Interactions of Annexins with the mu Subunits of the Clathrin Assembly Proteins†

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ABSTRACT: A number of biochemical and genetic studies have suggested that certain annexins play important roles in the endocytic pathway, possibly involving the generation, localization, or fusion of endocytic compartments. In a yeast two-hybrid screen for proteins that interact with the N-terminal domain of annexin A2 we identified the mu2 subunit of the clathrin assembly protein complex AP-2. The interaction depended upon two copies of a YxxΦ amino acid sequence motif (Y = tyrosine, x = variable residue, Φ = bulky, hydrophobic residue) in the annexin that is also characteristic of the binding site for mu2 on the cytoplasmic domains of transmembrane receptors. The interaction between mu2 and full-length annexin A2 was demonstrated in vitro to be direct, to require calcium, and to be functional in the sense that annexin A2 was able to recruit the mu2 to immobilized lipids. Examination of other annexins and mu subunits demonstrated that annexin A2 also binds the mu1 subunit of the AP-1 complex, that annexin A6 binds mu1 and mu2, and that annexin A1 binds only mu1. We propose that annexins can “masquerade” as transmembrane receptors when they are attached to membranes in the presence of calcium and that they might therefore function to initiate calcium-regulated coated pit formation at the cell surface or on intracellular organelles.

The annexins are a family of calcium-dependent, membrane-binding proteins that are expressed at high levels in most eukaryotic organisms (1, 2). Their abundance suggests they may play important roles in the overall structure, organization, and interactions of membranes when intracellular calcium levels are elevated. The “bivalent” ability of many of the annexins to bind to two membranes and form tight contacts between them led to the early suggestion that the annexins may act as “chaperones” of membrane fusion in processes such as exocytosis (3, 4). However, more recent cell biological studies involving in vitro membrane trafficking models, in vivo localization studies, and techniques to alter annexin expression or function in cells, have suggested that the annexins may also play important roles in the process of endocytosis and the movements, interactions, and localization of endosomes. For example, annexin A6 was shown to be an essential component of an in vitro model that recapitulates the formation of coated pits from isolated plasma membranes (5). Annexin A6 was also found to stimulate endocytosis when overexpressed in Chinese hamster ovary cells and to influence the trafficking of low-density lipoprotein to the prelysosomal compartment (6).

Annexin A2 was reported to be a major component of fusogenic endosomal vesicles that was transferred between vesicles during fusion in vitro (7). Inhibition of annexin A2 with an inhibitory peptide or specific antibodies inhibited calcium-dependent endosome fusion in vitro (8). The use of a dominant negative annexin A2 construct in cells led to the improper trafficking and localization of endosomes (9). Recent studies employing the use of inhibitory RNA to effectively reduce annexin A2 levels in cells have confirmed the importance of annexin A2 in the localization of transferrin receptor-containing recycling endosomes (10) as well as in the apical transport of vesicles in polarized epithelial cells (11) and in the formation of multivesicular transport intermediates following endocytosis (12). In addition, the trafficking of GLUT4 glucose transport vesicles to the plasma membrane in adipocytes in response to insulin was inhibited 60% by knockdown of annexin A2 levels by siRNA (13).

Annexin A1 also has been associated with vesicle trafficking in cells. In early studies, it was found to undergo phosphorylation during processing of the epidermal growth factor receptor in the multivesicular body (14). It was also found to be associated with early endosomes through an interaction that required its N-terminal domain (15), and it was found to be necessary for the recruitment of the S100C protein to early endosomes (16).

Particularly relevant to the studies reported here, annexins A2 and A6 were found to be components of isolated coated vesicles from the adrenal cortex. The association of these annexins with the coated vesicles was calcium independent and therefore did not appear to be related to the canonical property of annexins to bind lipids in a calcium-dependent fashion (17).

Although these prior studies provide a strong indication that annexins play an important role in these membrane trafficking events, they have not provided mechanistic insights into the molecular nature of this role. Working under the assumption that the functions of the annexins in these

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Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PIP2, phosphatidylinositol-4,5-bisphosphate; PE, phosphatidylethanolamine.
contexts would likely depend on the interactions of annexins with other proteins, we have been screening for binding partners for annexins, previously by biochemical methods (18, 19), and here by yeast two-hybrid technology. We have focused our attention on interactions with the N-terminal domains of the annexins since the C-terminal core domains may be primarily occupied with binding calcium and phospholipids in the cell. Here we report that the mu subunits of the AP clathrin assembly protein complexes are potential binding partners for annexins and suggest mechanistic roles annexins could play in coated pit formation and endosomal trafficking based on these observations.

EXPERIMENTAL PROCEDURES

**DNA Constructs.** Plasmids for the expression in yeast of human annexin A1 (20), annexin A2 (20), annexin A6 (21), T356 D mutant annexin A6 (21), and annexin A7 (20) were previously described. cDNA encoding residues 123–435 of human mu2 was obtained by screening a human fetal brain yeast two-hybrid library in vector pACT2 obtained from Clontech (Catalog No. HL4028AH). cDNAs for human mu1 and mu3 were obtained as EST (IMAGE) clones from the American Type Culture Collection. The portions of these cDNAs encoding residues 124–423 of mu1 and 123–418 of mu3 (isoform mu3a) were amplified by PCR and subcloned into the Eco RI and Xho I sites of the yeast two-hybrid library vector pGADT7 (Clontech).

cDNAs encoding the N-terminal domains of human annexins A1 (amino acid residues 1–43), A2 (residues 1–34), A6 (residues 1–21), and residues 327–364 of the interdomain linker), A7 (residues 1–145) and A11 (residues 1–201; ref 19) were amplified by PCR from the plasmids listed above and subcloned into the yeast two-hybrid bait vector pGBK7 (Clontech). All inserts included the initiating methionine (as residue number 1) of the original annexin cDNAs, now fused at the C-terminus of the GAL4 DNA binding domain.

The portions of the mu1, mu2, and mu3 cDNAs in the yeast two-hybrid vectors described above were subcloned after PCR amplification into the Sal I and Not I sites of the GST fusion vector pGEX-4T1.

**Proteins.** Human annexins A1, A2, A6 (native and T356D mutant), and A7 were produced as recombinant proteins in yeast using the plasmids described above and purified by calcium-dependent binding to phospholipid vesicles as described (20). Proteins extracted directly from lysates with the calcium chelator EGTA were used without further purification other than buffer exchange on Sephadex G25 or dialysis into appropriate experimental buffers. Bovine annexin A1 (22) and bovine annexin A2–S100A10 tetramer (23) were prepared from bovine lung as previously described.

GST fusion proteins of mu1, mu2, and mu3 were expressed in *Escherichia coli* and purified by binding to glutathione-substituted agarose as described previously for copine target proteins (24).

**Yeast Two-Hybrid Assay.** The Clontech system 3 vectors and AH109 host cells (similar to strain PJ69-4A; ref 25) were used for the two-hybrid assay. The annexin A2 N-terminal bait vector described above was used in two independent screenings of a human fetal brain cDNA library (Clontech Catalog No. HL4028AH) with approximately 200 000 trans-

forms in each screen. The library vector, pACT2, has the human cDNAs cloned to the C-terminus of the GAL4 activation domain. The host cell, AH109, (MATa, trp1-901, leu2-3,112, ura3-52, his3-200, gal4Δ, gal80A, LYS2:: GAL1 UAS−GAL1 TATA−HIS3, GAL2 UAS−GAL2 TATA−ADE2, URA3::MEL1 UAS−MEL1 TATA−lacZ) has been engineered so that reconstitution of an active GAL4 protein results in expression of HIS3 and ADE2. Transformants were plated on Leu−, Trp−, His−, Ade− selective plates. Colonies appearing on these plates after 3 or 4 days were streaked onto plates with the same medium to verify growth. After 3 or 4 days the plasmids from colonies growing on these plates were captured by transformation of *E. coli* in ampicillin-containing medium and DNA mini-preps were prepared for retransformation of the host cells harboring either the original annexin A2 bait vector or the bait vector without insert. The inserts of plasmids that promoted growth of the host cell harboring the annexin bait construct but did not promote growth of cells harboring the empty bait vector were scored as positive interactors.

**Mutagenesis of YxxΦ Motifs in Annexin A2.** Tyr24 and Tyr30 in the N-terminal domain of annexin A2 were replaced by alanines by amplifying the corresponding cDNA from the two-hybrid bait vector (pGBK7) with PCR primers containing codon replacements and then reinserting into the bait vector. Primers were synthesized that were the exact complements of the following sequences. The mutations are marked in bold:

**Native sequence**

\[
P_S A Y G S V K A Y T H F D * S A L I
\]

**Y24A mutant**

\[
P_S A A G S V K A Y T H F D * S A L I
\]

**Y30A mutant**

\[
P_S A Y G S V K A A T H F D * S A L I
\]

**Y24A, Y30A double mutant**

\[
P_S A A G S V K A A T H F D * S A L I
\]

**Expression of the Abl Kinase in the Yeast Two-Hybrid Host Cell.** The yeast two-hybrid assay was modified by expressing the Abl kinase in the host cell, AH109. The catalytic domain of the Abl tyrosine kinase with a 5′ nuclear localization sequence was integrated into the Ade2 locus of the AH109 chromosome using a yeast integration vector (YlpDCE1-Abi) kindly provided by Dr. Cynthia Corley Mastick of the University of Nevada. The modified host cell was transformed with the annexin A2 N-terminal bait vector and the mu2 library vector and the interaction was tested by selection on His− plates. Negative control experiments were performed with AH109 cells transformed with the YlpDCE1 vector without insert, and positive control experiments were performed with a copine I A domain bait and the N-terminal domain of protein phosphatase 5 as a binding protein (24).

**GST Pull-Down Assay.** The GST-mu fusion proteins (or GST alone in control experiments), bound to agarose beads were washed in binding buffer (50 mM Hepes-NaOH, pH
7.3, 150 mM KCl) plus 0.2% Triton X-100. Pull-down experiments were carried out by incubating approximately 20 μg of the fusion protein bound to beads with 20 μg of purified annexin A2 in a total volume of 500 μL of binding buffer plus 0.04% Triton X-100 and 5 mM EGTA or 5 mM CaCl₂ for 1–2 h on a shaker at room temperature. The volume of beads in each experiment was brought to 50 μL by the addition of glutathione agarose beads without bound protein if necessary. The incubations and washes were performed in “nonstick” microtubes from Research Products International. After incubation of the sample, the supernatant was withdrawn and the beads were washed three times with 1.5 mL of binding buffer containing 5 mM EGTA or 5 mM CaCl₂. Separation of beads from buffer was achieved by sedimentation at 500g for 1 min. Finally, proteins were eluted from the beads with electrophoresis sample buffer and loaded on SDS–PAGE gels. After electrophoresis, the proteins were electroblotted onto nitrocellulose paper, which was then stained with 0.2% Ponceau S in 3% trichloroacetic acid.

Recruitment Assay. To determine if annexins could recruit the mu subunits to a lipid surface an ELISA assay was designed. The first part of this assay involves the binding of the annexin to lipids immobilized in the well of a microtiter plate and is based on similar assays that have been used previously to assess lipid binding by annexins (Pollard, H. B., unpublished results, and refs 26–29). Nunc 96-well plates with a “Polysorb” coating were used as this coating is designed to bind hydrophobic molecules. “Macrosorb” coating has been used previously (26, 27), but we found in preliminary experiments that annexin binding was slightly enhanced with the “Polysorb” coating (data not shown). Lipids were obtained from Avanti Polar Lipids as chloroform solutions and mixed in appropriate ratios for experiments with mixed lipid systems. The following lipids were used: phosphatidylcholine from egg yolk, phosphatidylserine from brain, phosphatidylethanolamine from brain, phosphatidylinositol from bovine liver, phosphatidylinositol-4,5-bisphosphate from brain, and cholesterol from wool grease. After mixing of the samples, the lipids were diluted with methanol to 20 μg/mL and 100 μL of this solution was applied to each well of a 96-well Nunc plate. Lipids were dried overnight under house vacuum at room temperature. All subsequent steps were also conducted at room temperature. Wells were blocked for 30 min with 300 μL of 0.5% defatted bovine serum albumin (BSA, Sigma) dissolved in plate assay buffer (150 mM KCl, 40 mM Heps-NaOH pH7.4, 2 mM MgCl₂). Wells were then washed 2 times with 300 μL of plate assay buffer. Proteins were then applied (3 μg of annexin, 1 μg of mu–GST fusion protein) in 200 μL of plate assay buffer plus 0.5% BSA and a calcium/EGTA buffer consisting of 2.5 mM EGTA and appropriate concentrations of CaCl₂ to give the desired free calcium concentration as determined by calculation (3, 30). After 30–60 min incubation the wells were washed once with 300 μL of plate assay buffer containing the appropriate calcium/EGTA buffer and then twice with 300 μL of plate assay buffer containing 2.5 mM CaCl₂. All subsequent steps of the ELISA assay also included 2.5 mM CaCl₂ to maintain the binding of annexin–mu–GST complexes that bound during the initial experimental incubation. The wells were then incubated with 200 μL of a 1:2500 dilution of rabbit anti-GST antibodies (Sigma G-7781) in plate assay buffer plus 0.5% BSA, then washed three times with 300 μL of plate assay buffer, incubated with 200 μL of a 1:2500 dilution of goat anti-rabbit IgG coupled to horseradish peroxidase (American Qualex A102PN) in plate assay buffer plus 0.5% BSA, then washed three times with 300 μL of plate assay buffer. Finally, the bound GST was quantitated by incubation with 100 μL of 1 mM ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) in 70 mM citrate-phosphate buffer, pH 4.2, 0.03% H₂O₂ for 1 h, and the absorbance of the wells was read at 414 nm.

Analytical Methods. Protein concentrations were determined by the method of Bradford (31) with bovine serum albumin as standard, and SDS gels were run according to Laemmli (32). DNA sequencing by the Sanger chain termination method and oligonucleotide syntheses were performed by the University of Virginia Biomolecular Research Facility core laboratories.

RESULTS

Identification of the mu2 Subunit of the AP-2 Complex as a Binding Partner for the Annexin A2 N-Terminal Domain. To search for proteins that interact with the N-terminal domain of annexin A2, the N-terminal 34 residues were fused to the yeast GAL4 DNA binding domain and used as a bait in a yeast two-hybrid screen of a human fetal brain cDNA library. The bait construct was found not to cause auto-activation in control transformations. Approximately 400 000 colonies were screened in two independent transformations with the library DNA. Twenty-three colonies were isolated in these screens, and the library plasmids from these were isolated and sequenced. Six were discarded as containing noncoding portions of cDNAs or contaminating Alu sequences. The others were used to retransform the host cells containing either the GAL4 DNA binding domain alone or the complete bait construct. Eight plasmids interacted with the GAL4 DNA binding domain alone and were discarded, and three plasmids failed to induce host cell growth with either GAL4 alone or the bait construct and were also discarded. Six plasmids promoted growth with the bait construct only and were scored as true positives. Three of these six plasmids encoded portions of the mu2 subunit of the clathrin assembly complex AP-2: clone A2N-2, residues 139–435; clone A2N-9, residues 122–435, and clone A2N-20, residues 148–435. Thus, clone A2N-9 encoded the portion of the mu2 protein that has previously been shown to be soluble and to bind peptides corresponding to the cytoplasmic tails of transmembrane receptors (33, 34). It is also the portion of the mu2 subunit that was used to determine the crystal structure of mu2 bound to a peptide corresponding to a receptor tail (33). This clone was used for the subsequent studies reported here.

The mu2 protein is known to recognize a specific amino acid sequence motif of YxxΦ in the cytoplasmic tails of transmembrane receptors, where Y is tyrosine, x is variable, and Φ represents a bulky, hydrophobic residue (35–37). Upon inspection of the N-terminal domain of annexin A2, we found that two copies of this motif are present and were included in the bait construct, YGFS at residues 24–27, and YTNF at residues 30–33 (Figure 1). To determine if this motif may be a general feature of annexins, we examined the N-terminal domains of the other human annexins and found that this motif is present in the N-terminal domains...
Annexin A2
HSHTVVECLLCEOOGDQSTSEGG3G6YGSVAYTTP
Annexin A1
HSNXVGRDAVYRSEMIPDYDQTVVVGQVYYPPEW
Annexin A3
 KIIXVRIDHDFGTPDD
Annexin A6 N-terminal
AEQGPPPAAGYTCMVMLLYLFEYAPR
Annexin A6 Intervening
AEQGPPPAAGYVCMVMLLYLFEYAPR
Annexin A7
HSHTVVECLLCEOOGDQSTSEGG3G6YGSVAYTTP
TSVYKGGGPGQQELLQWDDQCTTSWTRTTQDIEQ
(TMSTOP=YPPV-Their)-AFYGRSTSPYTVNM
Annexin A11
HSNTVVECLLCEOOGDQSTSEGG3G6YGSVAYTTP
GSVYMVEQQPQLQDDQTFFQITDDVQDIQYGG
...
Annexins and Clathrin Assembly Proteins

**Figure 3:** Coexpression of the Abl kinase blocks interaction between mu2 and the annexin A2 N-terminal domain. Top plate: yeast strain AH109 was modified to express the Abl tyrosine kinase and then used in the two-hybrid assay to test the sensitivity of the annexin A2–mu2 interaction to tyrosine phosphorylation. The labels indicate the constructions that were used: abl/0/mu2: Abl expression plasmid, empty bait vector, mu2–GAL4 activation domain vector; 0/ANT/mu2: control expression plasmid without Abl, annexin A2 N-terminal domain bait vector, mu2–GAL4 activation domain vector; abl/ANT/mu2: Abl expression plasmid, annexin A2 N-terminal domain bait vector, mu2–GAL4 activation domain vector; 0/0/mu2: control expression plasmid without Abl, empty bait vector, mu2–GAL4 activation domain vector. Note that the interaction seen between annexin A2 and mu2 (0/0/mu2) is blocked by the expression of Abl (abl/ANT/mu2). Bottom plate: control experiment showing that the two-hybrid interaction between the copine I C-terminal core domain and the protein phosphatase 5 N-terminal tetratricopeptide repeat domain (0/COP/PP5) is not blocked by coexpression of the Abl kinase (abl/COP/PP5). The labels indicate the constructions used: abl/0/PP5: Abl expression plasmid, empty bait vector, protein phosphatase 5-GAL4 activation domain vector; 0/COP/PP5: control expression plasmid without Abl, copine I core domain bait vector, protein phosphatase 5-GAL4 activation domain vector; abl/COP/PP5: Abl expression plasmid, copine I core domain bait vector, protein phosphatase 5-GAL4 activation domain vector; 0/0/PP5: control expression plasmid without Abl, empty bait vector, protein phosphatase 5-GAL4 activation domain vector.

sequence for annexin A2 into the bait vector to determine if the intact protein would interact with mu2. No interaction was detected. However, we also found that when screening the full cDNA library with this full-length annexin A2 construct no signals were obtained, not even false positives representing proteins that interact with the GAL4 DNA binding domain in the bait construct. We therefore suspect that the GAL4–annexin A2 fusion protein does not enter the yeast cell nucleus and that this construction cannot be used to validate an interaction between mu2 and full-length annexin A2. Therefore, we tested whether the recombinant proteins would interact in vitro.

A GST pull-down assay was devised by subcloning the mu2 insert from the library screen (residues 122–435) into the GST fusion vector pGEX-4T1, and this construct was used to prepare a GST–mu2 fusion protein bound to glutathione-agarose beads. These beads were then incubated with recombinant full-length annexin A2. As shown in Figure 4, annexin A2 was found to cosediment with beads substituted with the fusion protein but not significantly with GST alone. Furthermore, the interaction was found to be dependent on calcium, since no interaction was seen in buffers containing EGTA.

Although these results verified that a direct interaction between mu2 and annexin A2 could occur, it was unexpected that the interaction would be calcium dependent. The N-terminus of annexin A2 is on the opposite side of the protein from the calcium binding sites, and the calcium binding sites function primarily to promote the binding of the core to phospholipids on the same side of the molecule as the calcium binding sites. If indeed the mu2 is binding to the core to phospholipids on the same side of the molecule as the calcium binding sites. If indeed the mu2 is binding to the core to phospholipids on the same side of the molecule as the calcium binding sites, then it appears that the binding of calcium to the annexin is leading to a conformational change in the N-terminal domain. Such a mechanism is compatible with observations that have been made on the related protein, annexin A1, where crystal structures of the protein in the presence of EGTA or calcium indicate that calcium causes the N-terminal domain to be ejected from an occluded state intertwined among the bundled alpha helices of the core to a position extended away from the core (43).

**Annexin A2 Recruits mu2 to a Phospholipid Monolayer.** To directly test whether the mu2 binds to the N-terminal face of annexin A2 and not at the calcium binding sites, we set up a solid-phase, lipid-recruitment assay to determine if mu2 would bind to the annexin when it is bound through its calcium-binding face to a lipid monolayer. Phospholipid
mixture were applied to the wells of plastic microtiter plates that had a “Polysorb” coating that binds hydrophobic molecules. Because of the hydrophobic nature of this coating, it is presumed that the fatty acyl chains of the phospholipids are oriented toward the plate surface, leaving the hydrophilic headgroups oriented toward aqueous media introduced into the well. In this orientation, the headgroups are presumed to be available for calcium-dependent interaction with annexin A2. As shown in Figure 5 the binding of annexin A2 to phospholipid treated wells [20% phosphatidylserine (PS), 80% phosphatidylcholine (PC)] could be detected by immunosassay using a peroxidase conjugated second antibody (PS), 80% phosphatidylcholine (PC)] could be detected by immunosassay using a peroxidase conjugated second antibody and a colorimetric substrate for peroxidase. The mu2-GST fusion protein construct did not bind to the plate wells by itself; however, it did bind if calcium and annexin A2 were also added to the well, indicating that annexin A2 was able to recruit the mu2 to the lipid monolayer. This suggested that the lipid-binding face of the annexin was functional when the annexin is complexed with mu2, and the interaction with mu2 is likely to be taking place at the “cytoplasmic” face of annexin A2, which is the location of the N-terminal domain.

Since the N-terminal domain of annexin A2 is also the binding site for the S100A10 protein with which the annexin forms a tightly bound tetramer consisting of two molecules of annexin A2 and two of the dimeric S100A10, we sought to determine if occupation of the annexin A2 N-terminal domain in the formation of this complex would block its ability to bind mu2. As shown in Figure 6, the annexin A2 tetramer was unable to recruit mu2 to a PS/PC monolayer. The figure also demonstrates that a preparation of annexin A1 was also unable to recruit mu2 to the monolayer, further confirming the specificity of the annexin A2–mu2 interaction. Both the annexin A2 tetramer and the annexin A1 proteins were isolated by calcium dependent binding to phospholipids, so the failure to recruit mu2 to the monolayer is not likely to have been due to a loss of this property in the experimental preparations. The failure of the annexin A2 tetramer to bind mu2 may underlie the earlier report that annexin A2, but not S100A10, was detected in isolated coated vesicles from the adrenal cortex (17).

The specificity of the interaction of mu2 for annexin A2 was further explored by testing for recruitment activity of annexins A6 and A7. Full-length recombinant human annexins A6 and A7 were expressed in yeast and purified by binding to phospholipids. In addition to the native annexin A6, an annexin A6 mutant that has a T356D amino acid substitution that mimics phosphorylation in the linker between the two core domains was also prepared (21). This mutation greatly increases the flexibility of the linker between the two core domains and presumably allows the molecule to adopt a greater range of conformations (21). Furthermore, a preparation of annexin A6 that was completely cleaved at Arg350 or Lys354 in the linker region so that the two core domains can function independently was also tested (21). Although the annexin A7 did not recruit mu2 to the lipid monolayer (not shown), the annexin A6 preparations were all active in this assay (Figure 7). Therefore, the result obtained in vitro with annexin A6 was different from the result obtained in the two-hybrid assay in which only the N-terminal domain of annexin A6, or the linker between the two domains, was tested and failed to demonstrate an interaction. The in vitro interaction may depend on different portions of the annexin A6 molecule or on the mode of presentation of the YxxΦ motif.

Lipid Dependence of mu2 Recruitment. The lipid composition of the monolayer required for annexin A2 or A6 to recruit mu2 was examined to determine if it reflected the expected requirement for the annexin to bind lipids or if the mu2 partner altered the overall lipid requirement. However, during these experiments it was found that if the monolayer was composed of 100% PS, the mu2 bound to the monolayer in the absence of the annexin. This may have been due to the high negative charge of this monolayer. To reveal the effect of the annexin, it was necessary to use monolayers with more physiological lipid compositions and lower charge densities. As shown in Figure 8, a number of different lipid compositions were tested. Pure PC was compared with 20%...
that would reflect the natural intracellular membrane surface

annexin above PC alone. To test a mixed lipid composition
cholesterol did not enhance recruitment activity of either
protein. Similarly, annexin A2 has been reported to bind
phosphatidylinositol-4,5-bisphosphate (PIP2) (42), but the addition of 20%
cholesterol did not enhance recruitment activity of either
annexin above PC alone. To test a mixed lipid composition
that approximates the composition of the intracellular
membrane: 21% PS, 38% PE, 8% PI, and 33% cholesterol (w/w mass percent).

The annexins would encounter in vivo, we used a mixture of lipids
that approximates the composition of the intracellular
leaflet of the red blood cell membrane: 21% PS, 38% PE, 8% PI, 33% cholesterol (w/w mass percent, corresponding
to 50% cholesterol, 30% PE, 15% PS, 5% PI mole percent).

Figure 8: Lipid specificity of mu2 recruitment by annexins A2 and A6. Microtiter plate wells were coated with the following lipids,
and the recruitment of the mu2-GST fusion protein was determined
by measurement of the absorbance of triplicate microtiter plate wells
at 414 nm ± standard deviation. Incubations were carried out in 1
mM free Ca²⁺, and the amount of the mu2-GST fusion protein bound was measured with an anti-GST antibody. 0, no lipid; PS, 20% phosphatidylserine, 80% phosphatidylcholine (w/w mass percent);
PE, 20% phosphatidylethanolamine, 80% phosphatidylcholine;
PI, 20% phosphatidylinositol, 80% phosphatidylcholine;
PIP2, 20% phosphatidylinositol-4,5-bisphosphate, 80% phosphatidylcholine; CH, 20% cholesterol, 80% phosphatidylcholine;
PC, 100% phosphatidylcholine; RC, a mixture of lipids corresponding
to the lipid composition of the inner leaflet of the red blood cell
membrane: 21% PS, 38% PE, 8% PI, and 33% cholesterol (w/w mass percent, corresponding to 50% cholesterol, 30% PE, 15% PS, 5% PI mole percent).

Annexin Concentration Dependence of mu2 Recruitment to Lipids. As seen above in Figure 8, annexin A2 consistently recruited more mu2 to the lipid monolayer than did similar amounts of annexin A6. To validate this comparison the concentration dependence of the recruitment activity was examined for each protein using the red blood cell lipid mixture that appeared optimal for the activity. As seen in Figure 9 the recruitment activity for both proteins is dose dependent and saturable, with annexin A6 only half as effective at saturation. Half-maximal saturation occurs at approximately 1:1 molar stoichiometry of the annexin to the mu2, suggesting a simple bimolecular interaction. If either
annexin concentration is increased above the saturation point, there is a loss of activity. We speculate that this might be
due to annexin—annexin interactions that occur at the side of the annexin facing the aqueous phase and that hinder the interaction with mu2.
Calcium Dependence of μ2 Recruitment by the Annexins.

The dependence of the recruitment activity of annexins A2 and A6 on calcium was examined using the red cell lipid mixture and a series of calcium/EGTA buffers. As illustrated in Figure 10, the annexin A2 activity had a fairly broad calcium dependence with significant activity seen at 1 mM calcium but increasing activity at higher levels up to 10 mM. The annexin A6 activity appeared to require higher levels of calcium with significant recruitment seen only above 10 μM calcium. Since the assay buffer contained 2 mM Mg²⁺, the recruitment activity is specifically activated by calcium rather than Mg²⁺. While the activity of both proteins is strictly dependent on calcium, the differences in calcium sensitivity suggest that in a cell the activity of annexin A6 may be restricted to regions of membrane close to sources of calcium, such as channels in the plasma membrane, while annexin A2 may be able to function in regions with more limited availability of calcium.

Interactions with μ1 and μ3 AP Subunits.

While the AP-2 complex is known to be involved in the process of coated vesicle formation at the plasma membrane, homologous AP complexes are involved in clathrin-dependent membrane trafficking in other regions of the cell. In particular, AP-1 is involved in trafficking between early and late endosomes and the trans-Golgi network, while AP-3 is involved in maturation from early to late endosomes (37). Previous studies of annexin A2 indicated that it may be involved in some of these intracellular pathways (6-12). Therefore, we tested the ability of several annexins to interact with the μ1 and μ3 subunits of these homologous complexes. The portions of human μ1 and μ3 (subtype μ3A) that correspond to the portion of μ2 used in the experiments above were subcloned into the yeast two-hybrid library vector and challenged with the annexin A2 N-terminal bait construct. After initially incubating the co-transformed yeast for 4 days, vigorous growth is seen with cells harboring μ2, confirming the interaction between μ2 and annexin A2. However, as shown in Figure 11, only after 10 days of incubation is a weak signal obtained with μ1 and μ3 represented by the appearance of a small number of colonies. Although this signal would have to be scored as quite weak compared to the interaction with μ2, it is nonetheless significant as many other irrelevant, noninteracting proteins examined with this technology failed to give any colony growth with extended incubation (data not shown).

Since the two-hybrid assay thus suggested μ1 and μ3 might also interact with annexin A2, we subcloned the portions of these proteins used in the two-hybrid assay into the pGEX-4T1 GST fusion vector so we could test the ability of annexins to recruit the μ1 and μ3 subunits to lipid monolayers in the microtiter plate assay. As shown in Figure 12, annexins A2 and A6 were able to recruit μ1 to the red
The presence of annexins A1, A2, or A6 was level to the lipid monolayer in the absence of an annexin A6 and mu2 (Figure 10). The recruitment of mu1 to the lipid monolayer by annexin A1 also required calcium annexin A1 and mu1 (not shown). The recruitment of mu1 (Figure 12), although when tested in the two-hybrid assay an interaction was not detected between annexin A1 and mu1 (not shown). The recruitment of mu1 to the lipid monolayer by annexin A1 also required calcium (Figure 13) with a sensitivity similar to that seen for annexin to the lipid monolayer by annexin A1.

The mu1 recruitment activity was detected for all three annexins, but mu3 recruitment activity could not be detected above the background level seen in the absence of annexin.

### DISCUSSION

As summarized in the introduction, annexins have been implicated in the processes of membrane trafficking at several points in the cell, based on localization studies, as well as manipulation of annexin expression or the use of dominant negative or interfering annexin constructs (1, 2, 6–14). Three annexins have in particular been associated with endocytosis or the trafficking or maturation of endosomes: annexins A1, A2, and A6. Interestingly, all three of these annexins harbor YxxΦ motifs that might promote interaction with the mu subunits of AP complexes, and these are the three annexins that were specifically found to interact with mu subunits in this study.

Table 1 summarizes the interactions between annexin family members and mu subunits revealed by our experiments. Although some of the interactions were detected in the yeast two-hybrid assay—a strong interaction between annexin A2 and mu2, and weak interactions between annexin A2 and mu1 and mu3—other interactions were not detected by this method, such as the interaction of annexin A1 with mu1, or annexin A6 with mu1 or mu2. The table therefore emphasizes interactions that were verifiable in in vitro studies with purified proteins. Although the weak interaction between annexin A2 and mu3 in the two-hybrid assay (Figure 11) could not be verified in vitro, it is possible that the use of a different lipid system might reduce the annexin-independent binding of mu3 to the lipid monolayer (Figure 12) and allow effects of annexins on recruitment of mu3 to be revealed.

The ability of annexins A2 and A6 to interact with both mu1 and mu2 suggests that they could be involved in trafficking events at both the plasma membrane, where mu2 functions, as well as in internal movements of vesicles between compartments in the endocytic pathway where mu1 is involved. In contrast, the failure of annexin A1 to interact with mu2 may be an indication that this annexin is only involved in trafficking between intracellular compartments.

No interaction was seen between annexin A7 and mu2. However, the annexin A7 constructs that were used in both the two-hybrid assay and the in vitro experiments were derived from the shorter annexin A7 isoform that is present in many tissues. Muscle and brain tissue express a larger isoform that includes a 22-residue splice insertion following residue 144 (see Figure 1, ref 47). It is striking that this 22-residue insert contains a YxxΦ motif, specifically YPVF. It would be of interest to determine if this larger isoform of annexin A7 can interact with mu subunits. Such a specific interaction might provide a biological explanation for the

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**Table 1: Summary of Interactions between Annexins and AP mu Subunits**

<table>
<thead>
<tr>
<th></th>
<th>mu1</th>
<th>mu2</th>
<th>mu3</th>
</tr>
</thead>
<tbody>
<tr>
<td>annexin A1</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>annexin A2</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>annexin A2−S100A10 tetramer</td>
<td>nd</td>
<td>–</td>
<td>nd</td>
</tr>
<tr>
<td>annexin A6</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>annexin A7</td>
<td>nd</td>
<td>–</td>
<td>nd</td>
</tr>
</tbody>
</table>

conservation and differential expression of this isoform. It also remains important to test the ability of annexin A3, the only human annexin with an N-terminal YxxΦ motif that was not studied here, to interact with mu subunits.

A “Masquerade” Hypothesis for the Role of Annexins in Endosome Trafficking. Despite a significant literature implicating annexin involvement in endosome generation or trafficking in vivo, no clear mechanistic or biochemical hypothesis has been put forward to explain their involvement. On the basis of our data, we propose the annexins may “masquerade” as transmembrane receptors in the process of coated pit formation (Figure 14). In the presence of calcium, the annexin binds a region of membrane containing acidic phospholipids. In the case of annexin A1 or A2, this causes the N-terminal domain of the annexin to be extended into the cytoplasm where the YxxΦ motifs are now accessible to the AP complex. In the case of annexin A6, exposure of the interdomain linker might be critical for AP recruitment. The interaction between the AP complex and the annexin would provide a driving force to initiate assembly of other soluble coated pit components. A key tenant of this hypothesis is that this mechanism provides a means for calcium to regulate the initiation of coated pit formation. While there is evidence in the literature that in some cases coated pit formation at the cell surface might involve regulation by calcium (48–54), there is not clear evidence that calcium is essential in all cases, and in particular, there is little evidence that calcium may be required for intracellular trafficking of endosomes that would involve coated pit formation on intracellular organelles such as the trans-Golgi network or early endosomes. It seems likely that the role of the annexin in these processes is not essential, but the annexin may provide a means for acceleration of trafficking when calcium levels are elevated. This may explain the observation, for example, that receptor mediated endocytosis proceeds in A431 cells that do not have detectable amounts of annexin A6 (55). It appears that critical experiments have yet to be performed to determine if the addition of annexin A6 to such a cell system might confer calcium sensitivity to endocytosis, or to other trafficking events.

In addition to a role for calcium in regulating annexin involvement in coated pit formation, our data suggest that phosphorylation of the annexin A2 N-terminal domain may provide another level of regulation of this mechanism. In particular, tyrosine phosphorylation of annexin A2 during cell transformation might block the ability of the membrane trafficking processes to be regulated by calcium. There are also protein kinase C phosphorylation sites near the YxxΦ motifs in annexins A1 and A2, as well as a site for tyrosine phosphorylation by the EGF receptor in annexin A1. Therefore, we speculate that activation of protein kinase C by a number of agonists, or activation of the EGF receptor, could also hinder the involvement of annexins A1 or A2 in membrane trafficking by adversely affecting interactions of the annexins with the clathrin assembly proteins.

Although this “masquerade” hypothesis suggests an appealing mechanistic role for annexins as critical initiators of coated vesicle formation, it is also possible that the annexins are simply “stowaways” after their successful masquerade: In response to a transient increase in calcium the annexins expose a YxxΦ motif and are picked up by the coated vesicle machinery, in substitution for a transmembrane receptor, and are then transported to other regions of the cell where they are released to perform other functions when the vesicle is uncoated. A careful study of the movements of labeled annexins within cells in comparison with vesicle trafficking routes might shed light on the possible importance of such a mechanism in annexin utilization.

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REFERENCES


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