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Annexin 2 has an essential role in actin-based macropinocytic rocketing

Christien J. Merrifield*, Ursula Rescher†, Wolfhard Almers*, Jezabel Proust‡, Volker Gerke†, Antonio S. Sechi§ and Stephen E. Moss¶

Annexin 2 is a Ca^{2+} binding protein that binds to and aggregates secretory vesicles at physiological Ca^{2+} levels [1] and that also associates Ca^{2+} independently with early endosomes [2, 3]. These properties suggest roles in both exocytosis and endocytosis, but little is known of the dynamics of Annexin 2 distribution in live cells during these processes. We have used evanescent field microscopy to image Annexin 2-GFP in live, secreting rat basophilic leukemia cells and in cells performing pinocytosis. Although we found no evidence of Annexin 2 involvement in exocytosis, we observed an enrichment of Annexin 2-GFP in actin tails propeling macropinosomes. The association of Annexin 2-GFP with rocketing macropinosomes was specific because Annexin 2-GFP was absent from the actin tails of rocketing *Listeria.* This finding suggests that the association of Annexin 2 with macropinocytic rockets requires native pinosomal membrane. Annexin 2 is necessary for the formation of macropinocytic rockets since overexpression of a dominant-negative Annexin 2 construct abolished the formation of these structures. The same construct did not prevent the movement of *Listeria* in infected cells. These results show that recruitment of Annexin 2 to nascent macropinosome membranes is an essential prerequisite for actin polymerization-dependent vesicle locomotion.

Addresses: *Vollum Institute, 3181 Sam Jackson Park Road, Oregon 97201, USA. †Institute for Medical Biochemistry, Zentrum für Molekularbiologie der Entzündung, University of Muenster, von-Esmarch-Strasse 56, Muenster D-48149, Germany. ‡Department of Physiology, University College London, Gower Street, London WC1E 6BT, United Kingdom. §Department of Cell Biology, Gesellschaft fuer Biotechnologische Forschung, Braunschweig D-38124, Germany. ¶Division of Cell Biology, Institute of Ophthalmology, University College London, Bath Street, London EC1V 9EL, United Kingdom.

Correspondence: Stephen E. Moss

E-mail: s.moss@ucl.ac.uk

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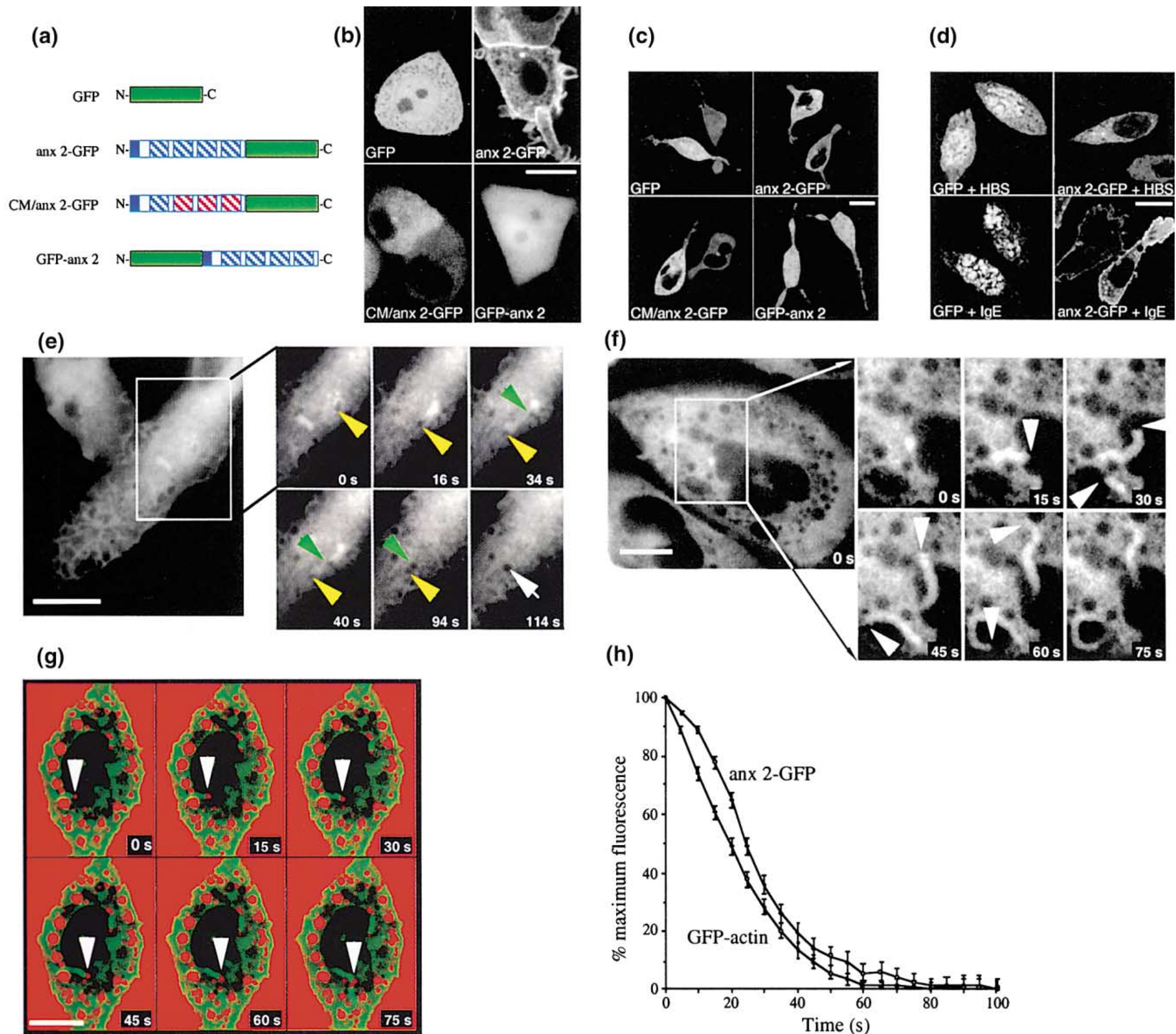
Results and discussion

To investigate the behavior of Annexin 2 in living cells, we tagged Annexin 2 with green fluorescent protein (Annexin 2-GFP) and used evanescent-field microscopy (EFM) to image the fusion protein in rat basophilic leukemia (RBL) cells. This technique allows the selective illumination of a thin peri-plasma membrane slice of living cells and has recently been used for the resolution of single exocytic events with fluorescent secretory granule markers [4]. To evaluate any anomalous effects of fusing GFP to Annexin 2 on intracellular targeting of the fusion protein, we expressed a range of constructs (Figure 1a) in PC12 and RBL cells. Endogenous Annexin 2 exists in part as a soluble monomer and as a heterotetrameric complex with the S100 protein p11 at the cell cortex [5]. Cortical targeting of Annexin 2 requires intact Ca^{2+} and p11 binding sites [6] and is thus a useful marker for the integrity of Annexin 2-GFP chimeras. When expressed in PC12 cells, GFP locates to both the cytoplasm and nucleus (Figure 1b). In contrast, fusion of GFP to the C terminus of wild-type Annexin 2 targeted GFP to the cell cortex with diffuse cytoplasmic fluorescence and exclusion from the nucleus (Figure 1b). This distribution is indistinguishable from that of native Annexin 2 as visualized with immunocytochemistry [7]. In contrast, mutation of the type II Ca^{2+} binding sites led to exclusively cytosolic expression of Annexin 2. These results show that correct targeting of Annexin 2-GFP to the plasma membrane of PC12 cells requires the core domain of Annexin 2 and intact Ca^{2+} binding sites.

In RBL cells Annexin 2-GFP was homogeneous throughout the cytoplasm and was excluded from the nucleus (Figure 1c). In stimulated chromaffin cells, Annexin 2 translocates from the cytosol to the cortex with similar kinetics to those of exocytosis [8]. We therefore tested whether Annexin 2-GFP would translocate to the cell cortex and/or secretory granules during exocytosis in RBL cells stimulated through the crosslinking of FcεR1. Similar to Annexin 2 in chromaffin cells, Annexin 2-GFP in RBL cells concentrated at the plasma membrane during exocytosis (Figure 1d). From these static images it is unclear whether the apparent enrichment of Annexin 2-GFP at the plasma membrane is due to selective loss from the cytoplasm during stimulation or genuine translocation. To address this question, we used EFM with time lapse image acquisition of Annexin 2-GFP in single sensitized RBL cells stimulated with DNP-albumin. Stimulation of cells expressing either Annexin 2-GFP or GFP alone

Figure 1



Hyperosmotic shock induces the formation of Annexin 2-GFP "comet tails" in RBL cells. **(a)** Constructs used in the studies included GFP alone, a fusion of GFP to the C terminus of Annexin 2 (anx 2-GFP), a fusion of GFP to the C terminus of an Annexin 2 mutant unable to bind Ca^{2+} (CM/anx 2-GFP), and GFP fused to the N terminus of Annexin 2 (GFP-anx 2). Hatched blue boxes represent native Annexin repeats; red boxes represent repeats with mutated Ca^{2+} binding sites; green boxes represent GFP; and solid blue boxes represent the Annexin 2 N terminus. **(b)** PC12 cells were transfected with the constructs in (a) and imaged by confocal microscopy. The fusion of GFP to the C terminus of Annexin 2 excluded GFP fluorescence from the nucleus and gave an enrichment of GFP fluorescence at the plasma membrane. The targeting of Annexin 2 to the plasma membrane in PC12 cells is Ca^{2+}-dependent since fusion of GFP with the Annexin 2 Ca^{2+} binding domain null mutant, CM/anx 2, fails to target fluorescence to the cell cortex. The scale bar represents 10 μm. **(c)** RBL cells were transfected as in (b) and imaged while alive. The scale bar represents 10 μm. **(d)** RBL cells were transfected with GFP or anx 2-GFP, incubated in HBS or HBS supplemented with DNP-albumin after sensitization with anti DNP-IgE, and fixed. They were then detergent extracted and mounted for confocal microscopy. GFP fluorescence is retained throughout both the nucleus and cytoplasm in resting cells but appears to be depleted from the cytoplasm in stimulated cells. The distribution of Annexin 2-GFP in (d) fixed resting cells is indistinguishable from that in (c) live resting cells, whereas stimulation leads to the concentration of Annexin 2-GFP at the plasma membrane. The scale bar represents 10 μm. **(e)** RBL cells expressing Annexin 2-GFP were challenged with hyperosmolar HBS and imaged by evanescent field microscopy (EFM). The time-resolved montage of the central area of the cell shows that at T = 0 s a vesicle appears in the evanescent field (yellow arrowhead) and moves in a manner parallel to the plane of the membrane (T = 0 s to T = 34 s) before stopping. A second rocketing vesicle (green arrow) appears at the same point, follows a similar path, and stops adjacent to the first vesicle, whereupon the two vesicles fuse (white arrow; see Movie 1 in the Supplementary material available with this article on the internet at http://images.cellpress.com/supmat/supmatin.htm). Note that Annexin 2-GFP is associated with the vesicles while they are motile but not when they become stationary. The scale bar represents 10 μm. **(f)** RBL cells transiently expressing Annexin

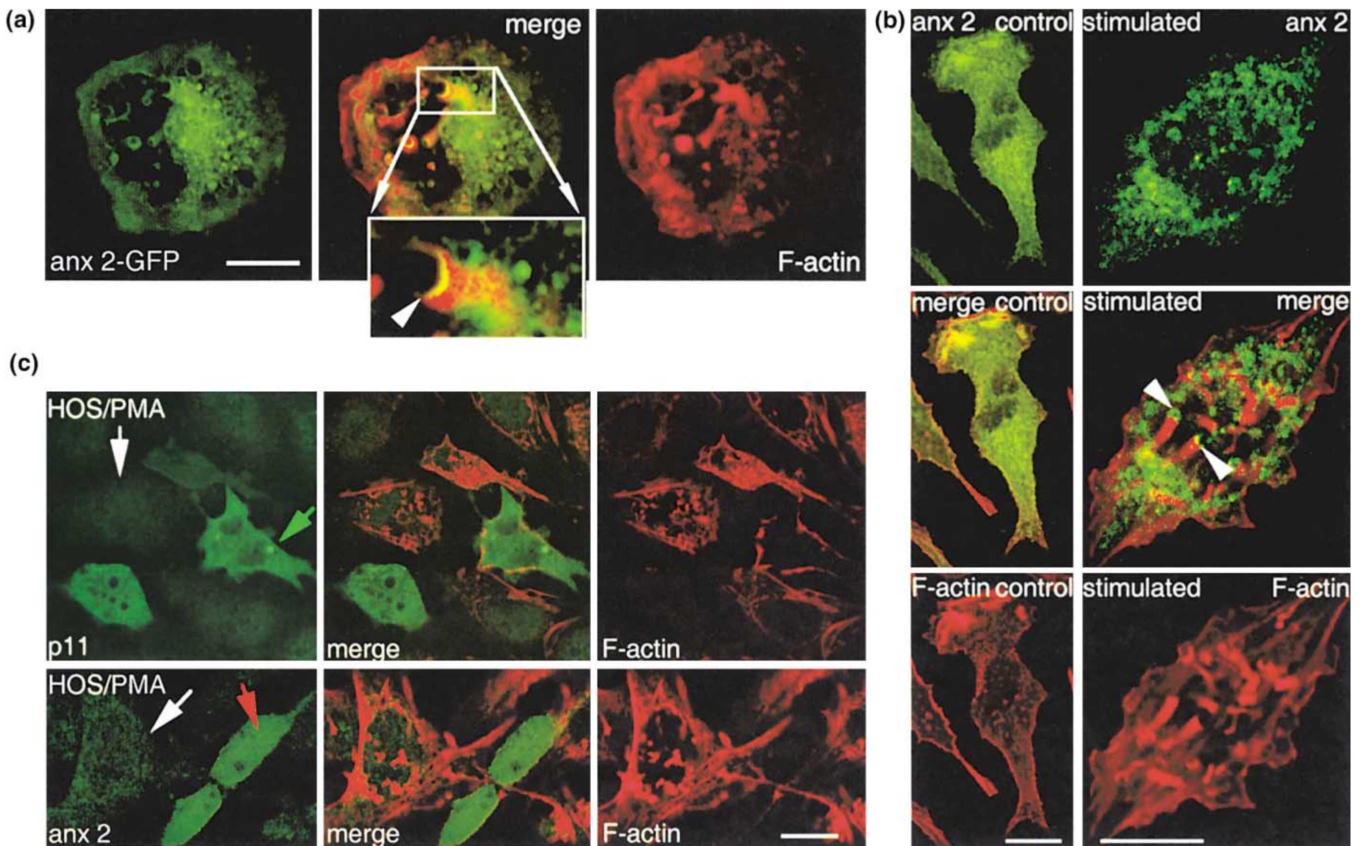
caused significant cell spreading (not shown), consistent with previous reports [9] and probably due to a decrease in cytoplasmic volume. To test whether the observed increases in membrane fluorescence were due to cell shrinkage, we challenged RBL cells expressing Annexin 2-GFP with hyperosmolar HBS. These conditions induced a similar increase in cellular fluorescence as that seen in stimulated cells without inducing any gross morphological changes. Unexpectedly, we also observed mobile vesicles that ranged in size from 100 nm to 2 μm and that moved in curved trajectories through the cytoplasm and were trailed by brightly fluorescent Annexin 2-GFP "comet" tails (Figure 1e). Since no such comets were seen in cells expressing GFP alone [10], the targeting of GFP to the comets was clearly dependent on its fusion to Annexin 2. Although there are no previous reports of Annexin 2 forming such structures, the Annexin 2-GFP comet tails superficially resembled the actin tails assembled by the intracellular bacterial pathogens *Listeria*, *Shigella*, and *Rickettsia* and the virus *Vaccinia* (for review see [11]).

We previously showed that comet tails induced in RBL cells by hyperosmotic shock are composed of F-actin and are nucleated by nascent macropinosomes at plasma membrane sites of pinocytosis [10]. We also showed that rocketing macropinosomes labeled with GFP-actin move at similar speeds to *Listeria* [12] and *Shigella* [13]. By costimulating cells with hyperosmotic shock and phorbol ester, we found that, as with GFP-actin, the incidence of Annexin 2-GFP-labeled rocketing vesicles was greatly enhanced (Figure 1f). Consistent with previous studies, dual wavelength confocal imaging of cells expressing Annexin 2-GFP immersed in Texas Red-dextran identified the vesicles as macropinosomes (Figure 1g). The characteristic tapered shape of actin comet tails arises from the rapid incorporation of actin at the cargo end of the tail followed by slower tail disassembly through actin depolymerization [14]. To compare the kinetics of dissociation from the tail region, we measured the fluorescence decay of both GFP-actin and Annexin 2-GFP tails (Figure 1h). This analysis revealed that Annexin 2-GFP tails decay with a slight lag when compared with GFP-actin tails ($t_{1/2}$ for GFP-actin tails = 21 s, $t_{1/2}$ for Annexin 2-GFP = 26 s). This could be because Annexin 2-GFP overexpression modulates the stability of the actin tail and retards disassembly through actin bundling [15], or it may be that Annexin 2 is not bound stoichiometrically to actin in the tail.

To examine the physical relationship between Annexin 2 and actin in macropinosome actin tails, we imaged Annexin 2, Annexin 2-GFP, and actin by using immunofluorescence. We hypothesized that if Annexin 2-GFP binds stoichiometrically to actin in comet tails, one would expect an even ratio of Annexin 2 to F-actin along the length of the tail. In fact, in stimulated RBL cells both Annexin 2-GFP (Figure 2a) and native Annexin 2 (Figure 2b) are concentrated at the interface between the actin tail and the attendant macropinosome, and this finding suggests that both endogenous Annexin 2 and Annexin 2-GFP in the actin tail are more unstable to fixation than the protein located at the pinosome-tail interface. To test whether Annexin 2 is necessary for the formation of rocketing macropinosomes, we transiently overexpressed the interfering XM mutant of Annexin 2 [16] in RBL cells before stimulating rocketing (Figure 2c). We identified cells overexpressing the XM protein by using an antibody specific to the human p11 part of XM or an antibody to the N terminus of Annexin 2; this latter antibody recognizes both XM and the endogenous protein. Rocketing macropinosomes were identified with rhodamine phalloidin. While 60% of untransfected cells had at least one rocketing macropinosome, consistent with our previous findings [10], not one of 200 cells (counted on 5 coverslips in 3 separate experiments) expressing the XM protein had any rocketing macropinosomes, and these results demonstrate that Annexin 2 is required in the formation of these structures. Collectively, these studies indicate an essential role for Annexin 2 in macropinocytic rocketing, possibly as a component of the molecular scaffold that tethers F-actin to the vesicle membrane. To test whether Annexin 2 associates directly with polymerized actin in vivo or requires the additional presence of plasma membrane, we investigated the localization of Annexin 2-GFP in cells infected with *Listeria* since *Listeria* activate actin polymerization downstream of a normal requirement for native cell membrane [17]. We observed that Annexin 2-GFP neither associates with actin clouds surrounding stationary bacteria nor with actin tails propeling *Listeria* (Figure 3a,b), and this observation demonstrates that Annexin 2-GFP association with actin tails indeed re-

2-GFP were challenged with hyperosmolar HBS containing 10 nM PMA and were imaged by EFM, resulting in multiple rocketing vesicles (arrowheads; see Movie 2 in the Supplementary material). The scale bar represents 10 μm. **(g)** RBL cells expressing Annexin 2-GFP were challenged as in (f) while immersed in medium containing Texas Red-dextran. Images were acquired by confocal microscopy and show at 15 s intervals the progress of a red-labeled pinosome (arrowhead) with a green Annexin 2-GFP tail (see Movie 3 in the Supplementary material). The scale bar represents 20 μm. **(h)** The analysis of image stacks of rocketing vesicles in cells expressing either GFP-actin [10] or Annexin 2-GFP allowed the determination of the decay profile for individual comet tails. A region of interest of similar diameter to the vesicle was defined in the path of a comet tail, and the average fluorescence in this region was measured over successive frames. The background of each data set was subtracted for local fluorescence, and 25 sets of data were pooled and aligned to give the curves shown. Each point is ± s.e.m. Annexin 2-GFP comet tails decay with a slight lag compared to GFP-actin tails.

Figure 2

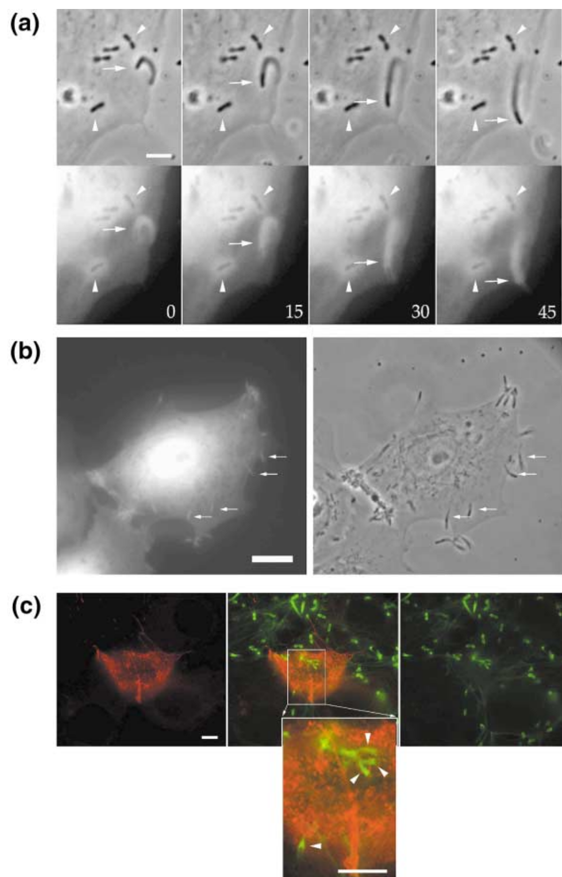


Annexin 2 is concentrated at the actin tail/pinosome interface and is essential for macropinocytic rocketing. **(a)** RBL cells were transfected with Annexin 2-GFP, stimulated, fixed, detergent extracted, and stained for F-actin with rhodamine phalloidin. Annexin 2-GFP is depleted from the rhodamine-labeled actin tail but is enriched at the interface of stained F-actin rockets and the attendant pinosome (yellow ring, indicated by arrowhead). The scale bar represents 10 μm. **(b)** Resting (left side panels) or stimulated (right side panels) wild-type RBL cells were fixed, permeabilized, and stained for Annexin 2 (green) and actin (red). Annexin 2 in resting cells is distributed evenly through the cytoplasm and colocalizes with F-actin in ruffles. In cells stimulated with hyperosmotic shock/PMA, the F-actin cytoskeleton reorganizes into rocket tails. Endogenous Annexin 2 is not present in these tails but is preferentially located at the vesicles at the rocket tips (indicated by arrowheads). The scale bars represent 10 μm. **(c)** RBL cells were transfected with the dominant-negative mutant XM, cultured overnight, washed in HBS, stimulated, fixed, and permeabilized as detailed earlier. The XM mutant (upper panels) and Annexin 2 (lower panels) were detected with H21 and HH7 monoclonal antibodies, respectively, and F-actin was detected with rhodamine-phalloidin. Stimulated nontransfected cells showed no XM staining (white arrow, upper panel) because the H21 antibody does not recognize rat p11, whereas expression of the XM mutant led to the formation of Annexin 2/p11 aggregates positive for the H21 antibody, which recognizes the XM protein (green arrow). The appearance of actin comets is completely inhibited in such cells. Stimulated nontransfected cells showed Annexin 2 staining through the cytoplasm (white arrow, lower panel), and small aggregates of Annexin 2 are visible in some transfected cells (red arrow). The scale bar represents 10 μm.

quires native pinosomal membrane. Because Annexin 2 is absent in *Listeria* actin tails but present in the tails of mobile macropinosomes, we hypothesized that Annexin 2 function would be dispensible for *Listeria* motility. Expression of the XM dominant mutant of Annexin 2 was found to have no effect on the motility of *Listeria* in infected cells (Figure 3c), and this finding supports the idea that the role of Annexin 2 lies upstream of actin polymerization.

Our understanding of the mechanisms responsible for the initiation and control of actin polymerization has accelerated through the investigation of intracellular pathogens that use actin polymerization to drive their own locomotion. Intracellular pathogens such as *Listeria* activate actin polymerization downstream of the normal physiological requirement for cellular membrane. While studies of rocketing pathogens have been instrumental in defining the minimal machinery necessary for actin polymerization [18], they reveal little about the upstream signaling events that control the polymerization motor and/or link the propelling machinery to cellular membranes. This gap has been partly filled by investigations of rocketing synthetic liposomes in vitro [19, 20]. These investigations revealed

Figure 3



Annexin 2-GFP does not associate with intracellular *Listeria*. **(a)** PtK2 cells transiently transfected with Annexin 2-GFP were infected with *Listeria monocytogenes* and imaged 4 hr later. **(b)** The slight background fluorescence of Annexin 2-GFP around nonmotile bacteria (arrowheads) and in the actin tails of motile *Listeria* (arrows) is also seen with GFP alone. The bottom panels show Annexin 2-GFP, and the top panels show the corresponding phase contrast images. Elapsed time (in seconds) is indicated in the bottom panels. The scale bar represents 2 μm. PtK2 cells were transiently transfected with GFP, then infected and imaged as above. The arrows point to actin tails in both the GFP and phase contrast images. The scale bar represents 5 μm. **(c)** PtK2 cells were transiently transfected with the dominant-negative Annexin 2 construct and infected with *Listeria monocytogenes*. Four hours after infection, cells were fixed, permeabilized, and costained with Texas Red-conjugated phalloidin and the antibody H21 that recognizes the XM protein. The enlarged inset shows that in cells expressing the XM mutant, *Listeria* still induce the formation of actin tails (arrowheads). The scale bar represents 2 μm.

a requirement for phosphoinositides and Cdc42 for the nucleation of actin polymerization at membrane surfaces. However, alternative mechanisms for nucleating actin polymerization exist, and Rho family G proteins may not control the initiation of polymerization in all cases. For example, the intracellular virus *Vaccinia* nucleates actin polymerization through a mechanism controlled by Src family tyrosine kinases [21]. It thus seems likely that alternative pathways—and probably unknown or unrecognized proteins—exist that control actin polymerization at membrane surfaces in vivo. Since Annexin 2 can bind both negatively charged phospholipids and actin at μM concentrations of Ca^{2+}, one might postulate a priori that Annexin 2 is a likely candidate for such a role. Indeed, a functional link between Annexin 2, the actin cortex, and early endosomes is supported by work showing that overexpression of the XM dominant-negative Annexin 2 mutant disrupts the cortical distribution of early endosomes in MDCK cells [16]. In conclusion, these data constitute the first in vivo evidence of a link among Annexin 2, an endosomal membrane, and polymerizing actin. Recruitment of Annexin 2-GFP to macropinosomes cannot be explained by promiscuous actin binding because the chimera is not recruited to intracellular *Listeria*. Furthermore, while Annexin 2 is neither involved in nor required for *Listeria* motility, it is essential for the formation of rocketing pinosomes since expression of a dominant-negative Annexin 2 mutant completely abolishes rocket tail formation. These results suggest a role for Annexin 2 in the regulation of actin polymerization at the actin-pinosome interface.

Supplementary material

Supplementary materials and methods as well as three supplementary movies are available with this article on the internet at http://images.cellpress.com/supmat/supmatin.htm.

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