isolated sos1 ph domain exhibits germinal vesicle breakdown-inducing activity in Xenopus oocytes

(Received for publication, February 20, 1996, and in revised form, May 2, 1996)

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Purified, bacterially expressed PH domains of Sos1, IRS-1, βARK, and PLCδ1 were analyzed functionally by means of microinjection into full grown, stage VI Xenopus laevis oocytes. Whereas the PH domains from IRS-1, βARK, or PLCδ1 did not show any effect in the oocytes, injection of the purified Sos1 PH domain resulted in induction of significant rates of germinal vesicle breakdown and meiotic maturation. Furthermore, the Sos1 PH domain exhibited also significant synergy with insulin or coinjected normal Ras protein in induction of germinal vesicle breakdown, although it did not affect the rate of progesterone-induced maturation. These results suggest that purified, isolated PH domains retain, at least in part, their functional specificity and that Xenopus oocytes may constitute a useful biological system to analyze the functional role of the Sos1 PH domain in Ras signaling pathways.

Pleckstrin homology (PH) domains are modular domains of about 100 amino acids present in a variety of signaling and cytoskeletal proteins, which were initially defined by their homology with the internal repeats of pleckstrin, a major protein kinase C substrate in platelets (1, 2). I included among the many PH-containing proteins described so far are several that participate in Ras signaling pathways. Despite having a rather limited primary sequence consensus identity, the PH domains from different proteins appear to share a common tertiary structure consisting of seven antiparallel β-sheets and a carboxyl-terminal amphipathic α helix (3–6).

The functional role of PH domains remains unclear. Some reports describe a role of PH domains in protein-protein interactions such as Akt homodimerization (7) and association with Gβγ subunits (8–10) or protein kinase C (11, 12), whereas others point to interactions with membrane phosphate such as phosphatidylinositol 4,5-biphosphate (13–17). An interesting hypothesis suggests that all those different interactions may represent alternative methods for directing the implicated proteins to membrane locations. Genetic evidence has confirmed the functional importance of PH domains since there is an established association between specific point mutations in the PH domain of Bruton tyrosine kinase and X-chromosome linked human agammaglobulinemia or mouse immunodeficiency (18–21). Regarding Ras signaling, recent reports point to the presence of positive regulatory elements in the region of the Ras guanine nucleotide exchanger Sos1 protein that encompasses its PH (and also the contiguous DH) domain (22).

Xenopus oocytes provide a useful experimental model for the functional analysis of signaling molecules, which are able to induce their meiotic maturation, i.e. germinal vesicle breakdown (GVBD). Xenopus oocytes possess at least two independent pathways leading to GVBD. In one, progesterone leads to decreased adenylate cyclase activity, with a resulting drop in overall cAMP levels and protein kinase A-dependent phosphorylations. In the other, insulin or IGF-1 triggers a cascade of phosphorylations initiated by tyrosine phosphorylation of their receptors (reviewed in Refs. 23–25). Ras and other oncogenic proteins also have been shown to induce meiotic maturation when microinjected into Xenopus oocytes (26–32). Several lines of evidence indicate that Ras proteins are essential components in insulin-induced maturation (33–35). The Xenopus system has also recently proven useful to analyze functionally the role of isolated, purified modular domains including the platelet-derived growth factor receptor kinase insert (36) and a variety of different SH2 domains (37–39).

In the present study, we utilized the Xenopus oocyte system to investigate possible in vivo function(s) of isolated, purified PH domains. Using polymerase chain reaction, we subcloned the PH domains of hSos1, rIRS-1, hβARK, and hPLCδ1 into appropriate bacterial expression vectors. The corresponding purified, bacterially expressed peptides were then analyzed functionally by means of microinjection into full grown, stage VI, Xenopus laevis oocytes. Whereas the PH domains from IRS-1, βARK, or PLCδ1 did not show any effect, purified hSos1 PH domain was able to induce maturation when injected alone and exhibited significant synergy with insulin or coinjected normal Ras protein in induction of GVBD, although it did not affect the rate of progesterone-induced maturation. These results suggest that purified, isolated PH domains retain, at least in part, their functional specificity and that Xenopus oocytes offer a useful biological system to analyze their functional roles in signaling pathways.

EXPERIMENTAL PROCEDURES

Oocyte Preparation and Microinjection—Adult female X. laevis were obtained from Xenopus I (Ann Arbor, MI) or Nasco (Fort Atkinson, WI) and stimulated to ovulate by injecting 100 units of pregnant mare serum gonadotropin (Calbiochem) 3 days before oocyte extraction. Ovarian fragments were surgically removed from frogs anesthetized by hypothermia. Fully grown stage VI oocytes were manually dissected into ND-96 medium (5 mM Hepes, 96 mM NaCl, 1 mM MgCl₂, 2 mM KCl, 1.8 mM CaCl₂, pH 7.8, and 10 μg/ml each of penicillin and streptomycin sulfate). The oocytes were allowed to recover overnight in the same...
buffer before further treatment and were always maintained at 20 °C.

For induction of meiotic maturation, groups of 10–30 oocytes were incubated in ND-96 without KCl in the presence of progesterone (15 μM, Sigma) or insulin (7.5 μM, Sigma), or were microinjected into the cytoplasm with 30–60 nl of purified PH domains (in 20 mM Tris/HCl, pH 7.4) or appropriate controls. In coinjection experiments, the oocytes were injected into ND-96 medium for 1–3 h after the first injection before the second treatment was performed. Total volume injected was always kept under 60 nl per oocyte. Control oocytes microinjected with either buffer alone or up to 8 molar excess (6 ng per oocyte) of the 6x His-tagged peptide (MRGSHHHHHHGSKLN) located at the amino terminus of the injected, 6x His-tagged PH domain did not undergo GVBD. Meiotic maturation was assayed by scoring the disappearance of the nucleus (GVBD) in oocytes fixed with 10% trichloroacetic acid. In most cases, the absence of the nucleus correlated with the appearance of a white spot in the animal pole.

Construction of Bacterial Plasmid Expression Vectors—DNA sequences corresponding to the human Sox1 PH domain (hSox1353–564) (Sox1 from ZAP human brain cDNA library, Stratagene), rat IRS-1 (rIRS-1390–1570) (rat liver cDNA library, Stratagene), human βARK (hβARK353–566) clone designated βARK-1, and hβARK553–569 clone designated βARK-3, were synthesized using the polymerase chain reaction as BamHI–HindIII (5′–3′) fragments that were then subcloned into pQE30 plasmid (Qiagen), encoding a six-histidine amino-terminal tag (40). Human PLC-δ1, PH domain (hPLC-δ1311–345) was generated by polymerase chain reaction as a NotI–EcoRI (5′–3′) DNA fragment that was subcloned into pET19b, encoding a ten-histidine tag at the amino terminus (15). The integrity of the PH-containing fragments was confirmed with the presence of a white spot in the animal pole.

PH Domain Expression and Purification—PH domain fusion proteins from hSox1, rIRS-1, and βARK were expressed in M15 Escherichia coli cells harboring plasmid pREP4 (Qiagen). Purification protocol was essentially as follows. Briefly, bacterial cells induced with 0.15 mM isopropyl-1-thio-β-D-galactopyranoside for 2 h were harvested and suspended in 20 mM Tris/HCl, pH 7.5, containing 8 mM urea, 1 mM dithiothreitol, and 2 mM phenylmethylsulfonyl fluoride. Supernatant material extracted by shaking for 1 h at room temperature was passed through a Ni-NTA resin column (Qiagen), and the proteins retained were eluted using 250 mM imidazole buffer (pH 6.3). Eluted protein fractions containing the purified PH domains were then submitted to stepwise dialysis in 20 mM Tris/HCl buffer (pH 7.5) containing 1 mM dithiothreitol and decreasing concentrations of urea. If needed, the peptides were further purified by fast protein liquid chromatography (Pharmacia) using a mono S column. The purified domains equilibrated in 20 mM Tris/HCl buffer (pH 7.5) in the absence of urea were finally concentrated using Centricon 3 filters (Amicon). The PH domain of PLC-δ1 was expressed and purified as described previously (15). Peptides purified for injection were always more than 95% homogenous as judged by SDS-polyacrylamide gel electrophoresis. Since freezing frequently resulted in aggregation and loss of material, purified PH domain aliquots were stored at 4 °C for several weeks without appreciable loss of biological activity.

RESULTS

Isolated Sox1 PH Domain Induces GVBD in Xenopus Oocytes—Histidine-tagged polypeptides representing the PH domains of Sox1, PLCδ1, IRS-1, and βARK were bacterially expressed, purified (see “Experimental Procedures” and Fig. 1A) and injected into full-grown Xenopus oocytes to test their biological activity.

Control injections with buffer or the His-containing amino-terminal peptide tag present in all injected peptides did not produce any effect in the oocytes (Fig. 1B). Injecting the purified PH domains of PLCδ1, IRS-1, and βARK did not produce any effect either (not shown). In contrast, various different preparations of a purified, 14.8-kDa peptide representing the PH domain of hSox1 (amino acids 450–569) (Fig. 1A) consistently resulted in GVBD induction, with a kinetics of maturation slightly slower than that induced by the hormones progesterone or insulin (Fig. 1B).

The ability of Sox1 PH to induce GVBD was also proportional to the concentration of injected peptide (Fig. 2). Fig. 2A shows a representative experiment showing the kinetics of GVBD produced by increasing amounts of Sox1 PH domain. The percentage of GVBD obtained correlated exponentially with the concentration of injected Sox1 PH peptide (Fig. 2B).

We also observed that cycloheximide treatment of the oocytes prior to injection resulted in complete blockade of the ability of injected Sox1 PH to induce GVBD (not shown), indicating that, as for progesterone- and insulin-induced meiotic maturation, protein synthesis may be required for completion of the process of GVBD induced by the injected Sox1 PH domain.

Sox1 PH Cooperates with Insulin but Not Progesterone in Induction of GVBD—Xenopus oocytes possess at least two distinct pathways (initiated by progesterone or insulin/IGF-1, respectively) that eventually converge in activation of the maturation-promoting factor kinase complex and subsequent GVBD (23–25). To determine if the Sox1 PH domain could interact with components of any of these pathways, oocytes were microinjected with either Sox1 PH domain or buffer alone and then treated with either insulin or progesterone. As shown in Fig. 3A, microinjected Sox1 PH domain produced a marked acceleration in the rate of GVBD induced by insulin. In contrast, no alteration of the rate of GVBD induced by progesterone was observed after microinjection of Sox1 PH domain (Fig. 3B).
synergistic effect of Sos1 PH and insulin was most obvious when suboptimal concentrations of the hormones (resulting in a delayed start of meiotic maturation) were used. Fig. 3, A and B, presents results obtained using, respectively, 1/10 and 1/200 of the standard optimal concentrations of insulin and progesterone normally used in oocytes.

The ability to stimulate insulin-induced GVBD (tyrosine kinase-dependent pathways) but not progesterone-induced GVBD (adenylate cyclase-dependent signaling pathways) suggests that the purified Sos1 PH domain retains structural and functional specificity, allowing it to interact with some still unidentified component of the tyrosine kinase signaling pathway involved in insulin induction of GVBD.

Sos1 PH Cooperates with Normal Ras Proteins and Is Blocked by Asn-17 Ras to Induce GVBD—Coinjection of Sos1 PH, but not any of the other PH domains tested, with normal c-Ras (Gly-12) protein (at a concentration at which c-Ras does not induce maturation; see Fig. 4) resulted in a rate of maturation that was very significantly accelerated over that produced by the microinjected Sos1 PH protein alone (Fig. 4). Since Ras proteins are essential elements of insulin signaling pathways in oocytes (33–35), these results are clearly consistent with the synergistic effects observed between insulin and Sos1 PH domain.

The synergistic effect between injected c-Ras (Gly-12) and Sos1 PH domains suggests that normal Ras and Sos1 PH domain induce mutually potentiating signals in Xenopus oocytes, which may either overlap or converge through common downstream effectors. This view was further confirmed when the effect of dominant negative Ras (Gly-12, Asn-17) proteins on the maturation produced by the Sos1 PH domain was studied (Fig. 5). As shown in this figure, under experimental conditions where insulin-induced maturation was blocked by microinjected, dominant negative Ras (Asn-17), the maturation induced by Sos1 PH domains was also totally blocked by the dominant negative Ras form. These results suggest that the Sos1 PH domain is acting upstream of Ras in the signaling pathway(s) involved in insulin-induced oocyte maturation.

**DISCUSSION**

We report in this paper that the isolated, purified PH domain of Sos1 exhibits functional biological activity in signal transduction processes involved in *X. laevis* oocyte maturation. Interestingly, a variety of PH domains from various other signaling molecules that were bacterially expressed and purified under similar conditions did not show any biological effect when injected in the oocytes, suggesting that the observed effect is specific to the Sos1 PH domain. Different PH domains appear to share a common tertiary structure, although the conservation of their primary sequence is rather limited (2–6). In this regard, the present results suggest that, in addition to the tertiary structure, the primary structure is an essential factor responsible for the observed biological activity. A variety of proteins able to interact with phosphoinositides through their PH domains share significant primary structure homology in regions around the β1-β2 and β3-β4 variable loops, which contain key residues involved in such interactions. Of particular importance appear to be a lysine (K) and a tryptophan (W) residue located, respectively, in the β1 and β2 regions, which are conserved in all inositide-interacting PH domains identified so far (13, 14, 16). Interestingly, the primary structure of Sos1 PH differs significantly from such inositide-binding consensus sequence (Fig. 6). This observation suggests that interactions with molecules other than phosphoinositides may account for the biological activity exhibited by Sos1 PH domain in oocytes. Consistent with this, preliminary in vitro experiments with the purified domains showed that, in sharp contrast to the PLCβ2 PH domain, the Sos1 PH was unable to interact with phosphoinositides using a liposome binding assay described previously (15) or a photocross-linking binding assay.2 On the other hand, it has also been shown that, in comparison with the other βARK and IRS-1 PH domains tested here, our purified Sos1 PH domain (i) binds negligibly to Gβγ
subunits and (ii) its CD spectrum reveals much less \( \alpha \)-helix content (40). These observations suggest that the Sos1 PH domain may be significantly divergent functionally from PH domains belonging to other signaling molecules. Our observation that only the Sos1 PH domain, but not any other PH domains belonging to other signaling molecules, exhibits biological activity in Xenopus oocytes suggests that the Sos1 PH domain may be significantly divergent functionally from PH domains involved in insulin signaling pathways in oocytes.

The injected Sos1 PH domain induced significant rates of GVBD that were only slightly slower than those produced by activated Ras oncogenes and about 60–70% of those produced by hormones. Furthermore, Sos1 PH-induced GVBD appeared to require protein synthesis since preincubation with cycloheximide blocked such process. A similar protein synthesis requirement has been previously described for hormone-induced GVBD (23–25, 32).

The ability of Sos1 PH domain to cooperate with insulin, but not progesterone, in induction of GVBD is noteworthy. Whereas injected Sos1 PH domain did not modify the kinetics of GVBD induced by various concentrations of progesterone, it showed a clear cooperative effect with insulin, in which both the speed of maturation and the percentage of GVBD were increased. This behavior suggests that the injected PH domain is not able to affect the adenylate cyclase-dependent signaling pathways involved in progesterone maturation but can probably interact with components of the tyrosine kinase-dependent signaling cascade(s) involved in insulin-induced maturation.

This view was further supported by the markedly synergistic effect observed when both Sos1 PH and normal Ras protein (Gly-12) which cannot induce maturation by itself were coinjected into the oocytes. We have previously observed a similar synergism between microinjected c-Ras protein and an isolated Vav SH2 domain (39). Since Ras proteins are thought to be essential components of insulin signaling pathways in oocytes (33–35), the cooperation of Sos1 PH with both insulin and normal Ras proteins is consistent. Such behavior would be consistent with models whereby the Sos1 PH domain is activating either some integral component of the insulin/Ras pathway itself or an alternative route eventually converging onto it. The observation that coinjection of a dominant negative (Asn-17) Ras protein completely blocked oocyte GVBD induced by the PH domain suggests that the first possibility is more likely and that the Sos1 PH domain is probably acting upstream of Ras in the insulin/Ras signaling cascade. A role of the Sos1 PH domain as a positive regulator of Ras signaling is also consistent with recent reports, indicating that the amino-terminal region of Sos1 encompasses the PH and DH domains is necessary to promote Sos catalytic domain activity in mammalian cells (22) and adequate R7 cell development in Drosophila (41).

If the isolated Sos1 PH domain retains the same specificity for molecular interactions that it has when it is integrated within the native Sos1 protein molecule, an interesting possibility is that the injected, isolated PH domain could be acting by up-regulating the endogenous Sos1 activity responsible for activating cellular Ras. An attractive hypothesis would be that the injected PH domain might be acting by titrating out endogenous Sos1 inhibitor(s), thus resulting in cellular Ras activation and subsequent GVBD. We should be able to test such a hypothesis by identifying endogenous molecule(s) with which the Sos1 PH domain interacts after injection into the oocytes.

Fig. 6. Sequence alignment and comparison of injected PH domains. Structure-based sequence alignment of PH domains used in this study is shown. Amino acid residues represented here correspond to positions hSos1148–396, hRbR115–189, hARK452–515, and hPLC-850–1152. Position of the main amino acid residues involved in the interaction of PLC with the PH domains (bold, large fonts) is indicated by the asterisks. Consensus amino acid residues conserved in most PH domains (1, 2) are shown in bold. PH secondary structure, consisting of seven \( \beta \) sheets and one \( \alpha \)-helix, is represented by boxes located under the primary sequence.

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