Annexin 2 Binding to Phosphatidylinositol 4,5-Bisphosphate on Endocytic Vesicles Is Regulated by the Stress Response Pathway*

Matthew J. Hayes, Christien J. Merrifield‡‡, Dongmin Shao, Jesus Ayala-Sanmartin‡, Crislyn D’Souza Schorey§, Tim P. Levine, Jezabel Proust**, Julie Curran, Maryse Bailly, and Stephen E. Moss‡‡

From the Division of Cell Biology, Institute of Ophthalmology, University College London, 11-43 Bath Street, London EC1V 9EL, United Kingdom, the Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, Connecticut 06520, and INSERM U538, Trafic membranaire et signalization dans les cellules épithéliales, CHU Saint Antoine, 27, rue Chaligny, 75012 Paris, France, the Department of Biological Sciences, University of Notre Dame, Notre Dame, Indiana 46556-0369, and **UMR 5546 CNRS/Université P. Sabatier, Pôle de Biotechnologies Végétales, 24, chemin de Borde Rouge, B.P. 17 Auziveille, 31326 Castanet-Tolosan, France

Annexin 2 is a Ca\textsuperscript{2+}-binding protein that has an essential role in actin-dependent macropinosome motility. We show here that macropinosome rocketing can be induced by hyperosmotic shock, either alone or synergistically when combined with phorbol ester or pervanadate. Rocketing was blocked by inhibitors of phosphatidylinositol 3-kinase (s), p38 mitogen-activated protein (MAP) kinase, and calcium, suggesting the involvement of phosphoinositol signalling. Since various phosphoinositides are enriched on inwardly mobile vesicles, we examined whether or not annexin 2 binds to any of this class of phospholipid. In liposome sedimentation assays, we show that recombinant annexin 2 binds to phosphatidylinositol 4,5-bisphosphate (PtdIns-4,5P\textsubscript{2}) but not to other poly- and mono-phosphoinositides. The affinity of annexin 2 for PtdIns-4,5P\textsubscript{2} (K\textsubscript{D} \approx 5 \mu M) is comparable with that reported for a variety of PtdIns-4,5P\textsubscript{2}-binding proteins and is enhanced in the presence of Ca\textsuperscript{2+}. Although annexin 1 also bound to PtdIns-4,5P\textsubscript{2}, annexin 5 did not, indicating that this is not a generic annexin property. To test whether annexin 2 binds to PtdIns-4,5P\textsubscript{2} in vivo, we microinjected rat basophilic leukemia cells stably expressing annexin 2-green fluorescent protein (GFP) with fluorescently tagged antibodies to PtdIns-4,5P\textsubscript{2}. Annexin 2-GFP and anti-PtdIns-4,5P\textsubscript{2} IgG co-localized at sites of pinosome formation, and annexin 2-GFP relocalized to intracellular membranes in Ptk cells microinjected with Arf6Q67L, which has been shown to stimulate PtdIns-4,5P\textsubscript{2} synthesis on pinosomes through activation of phosphatidylinositol 5 kinase. These results establish a novel phospholipid-binding specificity for annexin 2 consistent with a role in mediating the interaction between the macropinosome surface and the polymerized actin tail.

The vertebrate annexin family comprises a group of 12 unique genes that encode proteins which are biochemically characterized by their ability to bind to negatively charged phospholipids in the presence of calcium ions (for a review, see Ref. 1). Although differences exist for individual annexins with regard to preference for specific lipids, such as phosphatidylinerine and phosphatidylethanolamine, and also the Ca\textsuperscript{2+} requirement for half-maximal binding, this fundamental property has led to a paradigm for annexin function in which soluble cytosolic annexins translocate to intracellular membrane surfaces upon elevation of intracellular [Ca\textsuperscript{2+}]. Such Ca\textsuperscript{2+}-dependent membrane association has been demonstrated for several annexins in various cell types (2–4), although the functional consequences of reversible membrane binding by annexins are not well understood.

Annexin 2 is typical in this regard and has been shown to associate with early endosomes, macropinosomes, and phagosomes (5–7). However, the interaction between annexin 2 and endosomes is unusual in being Ca\textsuperscript{2+}-independent, instead relying at least in part on the presence of cholesterol in the endosomal membrane (8, 9), although the possible involvement of other protein-lipid and protein-protein interactions cannot be excluded. Annexin 2 is also an F-actin-binding protein, and since endosomes move from their sites of formation to the cell interior in an actin-dependent manner (10, 11), one possible role for annexin 2 in this process is at the interface between the endocytic vesicle and the associated actin filaments. Such a role would fit with the observations that although annexin 2 is a constituent of the actin tails that propel macropinosomes in rat basophilic leukemia (RBL)\textsuperscript{1} cells, it is enriched at the point of contact between the actin tail and the vesicle (6). Disruption of annexin 2 function by expression of a dominant negative mutant has been shown to disturb the trafficking of endosomes in HeLa cells (12) and to completely inhibit actin-based pinosome motility in RBL cells (6). In the latter study, it was noted that the same annexin 2 mutant had no effect on the actin-dependent motility of the intracellular pathogen, Listeria monocytogenes, demonstrating that the role of annexin 2 lies upstream of the actin polymerization machinery.

* This work was supported by the Wellcome Trust, Medical Research Council, Fight for Sight and the European Commission (contract number BIO4CT960083). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡‡ To whom correspondence should be addressed. Tel.: 020-7608-6973; Fax: 020-7608-4034; E-mail: s.moss@ucl.ac.uk.

The abbreviations used are: RBL, rat basophilic leukemia; PtdIns3P, phosphatidylinositol 3-phosphate; PtdIns4P, phosphatidylinositol 4-phosphate; PtdIns-4,5P\textsubscript{2}, phosphatidylinositol 4,5-bisphosphate; PtdIns-3,4,5P\textsubscript{3}, phosphatidylinositol 3,4,5-trisphosphate; MAP, mitogen-activated protein; GFP, green fluorescent protein; TMR, tetramethylrhodamine; TRITC, tetramethylrhodamine isothiocyanate; HOS, hyperosmolar saline; Perv, pervanadate; GTP/S, guanosine 5’-O-(thio)triphosphate; PMA, phorbol myristic acid; PBS, phosphate-buffered saline; HBS, HEPES-buffered saline; FITC, fluorescein isothiocyanate; DOPC, dioleoylphosphatidylcholine; BB, Dioleoylphosphatidylcholine.
These findings indicate that the binding of annexin 2 to endocytic vesicles is key to the function of this protein. Analysis of the lipid composition of endosome, pinosome, and phagosome membranes has revealed that these are enriched in mono- and polyphosphoinositides that are involved in the recruitment of proteins that mediate dynamic changes in the actin cytoskeleton (13–15). We show here that actin-dependent macropinosome rocketing is regulated by the stress response pathway, activation of which is known to lead to the synthesis of various phosphoinositides. We therefore examined whether or not annexin 2 could bind to any of this class of lipids, since such interactions may play an important role in the regulation of annexin 2 function. We show that annexin 2 binds to PtdIns-4,5P$_2$ in living cells. These findings, when placed in the context of existing knowledge of phospholipid-binding by annexins, demonstrate the versatility of lipid-binding by annexin 2 and support the idea that annexin 2 is a regulator of membrane-cytoskeleton dynamics in vesicle trafficking.

**EXPERIMENTAL PROCEDURES**

**Labeling Macropinosomes with Fluorescently Labeled IgE—Alexa-568 (Molecular Probes) was conjugated to anti-2,4-dinitrophenol IgE (Sigma) according to the manufacturers’ instructions and stored at −80 °C. Adherent RBL cells cultured as described previously (10) were chilled to 4 °C in HEPES-buffered saline (HBS). HBS (150 mM NaCl, 5 mM KCl, 1.8 mM CaCl$_2$, 0.8 mM MgCl$_2$, 20 mM HEPES, 1 mM glucose, pH 7.4) + 100 ng/ml IgE-A568 was added to the cells. After 30 min, cells were washed in ice-cold HBS to remove unbound IgE-A568 and then stimulated to produce actin rockets with hyperosmolar HBS (HBS supplemented with Perv (D), PMA (E), Perv/HOS (F), PMA/HOS (G), Perv/PMA/HOS (H). Stimulation with Perv led to the generation of a polarized F-actin cap (D, arrow), whereas co-stimulation with HOS induced F-actin rockets (F, arrow). Stimulation with PMA led to cell spreading, whereas co-stimulation with HOS led to the generation of rockets (G, arrow). Co-stimulation with PMA/Perv led to cell spreading and generation of rockets in the absence of HOS (H, arrows). Scale bar, 10 μm.
meabilized for 5 min using PBS supplemented with 10–100 μg/ml saponin, and washed a further three times in PBS. Cells were incubated overnight at 4 °C in the presence of primary probe and then washed in PBS before incubation with secondary probes for 1 h at 37 °C in a moist chamber. Coverslips were then washed again in PBS before incubation with tertiary probes for 1 h at 37 °C in a moist chamber. Coverslips were washed a further three times and mounted in 90% glycerol, 10% PBS, 0.01% n-propylgallate. To visualize actin rockets, cells were fixed and stained using FITC-phalloidin (Sigma) prior to mounting. To quantify the incidence of rocketing, 100 cells were examined, and the number of cells with one or more clearly defined F-actin rocket was scored. In experiments to examine the pharmacology of rocketing, potential inhibitors and activators were added to cells 20 min prior to stimulation and then maintained at the same concentration throughout stimulation.

**DNA Constructs**—The annexin 2-GFP and GFP-actin fusion constructs have been described previously (6, 10), as have constructs expressing the constitutively active (Q67L) and dominant negative (T27N) mutants of Arf6 (16).

**Fig. 2.** Macropinocytic rocketing is a stress response. Actin-based rocketing is inhibited by wortmannin (A), LY294002 (B), and SB202190 (C). In each experiment, RBL cells were incubated in the presence of the inhibitor at the range of concentrations indicated, before and during 45 min of stimulation with Perv/HOS. Cells were fixed and stained, and F-actin rockets counted as described in the legend for Fig. 1. The inset in C shows Western blots of p38 MAP kinase (p38) and phosphorylated p38 MAP kinase (p-p38) in RBL cells stimulated with Perv/HOS. M.S., molecular size. D and E, RBL cells were pretreated with 2 nM wortmannin for 20 min before incubation with either HBS (D) or Perv/HOS for a further 45 min (E). Cells were fixed, stained for F-actin, and viewed by confocal microscopy. Wortmannin inhibits rocket formation in stimulated cells; instead, cells have numerous F-actin-decorated vesicles (arrows in E). Scale bar, 10 μm. F and G, RBL cells were pretreated with 10 μM BAPTA-AM for 20 min before incubation with either HBS (F) or Perv/HOS for a further 45 min (G). Loading with BAPTA-AM had little effect on the cortical cytoskeleton in resting cells (F) but inhibited rocket formation in stimulated cells. Instead, cells contained numerous F-actin-decorated vesicles (arrows in G). Scale bar, 10 μm.
**RESULTS AND DISCUSSION**

We first established a protocol that preserved both actin tails and vesicles in fixed cells. The fluid phase markers used in live cell studies (10) were unstable during chemical fixation (not shown), so we used fluorescently labeled IgE to tag the vesicles at the heads of actin comet tails. RBL cells express the FceRI receptor, and IgE can be prebound to this receptor at the membrane surface and used to follow endocytosis stimulated by PMA (20). Unstimulated cells did not produce rockets, but following stimulation with PMA in hyperosmolar saline (HOS), macropinosomes and actin rockets were visualized using IgE conjugated to Alexa 568 and co-staining with FITC-phalloidin (Fig. 1A). To confirm that all rocket tails were nucleated at the surface of endosomes, a number of IgE-A568-labeled, -stimulated, -fixed, and FITC-phalloidin-stained cells were imaged using confocal microscopy. Of 80 rockets seen in 50 cells, 98% were associated with clearly labeled endosomes, consistent with our previous findings in living cells (10).

We previously showed that when used alone, mild HOS and PMA weakly stimulate macropinocytic rocketing but that the frequency of rocketing is enhanced when the agonists are used in combination (10), implying the activation of either two distinct signaling pathways or the synergistic activation of a single pathway. To test the possible involvement of protein-tyrosine phosphorylation/dephosphorylation, we quantified the induction of actin rockets in response to Perv, alone and in combination with HOS and PMA (Fig. 1B). Specifically, induction of rocketing pinosomes with either PMA or Perv was strictly dependent on co-stimulation with HOS, yet a combination of PMA and Perv bypassed this requirement. Although both PMA and Perv stimulated the production of macropinocytic rockets, they are unlikely to act in the same way, the
Annexin 2 Binds PtdIns-4,5P₂

Annexin 2 binds to liposomes containing PtdIns-4,5P₂. Annexin 2 but not annexin 5 shows calcium-dependent binding to immobilized phosphatidylinositols. Annexins 2 and 5 (0.5 μg/ml) were incubated overnight with PIP Strips in the presence of 1 mM EGTA or 50 μM CaCl₂. Annexin 2 demonstrated Ca²⁺-dependent adsorption to immobilized phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PI₄P), phosphatidylinositol 5-phosphate (PI₅P), phosphatidylinositol 3,4-bisphosphate (PI₃,4P₂), phosphatidylinositol 3,5-bisphosphate (PI₃,5P₂), phosphatidic acid (PA), and phosphatidylinositol 4,5-bisphosphate (PI₄,5P₂) but did not bind to inositol1,3,4,5-tetraphosphate (IP₄), phosphatidylethanolamine (PE), phosphatidylcholine (PC), or phosphatidylserine (PS). Coomassie Blue-stained gel showing annexin 2 in pellets co-sedimented only with DOPC liposomes containing 2.5% PtdIns-4,5P₂. Maximal binding of annexin 2 to liposomes did not exceed 80% of added protein, was observed in both the presence and the absence of calcium, and was enhanced in the presence of 50 μM calcium.

B, control experiment showing that annexin 2 does not sediment either in the absence of DOPC liposomes or with DOPC liposomes but in the absence of calcium.

d and e, histograms representing quantitative densitometric analysis of at least three independent liposome co-sedimentation assays exemplified in B. Annexin 2 but not annexin 5 shows both Ca²⁺-dependent and Ca²⁺-independent binding to PtdIns-4,5P₂-containing liposomes. F, the amount of annexin 2 which binds to liposomes is dependent on the concentration of PtdIns-4,5P₂, in the liposomes. There is significant annexin 2 binding in the absence of calcium, but binding is enhanced by the addition of 50 μM calcium. Shown is the mean ± S.E. of three independent experiments.

G, cooperativity of liposome-binding using lipid mixes. Binding of annexins 1 and 2 to DOPC liposomes containing both 10% phosphatidylserine and 25 μM PtdIns-4,5P₂ approximates to the sum of the percent annexin bound to either lipid in isolation. The data are the mean ± S.E. of four independent experiments.
Annexin 2 Binds PtdIns-4,5P$_2$

former probably acting through a protein kinase C-dependent pathway and the latter via protein tyrosine phosphorylation. This notion is supported by studies showing protein kinase C localized to rocketing endosomes in activated Xenopus eggs (21). These two signaling routes may be convergent, but both are clearly required to induce actin-basedrocketing. The low level induction of actin-based rocketing in the presence of HOS alone suggests simultaneous but weak activation of both pathways. The fact that Perv stimulates vesicle rocketing implicates tyrosine phosphorylation in this phenomenon. Indeed, it has been shown that tyrosine phosphorylation is required for actin-based propulsion of Vaccinia, although not for Listeria or Shigella (22). Despite the potent stimulatory effects of Perv on macroinocytic rocketing in vitro, we did not observe phosphotyrosine immunoreactivity associated with pinosomes or actin tails in fixed cells (results not shown), suggesting that different types of actin-based rocketing have different requirements for tyrosine phosphorylation. Despite the generation of new pinosomes under these conditions, cellular pinocytosis in the presence of HOS/PMA was significantly lower than with PMA alone (Fig. 1C). Thus, macroinocytic rocketing does not reflect stimulation of pinocytosis per se and is most likely due to the exaggeration of a step in pinocytosis not normally visible (10). Perv alone did not stimulate rocketing but instead induced a dramatic redistribution and polarization of F-actin (Fig. 1D). Other changes in cellular morphology elicited by these agonists are shown in Fig. 1, E–H.

These results are consistent with in vitro studies showing that vesicle rocketing is also strongly stimulated by Perv (23, 24). These studies, describing actin-based rocketing of both unidentified vesicles and synthetic vesicles in Xenopus egg extracts, revealed that rocketing in vitro was strongly stimulated by Perv and GTPγS and had an absolute requirement for the small GTP-binding protein Cdc42. The same studies also showed that the membrane composition of synthetic vesicles modulated their recruitment of actin and organization of actin tails. Specifically, it was found that incorporation of PtdIns-4,5P$_2$ or PtdIns-3,4,5P$_3$ into synthetic vesicle membranes relieved the requirement for GTPγS to promote rocketing (23). This is consistent with other studies reporting that co-stimulation of HEK-293 cells with Perv and HOS leads to a marked accumulation of PtdIns-3,4,5P$_3$ through the activation of PtdIns3-kinase(s) (25) and that PtdIns3-kinase has a role in cup closure during phagocytosis and macroinocytosis (26). Despite the observation that vesicle rocketing in vitro was insensitive to wortmannin, the demonstration in those studies of a requirement for phosphoinositides is consistent with a role for PtdIns3-kinase. This prompted us to ask whether or not rocketing in vivo required PtdIns3-kinase(s). Incubation of RBL cells with wortmannin or LY294002 during stimulation with HOS/Perv resulted in inhibition of actin tail formation (Fig. 2, A and B), confirming the requirement for PtdIns3-kinase(s) and supporting the idea that phosphoinositides are involved in macroinocytic rocketing.

To gain further insight into the signaling pathway that regulates macroinocytic rocketing, we tested a variety of other compounds for their ability to block the appearance of F-actin rockets (Table I). Because HOS is known to activate p38 MAP kinase in various cells (27–29) and components of this pathway are involved in actin remodeling, we evaluated inhibitors of this pathway. Inhibitors were considered not to have had a specific effect on rocketing unless they met two criteria. First, cells showed complete abolition of macroinocosome rocketing, and second, there was no obvious cell death or gross effects on cell morphology that might have indirectly affected macroinocytosis. In addition to LY294002 and wortmannin, SB202190 (Fig. 2C), latrunculin B, and BAPTA-AM all abolished macroinocytic rocketing. Inhibition of rocketing by SB202190 indicates the involvement of p38 MAP kinase, perhaps acting through the downstream effector Hsp27 (30), and is consistent with the appearance of phospho-p38 in RBL cells induced to rocket (Fig. 2C). Cortical actin and membrane ruffling in resting RBL cells treated with wortmannin appeared little different from controls (Fig. 2D), indicating that inhibition of phosphatidylinositol 3-kinase did not block recruitment of F-actin to the plasma membrane. However, RBL cells stimulated to produce rockets in the presence of wortmannin failed to do so, although the cells contained vesicles encircled by F-actin (Fig. 2E). These appear to be macropinosomes that budded from the plasma membrane but which then failed to rocket. Treatment of RBL cells with BAPTA-AM also abolished macroinocytic rocketing (Fig. 2, F and G), revealing the Ca$^{2+}$ dependence of this activity. Indeed, there are similarities in cells treated with wortmannin and BAPTA-AM. Rather than the characteristic F-actin rocks, cells contained numerous F-actin-decorated vesicles, which tended to be clustered close to the plasma membrane. These experiments establish the following. First, macroinocytic rocketing is dependent on actin polymerization. Second, macroinocytic rocketing requires the production of phosphoinositides. Third, conditions that lead to rocketing also activate and require p38 MAP kinase, and fourth, rocketing is Ca$^{2+}$-dependent.

Since vesicle rocketing in vitro can be stimulated by both PtdIns-3,4,5P$_3$ and PtdIns-4,5P$_2$ (23), and given that there may be interconversion between these lipids by kinases and phosphatases, we hypothesized that annexin 2 may bind to these or other phosphoinositides on endocytic vesicles. To obtain a broad view of phosphoinositide binding by annexin 2, we performed overlay blots using lipids immobilized on strips of nitrocellulose (Fig. 3A). Under these conditions, annexin 2 bound to most of the phosphoinositides tested, but binding was only observed in the presence of 50 μM Ca$^{2+}$. In contrast, annexin 5 failed to bind to any of the immobilized lipids. Unexpectedly, neither annexin 2 nor annexin 5 bound to phosphatidyserine under these conditions. This may be due to the low Ca$^{2+}$ concentration used in these experiments or to poor accessibility of the phospholipid headgroups in the planar array of the immobilized lipids. To examine phosphoinositide binding by annexin 2 in a more authentic model membrane, we performed lipidosome sedimentation studies in which annexin 2 was added to phospholipid vesicles composed of DOPC containing a 2.5% molar fraction of PtdIns3P, PtdIns4P, PtdIns3,4P$_2$, or PtdIns4,5P$_2$, in the presence and absence of Ca$^{2+}$ (Fig. 3, B and C). The results show that annexin 2 bound only to liposomes containing PtdIns4,5P$_2$, and binding occurred in both the presence and the absence of 50 μM Ca$^{2+}$. In contrast, annexin 5 failed to bind to any of the lipid mixes (Fig. 3, D and E).

Next we prepared DOPC liposomes containing a range of concentrations of PtdIns-4,5P$_2$ to assess the affinity of annexin 2 for this phospholipid (Fig. 3F). The results show that at low micromolar concentrations of PtdIns-4,5P$_2$, annexin 2 has an absolute requirement for Ca$^{2+}$ for binding, but that at concentrations of PtdIns-4,5P$_2$ greater than 10 μM, annexin 2 binds independently of Ca$^{2+}$. Quantitative densitometric scanning of Coomassie-stained gels revealed that in the presence of 50 μM Ca$^{2+}$, binding of annexin 2 to PtdIns-4,5P$_2$-containing liposomes is half-maximal at ~5 μM PtdIns-4,5P$_2$. To put these findings in a broader context, half-maximal binding to PtdIns-4,5P$_2$ has been reported in the range 1–300 μM for various pleckstrin homology domains (17, 31), whereas other PtdIns-4,5P$_2$-binding motifs, such as the ANTH (AP180 N-terminal homology) domain (13), bind with a similar affinity to annexin
but lack the high specificity of annexin 2 for PtdIns-4,5P2. Thus, the affinity of annexin 2 for PtdIns-4,5P2 is comparable with those reported for diverse PtdIns-4,5P2-binding domains, but the selectivity of annexin 2 for PtdIns-4,5P2 versus other phosphoinositides is high as compared with many PtdIns-4,5P2-binding proteins. Significantly, the affinity of annexin 2 for PtdIns-4,5P2 was increased in liposomes containing approximately physiological concentrations of phosphatidylserine (Fig. 3G), suggesting that in biological membranes, there may be cooperativity of binding. In this experiment we also compared annexin 2 with annexin 1, its closest relative in molecular phylogenetic terms and therefore the most likely among other annexins to exhibit binding to PtdIns-4,5P2. The results show that annexin 1 indeed displays similar characteristics to annexin 2, binding with high affinity to liposomes containing a mixture of PtdIns-4,5P2 and phosphatidylserine and with lower affinity to either lipid alone. Taken together, the results in Fig. 1 demonstrate that annexin 2 specifically binds PtdIns-4,5P2 and not to other polyphosphoinositides and show that the ability to bind this lipid is not a generic annexin property but is restricted to a subgroup within that family that also includes annexin 1.

There is increasing evidence that phosphoinositide lipids function at least in the initial recruitment and assembly of

Fig. 4. PtdIns-4,5P2 is enriched at sites of pinosome formation in RBL cells. A, F-actin rockets (which also contain annexin 2 (2)) accumulate PtdIns-4,5P2. RBL cells stimulated with hypertonic shock (HOS) and phorbol ester to induce F-actin rockets demonstrate co-localization of F-actin (FITC-phalloidin) and PtdIns-4,5P2 stained by indirect immunofluorescence (top panels). Fluorescently labeled PtdIns-4,5P2 introduced into the cell by means of Shuttle-PIPs also co-localizes with vesicles attached to F-actin rocket tails (lower panels, pink arrowhead). B, the left-hand y- and z-section show, by indirect immunofluorescence using anti-PtdIns-4,5P2 antibodies, the accumulation of PtdIns-4,5P2 at the apical surface of RBL cells (white arrowhead) exposed to hypertonic sucrose and phorbol ester. The right-hand y- and z-sections show, using transient expression of GFP-actin in RBL cells, that actin tail formation (yellow arrowheads) is initiated at the apical cell surface from where the motile pinosome travels toward the basal membrane. C, Cy3-labeled anti-PtdIns-4,5P2 antibodies were microinjected into RBL cells, which were then exposed to hypertonic sucrose and phorbol ester. Confocal sectioning at the apical surface of the injected cells reveals co-localization of annexin 2-GFP- with Cy3-labeled antibody (white arrowheads). Two representative cells are shown.

Fig. 5. Regulation of annexin 2 localization by Arf6. A, Ptk1 cells were microinjected with plasmids encoding GFP, annexin 2-GFP, and annexin 5-GFP. GFP and annexin 5-GFP localize to both cytosol and nucleus, whereas annexin 2-GFP exhibits any change in their localization pattern when co-expressed with the constitutively active Q67L mutant of Arf6. C, annexin 2-GFP redistributes dramatically to the plasma membrane and PtdIns-4,5P2-rich intracellular vesicles when co-expressed with Arf6Q67L. In contrast, the annexin 2-GFP fusion protein does not relocalize when co-expressed with the dominant negative mutant of Arf6 (Arf6T27N), although there is enrichment of annexin 2-GFP in perinuclear vesicles.
Annexin 2 Binds PtdIns-4,5P2

actin at sites of vesicle formation (15, 32) and that actin-dependent transport is responsible for the subsequent inward movement of endocytic, pinocytic, and phagocytic vesicles (11, 16, 33, 34). We therefore investigated whether or not PtdIns-4,5P2 is enriched in motile macropinosomes or at sites of pino-
some formation in the RBL experimental model. In RBL cells induced to generate rocketing macropinosomes, then fixed and processed for indirect immunofluorescence, we observed mod-
est co-localization of F-actin and PtdIns-4,5P2 (Fig. 4A). More-
striking association of PtdIns-4,5P2 with pinosomes was ob-
served in fixed cells preloaded with TMR-PtdIns-4,5P2. In per-
foming z-sections of fixed RBL cells, we consistently observed
concentration of PtdIns-4,5P2 immunoreactivity or TMR-

formings provide further evidence that annexin 2 is involved in
the dynamic remodeling of the membrane and actin cytoskel-
on that depends on PtdIns-4,5P2 and occurs during the formation of endocytic vesicles.

Acknowledgments—We are grateful to Volker Gerke for the regular
supplies of HH7 antibody and to Mark Shipman, William Hinkes, and
Keith Morris for help with the confocal microscopy.

REFERENCES

1. Gerke, V., and Moss, S. E. (2002) Physiol. Rev. 82, 331–371
2. Sjölin, C., Stendahl, O., and Dahlgren, C. (1994) Biochem. J. 300, 325–330
3. Chasserot-Golaz, S., Vitale, N., Sagot, I., Delouche, B., Dirrig, S., Pradel, L. A.,
Harder, T., Kellner, R., Parton, R. G., and Gruenberg, J. (1997) Mol. Biol. Cell 8,
535–545
4. Merrifield, C. J., Moss, S. E., Ballestrem, C., Imhoff, B. A., Giese, G.,
Wunderlich, I., and Almers, W. (1999) Nat. Cell Biol. 1, 72–74
5. Derijard, B., and Davis, R. J. (1994) EMBO J. 13, 357–365
6. Ma, L., Cantley, L. C., Janmey, P. A., and Kirschner, M. W. (1998) Science 280,
729–732
7. Wunderlich, I., and Almers, W. (1999) J. Cell Sci. 112, 1119–1132
8. Lemmon, M. A. (2003) Traffic 4, 201–213
9. Lemmon, M. A., and Ferguson, K. M. (2000) Biochem. J. 350, 1–18
10. Brown, F. D., Rozelle, A. L., Yin, H. L., Nalla, T., and Donaldson, J. G. (2001)
J. Cell Biol. 150, 599–607
11. Schafer, D. (2002) J. Cell Biol. 154, 1125–1136
12. Frischknecht, F., Cudmore, S., Moreau, V., Reckman, I., Rottger, S., and Way,
M. (1998) J. Cell Biol. 140, 603–616
13. Levine, T. P., and Munro, S. (2002) Curr. Biol. 12, 695–704
14. Regnouf, F., Rendon, A., and Pradel, L. A. (1991) J. Neurochem. 56, 1985–1996
15. Ma, L., Cantley, L. C., Janmey, P. A., and Kirschner, M. W. (1999) Biochemistry
38, 15190–15198
16. Ra, C., Furuchi, K., Rivera, J., Mullins, J. M., Isersky, C., and White, K. N.
(1999) J. Exp. Med. 189, 1771–1775
17. Taunton, J., Bougher, B. A., Coughlin, M. L., Wu, M., Moon, R. T., Mitchison, T. J.,
and Lababell, C. A. (2000) J. Cell Biol. 148, 519–530
18. Frischknecht, F., Cadmoure, S., Moreau, V., Reckman, I., Rottger, S., and Way,
M. (1999) Curr. Biol. 9, 89–92
19. Ma, L., Cantley, L. C., Janneny, P. A., and Kirschner, M. W. (1998) J. Cell Biol.
140, 1125–1136
20. Moreau, V., and Way, M. (1998) PERSPECTIVES 427, 353–356
21. Meier, T., Thelen, M., and Hemmings, B. A. (1998) EMBO J. 17, 7294–7303
22. Araki, N., Johnson, M. T., and Swanson, J. A. (1996) J. Cell Biol. 135, 1249–1260
23. Galcheva-Gargova, Z., Derijard, B., Wu, I. H., and Davis, R. J. (1994) Science
265, 806–808
24. Han, J., Lee, J. D., Bibbs, L., and Ulevitch, R. J. (1994) Science 265, 808–811
25. Kryczka, J. M., and Avruch, J. (1996) J. Biol. Chem. 271, 24313–24316
26. Guay, J., Lambert, H., Gingras-Breton, G., Lavoie, J. N., Huot, J., and Landry,
J. (1997) J. Biol. Chem. 272, 11369–11376
27. Seki, A., Suzuki, M., and Kano, Y. (1999) J. Cell Biol. 143, 1039–1049
28. Castellano, F., Chavrier, P., and Caron, E. (2001) Semin. Immunol. 13, 347–355
29. May, R. C., and Machesky, L. M. (2001) J. Cell Sci. 114, 1061–1077
30. Honda, A., Nomoto, M., Yokozeki, T., Yamazaki, M., Nakamura, H.,
Watanabe, H., Kawanoue, K, Nakayama, K., Morris, A. J., Frohman, M. A.,
and Kanaho, Y. (1999) Cell 99, 521–532

© J. Merrifield and S. E. Moss, unpublished observations.
Annexin 2 Binding to Phosphatidylinositol 4,5-Bisphosphate on Endocytic Vesicles Is Regulated by the Stress Response Pathway

Matthew J. Hayes, Christien J. Merrifield, Dongmin Shao, Jesus Ayala-Sanmartin, Crislyn D'Souza Schorey, Tim P. Levine, Jezabel Proust, Julie Curran, Maryse Bailly and Stephen E. Moss

J. Biol. Chem. 2004, 279:14157-14164.
doi: 10.1074/jbc.M313025200 originally published online January 20, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M313025200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 35 references, 20 of which can be accessed free at
http://www.jbc.org/content/279/14/14157.full.html#ref-list-1