Phosphatidylinositol polyphosphate binding to the mammalian septin H5 is modulated by GTP

Jianshe Zhang*, Chen Kong*, Hong Xie*, Peter S. McPherson†, Sergio Grinstein* and William S. Trimble*

Background: Septins are members of a conserved family of GTPases found in organisms as diverse as budding yeast and mammals. In budding yeast, septins form hetero-oligomeric filaments that lie adjacent to the membrane at the mother–bud neck, whereas in mammals, they concentrate at the cleavage furrow of mitotic cells; in both cases, septins provide a required function for cytokinesis. What directs the location and determines the stability of septin filaments, however, remains unknown.

Results: Here we show that the mammalian septin H5 is associated with the plasma membrane and specifically binds the phospholipids phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) and phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3). Deletion analysis revealed that this binding occurs at a site rich in basic residues that is conserved in most septins and is located adjacent to the GTP-binding motif. Phosphoinositide binding was inhibited by mutations within this motif and was also blocked by agents known to associate with PtdInsP_2 or by a peptide corresponding to the predicted PtdInsP_2-binding sequence of H5. GTP binding and hydrolysis by H5 significantly reduced its PtdInsP_2-binding capability. Treatment of cells with agents that occluded, dephosphorylated or degraded PtdInsP_2 altered the appearance and localization of H5.

Conclusions: These results indicate that the interaction of septins with PtdInsP_2 might be an important cellular mechanism for the spatial and temporal control of septin accumulation.

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Background

Septins are a family of proteins that were first identified in yeast as being required for the completion of the cell cycle [1–3]. Four septin proteins in the budding yeast *Saccharomyces cerevisiae*, CDC3, CDC10, CDC11 and CDC12, were all identified in mutants defective in the cell cycle that had a similar phenotype, namely abnormally elongated buds unable to complete cytokinesis [4]. Mutations in each of the genes encoding these proteins also disrupt a characteristic set of filaments, which closely associate with the plasma membrane in a series of concentric rings around the mother–bud neck [5,6]. Coupled with immunocytochemical studies, these observations indicate that septins form hetero-oligomeric filaments that contribute to bud-site selection and neck stability (reviewed in [2,3]). Demonstration that the *Drosophila melanogaster* cytokinesis mutation *peanut* occurs in a septin protein revealed that animal cells undergoing fission also require septins for this process [7]. Like yeast, *Drosophila* also have multiple septin isoforms, including Sep1 [8] and Sep2, which appear to assemble into hetero-oligomeric filaments [9] required for the completion of cytokinesis.

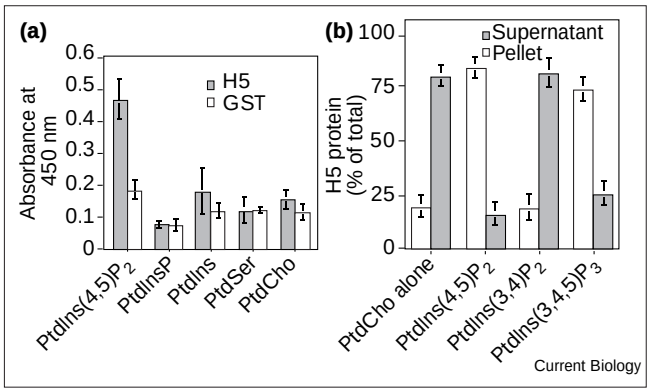
Ten septin isoforms have been identified to date in mammals. These are Nedd5 [10], hCDC10 [11], KIAA0128 [12], KIAA0202 [13], Diff6 [14], hCDCrel-1/PNUTL1 [15,16], eSEP [17], G-SEP (GenBank accession number AF111179), KIAA0991 [18] and H5 [19]. Most of these were identified through random sequencing strategies, subtractive screens or as candidate genes within disease loci. All septins share in common the presence of a GTP-binding domain near the amino terminus and almost all contain a predicted coiled-coil motif near the carboxyl terminus of the protein. The mammalian isoforms share between 41% and 76% amino acid sequence identity, with highest identity in the GTP-binding domain.

With the exception of Nedd5, very little is known about the function of mammalian septins. Nedd5 is broadly expressed and has been shown to concentrate at the cleavage furrow of mitotic cells. Mutations that prevent this protein from binding GTP disrupt its filamentous appearance in interphase cells. Microinjection of antibodies to Nedd5 impairs cytokinesis, as shown by the accumulation of multinucleated cells [20]. H5 is less well characterized but has many properties in common with Nedd5. We have

found that, in contrast to initial predictions based on its isolation from brain, H5 is also broadly expressed, with highest expression levels in the nervous system and significant expression levels in most tissues [21].

Preliminary fractionation studies have shown that both Nedd5 and H5 associate predominantly with membranes [21]. The mechanism underlying this association is obscure, because septins lack transmembrane domains and other obvious membrane-localization motifs. As an initial approach to establish how septins associate with membranes, we determined whether H5 could associate directly with phospholipids. Here, we show that H5 binds directly to phosphatidylinositol (4,5) bisphosphate (PtdIns(4,5)P_2) and phosphatidylinositol (3,4,5) trisphosphate (PtdIns(3,4,5)P_3) but not to a variety of other phospholipids including PtdIns(3,4)P_2. This interaction was found to occur at a site adjacent to the GTP-binding domain of H5 that is rich in lysine residues and similar to the PtdInsP$_2$-binding motifs found in several other proteins. Association of H5 with GTP decreased its binding to PtdIns(4,5)P_2, suggesting that functional interactions occur between its nucleotide-binding and lipid-binding domains. In cells treated with agents that block or hydrolyse PtdInsP$_2$, H5 filaments were reduced and cytokinesis, a cellular process requiring septin function, was blocked. These results suggest that PtdIns(4,5)P_2 and/or PtdIns(3,4,5)P_3 might be important in regulating septin localization and function.

Figure 1



Binding of PtdInsP$_2$ to the septin H5. (a) H5 binds to PtdIns(4,5)P_2 but not to several other phospholipids. An aliquot (1 μg) of PtdIns(4,5)P_2, PtdIns(4)P, PtdIns, PtdSer or PtdCho was dried onto an ELISA plate, washed extensively and then incubated with buffer containing either 1 μg GST or 1 μg of a fusion protein comprising GST and full-length H5 (H5). Bound proteins were detected with an anti-GST antibody and measured by ELISA using a secondary antibody conjugated with horseradish peroxidase. Quantitation of bound protein was determined by measuring absorbance at 450 nm. Values shown are the means ± standard errors (SEs) from triplicate experiments. (b) H5 specifically binds to PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3. A liposome sedimentation assay was used in which 30 μg of liposomes comprising 95% PtdCho and 5% of the indicated lipid were incubated with 1 μg H5 protein, then centrifuged to separate the bound (pellet) protein from the unbound (supernatant). Western blots were performed for each fraction from three independent experiments and quantified by densitometry.

Results

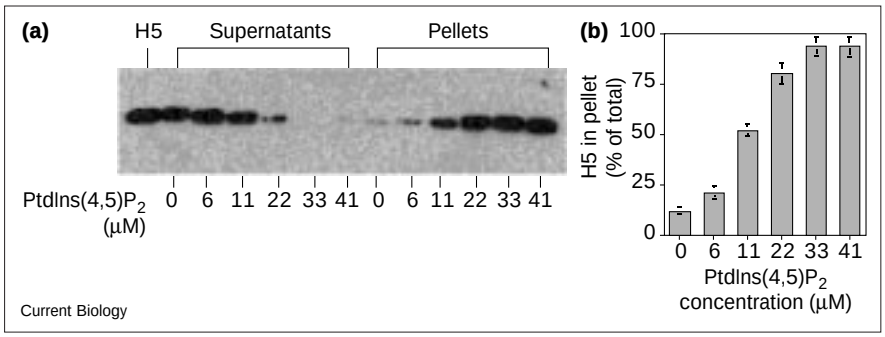
In yeast, septins appear to be tightly associated with the membrane of the mother–bud neck [5]. Our preliminary biochemical fractionation studies also suggest an association of mammalian septins with membranes [21]. To investigate the nature of this association, we examined the ability of the septin H5 to bind directly to membrane phospholipids. To this end, we used an enzyme-linked immunoabsorbent assay (ELISA) in which phospholipids were dried on solid substrates and incubated with recombinant H5 expressed as a glutathione-S-transferase (GST) fusion protein [22]. To control for nonspecific interactions, GST alone was also incubated with the phospholipids. Bound protein was detected by probing for the presence of GST with antibodies. As shown in Figure 1a, GST–H5 bound significantly better to PtdIns(4,5)P_2 than to phosphatidylinositol (4)monophosphate (PtdIns(4)P), phosphatidylinositol (PtdIns), phosphatidylcholine (PtdCho) or phosphatidylserine (PtdSer). GST alone bound very poorly to all of the lipids tested.

Dried lipids on a solid substrate might not represent the configuration of lipids found in biological membranes. Therefore, to more faithfully reconstitute the lipid environment of a membrane, and to further define the specificity of lipid binding to H5, we performed binding experiments using a liposome sedimentation assay [23]. As the ELISA assays suggested that H5 bound only marginally to PtdCho, we generated mixed liposomes containing 95% PtdCho and 5% of either PtdIns(3,4)P_2, PtdIns(4,5)P_2 or PtdIns(3,4,5)P_3. After incubation with recombinant H5, the liposomes were sedimented and the distribution of the protein between pellet and supernatant fractions was assessed by immunoblotting. As shown in Figure 1b, significant amounts of H5 associated with liposomes containing PtdIns(4,5)P_2 or PtdIns(3,4,5)P_3, but not with those composed of PtdCho alone or PtdCho and PtdIns(3,4)P_2.

The affinity of H5 for PtdIns(4,5)P_2 was also estimated using the sedimentation assay described above. A representative example is shown in Figure 2a and demonstrates that, when increasing concentrations of PtdIns(4,5)P_2 were included in the liposome mixture, the efficiency of H5 sedimentation increased proportionally. When the results of three independent experiments were quantified (Figure 2b), we found that H5 bound to PtdIns(4,5)P_2 with an apparent dissociation constant of approximately 10 μM.

PtdInsP$_2$ has been observed to interact with proteins containing pleckstrin homology (PH) domains, often promoting their association with the membrane [24]. Perusal of

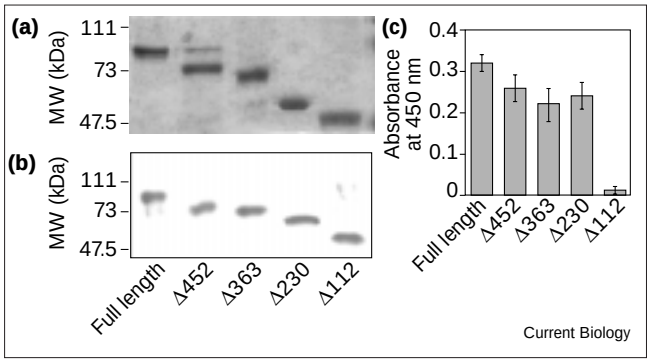
Figure 2



Saturable binding of H5 to PtdIns(4,5)P_2. (a) Western blot of a liposome sedimentation assay in which increasing concentrations of PtdIns(4,5)P_2 were used. Liposomes comprising PtdCho and increasing concentrations of PtdIns(4,5)P_2, as indicated, were incubated with 1 μg H5, then centrifuged to separate the bound (pellet) protein from the unbound (supernatant). (b) Quantitation of the sedimentation assays shown in (a). Western blots were performed for the pellet fractions from three independent experiments and quantified by densitometry. Values represent the mean ± SE from triplicate experiments.

the sequence of the septin H5 failed to reveal sequences related to PH domains, however. Nevertheless, PtdInsP_2 binding has also been demonstrated in many proteins lacking PH domains, suggesting that other protein motifs might also confer this property. A deletion analysis of gelsolin was the first to demonstrate that PtdInsP_2 can bind to sequences rich in basic amino acids, and that this binding is specific [25–30]. Like many other highly charged proteins, H5 contains a number of motifs that have a polybasic nature similar to the PtdInsP_2-binding site found in gelsolin. To determine whether any of these motifs were responsible for PtdInsP_2 binding by H5, we generated a series of GST–H5 constructs expressing successively truncated versions of the protein. Each of these constructs yielded stable proteins when expressed in bacteria, and the resulting purified proteins stained with Coomassie Brilliant Blue are shown in Figure 3a. For construct Δ452, in which the H5 protein was truncated after amino acid 452, a faint contaminating product was occasionally observed at the molecular weight of the full-length protein. This is, however, an immunologically unrelated product of unknown origin, as shown by western blot analysis (Figure 3b). As shown in Figure 3b, each of the truncated proteins reacted with an antibody raised to the amino terminus of H5, as would be expected given that even the shortest fragment contains the immunogenic motif.

Figure 3

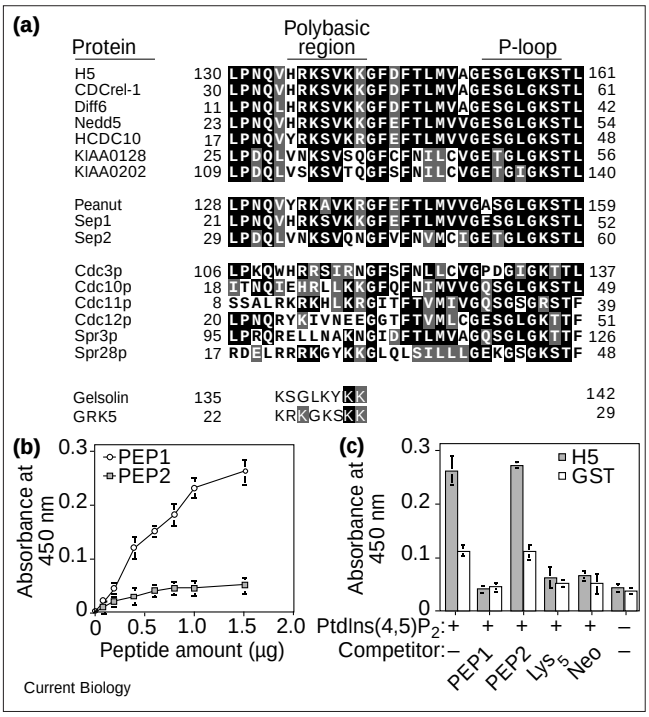


Deletion mapping of the PtdIns(4,5)P_2-binding site on H5. A series of carboxy-terminal truncations were constructed in the H5 coding sequence and expressed as GST fusion proteins. In each case, the name of the truncated protein represents the amino acid at which the protein terminated. (a) Purified GST–H5 and truncation mutants stained with Coomassie Brilliant Blue. Equivalent masses of fusion protein were electrophoresed and stained to demonstrate purity and stability of truncated forms. (b) Western blotting of truncated forms. Equal concentrations of GST–H5 and truncated proteins were electrophoresed, blotted and probed with an antibody generated against a peptide identical to amino acids 29–48 of H5. This peptide sequence is present in all truncated forms. (c) Binding of PtdIns(4,5)P_2 to truncated forms. ELISA was used to assay the binding of 1 μg PtdInsP_2 to 1 μg of each truncated protein, as described in Figure 2a. PtdIns(4,5)P_2 binding was unaffected until the carboxy-terminal truncations reached amino acid 112. Values represent the mean ± SE from triplicate experiments.

Each truncation product was then used in PtdInsP_2-binding ELISA assays similar to those shown in Figure 2a. As shown in Figure 3c, PtdIns(4,5)P_2 bound to all of the truncation mutants except Δ112, indicating that the binding site must reside between amino acids 112 and 230. Within these 118 residues lies a highly basic sequence between amino acids 135 and 141 that is very similar to polybasic sequences found in gelsolin and in members of the GRK4/5/6 family [26,29]. Related poly-basic sequences are found at the same location in septins from *Drosophila* and yeast, and in almost all of the mammalian septins (Figure 4a) [2].

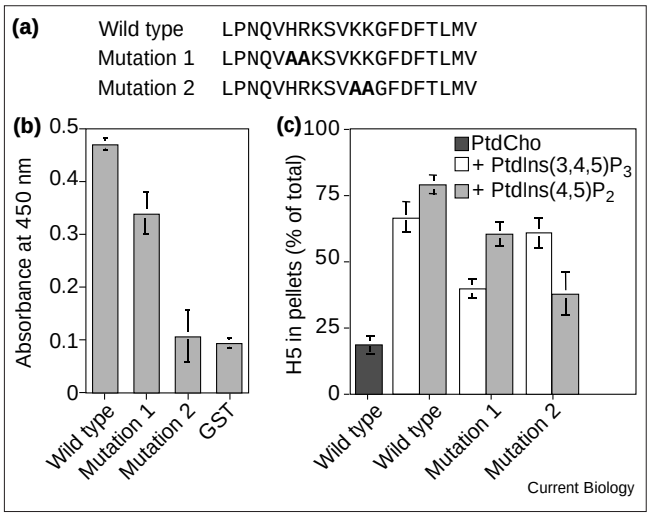
To determine whether this polybasic sequence is indeed capable of binding PtdInsP_2, a synthetic peptide was produced that corresponded to this region of the protein, and an unrelated sequence within H5 was used as a control. Various concentrations of these peptides were then immobilized on the surface of the ELISA plate and PtdInsP_2 binding was measured as before. As shown in Figure 4b, the peptide corresponding to the polybasic region (PEP1) bound PtdInsP_2 avidly, whereas the control peptide (PEP2)

Figure 4



A polybasic region in H5 can bind PtdIns(4,5)P_2. (a) Alignment of known septins reveals conservation of a polybasic motif near the P-loop sequence. Amino acid sequences of known mammalian (top group), *Drosophila* (middle group) and *S. cerevisiae* (bottom group) septins is shown. Aligned regions are indicated by amino acid numbers to the left and right. Identical amino acids are white on a black background, whereas those on a grey background are similar. The proximity of the polybasic and P-loop motifs are indicated above. Comparable polybasic motifs from gelsolin and GRK5 are indicated below. (b) A peptide derived from amino acids 136–149 (PEP1) but not a control peptide (PEP2) bound PtdIns(4,5)P_2 directly. ELISA wells coated with increasing amounts of PEP1, as indicated, displayed high PtdIns(4,5)P_2-binding capability, whereas wells coated with the same amount of PEP2 showed little if any binding. (c) The H5 polybasic sequence is an effective inhibitor of PtdIns(4,5)P_2 binding. Competitive inhibition of PtdIns(4,5)P_2 binding was performed by premixing the PtdIns(4,5)P_2 micelles with the indicated inhibitors before incubation with the immobilized GST–H5 or GST protein. PEP1 was as effective in competing for PtdIns(4,5)P_2 binding as the known PtdIns(4,5)P_2-binding agents pentalysine (lys_5) and neomycin (neo). Values represent the mean ± SE from triplicate experiments.

Figure 5

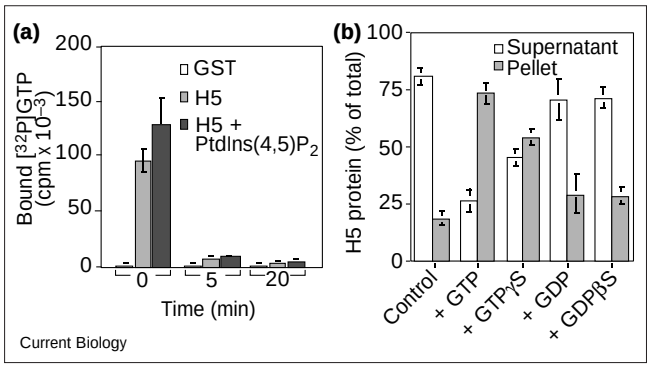


Distinct residues are important for PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 binding. (a) Site-directed mutagenesis strategy. Two mutations were generated that substituted alanine residues for residues 136 and 137 (mutation 1) or residues 140 and 141 (mutation 2). (b) PtdIns(4,5)P_2 binding is impaired by mutation 2. Using an ELISA assay, the amount of micellar PtdIns(4,5)P_2 bound to immobilized wild-type H5 or to the mutant proteins is shown. Values represent the mean ± SE from triplicate experiments. (c) Differential effects of mutations on PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 binding. Liposomes comprising PtdCho alone, or 95% PtdCho and 5% of either PtdIns(4,5)P_2 or PtdIns(3,4,5)P_3 were incubated with wild-type H5 or H5 mutants, then sedimented as described in the Materials and methods. The pellets were blotted for bound H5 protein and triplicate experiments were quantified. Binding of wild-type H5 to liposomes comprising 100% PtdCho was used as a control and is shown in the first lane (n = 6).

failed to bind. To determine whether the peptide could also bind to PtdInsP_2 in solution, an inhibition assay was performed. In this case the peptides, or other binding inhibitors, were mixed with the PtdInsP_2 micelles before their exposure to the immobilized full-length GST–H5 protein. To confirm that this assay could detect competitive inhibition, known antagonists of PtdInsP_2 binding were used. As shown in Figure 4c, both pentalysine [31–33] and neomycin [34] were effective at inhibiting the interaction of PtdInsP_2 with H5. The polybasic peptide (PEP1) also completely blocked the binding of PtdInsP_2 to H5, whereas a comparable concentration of the control peptide had no effect. Together, these results indicate that the polybasic sequence found between residues 135 and 141 of H5 is an efficient binding site for PtdIns(4,5)P_2.

We next used site-directed mutagenesis to demonstrate that the region encompassed by residues 135 and 141 was necessary for binding of PtdIns(4,5)P_2 to H5. Two different mutants were generated that converted pairs of basic amino acids to alanine residues, as shown in Figure 5a. Recombinant GST–H5 proteins containing these mutations were then immobilized on plates and used in an ELISA micellar assay, as described in Figure 2a, to quantify PtdIns(4,5)P_2 binding to the wild type and mutant forms of H5. As seen in Figure 5b, mutation of the arginine and lysine residues at positions 136 and 137 (mutation 1) had only a modest effect on the binding, whereas replacement of the lysines at positions 140 and 141 (mutation 2) ablated binding. The liposome sedimentation assay was used to compare the relative effects of the mutations on binding to PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3. In accordance with the ELISA assay, the amount of H5-mutation 2 found to associate with PtdIns(4,5)P_2 was markedly reduced, whereas the

Figure 6



(a) Binding and hydrolysis of GTP by H5 are not affected by PtdIns(4,5)P_2. Glutathione–agarose beads bound with either GST, GST–H5 or GST–H5 and PtdIns(4,5)P_2 were incubated with [γ-^{32}P]GTP on ice, then washed, resuspended in hydrolysis buffer and immediately assayed for bound [γ-^{32}P]GTP (0 min), or incubated for 5 or 20 min at 37°C before quantitation. Values represent the mean ± SE from quadruplicate experiments. (b) PtdIns(4,5)P_2 binding is reduced when H5 is in its GTP-bound form. Using the sedimentation assay described in Figure 1b, liposomes comprising PtdCho and PtdIns(4,5)P_2 at a ratio of 95:5 were prepared and mixed with 0.8 μg of freshly prepared GST–H5 protein that had previously been incubated with the indicated nucleotide (1 mM final concentration) for 30 min at room temperature. After mixing, the samples were incubated for a further 60 min at room temperature before centrifugation at 50,000 × *g* for 40 min. The supernatants and pellets from each sample were electrophoresed, blotted and probed for H5. The results shown are from three independent experiments. The control indicates the binding of wild-type H5 to liposomes comprising 100% PtdCho.

binding of H5-mutation 1 was intermediate (Figure 5c). In surprising contrast, binding of PtdIns(3,4,5)P_3 was not affected by mutation 2, but was significantly reduced by mutation 1, implying that distinct amino acids contribute to the binding of each lipid.

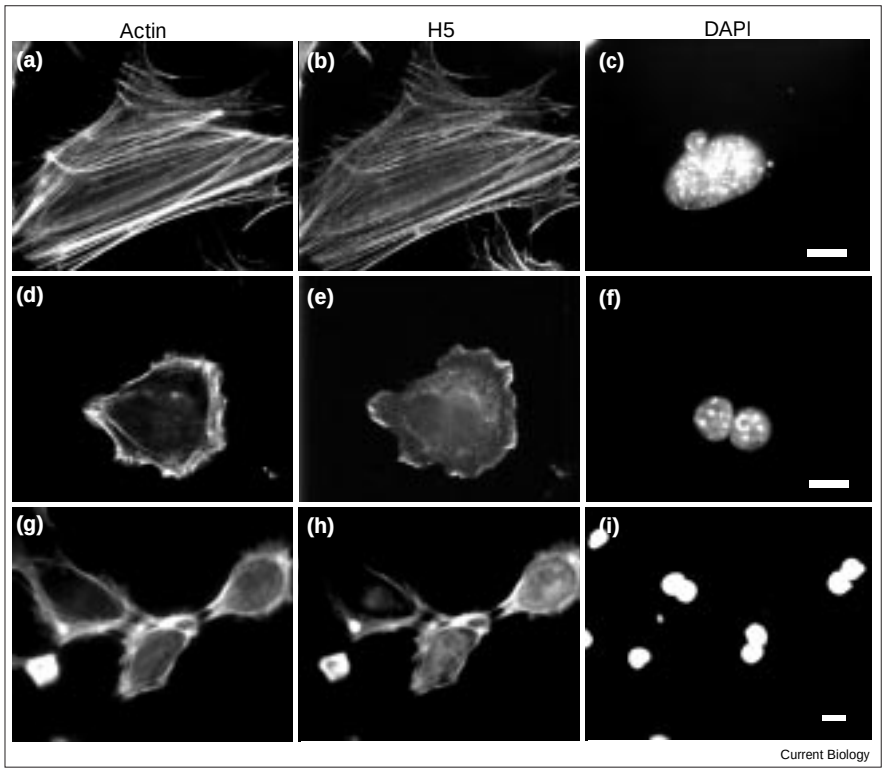
The proximity of the PtdInsP_2-binding motif to the GTP-binding P-loop raised the interesting possibility that PtdInsP_2 binding might influence the ability of H5 to bind or hydrolyze the nucleotide. In order to test this hypothesis, we measured [γ-^{32}P]GTP binding to the septin using the assay of Kinoshita *et al.* [20], in the presence or absence of PtdIns(4,5)P_2. Binding assays were performed by incubating GST–H5 or GST alone, bound to glutathione–agarose beads, with [γ-^{32}P]GTP at 0°C in the presence of EDTA to prevent hydrolysis. After washing, the amount of radioactivity associated with the beads was measured. As shown in Figure 6a, H5 bound significantly more [γ-^{32}P]GTP than the GST control but this binding was not influenced by the presence of PtdInsP_2. To measure hydrolysis of the nucleotide, beads treated in the same way were then washed with buffer containing Mg^{2+} but no EDTA, and warmed to 37°C. Hydrolysis leads to removal of the γ-phosphate from GTP with attendant loss of radioactivity from the beads. To prove that the loss of ^{32}P from the beads was due to hydrolysis and not dissociation, control samples were maintained for 20 min in either hydrolysis buffer at 0°C or in loading buffer at 37°C. Neither condition showed significant loss of ^{32}P during this period (data not shown). As shown in Figure 6a, complete hydrolysis of the bound GTP had occurred within 5 minutes either in the presence or absence of PtdIns(4,5)P_2, suggesting that within the limits of detection of this assay, PtdIns(4,5)P_2 had no effect on either the ability of H5 to bind or to hydrolyze GTP.

We next tested whether the inverse relationship existed, namely, whether PtdInsP_2 binding might itself be affected by binding of nucleotides to H5. To test this possibility, PtdIns(4,5)P_2 binding to H5 was analyzed using the liposome sedimentation assay. Liposomes were mixed with recombinant proteins that had been pre-incubated with the indicated nucleotides. As shown in Figure 6b, GDP and GDPβS had no significant effect on H5 binding. In contrast, GTPγS significantly reduced the amount of H5 found in the pellet. Most surprising was the reduction in binding found when H5 was bound to GTP. In this case, the vast majority of the H5 failed to bind to the liposomes. Together, these observations indicate that the association of H5 with PtdIns(4,5)P_2 is influenced by the nucleotide to which the septin is bound. Furthermore, as GTP inhibited binding more than GTPγS, this suggests that hydro-lysis of GTP might result in membrane dissociation.

If PtdInsP_2 is involved in modulating the association of H5 with the membrane and if this association is essential for its function, it should be possible to interfere with septin function *in vivo* by precluding its binding to PtdInsP_2 using competing compounds. Neomycin is known to permeate cell membranes slowly and to block PtdInsP_2 interactions with proteins. To evaluate whether competitive displacement of PtdInsP_2 would affect the location or filamentous appearance of H5, cells were incubated in 5 mM neomycin sulfate for various time periods, then fixed and immunostained with antibodies specific to H5. Consistent with earlier observations [35], neomycin treatment resulted in disruption of actin stress fibers from the central part of the cell, whereas peripheral fibers and cortical actin networks appeared unaffected (Figure 7). H5 filaments were also disrupted, causing the H5 immuno-reactivity to appear dispersed, with some concentrations near the edges of the plasma membrane.

Septin proteins have been shown in many species to have a requisite function in cytokinesis [4,6,7,20]. In *Drosophila* lacking the septin protein Peanut, developing tissues accumulate multinucleated syncytia, indicating inhibition of cytokinesis [7]. In mammalian cells, transfection of mutated Nedd5 proteins that are inhibited in GTP-binding

Figure 7



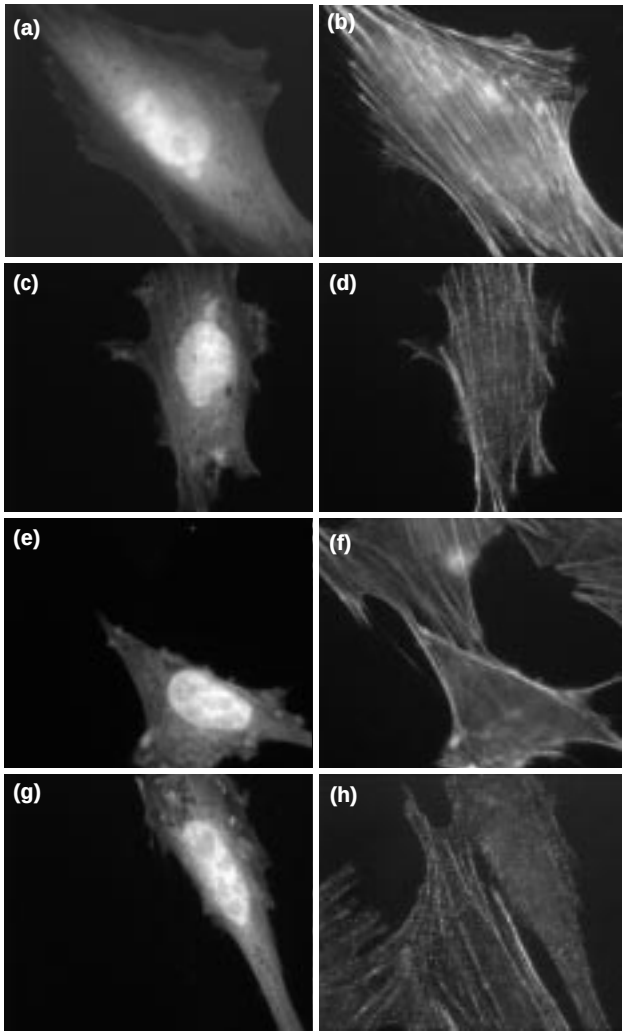
Neomycin disrupts H5 filaments and produces multinucleated cells. Swiss 3T3 cells grown to logarithmic phase in DMEM containing 10% fetal bovine serum were incubated for an additional 2 h in medium either with or without 5 mM neomycin sulfate. Cells were then fixed and processed for immunocytochemistry and triple-labeled with DAPI, FITC-conjugated phalloidin and primary rabbit antibodies to H5, which were detected with Cy3-conjugated goat anti-rabbit antibodies. **(a–c)** Control cells reflect the normal actin and H5 staining patterns in Swiss 3T3 cells. **(d–i)** Neomycin-treated cells have (e,h) dispersed H5 filaments, (d,g) loss of actin stress fibers, and (f,i) binucleation. (g–i) Lower magnification views demonstrate the high frequency of binucleation. The scale bar in (c,f,i) represents 10 μm.

disrupt the filamentous appearance of endogenous Nedd5 in interphase cells and injection of antibodies to Nedd5 led to binucleated cells through failure in cytokinesis [20]. In this study, we found that as many as 10% of the cells in the cultures treated with neomycin became binucleated, indicative of impaired cytokinesis (Figure 7i). In control cultures, binucleated cells were very rarely observed.

An alternative means to interfere with PtdInsP$_2$ availability in the cell is to reduce the overall levels of the phospholipid by transient transfection of the cells with the phosphatidylinositol 5-phosphatase synaptojanin [36]. A cDNA encoding synaptojanin was subcloned into the mammalian expression vector pcDNA3.1 and co-transfected into cells along with an enhanced green fluorescence protein (GFP) reporter plasmid (pEGFP). As before, indirect immunofluorescence was used to monitor the effects of synaptojanin expression on H5. As can be seen in Figure 8, GFP-positive cells contained significantly fewer actin stress fibers than untransfected control cells and, as in the case of cells treated with neomycin, the remaining fibers were displaced to the edge of the cell. This is consistent with previous studies in which synaptojanin was transfected into COS-7 cells [36]. In all GFP-positive cells, the filamentous appearance of H5 was disrupted and H5 appeared punctate. Occasionally, GFP-positive cells were found to be binucleated (data not shown), although at a lower frequency than in neomycin-treated cells.

As a third and more acute approach to interfere with the availability of PtdInsP$_2$, cells were treated with ionomycin in the presence of extracellular calcium to activate phospholipase C (PLC) [37]. In order to monitor PtdIns(4,5)P$_2$ levels during this treatment, cells were transiently transfected with a construct containing a fusion between the PH domain of phospholipase C-δ and GFP. This chimeric protein associates with PtdInsP$_2$ and has been used as an accurate index of the amount of this phospholipid present in intact cells [37]. In cells expressing moderate levels of the PH-domain–GFP chimera, the majority of the fluorescence was detected near the plasma membrane. Following activation of PLC by treatment with ionomycin for 10 minutes, the GFP construct shifted from the plasma membrane to the cytoplasm (data not shown). As seen in Figure 9, the H5 filaments were disrupted in cells treated with ionomycin, whereas actin stress fibers were only moderately reduced. Similar results were obtained in cells stained with antibodies to the septin Nedd5 (data not shown). Hence, the filamentous appearance of septins requires the continued presence of PtdInsP$_2$ and/or PtdInsP$_3$ within the cells and is not solely dependent on the filamentous appearance of actin.

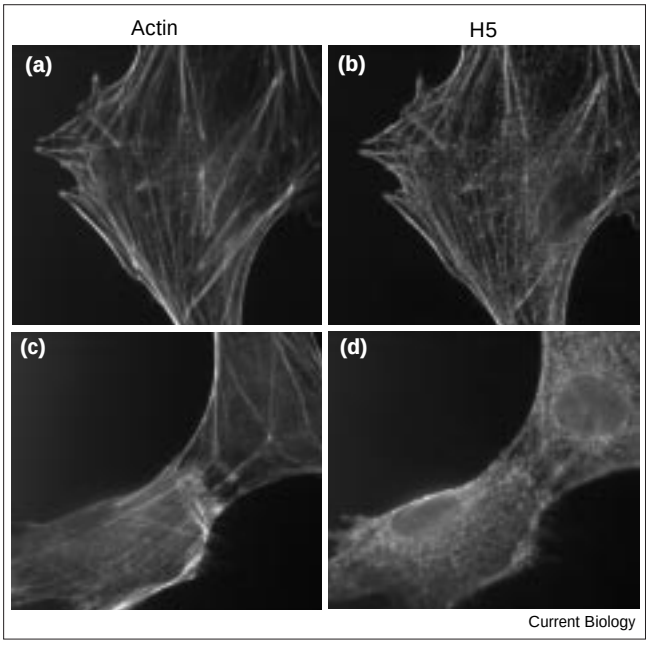
Figure 8



Expression of synaptojanin disrupts H5 filaments. Swiss 3T3 cells were transfected with (a–d) pEGFP alone or with (e–h) pEGFP and pCDNA-synaptojanin, then processed for immunocytochemistry. Cells immunostained with an antibody to H5 were detected with (c,d,g,h) a Cy3-conjugated secondary antibody or with (a,b,e,f) rhodamine–phalloidin to detect filamentous (F)-actin. The expression of GFP recombinant proteins for each set is shown to the left (a,c,e,g). Cells expressing synaptojanin (detected in e,g) had disruptions to actin and H5 filaments (f,h) not seen in neighboring cells in the same image.

While monitoring the effectiveness of the treatment with ionomycin, we noted that expression of the PH-domain–GFP chimera affected the distribution of H5. Even low levels of the chimera were sufficient to disrupt H5 filaments (data not shown), reminiscent of the chelation of $PtdInsP_2$ observed with neomycin (Figure 7). We believe that such disruption results from association of the chimera with $PtdInsP_2$, causing displacement of the septin from the membrane.

Figure 9



Ionomycin treatment causes rapid dissociation of H5 filaments. Swiss 3T3 cells were incubated in Dulbecco's MEM in the presence of 2 μM ionomycin for 10 min. (a,b) The actin and H5 patterns of untreated cells, respectively. (c,d) The effect of ionomycin treatment.

Discussion

The demonstration that the septin H5 binds directly and specifically to $PtdIns(4,5)P_2$ and $PtdIns(3,4,5)P_3$ provides the first insights into the possible signaling mechanisms that lead to filament-like accumulations of septins. H5 and several other mammalian septins contain a polybasic sequence similar to those of a variety of $PtdInsP_2$-binding proteins. Here, we have shown that this motif is capable of specific interactions with $PtdInsP_2$. Polybasic $PtdInsP_2$-binding sites were first identified in the actin-severing protein gelsolin [26] and later in a variety of proteins, including profilin [27,30], G-protein-coupled receptor kinases [29], inward rectifying potassium channels and Na^+–Ca^{2+} exchangers [28]. In general, these sequences are rich in lysine and arginine, and bind $PtdInsP_2$ with a lower affinity than has been observed for PH domains. This suggests that other residues within the motif also contribute to secondary structures that enhance the binding affinity. For example, the protein mSOS1 has both a PH domain and a polybasic motif. The former was found to bind with a K_d of 1.8 μM whereas the latter had a K_d of 53 μM [23]. The apparent K_d of $PtdIns(4,5)P_2$ binding to H5 was 10 μM, reflecting a lower binding affinity than a typical PH domain but higher than that of the related polybasic site in mSOS1.

Both the location and charge distribution of the $PtdInsP_2$-binding site identified in H5 are highly conserved

throughout evolution, with similar motifs being found in septins from yeast to mammals. In the mammalian septins almost all of the isoforms contain an identical motif. Only the two uncharacterized mammalian proteins, KIAA0128 [12], and KIAA0202 [13], have poor conservation of this motif, but these proteins have not yet been shown to have septin functions and might not be true septins. Alternatively, septins lacking PtdInsP$_2$-binding sites might be under other forms of regulation.

The septins are unique among proteins containing the polybasic PtdInsP$_2$-binding motif, in that this site is located in the proximity of another functional site, the P-loop of the GTP-binding domain. This proximity raises the prospect that occupancy of the two sites by their respective ligands might be in some way interdependent. Indeed, although we found that GTP binding and hydrolysis by H5 were not affected by the presence of PtdInsP$_2$ micelles, binding to the phospholipid was significantly inhibited by the presence of GTP. The simplest explanation that could account for this interdependence is that occupancy of one site sterically interferes with occupancy of the other. While the P-loop interacts with the α- and β-phosphates of the nucleotide [38], only GTP interfered with the binding of PtdInsP$_2$. It is therefore conceivable that the γ-phosphate of GTP extends to the vicinity of the phospholipid-binding domain, where it could obstruct the interaction with PtdInsP$_2$. Alternatively, binding of GTP, but not GDP, could cause a conformational change in H5 that masks or alters the PtdInsP$_2$-binding site, making it inaccessible. GTPγS inhibited PtdInsP$_2$ binding less effectively than GTP, however, indicating that steric interference by the γ-phosphate is not the principal cause of binding differences and suggesting that dissociation results as a consequence of conformational changes that occur during the process of GTP hydrolysis. In this context, it is of interest to note that PtdInsP$_2$ can stimulate the dissociation of GDP from the small GTPases Rho, CDC42, and Rac [39], as has previously been shown for Arf1 [40]. In these cases, PtdInsP$_2$ is thought to bind to a carboxy-terminal polybasic region [39] that is remote from the nucleotide-binding site, suggesting that displacement of GDP is the result of a conformational change in the protein.

Preferential binding of PtdInsP$_2$ to the GDP-associated form of H5 could provide a means by which the location of the septin within the cell is regulated. Previous studies of immunoisolated *Drosophila* and mammalian septins revealed that they exist as filamentous 'rodlets' and that they are predominantly associated with GDP. The short filaments isolated from *Drosophila*, and predicted to comprise a set of the three *Drosophila* septin proteins, have a GDP:GTP ratio of about 2.6 [9]. If the isolated septin complexes are representative of *in vivo* septin complexes, this suggests that septin complexes should have the potential to interact with PtdInsP$_2$ *in vivo*. Moreover, one study has suggested that GTP hydrolysis is associated with the filamentous appearance of septins in mammals [20]. In this study, it was shown that injection of a non-hydrolyzable GTP analog or transfection of mutant Nedd5 proteins unable to bind GTP disrupts the filamentous appearance of endogenous Nedd5 [20]. If mammalian septins exist in higher-order filaments, as they do in yeast, then it is possible that the filamentous structures seen by microscopy might reflect septin polymerization. In such a case, GTP-bound forms of H5, which in our study did not bind to PtdIns(4,5)P$_2$, would be unable to bind PtdIns(4,5)P$_2$, whereas GDP-bound forms in filament-like structures would. Preferential PtdIns(4,5)P$_2$ binding by the GDP-bound forms along the filament core could be responsible for the anchorage and accumulation of filaments at the regions where PtdIns(4,5)P$_2$ is concentrated.

Continuous interaction of the septin proteins with PtdIns(4,5)P$_2$ appears to be necessary for the maintenance of their filamentous appearance. In non-mitotic cells the H5 protein is normally found in a filamentous pattern similar to that of actin, but in cells treated with neomycin, in those transfected with the PtdIns 5-phosphatase synaptojanin, or in those treated with ionomycin to activate phospholipase C, H5 filaments disappeared and punctate staining was observed. In the former two cases, these effects correlated with loss of actin filaments, and septin appearance could have been altered as a consequence of alterations in actin. In contrast, ionomycin treatment resulted in a disruption of the H5 filaments that preceded changes in actin stress fibers, indicating that their filamentous appearance is not solely due to the filamentous arrangement of actin. If the septins are true filaments, then their filamentous properties depend on binding to PtdIns(4,5)P$_2$. Alternatively, if H5 proteins or septin rodlets simply associate with actin and thereby appear filamentous, then this association is dependent on the continued presence of PtdIns(4,5)P$_2$.

Septin function is required for cytokinesis and inhibition of this function induces the formation of multinucleated cells. Interestingly, treatment of cells with agents, such as neomycin, that interfere with PtdInsP$_2$ also altered the appearance of H5 and inhibited cytokinesis. A high percentage of binucleated cells were observed after interference with PtdInsP$_2$ binding; binucleated cells occurred very rarely when cells were untreated. Cells that became binucleated were probably within 2 hours of undergoing cytokinesis and underwent mitosis without cytokinesis. Failure to observe increasing numbers of binucleated cells accumulate upon longer treatment implies that additional PtdInsP$_2$-dependent steps earlier in the cycle might also be required for progression into mitosis. Nevertheless, our results raise the possibility that one of the PtdInsP$_2$-dependent steps late in the cell cycle might be

the interaction of PtdIns(4,5)P_2 with H5, and this might be involved in the recruitment of septins to the cleavage furrow, where they can carry out their essential role in cell cleavage.

Materials and methods

PtdInsP_2-binding assays

The ELISA procedures for PtdInsP_2 binding assays were developed by Miki *et al.* [22], and used with some modifications. Briefly, GST, or GST–H5 fusion proteins or peptides were absorbed to 96-well plates in 50 ml of bicarbonate buffer (50 mM sodium bicarbonate, pH 9.6) at 1 μg/well and incubated at room temperature for 4 h or overnight at 4°C. After washing three times with PBS, PtdInsP_2, resuspended in PBS, was added to the well at 1 μg per well and incubated at room temperature for 2 h. After washing three times in PBS, the wells were blocked with 3% powdered milk dissolved in PBS, washed once in PBS, and then incubated with primary antibody. Anti-PtdInsP_2 (PerSeptive Biosystems) was diluted 1:500 in blocking solution and incubated for 2 h at room temperature. The wells were again washed three times and a secondary goat anti-mouse antibody conjugated with horseradish peroxidase (HRP) was used at a dilution of 1:1000 in 50 μl PBS and incubated for 1 h at room temperature. After three washes in PBS, the wells were incubated with 50 μl developing solution containing orthophosphate diamine as substrate and the absorbance at 450 nm was measured in the ELISA plate reader.

To determine the specificity of lipid interactions, 1 μg of each of the following phospholipids was coated in the wells of a 96-well plate and allowed to dry: PtdIns(4,5)P_2 (sodium salt, Sigma), PtdIns(4)P (sodium salt, Sigma), PtdIns (sodium salt, Sigma), PtdSer (Sigma), and PtdCho (chloroform solution, Sigma). After washing the wells three times in PBS, they were blocked with milk powder solution, as above, washed, and incubated with GST or GST–H5 fusion protein at 1 μg per 50 μl at room temperature for 2 h. After three washes, the wells were developed as above, this time probing with rabbit anti-GST antibody followed by a secondary HRP-conjugated goat anti-rabbit antibody.

In competition experiments, neomycin sulfate, pentalysine or blocking peptides were preincubated with the PtdInsP_2 micelles in PBS at room temperature for 30 min. The mixture (1 μg of each in 50 μl) was then added to the protein-coated wells and developed as above. Synthetic peptides (Sheldon Biotechnology) were produced: the first was of a region that included the polybasic motif of H5 and was identical to amino acids 136–149 (PEP1, sequence Ac-RKSVKKGFDFTLMVC-NH_2); the second was a control peptide (PEP2) generated from the amino-terminus amino acids 26–45 of H5 (Ac-EDSSDDDAELSKFVKDFPGC-NH_2).

Liposome sedimentation assays were performed as follows. Lipid vesicles comprising 95% PtdCho and 5% of either PtdIns(3,4)P_2, PtdIns(4,5)P_2 or PtdIns(3,4,5)P_3 were prepared as described by Kubiseki *et al.* [23]. Briefly, the lipids were mixed in buffer containing 10 mM HEPES pH 7.0 and 10 mM sucrose, vortexed for 1 min, then subjected to five freeze–thaw cycles on dry ice. Liposomes were then used immediately by mixing with 1 μg H5 protein and incubating at room temperature with shaking for 1 h. Liposomes were then pelleted by centrifugation at 50,000 × *g* for 40 min at 25°C. The resultant supernatants and pellets were analyzed by SDS–PAGE and western blotting with anti-H5 antibody. Relative H5 levels were determined from densitometric scans of linear exposures from three independent experiments.

Deletion constructs of H5 and site-directed mutagenesis

Four carboxy-terminal truncations were produced in H5 using the bacterial expression vector pGEX, as follows: D452 was generated by partial digestion of pGEX-H5 with *Hind*III, which cleaved at nucleotide 1356 after the initiation methionine and 3′ to the cDNA in the pGEX-KG vector. D363 was produced by cleavage with *Sal*I, which cleaved at nucleotide 1089 and in the vector 3′ of the insert. D230 was generated by cutting the insert with *Bcl*I and cutting pGEX-KG with *Xho*I, followed by blunt-ending with Klenow and ligation. D112 was produced with a complete *Hind*III digestion by cleavage at nucleotide 336 and in the pGEX-KG polylinker.

Site-directed mutagenesis was performed by using the QuickChange Kit (Stratagene) following the manufacturer's recommendations. PGEX-H5 DNA was used as template and the following two pairs of mutagenic oligonucleotides were used: P1A, 5′-CCCAACCAAGTC-CACGCAGCGTCCGTGAAGAAAGGC-3′; P1B, 5′-GCCTTTCTT-CACGGACGCTGCGTGGACTTGGTTGGG-3′; P2A, 5′-CACCGA-AAGTCCGTGGCGGCAGGCTTTGCATTTACC-3′; P2B, 5′-GGT-AAAGTCAAAGCCTGCCGCCACGGACTTTCGGTG-3′.

Assays of GTP binding and hydrolysis

Extracts of bacteria expressing either GST or GST–H5 were purified on glutathione–agarose beads and left bound to the beads for GTP-binding assays. The GTP-binding and -hydrolysis assays were performed as described by Kinoshita *et al.* [20], with slight modification. Briefly, beads containing 0.5 μg or 1 μg GST–H5 were incubated with 1 μg PtdInsP_2 at 4°C overnight in PBS. The beads were then washed in PBS before incubation with 10 nM [γ-^{32}P]GTP in GTP-binding buffer containing 20 mM Tris–HCl pH 8.0, 5 mM $MgCl_2$, 100 mM NaCl, 10 mM 2-mercaptoethanol, 10% glycerol, 5 mM EDTA and 100 μg/ml bovine serum albumin. The binding was performed for 20 min at 4°C. Hydrolysis was assayed by first washing the beads three times in binding buffer at 4°C before the buffer was exchanged with 50 μl reaction buffer (binding buffer lacking EDTA) and incubation for 0, 5 or 20 min at 37°C. The reaction was stopped by washing the beads with the same buffer and the radioactivity bound to the beads was determined by counting in scintillation fluid. The amount of [γ-^{32}P]GTP remaining bound to the beads after three washes in the same buffer was the bound fraction and loss of radioactivity on the beads reflected the hydrolysis of GTP. Control experiments involved the incubation of the beads at 37°C but in the presence of EDTA and in this case, little radioactivity was lost from the beads. Experiments were performed in quadruplicate.

Cell culture and experimental treatments

Swiss 3T3 cells, obtained from the ATCC, were grown on coverslips to 60% confluence in DMEM containing 10% fetal calf serum (FCS) at 37°C. For neomycin treatments, cells grown as above were incubated for 2–24 h in 5 mM neomycin sulfate in DMEM + 10% FCS. Transfections were performed essentially as described [41]. After 48 h, the cells were fixed and processed for immunocytochemistry. For the synaptojanin-expression construct, the 5′ end of a synaptojanin cDNA [42] was ligated to a partial synaptojanin cDNA construct [43] (a gift of Rudiger Woscholski and Peter Parker, Imperial Cancer Research Fund, London) at a unique *Nhe*I site. The complete cDNA clone was then subcloned into the mammalian expression vector pcDNA 3.1 at the *Eco*RI site. Transfections were carried out either with the pEGFP vector (Clontech) alone, or with a mixture of pEGFP and pCDNA3.1 synaptojanin.

For ionomycin treatments, cells were transfected with a vector encoding a fusion construct comprising the phospholipase C-δ PH domain and GFP [37] and cultured on coverslips in Dulbecco's MEM overnight, then incubated in the same medium (containing 1.7 mM Ca^{2+}) in the presence of 2 μM ionomycin (Calbiochem) for 10 min at room temperature. The cells were then immediately fixed and processed for immunocytochemistry.

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