Annexin 2 is a phosphatidylinositol (4,5)-bisphosphate binding protein recruited to actin assembly sites at cellular membranes

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Summary

Annexin 2 is a Ca^{2+}-regulated membrane protein and an F-actin-binding protein enriched at actin assembly sites both, on the plasma membrane and on endosomal vesicles. Here, we identify annexin 2 as a phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P_2)-interacting protein, thereby explaining this specific membrane association. Using the pleckstrin-homology (PH) domain of phospholipase Cδ1 fused to yellow fluorescent protein as a marker for PtdIns(4,5)P_2, we show that annexin 2 and its ligand p11 (S100A10) are targeted to sites of PtdIns(4,5)P_2 enrichment where F-actin accumulates. At the plasma membrane, adhesion of pedestal-forming enteropathogenic *Escherichia coli* induces a recruitment of 1-phosphatidylinositol-4-phosphate 5-kinase (PtdIns4*P* 5-kinase) and an enrichment of PtdIns(4,5)P_2 and annexin 2-p11 at sites of bacterial adhesion. Induction of PtdIns(4,5)P_2-enriched ruffles and PtdIns(4,5)P_2-positive, actin-coated vacuoles by Arf6-mediated activation of PtdIns4*P* 5-kinase also leads to a concomitant accumulation of the annexin 2-p11 complex and the PH domain. Binding studies with immobilized phosphoinositides and phosphoinositide-containing liposomes reveal that the purified annexin 2-p11 complex directly and specifically binds to PtdIns(4,5)P_2 with an affinity comparable to that of the PH domain of phospholipase Cδ1. Experiments using individual subunits identify annexin 2 as the PtdIns(4,5)P_2-binding entity. Thus, the direct interaction of annexin 2 with PtdIns(4,5)P_2 is a means of specifically recruiting the annexin 2-p11 complex to sites of membrane-associated actin assembly.

Key words: Annexin 2-p11 complex, Actin dynamics, PtdIns(4,5)P_2-binding, Arf6

Introduction

Annexins are a family of peripheral membrane-binding proteins implicated in the regulation of membrane organization and membrane traffic (for reviews, see Gerke and Moss, 2002; Raynal and Pollard, 1994). They cycle between the cytosol and the cytosolic surface of cellular membranes in a Ca^{2+}-regulated manner which is mediated through the annexin core domain, a conserved Ca^{2+}-lipid-binding module. Although all annexins share this core domain, they show a certain degree of non-overlapping specificity with respect to their cellular target membrane. This specificity depends most probably on certain sequences within the core domain and, at least in some cases, also on the N-terminal annexin domain which is unique for a given member of the family and can harbor sites for protein interaction and posttranslational modification.

Annexin 2 is also capable of interacting with polymerized actin (for reviews, see Gerke and Moss, 2002; Waisman, 1995). The F-actin-binding site maps to the core domain whereas the N-terminal domain harbors the binding site for another protein ligand, the S100 protein p11 (S100A10). p11 is a dimer, and complex-formation leads to a heterotetrameric annexin 2_2-$p11_2$ entity. This heterotetramer, which is highly symmetric and contains two membrane- and F-actin binding sites (Lewit-Bentley et al., 2000), has been implicated in providing membrane-cytoskeleton linkages (for a review, see Gerke and Moss, 2002). In support of this view, annexin 2 is found at membrane sites that function as assembly points for actin filaments. These include nascent pinosomes which assemble a tail of actin filaments and it was shown recently that annexin 2 is involved in the generation of such actin comet tails but not in the formation of actin tails propelling pathogenic *Listeria monocytogenes* (Merrifield et al., 2001). Moreover, annexin 2 and its ligand p11 are recruited to actin assembly sites at the plasma membrane of HeLa cells, infected with non-invading entheropathogenic *Escherichia coli* (*E. coli*) (EPEC). EPEC utilize a type III secretion system to inject a bacterial protein into host cells, the translocated intimin receptor (Tir). Tir recruits the adaptor protein Nck and in turn a number of actin-regulating proteins such as N-WASP and the Arp 2/3 complex. These cause actin filaments to assemble into pedestal-like structures that form underneath the adhering bacteria (for reviews, see Frischknecht and Way, 2001; Goosney et al., 2000). Tir is required for full pedestal formation, whereas a less pronounced actin assembly also occurs when bacteria that lack a functional Tir protein adhere to their host cells. Both wild-type and Tir^- EPEC also induce a clustering of membrane cholesterol and GPI-anchored proteins concomitant with the recruitment of annexin 2-p11 (Zobiack et al., 2002).

Together, these observations indicate that annexin 2-p11 can function at actin-assembly sites on the plasma membrane and on endosomal membranes, and that it requires the presence of a cellular membrane for this activity. Polymerized actin itself, as present in the *Listeria* tails, is not sufficient to recruit annexin 2-p11, which strongly indicates that specific membrane components are required for the annexin 2-p11 recruitment. To identify these membrane components, we focused on phosphoinositides – especially phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P_2) – which play a central role in the remodeling of the subplasmalemmal actin cytoskeleton (Cullen et al., 2001; Martin, 2001). By using EPEC infection to cluster PtdIns(4,5)P_2 within the plasma membrane, or Arf6 activation to stimulate PtdIns(4,5)P_2 synthesis at the plasma membrane and on internal pinosomes, we identified sites of PtdIns(4,5)P_2 accumulation as specific annexin 2-p11 recruitment sites. Binding experiments using purified components reveal a direct interaction between PtdIns(4,5)P_2 and the annexin 2-p11 complex, with the binding site mapping to the annexin 2 subunit. These results reveal a novel type of annexin-membrane interaction and identify PtdIns(4,5)P_2 as the membrane lipid responsible for recruiting annexin 2-p11 to submembraneous actin-assembly sites.

Materials and Methods

Expression plasmids

To generate the pleckstrin homology (PH) domain-yellow fluorescent protein (YFP) expression plasmid (PHD-YFP), the cDNA encoding the N-terminal PH domain of human PLC-δ1 (185 amino acids) was amplified by PCR from a human placenta cDNA library (Clontech), using the primers 5′-GATCGAATTCGGCATGGACTCGGGCCG-GGACTTCCTG-3′ and 5′-GATCGTCGACAAGATCTTCCGGG-CATAGCTGTCG-3′. After digestion with *Eco*RI and *Sal*I, the PCR product was inserted into the appropriately digested pEYFP-N1 eukaryotic expression vector (Clontech). For expression of C-terminally His-tagged protein in *E. coli*, PCR was performed with the primers 5′-GATCGAATTCGGCATGGACTCGGGCCGGGACTT-CCTG-3′ and 5′-GATCGTCGACGAAGATCTTCCGGGCATAGC-TGTCG-3′ and the product cloned via the flanking *Eco*RI and *Sal*I restriction sites into the pET-23a(+) vector (Novagen). All constructs were confirmed by sequencing (SEQLAB, Göttingen, Germany). The annexin 2-GFP and YFP-p11 expression constructs were described previously (Rescher et al., 2000; Zobiack et al., 2001). Expression plasmids encoding Arf6, Arf6 Q67L, and the α isoform of the type I 1-phosphatidylinositol-4-phosphate 5-kinase (PtdIns4*P* 5-kinase) were kindly provided by P. Chavrier (Institut Curie CNRS, Paris) and L. Machesky (University of Birmingham), respectively. The GFP-actin-expressing plasmid was obtained from M. Bähler (University of Münster).

Expression and purification of His-tagged PH domain

E. coli expression-strain BL21(DE3)pLysS (Stratagene) was transformed with the pET-23a(+) expression vector encoding the His-tagged PH domain of phospholipase C δ1 (PLC-δ1). Transformed bacteria were grown at 37°C in Luria-Bertani (LB) medium supplemented with 150 μg/ml ampicillin, to an OD_{600} of 0.6 and induced with 0.5 mM isopropyl β-D-thiogalactoside at 26°C for 16 hours. Cells were lysed by freeze-thaw cycles and sonication in lysis buffer [50 mM NaH_2PO_4, 10 mM imidazole pH 8.0, 300 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride (PMSF)]. Lysates were clarified by centrifugation at 30,000 ***g*** for 30 minutes at 4°C. The supernatant was incubated with Ni-NTA-agarose beads (Quiagen) for 1 hour at room temperature (RT). The beads were then washed twice with washing buffer (50 mM NaH_2PO_4, 20 mM imidazole pH 8.0, 300 mM NaCl, 0.5 mM PMSF), and bound protein was eluted with washing buffer containing 250 mM imidazole.

Cell culture and transfection

HeLa cells were cultured in DMEM with 10% fetal bovine serum, 2 mM glutamine, penicillin and streptomycin at 37°C and 7% CO_2. Cells grown on coverslips were transfected using the Effectene transfection kit (Life Technologies) according to the manufacturer's protocol.

Fluorescence microscopy

Sixteen to 24 hours after transfection, cells grown on coverslips were fixed with 4% PFA in PBS for 10 minutes at RT, permeabilized for 2 minutes with 0.2% Triton X-100 and treated with 50 mM NH_4Cl in PBS to quench free aldehydes. Cells were then incubated with primary antibodies diluted in PBS with 2% BSA for 45 minutes at RT. Following washing with PBS, Texas Red-labelled secondary antibodies (Abs) (Dianova) were applied for 45 minutes at RT. Anti-myc monoclonal antibodies (mAbs) (clone 9E10, Santa Cruz Biotech) were used to detect myc-tagged PtdIns4*P* 5-kinase, HA-tagged wild-type Arf6 or the Q67L mutant and were visualized with anti-HA mAbs (clone Y-11, Santa Cruz Biotech). Filamentous actin was stained with Texas Red-coupled phalloidin (Molecular Probes) that was added during incubation with the secondary antibodies. To label the plasma membrane, the lipophilic tracer CM-DiI (Molecular Probes) was used. Arf6-PHD-YFP co-transfected cells grown on coverslips were activated with AlF and then incubated for 5 minutes at 37°C with 2 μM CM-DiI in culture medium without serum. After labeling, cells were washed for 5 minutes with ice-cold PBS and fixed with 4% PFA in PBS for 10 minutes. Cells were inspected with a Leica DM RXA epifluorescence microscope or a Zeiss LSM510 Meta confocal microscope, after being mounted in mowiol containing 4% n-propyl-gallate as antifade agent.

Quantitative analyses of DiI-membrane labeling in Arf6-activated cells were carried out essentially as described (Mukherjee et al., 1999). Confocal stacks of transfected cells showing a well spread morphology and a distinct DiI-labeling of the plasma membrane were obtained by exciting the cells with a 30 mW argon laser emitting at 488 nm and a 1 mW helium/neon laser emitting at 543 nm. Emissions were collected using a 505-530 nm band-pass filter and a 560 nm long-pass filter to collect YFP and DiI fluorescence signals, respectively. Since signals were obtained using the multitrack function, crossover of signals is prevented and correcting by crossover measurements is therefore not necessary. Based on the PHD-YFP signal, the confocal slice of each stack showing sites of ruffling at the plasma membrane was selected. After transferring these eight-bit images (chosen from the LSM510 software) to the MetaMorph software the channels were not mixed. Several ruffled and non-ruffled regions along the plasma membrane of a given cell were morphologically defined in the PHD-YFP image. The outlines of the selected regions were boxed (25×25 pixels) and copied onto the DiI image. Punctate structures indicating internal vesicles or lipid dye aggregates were excluded. For both the PHD-YFP and the DiI signals, the mean fluorescence intensity per pixel of the boxed areas was measured. To correct for cell-to-cell variations in PHD-YFP expression levels and DiI staining, the ratios of signal intensities for ruffled to non-ruffled regions were determined for each cell for PHD-YFP and DiI. Data were collected from ten cells following three independent transfection and labeling experiments and the results are presented in percent ratios ± standard error of the means (s.e.m.). Statistical significance of the results was evaluated by unpaired Student's *t* tests.

HeLa infection

Transfected cells grown on coverslips were infected with EPEC strain 2348/69 (wild type) or the different mutant strains as described previously (Zobiack et al., 2002). Briefly, HeLa cells were infected with an overnight EPEC culture (~100 bacteria/cell) in DMEM, 2% fetal calf serum, 1 mM glutamine, 10 mM HEPES and 1% methyl-α-D-mannose at 37°C in a 10% CO_2 incubator. Three hours after infection, cells were intensively washed with PBS, fixed, permeabilized and analyzed for the distribution of the respective ectopically expressed protein.

Co-sedimentation assays

Annexin 2-p11 complex was purified from porcine intestine as described previously (Gerke and Weber, 1984). Liposomes (5 mg/ml) were generated by sonifying brain extract (Folch fraction I) (Sigma) supplemented with 0.5% (w/w) of the respective phosphoinositides [PtdIns(3,4)P_2, PtdIns(4,5)P_2, PtdIns(3,4,5)P_3] (Sigma) in PBS and mixed with 2.5 μg annexin 2-p11 complex/40 μl liposome suspension in the presence of 1 mM Ca^{2+} or 1 mM EGTA. Liposomes were collected by ultracentrifugation (75,000 ***g***, 15 minutes, 4°C). After three rounds of washing and sedimentation, liposome pellets were subjected to SDS-PAGE, and bound annexin 2-p11 complex was detected by immunoblotting with polyclonal antibodies specific to the annexin 2 subunit (Gerke and Weber, 1984). Signal intensities of the immune-reactive bands were densitometrically scanned and calculated using a Lumi-Imager from Boehringer (Mannheim, Germany).

Lipid plate binding assays

Wells of 96-well microtiter plates (F96 Maxisorp, Nunc) were coated with 2 μg/ml of the respective lipid and air-dried overnight. After blocking the wells with 0.5% BSA in PBS for 1 hour, purified annexin 2-p11 complex (800 ng/well) in Ca^{2+}- or EGTA-containing PBS was added for 1 hour at room temperature. Subsequently, wells were washed several times with PBS containing 0.05% Tween in an ELISA plate washer (Tecan). The amount of bound complex was determined by a colorimetric reaction using annexin 2-specific antibodies, peroxidase-coupled secondary antibodies (Dianova) and TMB substrate (Perbio) measured at 450 nm in an ELISA plate reader (MWG-Biotech). To investigate the binding of the individual subunits to immobilized PtdIns(4,5)P_2, the annexin 2-p11 complex was dissociated as described (Gerke and Weber, 1985) and the individual subunits were detected with annexin 2- (Thiel et al., 1992) or p11-specific antibodies (Gerke and Weber, 1984; Osborn et al., 1988), respectively. For competition assays, purified His-tagged PH-domain of human PLC-δ1 was added at the molar ratios indicated.

Results

The annexin 2-p11 complex is recruited to PtdIns(4,5)P_2-rich membrane sites induced by EPEC infection or Arf6 activation

Since direct interaction with PtdIns(4,5)P_2 triggers the recruitment of a set of proteins required for membrane and cytoskeleton dynamics to sites of active cytoskeletal and membrane rearrangement (Caroni, 2001; Sechi and Wehland, 2000), we speculated that PtdIns(4,5)P_2 might be responsible for targeting annexin 2-p11 to sites of F-actin assembly. Therefore, we recorded the distribution of PtdIns(4,5)P_2 during EPEC infection, which is known to induce annexin 2-p11 recruitment and F-actin assembly underneath bacterial adhesion sites. PtdIns(4,5)P_2 was visualized by monitoring the intracellular localization of the PH domain of phospholipase Cδ1 fused to YFP (PHD-YFP). This domain has been shown to serve as a high affinity marker for membrane-associated PtdIns(4,5)P_2 (Varnai and Balla, 1998; Watt et al., 2002) and has also been used in previous studies on EPEC-infected cells (Celli et al., 2001).

HeLa cells transfected with PHD-YFP showed a plasma membrane staining, whereas GFP alone was distributed evenly throughout the cell (not shown). When PHD-YFP expressing cells were infected with EPEC, intensely fluorescent signals were observed at the sites of F-actin enrichment beneath adhering bacteria. This accumulation is identical to the one seen for the subunits of the annexin 2-p11 complex, which were visualized by expression of GFP- or YFP-tagged versions previously shown to be capable of complex formation (Zobiack et al., 2001) (Fig. 1). A similar recruitment of both annexin 2-p11 and PHD-YFP was seen when mutant EPEC, lacking a functional Tir protein [EPEC*tir*$^-$ (SE896)] were used to infect HeLa cells (not shown). By contrast, bacteria deficient in secretion of all proteins requiring the type III secretion system, EPEC CVD452, failed to induce an accumulation of PHD-YFP or annexin 2-p11 and did not trigger any actin recruitment (not shown). The elevation of PtdIns(4,5)P_2 levels at sites of EPEC attachment is possibly mediated through the enzymatic activity of PtdIns4*P* 5-kinase because transiently expressed, myc-tagged PtdIns4*P* 5-kinase was also present at the actin-rich EPEC attachment sites (Fig. 1).

To further correlate the heterotetrameric annexin 2-p11 complex with sites of PtdIns(4,5)P_2 enrichment, we specifically manipulated the level of plasma membrane PtdIns(4,5)P_2 through activation of Arf6, a GTPase involved in regulation of membrane trafficking (Chavrier and Goud, 1999; D'Souza-Schorey et al., 1995; Song et al., 1998). Arf6 mediates the recruitment and activation of PtdIns4*P* 5-kinase at sites of membrane ruffling, thereby locally elevating the PtdIns(4,5)P_2 level (Honda et al., 1999; Skippen et al., 2002). First, we activated Arf6 by treating Arf6-transfected cells with aluminum fluoride (AlF) (Brown et al., 2001). Consistent with previous reports (Brown et al., 2001; Honda et al., 1999; Radhakrishna et al., 1996; Radhakrishna et al., 1999), the majority of AlF-treated cells displayed plasma membrane protrusions enriched in PtdIns(4,5)P_2 as revealed by co-labeling with PHD-YFP (not shown). Annexin 2 showed a marked colocalization with Arf6 in these structures (Fig. 2A). Labeling with the membrane lipid dye CM-DiI confirmed that the enrichment of PHD-YFP and Arf6 as well as annexin 2 and p11 (not shown) at sites of membrane ruffling is not the result of a general increase in membrane thickness but reflects the specific and significant accumulation of PtdIns(4,5)P_2 (Fig. 2A). Next, we expressed the GTP hydrolysis-resistant and therefore constitutively active Arf6 mutant Q67L, which also induces extensive membrane ruffling. Following longer expression times the mutant triggers the formation of PtdIns(4,5)P_2-positive vacuoles, presumably resulting from pinocytic membrane internalisation (Honda et al., 1999) and the subsequent fusion of smaller vesicles into larger vacuoles (Brown et al., 2001). In most of the Arf6 Q67L expressing cells, aggregated vacuoles that differed in size and number but often were clustered on one side of the cell, were evident (Fig. 2B). As described previously (Brown et al., 2001), these vacuoles were coated with actin and strongly labeled for PHD-YFP (Fig. 2B). Significantly, and in line with the EPEC

infection data, annexin 2 and p11 were both enriched on the PtdIns(4,5)P_2-positive structures induced by active Arf6 (Fig. 2B).

Annexin 2 directly interacts with PtdIns(4,5)P_2

Since annexin 2 does not contain known phosphoinositide binding domains (Lemmon, 2003), the accumulation at sites of increased PtdIns(4,5)P_2 levels might be caused either indirectly through other proteins or via a novel binding motif. We therefore investigated whether the annexin 2-p11 complex binds directly to phosphoinositides. First, we assayed the binding of purified annexin 2_2p11_2 to brain extract phospholipid liposomes that contained 0.5% (w/w) of different phosphoinositides (Fig. 3A). Since loading annexin 2 with Ca^{2+} might influence the binding, the assays were carried out in the presence or absence of Ca^{2+}. As previously shown (Powell and Glenney, 1987), Ca^{2+} strongly enhanced co-sedimentation of the annexin 2-p11 complex with control liposomes lacking phosphoinositides, although some complex was pelleted even in the presence of 1 mM EGTA. This is in line with previous experiments, showing a substantial binding of annexin 2-p11 to phosphoinositide-free liposomes at submicromolar Ca^{2+} concentrations (König et al., 1998). When the liposomes contained PtdIns(4,5)P_2, increased co-sedimentation was observed, in particular in the absence of Ca^{2+} (Fig. 3A). However, because the annexin 2-p11 complex shows general binding to acidic phospholipids present in the brain-extract liposomes (for review see Creutz, 1992), the liposome pelleting experiments only reveal a phosphoinositide-induced increase over the general ('background') binding to the brain-extract liposomes. Therefore, to unequivocally show that the increased co-sedimentation reflects a direct interaction between annexin 2_2p11_2 and PtdIns(4,5)P_2, and to elucidate whether the interaction is specific for PtdIns(4,5)P_2 or whether it is also seen with other phosphoinositides, we carried out lipid plate binding assays. Phosphoinositides immobilized on microtiter wells were incubated with annexin 2_2p11_2 and the binding was measured by ELISA using anti-annexin 2 antibodies. As shown in Fig. 3B, only minor background binding was observed to wells containing no phosphoinositides. The signal markedly increased in the presence of different phosphoinositides with PtdIns(4,5)P_2 being much more efficient than PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2. Although Ca^{2+} enhanced the PtdIns(4,5)P_2 interaction, a substantial degree of binding is already observed in the presence of EGTA.

We next examined whether the direct interaction with PtdIns(4,5)P_2 required the assembled heterotetramer or whether it is assigned to one of the two subunits. Therefore, the complex was dissociated and the annexin 2- and p11-subunits were separately tested for binding to those phosphoinositides that elicited significant annexin 2_2p11_2 binding, i.e. PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3. As shown in Fig. 3C, monomeric annexin 2 interacted with PtdIns(4,5)P_2 and, to a much lesser extent, with PtdIns(3,4,5)P_3, whereas p11 bound neither to PtdIns(4,5)P_2 nor to PtdIns(3,4,5)P_3. Therefore, the isolated annexin 2 subunit is not only responsible for the observed phosphoinositide binding capacity but also for the observed specificity. This novel type of PtdIns(4,5)P_2 binding is specific for annexin 2 because its closest relative in the annexin family, annexin 1, did not show any interaction (not shown). To confirm the observed PtdIns(4,5)P_2 specificity, competition assays were carried out with the His-tagged PtdIns(4,5)P_2-specific PH domain of PLC-δ1 (Varnai and Balla, 1998; Watt et al., 2002). As shown in Fig. 3D, binding of annexin 2 to PtdIns(4,5)P_2 decreased in the presence of increasing amounts of PH domain, revealing the specificity of annexin 2 binding. A several-fold molar excess

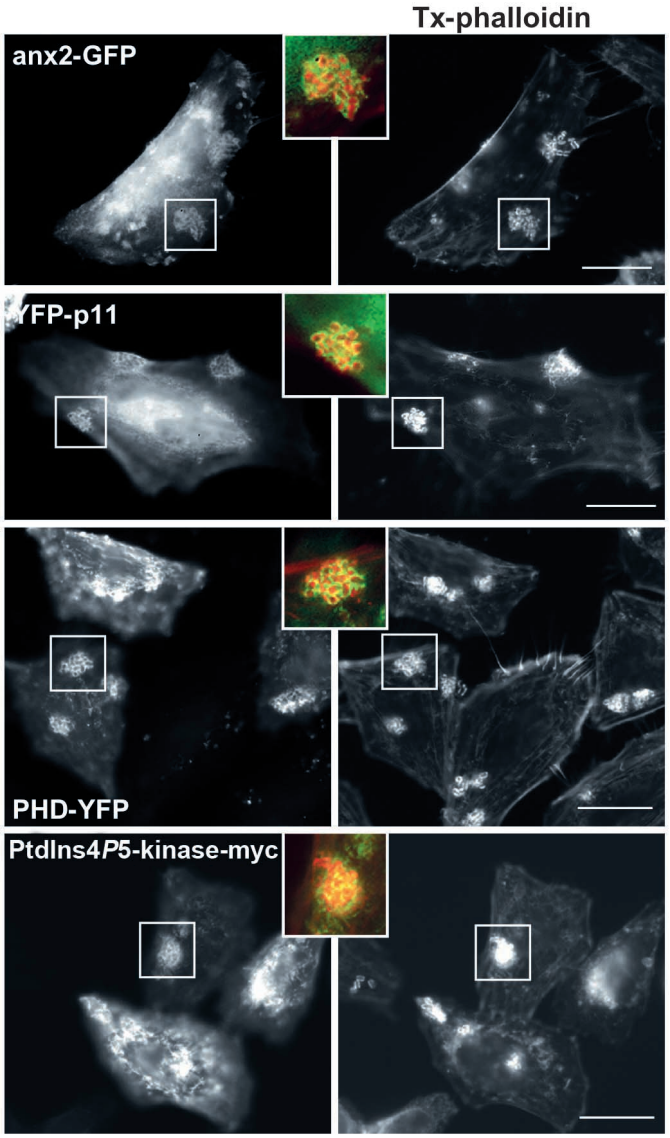

Fig. 1. EPEC-infection induces annexin 2-p11 accumulation at PtdIns(4,5)P_2-enriched sites of actin pedestal formation. HeLa cells, transiently expressing the different tagged proteins indicated, were infected with EPEC. Three hours after infection, cells were fixed, permeabilized and analyzed for the distribution of the respective, ectopically expressed protein. Staining with Texas Red-phalloidin (Tx-phalloidin) revealed the characteristic reorganization of the actin cytoskeleton beneath micro-colonies of adhering bacteria. Annexin 2 (anx2) -GFP and YFP-p11 accumulate at sites of EPEC attachment. PHD-YFP localizes to the same actin-rich structures, indicating elevated PtdIns(4,5)P_2 levels at sites of pedestal formation. Since PtdIns4*P* 5-kinase is also enriched, the rise in PtdIns(4,5)P_2 is possibly caused by its enzymatic activity. Bars 10 μm.

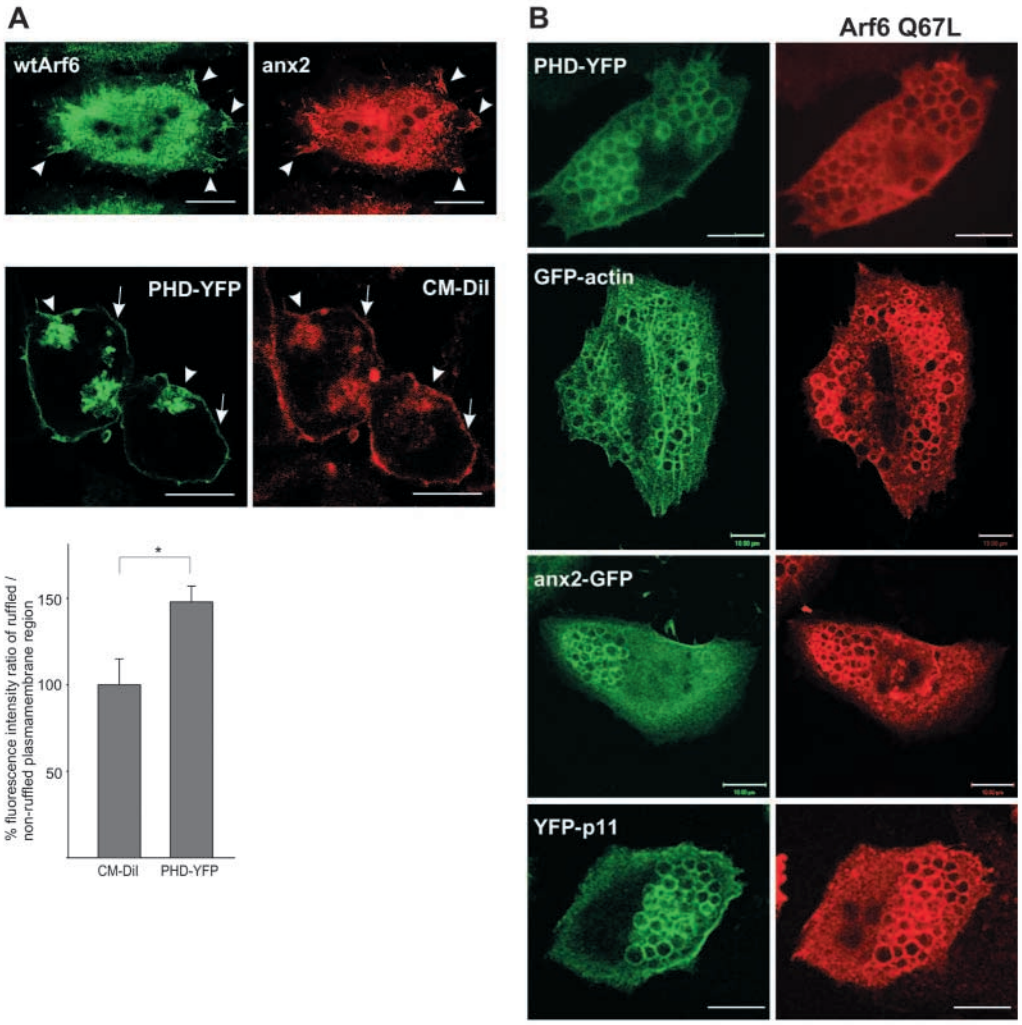

Fig. 2. Annexin 2 is recruited to PtdIns(4,5)P_2-rich structures elicited by active Arf6. (A) HeLa cells, transiently expressing wild-type Arf6 or co-expressing Arf6 and PHD-YFP, were treated for 30 minutes with AlF (30 mM NaFl and 50 μM $AlCl_3$) and processed for annexin 2 immunostaining or plasma membrane labeling with CM-DiI, respectively. Both annexin 2 (anx2) and PHD-YFP localize to plasma membrane protrusions induced by AlF-mediated Arf6 activation (arrowheads). To ascertain that increased signals at sites of membrane ruffling are not owing to a general increase in membrane thickness, mean fluorescence intensity ratios for signals in ruffled (arrowheads) to non-ruffled (arrows) regions were determined for PHD-YFP and DiI, respectively. Results are presented as percent-ratio of the mean fluorescence intensity ± s.e.m. *P<0.05, bars 10 μm. (B) HeLa cells were co-transfected with constitutively active Arf6 Q67L and PHD-YFP, GFP-actin, annexin 2 (anx2)-GFP, or YFP-p11. Annexin 2-GFP and YFP-p11 both clearly localize to the GFP-actin-positive, PtdIns(4,5)P_2-rich vacuoles that were induced by Arf6 Q67L expression. Bars 10 μm.

of GST-PHD is required to significantly decrease annexin 2 binding, indicating that the binding affinity of annexin 2 is at least comparable to that of the PtdIns(4,5)P_2-binding domain of PLC-δ1. Thus, in addition to directing the annexin 2-p11 complex to cellular membranes through the well known Ca^{2+} and lipid binding sites in the protein core domain, the annexin 2 subunit also targets the complex to PtdIns(4,5)P_2-enriched microdomains through direct interaction with PtdIns(4,5)P_2. Interestingly, monomeric annexin 2 requires higher Ca^{2+} concentrations for PtdIns(4,5)P_2 interaction and shows a somewhat reduced binding when compared with the annexin 2-p11 complex.

Discussion

Because of its Ca^{2+}-regulated actin and membrane binding properties, annexin 2 is a probable candidate for participating in the regulation of actin assembly and/or the formation of membrane-cytoskeleton links at certain membrane sites. This is supported by several reports revealing a relationship between annexin 2 and the actin cytoskeleton that underlies cellular membranes (Babiychuk and Draeger, 2000; Harder and Gerke, 1993; Harder and Gerke, 1994; Merrifield et al., 2001; Oliferenko et al., 1999; Zobiack et al., 2002). Recently, annexin 2 was found to be specifically enriched at the membrane–F-actin interface of motile pinosomes (Merrifield et al., 2001). The formation of macropinosome actin tails was inhibited by the overexpression of a trans-dominant annexin 2 mutant protein, which revealed an essential role for annexin 2 in the generation of these structures. However, the finding that annexin 2 is not recruited to actin tails assembled behind moving *Listeria*, shows that annexin 2 is not generally targeted

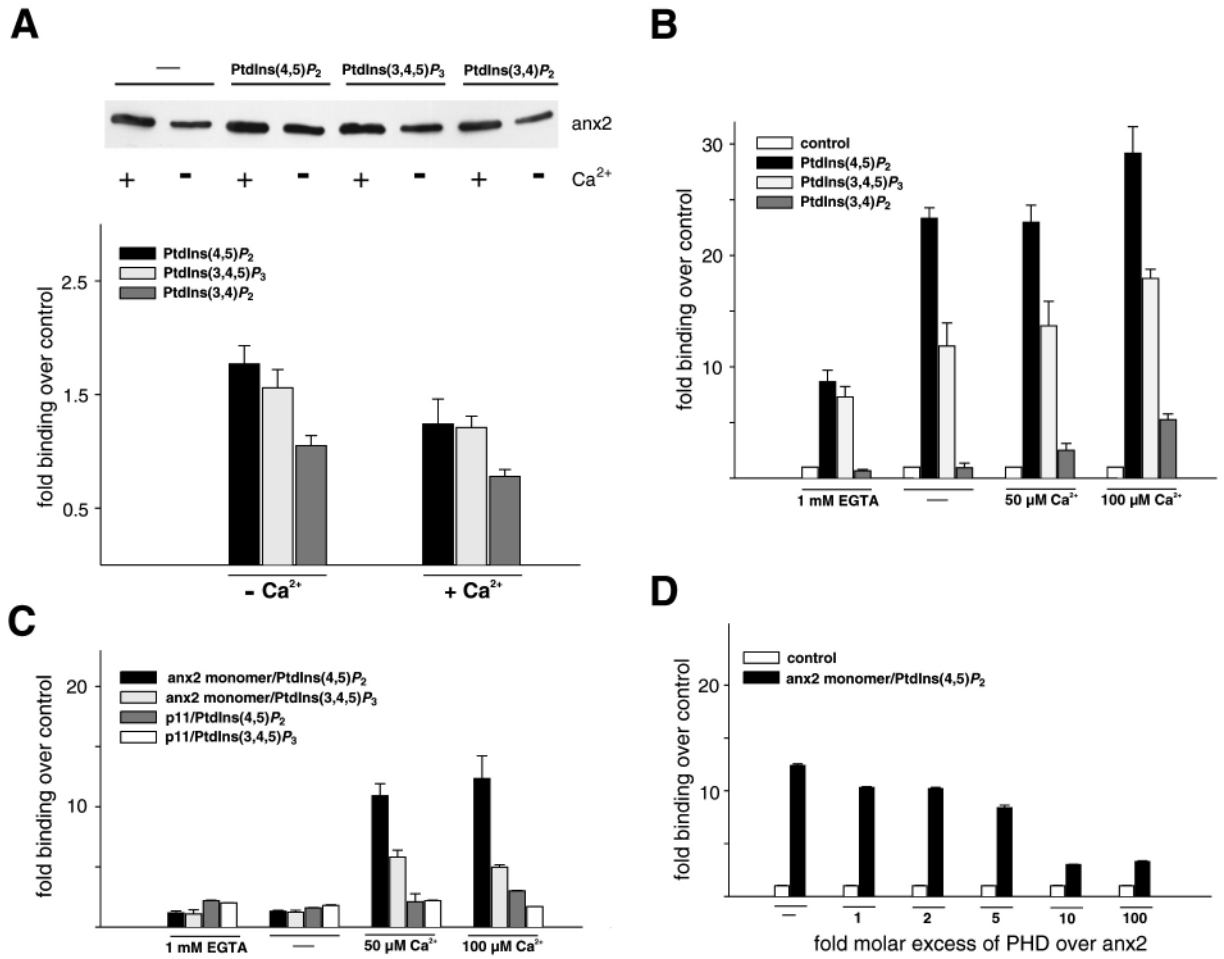

Fig. 3. Annexin 2 binds directly to PtdIns(4,5)P_2. (A) Co-sedimentation assays, employing the annexin 2-p11 heterotetramer and phospholipid liposomes. Annexin 2-p11 complex was mixed with brain-extract liposomes containing no (–) or 0.5% (w/w) of the indicated phosphoinositides in the presence of 1 mM Ca^{2+} (+) or in the presence of 1 mM EGTA (–). Liposomes were collected by ultracentrifugation and bound annexin 2-p11 complex was detected by immunoblotting with an antibody specific for the annexin 2 subunit. Experiments were carried out several times and a representative blot is shown. Signal intensities of blots of three independent experiments were quantified by densitometric scanning. Relative intensities are presented as fold-binding (mean value±s.e.m.) over control, i.e. brain-extract liposomes without added phosphoinositides, in the diagramm below the blot. Note the increase in co-pelleted annexin 2 in the case of liposomes containing PtdIns(4,5)P_2 which is seen in the absence of Ca^{2+}. (B) Lipid-plate binding-assays. Microtiter wells were coated with the indicated lipids and blocked with BSA. Purified annexin 2-p11 complex was added in the presence of Ca^{2+} or EGTA as indicated; (–) denotes reactions carried out in buffer alone. The amount of bound complex was determined by a colorimetric reaction using annexin 2-specific antibodies and peroxidase-coupled secondary antibodies. In the absence of phosphoinositides (control), a weak background signal was detected. Binding assays were performed at least three times and the bar graphs give the mean value ± s.e.m. calculated from triplicate samples of a representative individual experiment. (C) The annexin 2-p11 complex was dissociated and the binding of the individual subunits to both, immobilized PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 was investigated with the experimental setup described in (B), using either annexin 2- or p11-specific antibodies. (D) The binding of monomeric annexin 2 to immobilized PtdIns(4,5)P_2 was compared with that of the recombinantly expressed PH domain of human PLC-δ1. Experiments were carried out using increasing molar ratios of PHD-PLC to annexin 2 and bound annexin 2 was determined as described in (B).

to sites of dynamic actin rearrangement but requires the presence of a cellular membrane. Previous analyses have observed that the subcellular membrane association of annexin 2 seems to be dynamically regulated by changes in the cholesterol distribution of membrane. Annexin 2 is extracted by cholesterol sequestering agents from cell membranes, together with a set of actin binding proteins involved in the regulation of the cortical cytoskeleton (Harder et al., 1997; Zeuschner et al., 2001). Moreover, cholesterol accumulation in late endosomes caused by the genetic disorder Niemann-Pick C or by drug treatment is paralleled by a relocalization of annexin 2 to these altered structures (Mayran et al., 2003). Based on the observation that annexin 2 associates with detergent-resistant cholesterol-rich membrane domains, the protein has been proposed to play a role in the organization and dynamics of membrane rafts (Harder et al., 1997; Oliferenko et al., 1999). However, the precise nature of the ‘annexin 2 receptor’ responsible for the selective targeting of the protein to such lipid microdomains has remained elusive.

In addition to serving as a precursor of signaling molecules

such as inositol triphosphate and diacyl glycerol, membrane phosphoinositides such as PtdIns(4,5)P_2 participate in a number of fundamental cellular processes including vesicular trafficking and cell motility (Cullen et al., 2001; Martin, 2001). Interestingly, PtdIns(4,5)P_2 seems to be clustered in raft-like structures; similar to sphingolipid/cholesterol-rich rafts, the PtdIns(4,5)P_2-rich microdomains depend on cholesterol. Depletion of cholesterol disperses PtdIns(4,5)P_2 from sphingolipid-cholesterol microdomains (Pike and Miller, 1998). Sphingolipid/cholesterol-rich rafts were shown to be preferential sites of membrane-linked, PtdIns(4,5)P_2-mediated actin-assembly, such as that occuring during the formation of actin comets behind raft-enriched vesicles (Rozelle et al., 2000). This prompted us to investigate whether annexin 2 is directed to sites of actin assembly by an interaction with PtdIns(4,5)P_2. In a previous study, we have identified sites of EPEC adhesion as being enriched in components of lipid rafts such as cholesterol (Zobiack et al., 2002). We therefore imaged the PtdIns(4,5)P_2 dynamics during EPEC infection. Our data indicate that PtdIns(4,5)P_2 and annexin 2-p11 are recruited to actin assembly sites underneath adhering EPEC, and that this occurs independently of Tir and thus the recruitment of N-WASP and Arp 2/3. To further correlate sites of local PtdIns(4,5)P_2 enrichment with annexin 2 accumulation, we made use of the Arf6 Q67L mutant protein to manipulate the endogenous PtdIns(4,5)P_2 levels (Brown et al., 2001; Honda et al., 1999). As expected, overexpression of this mutant resulted in the formation of actin/PtdIns(4,5)P_2 positive large vacuoles that also labeled strongly for annexin 2 and its ligand p11.

Although the presence of the PHD-YFP signal clearly indicates elevated PtdIns(4,5)P_2 levels, changes in the lipid composition caused by Arf6 activation are complex and do not allow simple conclusions. We therefore carried out direct binding experiments to test a physical interaction of annexin 2 with PtdIns(4,5)P_2. The specificity of the protein for PtdIns(4,5)P_2 as compared with other phosphoinositides and the ability of the PH domain of PLC-δ1 to compete with annexin 2 for PtdIns(4,5)P_2-binding, strongly suggests that it is an enrichment of PtdIns(4,5)P_2 that recruits annexin 2-p11 to membrane sites which function as actin assembly points. Annexin 2 forms heterotetrameric complexes with p11 via its N-terminal domain which harbours the p11 binding site. The PtdIns(4,5)P_2 interaction is obviously mediated through the annexin 2 subunit of the complex, whereas the heterotetramer needs less Ca^{2+} for the binding. This indicates that the PtdIns(4,5)P_2 binding site in annexin 2 is somewhat altered and possibly more exposed in the p11-complexed form and the Ca^{2+}-bound form. The architecture of the PtdIns(4,5)P_2 binding site in annexin 2 is not known at present and remains to be investigated in further detail. It is, however, probably different from the Ca^{2+}/lipid binding sites in the core domain that is shared by annexin 2 and annexin 1, because the latter is not capable of specific PtdIns(4,5)P_2-binding. Moreover, the PtdIns(4,5)P_2-binding of annexin 2 depends at least in part on its unique N-terminal domain, because the C-terminal annexin core domain does not efficiently compete with full-length annexin 2 for PtdIns(4,5)P_2-binding (not shown). Based on these findings it appears plausible that the annexin 2-p11 complex uses the Ca^{2+}-dependent membrane-binding ability of the annexin 2 protein-core to dock onto membranes and, subsequently, uses the binding to PtdIns(4,5)P_2 for the specific accumulation at PtdIns(4,5)P_2-rich membrane sites. Thus, annexin 2, identified here as a member of the increasing group of PtdIns(4,5)P_2-binding proteins, is a probable candidate for affecting in its p11-complexed form membrane-cytoskeleton contacts at sites, specified by an enrichment of PtdIns(4,5)P_2 that resides in cholesterol-rich rafts.

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