arrows denote the high concentration of PH-Sos at the leading edge.
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Fig. 5. Purification and localization of GST–PTB from Shc. (A) A GST–PTB preparation which was expressed in E. coli and purified using glutathione–agarose beads. (B) Subcellular distribution of the GST–PTB domain in COS cells. Purified GST–PTB was microinjected into COS cells and the localization of the injected protein was examined by indirect immunofluorescence staining using anti-GST mouse monoclonal antibody and rhodamine-conjugated goat anti-mouse IgG secondary antibody. Injected cells were viewed by conventional fluorescence microscopy (panel a) or by phase-contrast microscopy (panel b). The cell boundaries are delineated by the broken lines (panel c) which were hand-drawn by overlapping the phase and fluorescence micrographs. Note that the fluorescent staining does not coincide with the cell periphery.

The Shc PTB domain displays a localization pattern distinct from that of PH-Sos

The PTB or PI domains of Shc and IRS-1 recently have been shown by structural studies to adopt the same structural folds as the PH domains and therefore could be perceived as bona fide PH domains. To test whether membrane localization is a general property of PH domains, a GST fusion protein containing the PTB domain from Shc was purified (Figure 5A) and microinjected into COS cells. Immunofluorescence staining of the injected cells with anti-GST antibody showed a patchy distribution pattern organized in a ring-like structure that does not correspond to the cell periphery (Figure 5B). Confocal microscopy analysis of the Shc PTB staining indicated that the protein was present in the ventral part of the cell close to areas of contact with the substratum (data not shown). This localization pattern is markedly different from that observed for PH-Sos. Moreover, in contrast to PH-Sos, the distribution of Shc PTB was not affected by serum stimulation. Thus the regulated membrane localization of PH-Sos most likely reflects properties that are specific for this protein.

Role of the PH domain in Sos activation

To investigate the functional significance of the PH domain of Sos, we analyzed the effects of different deletion mutants of Sos on serum-induced activation of mitogen-activated protein (MAP) kinase. COS cells were co-transfected with plasmids encoding hemagglutinin (HA)-tagged ERK2 MAP kinase and HA-tagged deletion mutants of Sos. ERK2 was immunoprecipitated with the anti-HA antibody and MAP kinase activity was measured by in vitro kinase assay using myelin basic protein (MBP) as an exogenous substrate. As illustrated in Figures 6A, the expression levels of ERK2, as detected by immunoblotting, were similar among the various transfected cells. Under the experimental conditions we have used, serum-induced activation of MAP kinase is predominantly Sos dependent. This is indicated by the fact that the expression of a dominant interfering mutant of Sos lacking the catalytic domain (ΔCat) resulted in a 75% inhibition of MAP kinase activity (Figure 6B). Significantly, the expression of the PH domain of Sos (PH-WT) alone was also accompanied by a marked inhibition (75%) of serum-induced MAP kinase activation (Figure 6B). This interfering effect was independent of PIP2 binding (Figure 6B, PH-KR→EE). This suggests that the PH domain can compete with endogenous Sos molecules for a cellular target that is critical for Sos activation. It could be argued that, when overexpressed, the PH domain might interfere with MAP kinase activation by affecting other components of the MAP kinase cascade. However, the expression of the PH domain did not interfere with the ability of Raf-CAAX, a constitutively active membrane-targeted form of Raf, to activate MAP kinase (Figure 6B). Thus the dominant-negative effect of the overexpressed PH domain is exerted upstream of Ras and most likely at the level of Sos itself.

Discussion

To study the function of the PH domain of Sos, we generated a recombinant protein corresponding to the PH domain of hSos1. Biochemically, the purified PH domain of hSos1 displays characteristics that are similar to other PH domains in that it binds with high affinity to PIP2. It is noteworthy that, with the exclusion of the PH domain of PLC-δ1, the interaction of PH-Sos with PIP2 is stronger than that seen in studies with any of the other PH domains. For PH domains of known
Sos which abolish PIP₂ binding do not interfere with the ability of the protein to target itself to the plasma membrane. Thus, it appears that PIP₂ binding is not necessary for the anchoring of PH-Sos to the membrane. Moreover, the binding of PH domains to PIP₂ does not seem to be sufficient for membrane association, as the isolated PTB domain, which has been shown to bind PIP₂ with high affinity, failed to interact with the plasma membrane. Thus, although we cannot definitively rule out the possibility that interactions with negatively charged lipids other than PIP₂ might be necessary for membrane localization, our data are most consistent with the interpretation that the recruitment of PH-Sos to the membrane is mediated by a specific ligand that is distinct from PIP₂. The identity of such ligand(s) is not known. Whereas the PH domain of oncogenic Dbl has been shown to mediate interaction with the detergent-resistant cytoskeletal matrix (Zheng et al., 1996b), both the isolated PH domain of Sos and the intact Sos protein are detergent soluble (R.-H.Chen and D.Bar-Sagi, unpublished observations). Thus the differential interactions of PH domains with cellular ligands might constitute a mechanism for the targeting of PH domain-containing signaling molecules to distinct subcellular locations. In this context, it is of interest to note that the PH domain of Sos appears to be recruited preferentially to the leading edge of motile cells. Since several cell surface receptors have been shown to be concentrated at the leading edge (Regen and Horwitz, 1992; Warn et al., 1993), the enrichment of Sos in this region might provide the means by which signals could be generated in a specific spatial pattern.

Given their postulated function in the cellular targeting of signaling molecules, PH domains would be predicted to function in a signal-dependent fashion. Consistent with this view, we found that the recruitment of the PH domain of Sos to the membrane is serum dependent. The mechanisms by which serum growth factors might modulate the membrane anchoring of the PH domain remain to be determined. The presence of several potential phosphorylation sites for kinases within the PH domain of Sos involved in signaling (e.g. protein kinase C and casein kinase II) raises the possibility that phosphorylation of the PH domain itself might be the target for modulation by extracellular signals. However, it is also possible that these signals act on the ligand(s) of the PH domain of Sos to enhance their accessibility and/or affinity.

The activation of Ras by Sos in response to growth factor stimulation is not mediated by a change in the catalytic activity of Sos (Buday and Downward, 1993; McCormick, 1993; Olivier et al., 1993). Rather, it appears that the functional activity of Sos depends on its accessibility to Ras (Aronheim et al., 1994; Quilliam et al., 1994). Since Grb2 mediates the recruitment of Sos to the activated receptor, the initial view has been that the interaction of Sos with Grb2 serves as the principle mechanism to place Sos in spatial proximity to Ras (Buday and Downward, 1993; Chardin et al., 1993; Egan et al., 1993; Li et al., 1993). However, more recent genetic and biochemical studies have indicated that activation of Ras by Sos can occur even in the absence of interaction with Grb2 (Karlovich et al., 1995; McCollam et al., 1995; Wang et al., 1995).
Under these conditions the N-terminal domain of Sos appears to be the critical determinant for activation (Karlovich et al., 1995; McCollam et al., 1995; Wang et al., 1995). Our observation that the PH domain of Sos can target itself to the plasma membrane, suggests a mechanism by which the C-terminal domain of Sos can interact with cellular membranes thereby gaining access to membrane-bound Ras. The ability of overexpressed PH domain of Sos to exert a dominant-negative effect lends further support to this concept and suggests the existence of specific cellular targets that contribute to the regulation of Sos activity by interacting with this domain.

It is becoming increasingly evident that the proper presentation of Sos to Ras is achieved through multiple intermolecular interactions involving both the N- and C-terminal domains of the protein. Thus, further analysis of the structure of the PH domain of Sos and its functional interaction with ligands should contribute to the elucidation of the mechanisms that govern the coupling of receptor tyrosine kinases to Ras activation.

Materials and methods

Construction of expression plasmids

The DNA fragments corresponding to the PH domain of human Sos1 (residues 422–551) were amplified using PCR. The PCR products were digested by the restriction enzymes BamHI and SalI and then ligated into the PET28b(+) bacterial expression vector, which contains a 6-His tag and a T7 epitope tag (Novagen). Mutants of the PH domain were generated using PCR mutagenesis and cloned into the same expression vector. All the constructs were confirmed by DNA sequencing.

Expression and purification of the PH and PTB domains

The PET28b(+) plasmid containing the wild-type or mutant PH domains was transformed into BL21(DE3) Escherichia coli cells. The bacterial cultures (OD600 0.6) were induced with 0.1 mM IPTG at 30°C with 1 mM PMSF, 10 μg/ml of pepstatin, 50 mM NaF, 1% aprotinin, 10 μg/ml of trypsin inhibitor. The soluble fraction of the lysate was centrifuged at 100 000 g for 30 min prior to being added to the culture medium.

Twelve hours after transfection, media were aspirated and cultures were fed with 10 ml of DMEM supplemented with 5% FBS.

Indirect immunofluorescence

Cells were fixed with 3.7% formaldehyde in PBS for 1 h at room temperature or overnight at 4°C and then permeabilized with 0.1% Triton X-100 in PBS for 3 min at room temperature. The coverslips were incubated for 1 h at 37°C with the primary antibody (mouse anti-T7 antibody or mouse anti-GST antibody) diluted in 1% bovine serum albumin (BSA) in PBS, followed by 1 h incubation with the secondary antibody (affinity-purified rhodamine-conjugated goat anti-mouse IgG). The stained cells were observed by conventional fluorescence and confocal microscopes. For confocal fluorescence microscopy, cells were viewed using an Odyssey confocal system (NORAN Instrument Inc.). Each image shown represents a 0.5 μm optical section (x-y) taken through the vertical region of the cell.

Lipid binding assay

Lipid vesicles were generated by mixing equimolar solutions of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine (POPS) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) (1:1:1) with or without 2% FBS, respectively. The beads were washed with phosphate-buffered saline (PBS) PMSF, 10 mg/ml pepstatin, 50 mM NaF, 1% aprotinin, 10 μg/ml trypsin inhibitor, and 10 μg/ml of leupeptin, 10 μg/ml of aprotinin, 10 μg/ml of pepstatin A and 10 μg/ml of trypsin inhibitor [PH-S]f is the fraction of PH domain free in solution after centrifugation, [PH-S]b is the fraction of PH domain bound to the lipid vesicles after centrifugation. The reciprocal of these numbers can be taken as an apparent dissociation constant.

Membrane targeting of Sos by the PH domain

Subconfluent or confluent cells were starved in serum-free DMEM at least 16 h prior to microinjection. Proteins (2–4 mg/ml) in either 0.5× PBS or microinjection buffer (20 mM Tris-acetate pH 7.4, 20 mM NaCl, 1 mM MgCl2, 0.1 mM EDTA and 5 mM β-mercaptoethanol) were injected into the cytoplasm of cells in selected squares of the coverslips. For serum stimulation, cells were stimulated with DMEM containing 20% FBS, 2 h after injections. Transfections of COS-1 cells were performed using calcium phosphate as described by Wigler et al. (1977). Brieﬂy, DNA was diluted in Tris/EDTA buffer and 2 M CaCl2 was added to a final concentration of 125 mM. The mixture was then diluted in HEPEs/NaHP04 buffer and allowed to stand at room temperature for 30 min prior to being added to the culture medium. Twelve hours after transfection, media were aspirated and cultures were fed with 10 ml of DMEM supplemented with 5% FBS.

ERK2 immune complex kinase assay

COS-1 cells were transiently transfected with epitope (HA)-tagged constructs of 0.5 μg of ERK2 and 2 μg of PH-PTB (2 μg of PH–PTB domain was co-transfected with epitope (HA)-tagged plasmid containing the wild-type or mutant PH domains) and 2 μg of PH–PTB domain was co-transfected with epitope (HA)-tagged plasmid containing the wild-type or mutant PH domains). The cells were lysed with lysis buffer containing 20 mM imidazole. The bound fractions were eluted with ProBond Ni beads (Invitrogen) for at least 2 h at 4°C. After washing with the same buffer containing 50, 200 and 500 mM imidazole, the protein was eluted using 200 mM imidazole. The bound fractions were eluted with the same buffer containing 50, 200 and 500 mM imidazole. The GST–PTB domain of Shc was a gift from Ben Margolis (Yajnik et al., 1996). The construct was transformed into HB101 E.coli cells. The bacterial cultures (OD600 0.6) were induced with 0.1 mM IPTG for 5 h at 30°C. The cells were lysed by sonication in lysis buffer (50 mM phosphate buffer, pH 8.0 and 300 mM NaCl) containing protease inhibitors [10 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml of leupeptin, 10 μg/ml of aprotinin, 10 μg/ml of pepstatin A and 10 μg/ml of trypsin inhibitor]. The lysate was clarified by centrifugation and the soluble fractions were incubated with ProBond Ni beads (Invitrogen) for at least 2 h at 4°C. The Ni beads were washed extensively with lysis buffer and then with lysis buffer containing 20 mM imidazole. The bound fractions were eluted with the same buffer containing 50, 200 and 500 mM imidazole.

Cell culture, transfection and microinjection

COS-1 or REF-52 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5 or 10% fetal bovine serum (FBS), respectively. Cells were plated onto grided glass coverslips.

Subconfluent or confluent cells were starved in serum-free DMEM at least 16 h prior to microinjection. Proteins (2–4 mg/ml) in either 0.5× PBS or microinjection buffer (20 mM Tris-acetate pH 7.4, 20 mM NaCl, 1 mM MgCl2, 0.1 mM EDTA and 5 mM β-mercaptoethanol) were injected into the cytoplasm of cells in selected squares of the coverslips. For serum stimulation, cells were stimulated with DMEM containing 20% FBS, 2 h after injections. Transfections of COS-1 cells were performed using calcium phosphate as described by Wigler et al. (1977). Brieﬂy, DNA was diluted in Tris/EDTA buffer and 2 M CaCl2 was added to a final concentration of 125 mM. The mixture was then diluted in HEPEs/NaHP04 buffer and allowed to stand at room temperature for 30 min prior to being added to the culture medium. Twelve hours after transfection, media were aspirated and cultures were fed with 10 ml of DMEM supplemented with 5% FBS.

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Cell culture, transfection and microinjection

COS-1 or REF-52 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5 or 10% fetal bovine serum (FBS), respectively. Cells were plated onto grided glass coverslips.
4°C. The immune complexes were collected by centrifugation for 15 s of protein kinase C zeta.

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References


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