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Annexin A8 displays unique phospholipid and F-actin binding properties

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Abstract Annexin A8 is a poorly characterized member of the annexin family of Ca^{2+}-regulated membrane binding proteins. Initially only identified at the cDNA level it had been tentatively linked to acute promyelocytic leukaemia (APL) due to its high and regulated expression in APL-derived cells. Here we identify unique properties of the annexin A8 protein. We show that it binds Ca^{2+}-dependently and with high specificity to phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P_2) and is also capable of interacting with F-actin. In line with these characteristics annexin A8 is recruited to F-actin-associated PtdIns(4,5)P_2-rich membrane domains formed in HeLa cells upon infection with non-invading enteropathogenic *Escherichia coli*. These properties suggest a role of annexin A8 in the organization of certain actin-associated membrane domains.

Keywords: Calcium; Cytoskeleton; Membrane binding; Phosphoinositides

1. Introduction

Annexins are a multigene family of proteins characterized by their reversible, Ca^{2+}-dependent interaction with membrane surfaces. They share as a common structural principle a compact, slightly curved domain, the so-called annexin core. This is built of four annexin repeats, segments of 70–80 amino acids showing intra- and intermolecular sequence homologies [1]. Within the core, annexins can harbour specific, non-EF hand Ca^{2+} sites, so called type II sites, which mediated an interaction with phospholipids headgroups in membrane-bound annexins. Although the core domain is structurally well conserved in all annexins, different members of the family show different Ca^{2+} sensitivities and phospholipid headgroup specificities (for review, see [2]).

Within cells, several annexins have been shown to shuttle between the cytosol and membrane surfaces with intracellular Ca^{2+} rises being a prime regulator of membrane association. Membrane binding occurs not only in a regulated but most likely also in a specific manner since different annexins interact with different target membranes. The N-terminal domains, which precede the core and are unique for a given annexin, play an important role in conferring target membrane specificity. They can also harbour sites for posttranslational modification and protein ligand binding (for review, see [3,4]). Some annexins have been shown to interact with components of the actin cytoskeleton. Annexin A2, for example, can bind to and under certain conditions even bundle F-actin filaments in a Ca^{2+}-dependent manner [5–7]. Therefore, at least some annexins are thought to function as membrane scaffolds organizing and/or stabilizing certain membrane domains, e.g., by providing a link to an underlying actin cytoskeleton (for review, see [8]).

Upon screening of a human placental cDNA library with a probe for human vascular anticoagulant-α (VAC-α), now known as annexin A5, Hauptmann et al. discovered cDNA clones comprising an open reading frame 56% identical to VAC-α [9]. The deduced protein was named VAC-β and is now referred to as annexin A8. In contrast to many other annexins, annexin A8 is a poorly characterized member of the family expressed at rather low levels in a tissue-specific manner [10,11]. However, acute promyelocytic leukemia (APL) cells and APL-derived NB4 cells constitute a notable exception since they contain high amounts of annexin A8 mRNA [12]. Moreover, expression of annexin A8 is downregulated after all-*trans* retinoic acid-induced differentiation of APL cells and the protein has therefore been linked to the regulation of proliferation and cellular differentiation [13]. Here we describe for the first time unique biochemical properties of annexin A8 and show that it interacts in a Ca^{2+}-dependent manner with PtdIns(4,5)P_2 and actin filaments. In line with these characteristics it is recruited to PtdIns(4,5)P_2-rich sites of dynamic actin rearrangement induced by infection of HeLa cells with non-invading enteropathogenic *Escherichia coli*.

2. Materials and methods

2.1. Expression and purification of human annexin A8

The full-length cDNA encoding human annexin A8 was amplified by PCR from a human placental MATCHMAKER cDNA library (BD Clontech, Heidelberg, Germany) and cloned into the *Eco*RI-linearized bacterial expression vector pET-23a(+) (Novagen, Heidelberg, Germany) that had been modified to allow expression of full-length annexin A8 without the T7 tag [14]. For protein expression, BL21(DE3)pLysS (Stratagene, Amsterdam, The Netherlands) bacteria transformed with the vector pET-23a(+)$_{mod}$/*anx*A8 were grown at 37 °C in LB-medium containing 150 μg/ml ampicillin to an OD_{600} of 0.6. 1 mM isopropyl-β-D-thiogalactose (IPTG) was then added to induce protein expression, and bacteria were harvested by centrifugation 3 h after induction. The bacterial cell pellet was resuspended in 1/10 volume of lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM DTT, 1 mM EGTA, 0.5 mM PMSF) and lysed by repeated freeze/thawing cycles (3 times) and sonication. The annexin A8-containing lysate was cleared by high-speed centrifugation and then dialyzed against liposome binding buffer (20 mM Imidazol–HCl, pH 7.4, 100 mM NaCl, 1 mM DTT, 0.5 mM PMSF).

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Annexin A8 was purified by exploiting its property to bind Ca^{2+}-dependently to phospholipid liposomes. Therefore, liposomes were generated by sonicating brain extract (Folch fraction I from bovine brain, Sigma B-1502, Steinheim, Germany) in water and then added at a final concentration of 2 mg/ml to the annexin A8-containing dialysate in the presence of 2 μM E64 and 1 mM $CaCl_2$. Following incubation for 1 h at room temperature the annexin A8-bound liposomes were pelleted by centrifugation at 100 000 × *g* for 30 min at 4 °C, resuspended in liposome buffer containing 50 mM EGTA and repelleted. Annexin A8 was released into the supernatant, dialyzed against liposome binding buffer and then subjected to a second round of liposome purification.

2.2. Phospholipid binding assay

Phospholipid binding assays [15] were performed in liposome buffer containing different Ca^{2+} concentrations (1 mM EGTA, 40 and 50 μM Ca^{2+}) using purified annexin A8 and phospholipids liposomes (2 mg/ml) generated as described above. Reaction mixtures were incubated for 1 h at room temperature and the liposomes were then spun down at 100 000 × *g* for 30 min at 4 °C. Pelleted liposomes were resuspended in liposome buffer containing 50 mM EGTA and again pelleted to release protein bound in a Ca^{2+}-dependent and reversible manner into the supernatant (EGTA eluate). Equal amounts of first supernatants, the EGTA eluates and pelleted liposomes were analyzed by SDS–PAGE and subsequent Coomassie staining.

2.3. Actin co-sedimentation assay

F-actin binding was analyzed as described [7] by co-sedimentation in reaction buffer containing 2 mM Tris–HCl, pH 7.5, 50 mM KCl, 1 mM $MgCl_2$, 0.33 mM ATP, 0.5 mM DTT. To generate F-actin, rabbit skeletal muscle actin (Cytoskeleton, Denver, USA) was diluted to 1 mg/ml in G-actin buffer (5 mM Tris–HCl, pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM ATP) and polymerisation was then induced by adding 1/50 volume of a polymerisation solution (2.5 M KCl, 100 mM $MgCl_2$, 50 mM ATP) and incubation for 1 h at room temperature. In a final volume of 200 μl, 25 μg of purified annexin A8 was incubated for 15 min at room temperature in the presence of 200 μM Ca^{2+} or 2 mM EGTA and precleared by centrifugation at 350 000 × *g* for 15 min at 4 °C. 25 μg of F-actin was added to the supernatant, the reaction was incubated for 1 h at room temperature and actin filaments were then spun down at 350 000 × *g* for 30 min at 4 °C. The remaining pellet was washed in reaction buffer containing Ca^{2+} or EGTA and repelleted. Equal amounts of the different samples (non-bound fraction, F-actin pellet) were analyzed by SDS–PAGE and Coomassie staining.

2.4. Lipid-plate binding assay

Phosphoinositide binding was analyzed employing a lipid-plate assay [16]. Briefly, wells of 96-well microtiter plates (F96 Maxisorp, Nunc, Wiesbaden, Germany) were coated with 2 μg/ml of the respective lipid, air-dried overnight, and then blocked for 1 h with 0.5% bovine serum albumine in phosphate-buffer saline (PBS). Purified annexin A8 (800 ng/well) in Ca^{2+}- or EGTA-containing PBS was added for 1 h at room temperature. Subsequently, wells were washed three times with PBS containing 0.05% Tween and the amount of bound protein was determined by a colorimetric reaction using polyclonal anti-annexin A8 antibodies (Santa Cruz Biotechnology, Heidelberg, Germany), peroxidase-coupled secondary antibodies and TMB substrate (Perbio, Bonn, Germany). For competition assays, the HIS-tagged PH-domain of human phospholipase C-δ_1 was purified as described [16] and added at the molar ratios indicated.

2.5. Mammalian expression

The cDNA coding for human annexin A8 was cloned into the *Eco*RI/*Sal*I-linearized mammalian expression vector pEGFP-N3 for in frame fusion to GFP (BD Clontech, Heidelberg, Germany). The correct sequence was verified by sequence analysis.

HeLa cells were maintained in Dulbecco's modified Eagle's medium (PAA, Pasching, Austria) with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin in a 7% CO_2 incubator at 37 °C. The Effectene system (Qiagen, Heidelberg, Germany) was used according to the manufacturer's protocol to introduce the annexin A8-GFP construct and cells were analyzed 24 h after transfection.

2.6. EPEC infection of HeLa cells

Transfected cells grown on coverslips were infected with enteropathogenic *E. coli* (EPEC) strain E2348/69 (wild-type) [17] or EPEC strain 2348/69 CVD452 (a mutant defective in type III secretion system) [18]. Infections were carried out 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 washed with PBS, fixed with 4% paraformaldhyde (PFA) in PBS, permeabilized using 0.2% Triton

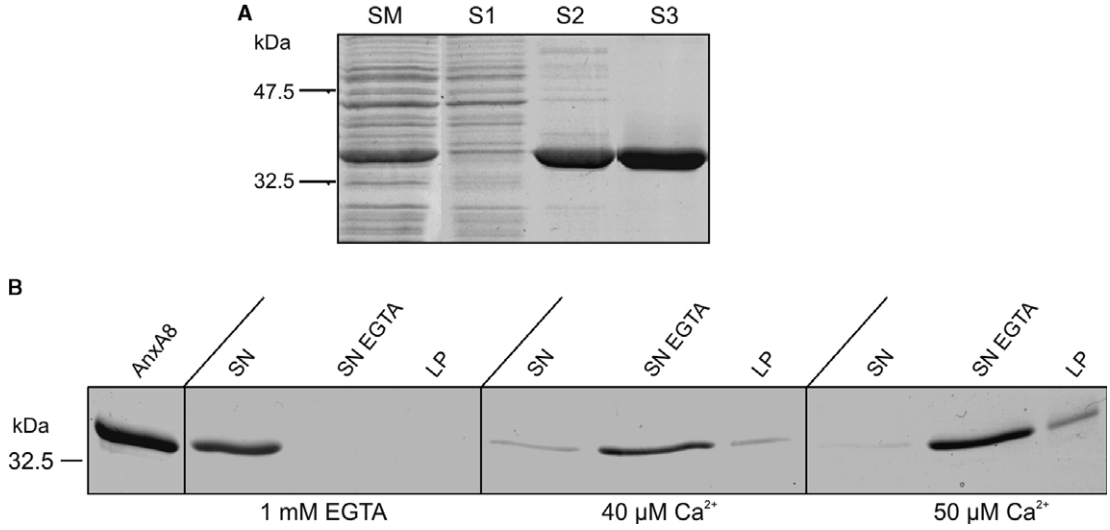

Fig. 1. Expression, purification and Ca^{2+}-dependent phospholipid binding of human annexin A8. (A) Recombinant protein expression and purification. Annexin A8 was expressed in *E. coli* BL21(DE3)-pLysS and purified from the soluble protein pool (SM) by two subsequent Ca^{2+}-dependent liposome binding steps. Protein samples of the different purification steps were analyzed by SDS–PAGE and subsequent Coomassie staining. S1, Ca^{2+}-containing supernatant after first liposome binding; S2, first EGTA eluate of pelleted liposomes; S3, purified annexin A8 (second EGTA eluate). (B) Purified annexin A8 was incubated with phospholipid vesicles in the presence of either EGTA (1 mM) or increasing Ca^{2+} concentrations (40 and 50 μM Ca^{2+}). Liposomes were pelleted, re-extracted with 50 mM EGTA and pelleted again. Supernatants of the first and second centrifugation containing the unbound material (SN), liposome-bound and EGTA-released annexin A8 (SN EGTA) as well as the liposome pellets containing irreversibly bound protein (LP) were analyzed by SDS–PAGE and Coomassie staining.

X-100 in PBS and treated with 50 mM NH_4Cl in PBS to quench free aldehydes. Filamentous actin was stained using TRITC-conjugated Phalloidin and coverslips were examined using a DM RXA fluorescence microscope (Leica, Wetzlar, Germany).

3. Results and discussion

3.1. Bacterial expression, purification and Ca^{2+}-dependent phospholipid binding of annexin A8

Biochemical studies performed with other bacterially expressed annexins revealed that they retained their typical properties such as the Ca^{2+}-dependent binding to negatively charged phospholipids (for example, see [19,20]). Therefore, we chose a bacterial expression system to produce full-length authentic annexin A8 (molecular weight of approximately 36 kDa, Fig. 1A). Recombinant annexin A8 was purified by preparative Ca^{2+}-dependent liposome binding. This protocol yielded 50 mg of purified annexin A8 from 1 L bacterial culture and produced the authentic, i.e., non-tagged, version of the protein, which is of particular importance since tags could easily alter biochemical properties. Employing this purified annexin A8, we characterized in more detail its Ca^{2+}-dependent phospholipid binding. Liposome pelleting experiments carried out with increasing Ca^{2+} concentrations revealed a nearly complete annexin A8 binding to negatively charged liposomes at 40 μM free Ca^{2+} which could be reversed by addition of the Ca^{2+} chelator EGTA (Fig. 1B). These binding data indicate that annexin A8 can respond to Ca^{2+} concentrations in the low micromolar range and thus is most likely regulated by Ca^{2+} fluctuations occurring within cells.

3.2. Annexin A8 binds F-actin and PtdIns(4,5)P_2 in vitro

Several members of the annexin protein family have been reported to bind F-actin in a Ca^{2+}-dependent manner, although in most cases this has only been demonstrated in vitro. For example, annexin A2 is able to bind and even bundle F-actin filaments in the presence of Ca^{2+} [5,21]. For annexin A2, two binding motifs were identified as being important for actin binding/bundling. One is located at the very C-terminus of the protein comprising the amino acid sequence LLYLCGGDD. A different motif that apparently mediates actin bundling is found in the fourth annexin repeat (VLIRIMVSR) [6,7]. Although, the C-terminal actin binding motif is conserved in many other annexins, e.g., annexin A4, an interaction of these annexins with F-actin has not been detected. Therefore, the existence of this motif is not the only prerequisite for F-actin binding [2,8]. Because the C-terminal actin binding motif is also found in annexin A8 we analyzed wether purified human annexin A8 can bind F-actin by employing co-sedimentation assays in the presence or absence of Ca^{2+}. As shown in Fig. 2, some annexin A8 co-sediments with F-actin in the presence of Ca^{2+}. Albeit being a minor fraction of the annexin A8 present in the reaction mixture under these conditions, a significant co-pelleting of annexin A8 with the actin filaments is observed consistently.

Within cells actin filaments often assemble at membrane sites characterized by an elevated content of certain phosphoinositides and one annexin, annexin A2, has been implicated recently to participate in mediating this assembly due to its capacity to bind F-actin and PtdIns(4,5)P_2 [16,22]. Since annexin A8 also displays some F-actin binding and could therefore act in a manner similar to annexin A2, we next analyzed wether annexin A8 can directly bind to phosphoinositides

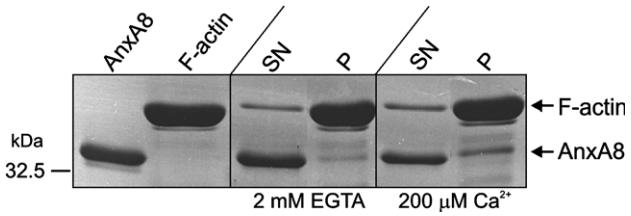

Fig. 2. F-actin co-sedimentation assay. Purified annexin A8 (Anx A8) was mixed with F-actin (F-actin) in the presence of either 200 μM Ca^{2+} or 2 mM EGTA and actin filaments were collected by centrifugation. Equal amounts of the non-bound fractions (SN) and the F-actin-containing pellet fractions (P) were analyzed by SDS–PAGE and subsequent Coomassie staining.

immobilized on microtiter wells. Fig. 3A reveals that annexin A8 indeed shows Ca^{2+}-dependent phosphoinositide binding with a clear preference for PtdIns(4,5)P_2. To confirm the specificity of the observed interaction with PtdIns(4,5)P_2, we carried out competition assays using the PtdIns(4,5)P_2-specific

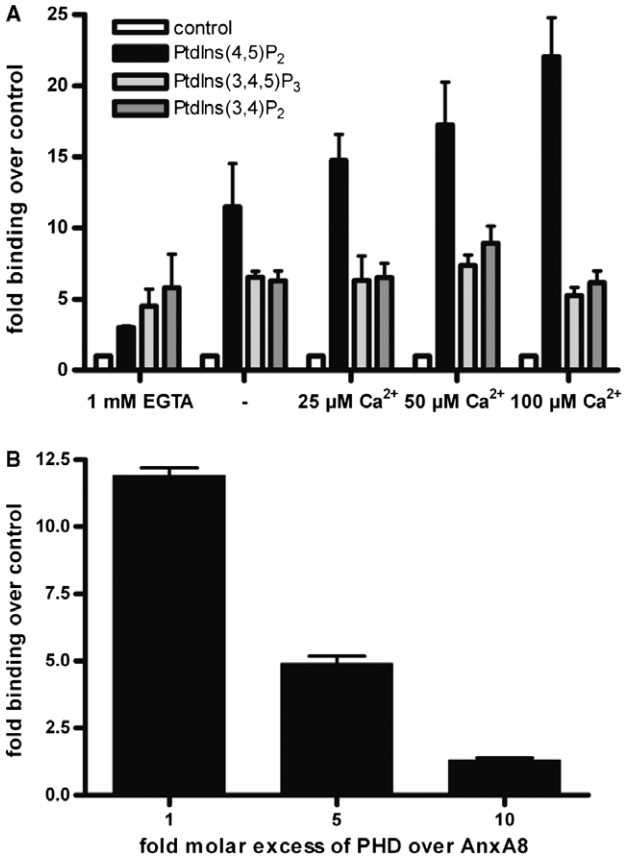

Fig. 3. Ca^{2+}-dependent binding of annexin A8 to phosphoinositides. (A) Wells of 96-well microtiter plates were coated with the indicated phosphoinositide and incubated with purified annexin A8 in the presence of EGTA or increasing Ca^{2+} concentrations as indicated. (−) denotes reactions carried out in buffer containing neither EGTA nor Ca^{2+}. After washing bound annexin A8 was detected by a colorimetric reaction using annexin A8-specific antibodies. Bar graphs show the mean value ± S.E.M. calculated from triplicate samples of one representative individual experiment. (B) The binding of annexin A8 to PtdIns(4,5)P_2 was compared to that of the PtdIns(4,5)P_2-binding PH-domain of phospholipase C-δ_1 (PHD). Therefore, increasing molar ratio of PHD to annexin A8 were added to the wells in the presence of 100 μM Ca^{2+} and bound annexin A8 was measured as described in A.

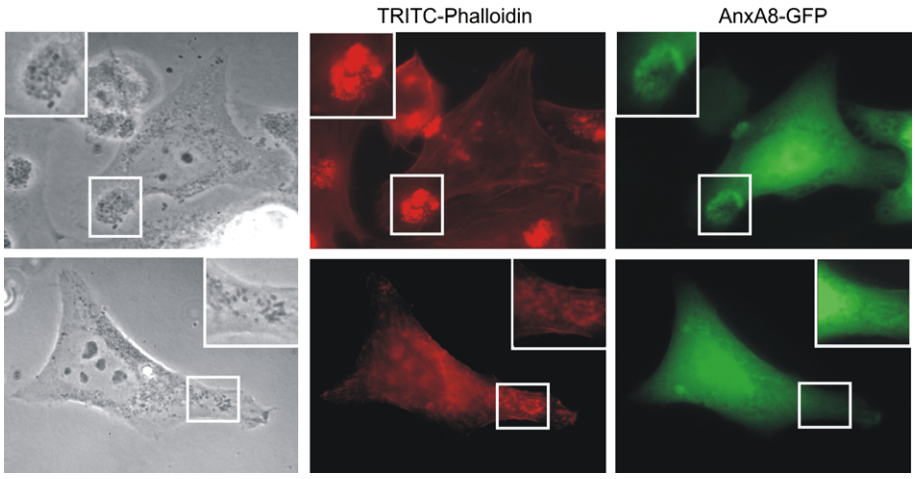

Fig. 4. EPEC infection induces annexin A8-GFP accumulation at sites of adhering bacteria. HeLa cells transiently expressing annexin A8-GFP were infected with either wild-type enteropathogenic *E. coli* (upper panels) or mutant EPEC CVD452 defective in type III secretion (lower panels). Three hours after infection, cells were fixed, permeabilized and analyzed for the distribution of annexin A8-GFP. Phase contrast images show the adhering bacteria. Staining with TRITC-Phalloidin reveals the characteristic reorganization of the actin cytoskeleton beneath adhering EPEC. Inset shows a higher magnification of adhering EPEC microcolonies.

PH-domain of phospholipase C-δ_1 [23]. As shown in Fig. 3B, a several-fold molar excess of the PH-domain can displace annexin A8, suggesting direct competition. Thus, similar to annexin A2, annexin A8 is capable of binding F-actin and PtdIns(4,5)P_2 and could play a role in organizing certain membrane-F-actin interfaces. However, significant differences between annexin A2 and A8 are discernible arguing for non-overlapping functions of these annexins. First, annexin A2 binds to F-actin at a ratio of 1:1.9 (annexin A2:actin) [24] whereas the interaction of annexin A8 with actin filaments occurs substoichiometrically. Second, while a significant interaction of annexin A2/S100A10 with PtdIns(4,5)P_2 is observed in the absence of Ca^{2+} [16], binding of annexin A8 to this phosphoinositide is strictly Ca^{2+}-dependent.

3.3. Annexin A8 is recruited to PtdIns(4,5)P_2-enriched sites of F-actin accumulation in vivo

To elucidate whether annexin A8 can associate with F-actin- and PtdIns(4,5)P_2-rich sites in vivo, we chose to locally induce areas of dynamic actin rearrangement by infecting HeLa cells with enteropathogenic *E. coli* (EPEC). Upon host cell attachment, EPEC use a type III secretion system to deliver *E. coli*-secreted proteins (Esps) into the host cells, including a receptor for the EPEC outer membrane protein intimin. This translocated intimin receptor (Tir) recruits host cell proteins involved in actin dynamics such as α-actinin, ezrin, Wiskott–Aldrich syndrome protein (WASP) and the actin-related proteins 2 and 3 (Arp2/3) complex leading to the formation of actin-rich protrusions, so-called pedestals, beneath the attached EPEC microcolonies [25,26]. In EPEC-infected HeLa cells the dynamic intracellular distribution of annexin A8 was monitored by employing annexin A8 fused N-terminally to GFP. In previous studies such annexin–GFP fusions have been shown to faithfully maintain the correct localization of the endogenous proteins and display the same biochemical properties as the authentic protein (for review, see [3]).

Fig. 4 reveals that annexin A8-GFP accumulates at sites of EPEC attachment which are easily discernible because of the characteristic reorganization of the actin cytoskeleton. EPEC CVD452, a mutant strain that lacks a functional type III secretion system and does not induce pedestal formation, fails to recruit annexin A8-GFP. As shown previously, EPEC attachment also causes an accumulation of components of lipid microdomains, e.g., cholesterol and GFP-GPI, together with an enrichment of PtdIns(4,5)P_2 [16,27]. Thus the recruitment of annexin A8 to sites of EPEC attachment is likely to result from an interaction with PtdIns(4,5)P_2 and/or F-actin. Most likely, both factors act in concert since one (PtdIns(4,5)P_2) typically functions upstream of the other (membrane recruitment of F-actin) in the regulation of cortical actin assemblies.

In summary, the observation that annexin A8 localizes to PtdIns(4,5)P_2-rich membrane sites of F-actin pedestal formation underneath adherent EPEC along with our biochemical data demonstrating F-actin and PtdIns(4,5)P_2 binding lead to the notion that annexin A8 could play a role in the organization of specific membrane/cytoskeleton contacts.

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