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Regulation of ASAP1 by phospholipids is dependent on the interface between the PH and Arf GAP domains

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Abstract

ASAP1 is an Arf GAP with a PH domain immediately N-terminal to the catalytic Arf GAP domain. PH domains are thought to regulate enzymes by binding to specific phosphoinositide lipids in membranes, thereby recruiting the enzyme to a site of action. Here, we have examined the functional relationship between the PH and Arf GAP domains. We found that GAP activity requires the cognate PH domain of ASAP1, leading us to hypothesize that the Arf GAP and PH domains directly interact to form the substrate binding site. This hypothesis was supported by the combined results of protection and hydrodynamic studies. We then examined the role of the PH domain in the regulation of Arf GAP activity. The results of saturation kinetics, limited proteolysis, FRET and fluorescence spectrometry support a model in which regulation of the GAP activity of ASAP1 involves a conformational change coincident with recruitment to a membrane surface, and a second conformational change following the specific binding of phosphatidylinositol 4,5-bisphosphate.

Keywords: ADP-ribosylation factor; PH domains; GTPase-activating proteins

1. Introduction

Pleckstrin homology (PH) domains are common to many signalling and membrane trafficking proteins (reviewed in [1–6]). This protein motif is comprised of approximately 100 amino acids and, although the primary sequences of different PH domains often have low levels of identity, the protein fold is conserved. PH domains form a seven-stranded β sandwich capped by an α-helix. Basic residues on several loops cluster into a patch of positive charge, which mediates binding through electrostatic interactions to both lipids and proteins. The ability to bind specific components of membranes is central to the proposed functions of PH domains.

One function that has been extensively examined is site-specific targeting through binding of low abundance

Abbreviations: DH, dbl homology; FRET, fluorescence resonance energy transfer; GAP, GTPase-activating protein; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); NHS, *N*-hydroxysuccinimide; PA, phosphatidic acid; PdZA, a chimeric protein consisting of the PH domain of PLC δ1 and the Arf GAP and ANK repeat domains of ASAP1; PH, pleckstrin homology; PI, phosphatidylinositol; PI(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PI(3,4)P2, phosphatidylinositol 3,4-bisphosphate; PI(3,5)P2, phosphatidylinositol 3,5-bisphosphate; PI(3,4,5)P3, phosphatidylinositol 3,4,5-triphosphate; PLC, phospholipase C; PS, phosphatidylserine; PZA, protein comprised of the PH, Arf GAP and ANK repeat domains of ASAP1; PZA2, protein comprised of PH, Arf GAP and ANK repeat domains of ASAP2/PAP; ZA, protein comprised of Arf GAP (which contains zinc binding motif) and ANK repeats of ASAP1.

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membrane components derived from the phosphorylation of phosphatidylinositol (PI) [1,3,5]. Kinases that catalyze the production of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2), phosphatidylinositol 3,4-bisphosphate (PI(3,4)P2), phosphatidylinositol 3,5-bisphosphate (PI(3,5)P2) and phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P3) are regulated. Production of a particular phosphoinositide within a membrane compartment results in the recruitment of proteins containing PH domains specific for that phosphoinositide [4,7–14]. Localization dependent on PH domain interaction with specific proteins has also been studied [15–17]. For instance, the PH domain of PLC β2 and PLC β3 binds Rac•GTP [18] and the PH domain of four phosphate adaptor protein (FAPP) binds Arf1•GTP [17]. Coincident binding of lipids and proteins may increase the spatial and temporal specificity of targeting.

Studies of guanine nucleotide exchange factors for Rho-family proteins support the idea that PH domains have roles in addition to a targeting function [19–21]. Rho-family GTP-binding proteins [22–24] are members of the Ras superfamily that regulate actin cytoskeleton remodeling and cell growth. The function of Rho proteins depends on the controlled binding of GTP [25–27], which, in turn, is catalyzed by guanine nucleotide exchange factors. Those exchange factors specific for Rho-family proteins contain a catalytic dbl-homology (DH) domain and, invariably, a PH domain occurs C-terminal to it [19,20]. Crystallographically determined structures reveal that the PH domain folds together with the DH domain and, further, that the PH domain contributes residues that interact with the substrate Rho family protein [20], so it can be considered to form part of the substrate binding site.

AZAP family Arf GAPs are another example of proteins containing PH domains that may function in a capacity other than site-specific targeting. The substrates of Arf GAPs are Arfs, which, like Rho proteins, are members of the Ras superfamily of GTP binding proteins [28–31]. Arfs regulate membrane traffic and also contribute to actin remodeling [32–34]. As for other Ras family proteins, the function of Arfs is dependent on the controlled binding and hydrolysis of GTP. Because Arfs have no detectable intrinsic GTPase activity, GTPase-activating proteins (GAPs) are critical to their function. The first Arf GAP identified, Arf GAP1, was found to contain a catalytic domain comprised of a zinc finger motif [35,36] and subsequently, 24 genes have been found to encode the Arf GAP domain [37,38]. Of these, the products of 6 gene subtypes have been found to have GAP activity. Two subtypes, Arf GAP1/3 and Git1/2, have the catalytic domain at the N-terminus of the proteins; four subtypes (ASAPs, ACAPs, AGAPs and ARAPs), collectively called AZAPs, have a PH domain immediately N-terminal to the Arf GAP domain. The PH domain of AZAP proteins has a critical role in regulating Arf GAP activity. The most extensively studied, in this regard, is ASAP1, which was first identified as a PI(4,5)P2-dependent Arf GAP. Efficient stimulation of GAP activity was found to require PI(4,5)P2 in the context of an acidic lipid environment that could include phosphatidylserine or phosphatidic acid [39,40]. A combination of PA and PI(4,5)P2 activates ASAP1 10,000-fold [40].

The PH domain of ASAP1 was also found to be necessary for the protein to have any GAP activity, independent of the presence of phosphoinositides. Truncation of the PH domain did not appear to affect the folding of the Arf GAP domain. ASAP1 truncation mutants were soluble and did not have aberrant chromatographic behavior. Furthermore, the Arf GAP and ANK repeats of an ASAP1 homolog, ASAP2/PAP, were found to be structurally similar to Arf GAP1 in crystallographic analysis [41]. However, ASAP1 and PAP mutants lacking the PH domain had less than 10^{-5} the activity of protein with the PH domain [40]. The activities of other AZAPs, including ACAP1, ARAP1 and AGAP1, are also highly dependent on the presence of the PH domain immediately N-terminal to the Arf GAP domain. These results have led us to consider that the PH domain of ASAP1 may interact with the Arf GAP domain to form the catalytic interface with the substrate Arf1•GTP, in a manner similar to that described for Rho GEFs [20]. With the PH and Arf GAP domains closely apposed, we also propose that conformational changes in the PH domain that occur on binding the ligand [42] could be directly transmitted to the Arf GAP domain.

Here we test the hypotheses that (i) an interface between the PH and Arf GAP domains contributes to formation of the substrate binding site, and that (ii) PIP2 binding to the PH domain of ASAP1 induces a conformational change resulting in stimulation of GAP activity. We tested for an interface using protection assays, modeling and hydrodynamic analysis. Conformational changes were detected by changes in protease sensitivity, FRET and fluorescence spectrometry. The results are consistent with a model in which ASAP1 is recruited to a surface with a coincident conformational change. On specifically binding PIP2, ASAP1 then undergoes an additional conformational change that results in maximum stimulation of GAP activity.

2. Experimental procedures

2.1. Plasmids

Bacterial expression vectors for Arf1 [43], [L8K]Arf1 [44], [338–431]ASAP1 (PH), [325–724]ASAP1 (PZA) and [452–724]ASAP1 (ZA) [40] have been previously described. An expression vector for a chimeric protein consisting of residues 1 to 134 of PLC δ1 [45] and residues 441 to 724 of ASAP1 was constructed by amplifying the reading frame encoding residues 441 to 724 of ASAP1 with *Nde*I and *Not*I restriction sites on the 5′ end of the ORF and