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Specific Release of Membrane-bound Annexin II and Cortical Cytoskeletal Elements by Sequestration of Membrane Cholesterol

Thomas Harder,*† Roland Kellner,‡ Robert G. Parton,§ and Jean Gruenberg*‖

*Department of Biochemistry, Sciences II, 1211, Geneva 4, Switzerland; ‡Institut für Physiologische Chemie und Pathobiochemie, University of Mainz, Mainz, Germany; and ‖Department of Physiology and Pharmacology, §Centre for Microscopy and Microanalysis and Centre for Molecular and Cellular Biology, University of Queensland, Brisbane, Queensland 4072, Australia

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Annexin II is an abundant protein which is present in the cytosol and on the cytoplasmic face of plasma membrane and early endosomes. It is generally believed that this association occurs via Ca^{2+}-dependent binding to lipids, a mechanism typical for the annexin protein family. Although previous studies have shown that annexin II is involved in early endosome dynamics and organization, the precise biological role of the protein is unknown. In this study, we found that approximately 50% of the total cellular annexin was associated with membranes in a Ca^{2+}-independent manner. This binding was extremely tight, since it resisted high salt and, to some extent, high pH treatments. We found, however, that membrane-associated annexin II could be quantitatively released by low concentrations of the cholesterol-sequestering agents filipin and digitonin. Both treatments released an identical and limited set of proteins but had no effects on other membrane-associated proteins. Among the released proteins, we identified, in addition to annexin II itself, the cortical cytoskeletal proteins α-actinin, ezrin and moesin, and membrane-associated actin. Our biochemical and immunological observations indicate that these proteins are part of a complex containing annexin II and that stability of the complex is sensitive to cholesterol sequestering agents. Since annexin II is tightly membrane-associated in a cholesterol-dependent manner, and since it seems to interact physically with elements of the cortical actin cytoskeleton, we propose that the protein serves as interface between membranes containing high amounts of cholesterol and the actin cytoskeleton.

INTRODUCTION

The annexin protein family comprises at least 18 members that have been identified in organisms from plants to mammals, indicating a wide distribution throughout the eukaryotic kingdom (Moss, 1992; Raynal and Pollard, 1994; Morgan and Fernandez, 1995). All annexins that have been characterized so far are found both in the cytosol and associated with the cytoplasmic face of cellular membranes, and share the capacity to bind liposomes containing negatively charged phospholipids in a Ca^{2+}-dependent manner. Ca^{2+}-dependent lipid binding is mediated by a highly conserved C-terminal core domain, which is defined biochemically by its resistance toward limited proteolysis. In contrast to the core domain, the protease sensitive N-terminal head region is highly divergent in length and sequence within the family and contains features specific for each annexin, including sites for posttranslational modifications and/or binding sites

† Present address: European Molecular Biology Laboratory, Meyerhofstrasse 1, Heidelberg 69012, Germany.
‖ Corresponding author: Department of Biochemistry, Sciences II, 30 quai E. Ansermet, 1211 Geneva 4, Switzerland.

for specific ligands (Moss, 1992). Although this Ca^{2+}-dependent lipid binding is generally thought to reflect the mechanism mediating annexin membrane association, the binding of most annexins to lipids requires relatively high Ca^{2+} concentrations (≥ 1 μM) beyond the free Ca^{2+} concentrations observed in resting cells. This suggests that additional mechanisms may be involved in annexin association with intracellular membranes.

The specific association of annexins to intracellular membranes observed in vivo has led to the view that annexins play a role in membrane-related processes. Indeed, the function of annexins has been linked to the dynamics and structure of cellular membranes (Creutz, 1992; Gruenberg and Emans, 1993). Annexin I has been shown to be phosphorylated via the epidermal growth factor receptor and to be associated with endosomal membranes (Fava and Cohen, 1984), and has been proposed to play a role in multivesicular body biogenesis (Futter *et al.*, 1993). More recently, annexin XIIIb has been identified as a major component of trans-Golgi network-derived vesicles destined for the apical membrane of MDCK cells (Fiedler *et al.*, 1995). In this study, transport to the apical membrane could be inhibited by anti-annexin XIIIb antibodies, suggesting that the protein may be involved in polarized vesicular transport.

Annexin II is an extensively studied member of the family (Kaetzel and Dedman, 1995; Waisman, 1995). Its N-terminal head region contains a binding site for the dimer of p11, the cellular ligand of annexin II, and is therefore responsible for mediating the formation of an annexin II_2p11_2 heterotetramer found in many cell types. P11 itself belongs to the S100 protein family of Ca^{2+}-binding proteins (Schaefer and Heizman, 1996), but has suffered critical mutations in the EF hand loops of the Ca^{2+}-binding sites, and is thus incapable of binding Ca^{2+} (Gerke, 1991). In addition to the p11-binding site, the annexin II N-terminal region also contains phosphorylation sites for both src kinase and protein kinase C.

Several lines of evidence indicate that annexin II is involved in both the regulated exocytic pathway (Creutz, 1992) and the endocytic pathway (Gruenberg and Emans, 1993). This protein can partially restore Ca^{2+}-dependent secretory activity in digitonin/SLO-permeabilized chromaffin cells, and annexin II can aggregate chromaffin granules at Ca^{2+} concentrations in the μM range (Ali *et al.*, 1989; Drust and Creutz, 1988; Sarafian *et al.*, 1991; Chasserot-Golaz *et al.*, 1996). In the endocytic pathway, immunoelectron microscopic studies have shown that annexin II is associated with plasma membrane and early endosomes of BHK cells (Emans *et al.*, 1993) and with the apical and basolateral plasma membranes and early endosomes of polarized MDCK cells (Harder and Gerke, 1993). Since annexin II is a major component of BHK early endosomes and since it is transferred with high efficiency from a donor to an acceptor endosomal membrane in vitro, it has been proposed that the protein may play a role in early endosome dynamics (Emans *et al.*, 1993). Anti-annexin II antibodies were shown to inhibit Ca^{2+}-dependent early endosomes fusion occurring in the absence of cytosol (Mayorga *et al.*, 1994). A role for annexin II in early endosome structure and dynamics was demonstrated in vivo after expression of an annexin II/p11 chimera in polarized MDCK cells (Harder and Gerke, 1993). Altogether, these observations strongly suggest that annexin II contributes to the regulation of the structure and/or dynamics of a defined set of intracellular membranes, although the molecular mechanism of this process remains to be elucidated.

In the present article, we have used biochemical approaches to reveal the existence of a novel mechanism responsible for annexin II-membrane association. This mechanisms is Ca^{2+} independent, in contrast to other annexins, and sensitive to low concentrations of cholesterol-sequestering agents. These agents efficiently release annexin II from membranes in combination with a limited set of proteins, including elements of the cortical actin cytoskeleton. In addition, coimmunoprecipitation experiments from detergent-solubilized membranes suggest that annexin II interacts with these proteins in vivo. These findings strongly suggest that annexin II is associated with cholesterol-rich membrane domains and may serve as interface to the cortical actin cytoskeleton.

MATERIALS AND METHODS

Cells and Immunological Reagents

Monolayers of BHK cells were grown and maintained as described (Gruenberg *et al.*, 1989). Cells were metabolically labeled by incubation for 16 h before the experiment with 0.5 mCi/ml [^{35}S]methionine/[^{35}S]cysteine ExpreSS label (DuPont New England Nuclear, Boston, MA) in low Met/Cys medium (1.5 mg/l Met, 3.1 mg/l Cys). Antibodies against annexin II, two monoclonal (HH7 and H28) and one polyclonal rabbit serum, were described previously (Gerke and Weber, 1984; Osborn *et al.*, 1988; Thiel *et al.*, 1992) and were gifts from Volker Gerke (University of Münster, Münster, Germany). Anti-moesin and anti-ezrin antisera were gifts from Anthony Bretscher (Cornell University, Ithaca, NY). A mouse monoclonal antibody against α-actinin was obtained from Sigma (St. Louis, MO), and horseradish peroxidase- (HRP) conjugated secondary antibodies were obtained from Amersham (Buckinghamshire, England).

Subcellular Fractionation

We previously established a step sucrose flotation gradient in D_2O, which separates early from late endosomes (Gorvel *et al.*, 1991). Here, we used a modified version of this gradient in H_2O (Aniento *et al.*, 1996) to prepare an annexin II-enriched fraction. Briefly, cells were gently homogenized in 250 mM sucrose, 3 mM imidazole (pH 7.4), and 0.5 mM EDTA (HB) by passage through a 22-gauge injection needle, and a post-nuclear supernatant was prepared by centrifugation. The post-nuclear supernatant was adjusted to 40.6%

sucrose and 3 mM imidazole (pH 7.4) and overlaid sequentially with 35% sucrose in 3 mM imidazole (pH 7.4), 0.5 mM EDTA, 25% sucrose in 3 mM imidazole (pH 7.4), 0.5 mM EDTA, and HB. The gradient was centrifuged at 35,000 rpm in a SW60 rotor for 60 min at 4°C. The 40.6%/35% (membrane fraction 1), the 35%/25% (membrane fraction 2), and the 25%/HB interphases (membrane fraction 3) were collected, as well as the corresponding sucrose cushions, and analyzed. In these experiments, we used HRP, as early endosomal marker, internalized from the medium for 5 min at 37°C (Gruenberg *et al.*, 1989). To provide a general marker of the plasma membrane, the cell surface was biotinylated with 0.5 mg/ml NHS-LC-biotin (Pierce Chemical, Rockford, IL) for 30 min at 4°C (Graeve *et al.*, 1989). The cells were then incubated for 30 min on ice with 10 μg/ml HRP-avidin conjugate and washed three times for 15 min on ice with phosphate-buffered saline (PBS) containing 2% bovine serum albumin. Amounts of HRP-avidin nonspecifically associated with the plasma membrane were negligible, since no HRP activity could be detected when the biotinylation step was omitted. Thirty to 40% of each marker was typically lost to the nuclear pellet (Gruenberg *et al.*, 1989). Amounts of each marker were quantified in all fractions of the gradient, and the sum was normalized to 100%.

Labeling with [^{3}H]-labeled Cholesterol

For cholesterol labeling, cells were washed, transferred into serum-free medium containing 8 ng/ml and 10 μCi/ml [1α-,2α-^{3}H]Cholesterol (Amersham, 35–50 Ci/mmol in an ethanol solution; ethanol final concentration was 0.1% vol/vol), and incubated for 3 h at 37°C in 5% CO_2. The medium was then replaced by unlabeled serum-free medium, and cells were further incubated for 90 min at 37°C to allow equilibration of the labeled cholesterol in intracellular membranes. Cellular fractionation was performed as described above. TLC analysis showed no esterification during the incubation times (our unpublished observations). Cholesterol quantitation of subcellular fractions showed that values obtained after labeling were identical to those obtained by colorimetric determination using the cholesterol oxidase method (kit from Boehringer Mannheim, Mannheim, Germany). As for endosomes and plasma membrane, 30–40% of the cholesterol was lost to the nuclear pellet. Amounts of cholesterol were quantified in all fractions of the gradient, and the sum was normalized to 100%.

Gel Filtration

Membranes of fraction 2 (200 μg of protein) were washed in 1 ml of HBSE (20 mM HEPES buffer, pH 7.2, 150 mM NaCl, 2 mM EGTA) by centrifugation at 100,000 × *g* in a TL100.2 rotor in a TL-100 centrifuge (Beckman Instruments, Fullerton, CA) for 15 min. The membrane pellet was then resuspended at 4°C in 50 μl of HBSE containing either 0.2% Nonidet P-40 (NP40), 0.05% digitonin, or both, and the mixture was submitted to brief pulses of sonication in a bath-type sonicator. After 15 min on ice, the mixture was centrifuged as above for 10 min at 100,000 × *g*. In the presence of NP40, >90% of the total protein was solubilized by the treatment. The supernatants were immediately subjected to gel filtration on a Superose 12 column, which had been preequlibrated with the corresponding solubilization buffer at 4°C using the SMART FPLC system (Pharmacia, Uppsala, Sweden). Flow rate was 40 μl/min and fractions of 100 μl were collected. Proteins in the fractions were then precipitated using chloroform/methanol, as above, and analyzed with SDS gels and Western blotting. The elution volume of free annexin was determined for purified annexin heterotetramer II_2p11_2 and for recombinant (monomeric) annexin II (Thiel *et al.*, 1991).

Extraction of Annexin II and Sequestration of Membrane Cholesterol

Several conditions were tested to extract annexin II from membranes. Fraction 2 (20 μg of protein) was diluted in 200 μl of HBSE and harvested by centrifugation at 100,000 × *g* for 10 min at 4°C in a Beckmann TL-100 table-top ultracentrifuge. The pellets were then resuspended in either 150 μl of HBSE containing 1 or 2 M KCl, 150 μl of 0.1 M $NaHCO_3$ buffer (pH 11), or 150 μl of HBSE containing 0.2% NP40. After incubation for 10 min on ice, membranes were recovered by centrifugation at 100,000 × *g* for 10 min at 4°C, as above, and supernatant proteins were precipitated in chloroform/methanol. All samples were analyzed by SDS gel electrophoresis followed by Western blotting using anti-annexin antibodies.

Alternatively, fraction 2 (10 μg to 1 mg) was resuspended in HBSE, and then the membranes were sedimented at 4°C by centrifugation for 20 min at 35,000 rpm in a SW60 rotor onto a cushion of 45% sucrose and 3 mM imidazole (pH 7.4). The membranes were collected and brought to a final volume of 150 μl with HBSE buffer. Filipin was then added dissolved in tissue culture quality dimethyl sulfoxide (DMSO, Sigma) to a final concentration of 10 μg/ml (wt/vol) or 0.2 μg/mg protein (wt/wt). As a control for nonspecific effects of DMSO, mock extractions were carried out at the same final DMSO concentration (1% vol/vol). We also used 0.01% digitonin instead of filipin. All extractions were performed at 4°C for 15 min. When large amounts of membranes (>200 μg of protein) were used, the minimal amounts of each drug necessary for solubilization were titrated. After the treatment, membranes were collected by centrifugation at 100,000 × *g* for 10 min at 4°C in a TL100 centrifuge. Supernatant proteins were precipitated in chloroform/methanol. All samples were then analyzed by gel electrophoresis in one or two dimensions.

Analysis in Sucrose Gradient at Equilibrium and Immunoprecipitations

Fraction 2 (240 μg of ^{35}S-labeled protein at 100,000 cpm/μg protein) was diluted in 1 ml of HBSE centrifuged at 100,000 × *g* for 30 min. Membranes were collected and solubilized in 50 μl of HBSE containing 0.2% NP40 as described above. Nonsoluble material was removed by centrifugation for 5 min at 100,000 × *g*. The supernatant was diluted to 1 ml with HBSE, loaded onto a linear sucrose gradient from 8% to 30% in HBSE, and centrifuged at 4°C in a SW60 rotor for 16 h at 35,000 rpm. Fractions of 500 μl were collected and, after determination of the sucrose density, analyzed by SDS gel and autoradiography. Based on this analysis, 100 μl of each fraction (20%, 15%, 12%, and 8% sucrose) were adjusted to 0.05% digitonin and incubated for 30 min on ice or mock treated. Then, each sample was diluted to 1 ml with HBSE (when present, the digitonin final concentration was then 0.01%) and processed for immunoprecipitation by adding 10 μl of anti-annexin II antiserum. The mixture was then incubated overnight at 4°C. The immune complex was retrieved with protein A-Sepharose (Pharmacia), washed with HBSE followed by HBSE containing 450 mM NaCl, and analyzed by SDS PAGE and autoradiography.

Two-Dimensional (2D) Gel Electrophoresis

A combination of isoelectric focusing and SDS-PAGE was used to resolve proteins in two dimensions as described (Bravo, 1984). The samples were dissolved 9.8 M urea, 4% wt/vol NP40, 2% wt/vol ampholines (pH 7–9), 100 mM dithiothreitol, and 2 mM iodacetamide. Tube gels were 25 cm long and 2.5 mm in internal diameter. Isoelectric focusing gels were run at 1200 V for 17 h. The pH gradient was linear between pH 4.5 and 7.5. The second dimension resolving gel was 15% (wt/vol) acrylamide and 0.075% (wt/vol) *N,N'*-methylenebisacrylamide and the stacking gels were 5% (wt/vol) acrylamide and 0.25% (wt/vol) *N,N'*-methylenebisacrylamide. After electrophoresis, the gels were fixed and prepared for autoradiography using intensifying scintillation reagents (DuPont New England Nuclear).

Microsequencing

Fraction 2 (2 mg of protein) was extracted by filipin as described. Extracted proteins were precipitated by chloroform/methanol, as above, resolved in SDS gels, transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA), and stained with Coomassie Blue R-250 (Herwald *et al.*, 1996). Protein bands were excised and subjected to NH_2-terminal sequence analysis by automated Edman degradation using a 477A sequenator connected to a model 120 on-line phenylthiohydantion analyzer (Applied Biosystems, Weiterstadt, Germany). Computer sequence analysis was done searching for homologous sequences in SwissProt release 32, EMBL release 43, and GenBank release 89.

Immunolocalization of Annexin II in Perforated MDCK Cells

MDCK cells grown on a filter support were incubated with 10 mg/ml HRP (Sigma) for 10 min at 37°C. The cells were then washed at 4°C in cold PBS and then permeabilized mechanically using nitrocellulose exactly as described previously (Ikonen *et al.*, 1996). The cells were incubated at 4°C for 15 min in acetate transport buffer to allow cytosol to leak out and then were fixed in 0.5% glutaraldehyde in PBS. The perforated cells were immunolabeled and processed for Epon embedding exactly as described previously (Ikonen *et al.*, 1996).

Other Methods

HRP activity was quantified as previously described (Gruenberg and Howell, 1989). Protein was quantified using the method of Bradford (1976). Protein precipitation using chloroform/methanol was according to Wessel and Flügge (1984). For SDS gel electrophoresis (Laemmli, 1970), we used a Bio-Rad Protean gel II minigel system, and, for Western blotting, proteins were transferred onto nitrocellulose (0.4-μm pore size; Schleicher & Schuell, Keene, NH) using a Bio-Rad Semi dry transfer chamber.

RESULTS

Ca^{2+}-dependent binding of annexins to phospholipid bilayers is believed to occur via simultaneous chelation of Ca^{2+} ions by specific annexin residues and by the negatively charged head groups of phospholipids (Huber *et al.*, 1990). Although it is generally believed that the same mechanism mediates the association of annexins to the cytoplasmic face of cellular membranes, it is difficult to envision how such an unspecific mechanism could account for the specific distribution of annexin II in vivo. Indeed, fractionation, immunofluorescence, and immunoelectron microscopy have demonstrated that annexin II is specifically associated with a subset of all intracellular membranes in MDCK and BHK cells, i.e., plasma membrane and early endosomes (Emans *et al.*, 1993; Harder and Gerke, 1993).

Using a sucrose step flotation gradient, we prepared a membrane fraction significantly enriched (approximately 10-fold) in membrane-bound annexin II (Figure 1). We analyzed the distribution in the gradient of the two membrane populations to which annexin II is shown to be associated in vivo (Table 1). To label the plasma membrane, the cell surface was biotinylated on ice, and biotin residues were then quantified with an avidin-HRP conjugate. Early endosomes were labeled after internalization of HRP for 5 min at 37°C from the medium (Gruenberg and Howell, 1989). Quantitation of yields and enrichments show that both early endosome and plasma membrane markers were coenriched ≈10-fold in the same fraction as annexin II (Table 1). We have used this fraction, referred to as membrane fraction 2 in the text, to study the properties of annexin II association with its cellular target membranes.

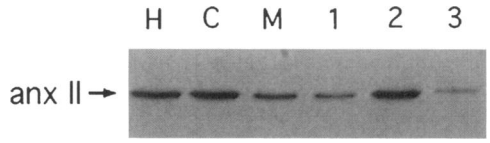

Figure 1. Distribution of annexin II in subcellular fractions of BHK cells. The proportion of annexin II associated with cellular membranes (M) or free in the cytosol (C) was determined by centrifugation of a post-nuclear supernatant at 100,000 × *g*. For comparison, the total cell homogenate (H) is shown. Alternatively, the post-nuclear supernatant was fractionated on a discontinuous sucrose gradient. Fractions were collected from the 40.6%/35% (lane 1), 35%/25% (lane 2), 25%/8.5% (lane 3) sucrose interfaces, diluted in HBSE buffer, and then sedimented at 100,000 × *g*. In all cases, 5 μg of protein were analyzed by SDS gel electrophoresis followed by Western blotting. Annexin II was detected using a 1:100 dilution of HH7 hybridoma supernatant followed by anti-mouse antibodies coupled to HRP.

Table 1. Distribution of cholesterol, plasma membrane and early endosomes on the gradient

	Plasma membrane		Early endosomes		[^{3}H]-cholesterol		Protein
	Y (%)	E (fold)	Y (%)	E (fold)	Y (%)	E (fold)	Y (%)
Fraction 1	12 ± 4	1	15 ± 3	1	8 ± 2	2	16 ± 4
Fraction 2	43 ± 6	9	46 ± 2	9	45 ± 5	9	5 ± 1
Fraction 3	3.5 ± 1	2.5	4 ± 1	3	5 ± 1	3	1 ± 0.5

Plasma membrane and early endosomes were labeled after cell surface biotinylation and endocytosis of HRP for 5 min, respectively (see MATERIALS AND METHODS). Cells were homogenized, and the distribution of the markers was analyzed using a step flotation gradient. The interfaces were then collected and analyzed (fraction 1, 40.6/35% interface; fraction 2, 35%/25% interface; fraction 3, 25%/HB interface). Yields (Y) in percentage of total gradient and enrichments (E) over protein are indicated for each marker. To determine the distribution of cholesterol, cells were incubated in vivo with ^{3}H-labeled cholesterol, processed as above, and then amounts of ^{3}H-labeled cholesterol were quantified in each fraction. Measurements were repeated three times, and SE are indicated. In the three fractions, the distribution of phospholipids was ≈1:1:0.5 (fraction 1:fraction 2:fraction 3). This analysis shows that cholesterol cofractionates with plasma membrane and endosome markers..

Ca^{2+}-independent Annexin II-Membrane Interaction

Surprisingly, we found that the annexin II-membrane association was preserved after treatment of membrane fraction 2 with EGTA, and thus independent of free Ca^{2+}. We also found that annexin II was remarkably tightly membrane associated, as illustrated in Figure 2. Association of annexin II with membranes was resistant to treatments with high salt concentration, up to 2 M KCl, in the presence of EGTA, and was only partially released in the presence of 0.1 M carbonate (pH 11). To solubilize annexin II, treatments with detergents, such as NP40, were necessary. These properties are in line with observations that chromaffin granules and chromaffin cell plasma membrane contain a pool of annexin II resistant to Ca^{2+} chelation (Drust and Creutz, 1991). These data show the existence of a tight annexin II/membrane interaction of a nature entirely different from the previously described Ca^{2+}-dependent binding of annexins to negatively charged phospholipids. Moreover, upon annexin II addition, membrane binding did not appear to be saturable (our unpublished data), in agreement with our previous observations that annexin II is more abundant than any membrane protein which could serve as a putative receptor (Emans *et al.*, 1993).

Sequestration of Membrane Cholesterol Releases Annexin II from the Membranes

Solubilization experiments using different detergents showed that low digitonin concentrations were sufficient to release annexin II from membranes (Figure 3). At these concentrations (0.01%), clearly below the CMC (0.086%), digitonin is a very poor solubilizer of membrane proteins. We thus reasoned that annexin II extraction may not be due to the detergent properties of digitonin but to its known capacity to cluster membrane cholesterol (Elias *et al.*, 1978). We then tested the effects of independent compounds known to react specifically with membrane cholesterol. The polyene antibiotic filipin, chemically unrelated to digitonin, but with similar cholesterol-sequestering properties (Bolard, 1986), also released annexin II at low concentrations (10 μg/ml; Figure 3). Similar sub-CMC concentrations of Triton X-100 did not release membrane-bound annexin II, indicating that the effects of cholesterol-clustering agents were specific (our unpublished observation). In agreement with the notion that cholesterol is directly or indirectly involved in annexin II-membrane interactions, cholesterol cofractionated with annexin II and its known target membranes in our gradient (Table 1). Our data thus show that annexin II interacts with membranes in a Ca^{2+}-independent manner and strongly suggest that membrane cholesterol plays a role in this interaction.

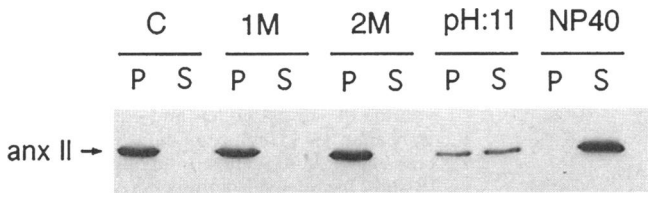

Figure 2. Extraction of annexin II from membranes. Membranes present in fraction 2 (20 μg of protein; Figure 1) were diluted in HBSE and washed once by centrifugation. Pellets were resuspended in 150 μl of HBSE (C), 1 M KCl in HBSE (1M), 2 M KCl in HBSE (2M), 0.1 M carbonate buffer pH 11 (pH 11), or HBSE containing 0.2% NP40. After a 10-min incubation on ice, membranes were collected by high-speed centrifugation; supernatant proteins were precipitated in chloroform/methanol. For each condition, membrane pellets and precipitated supernatant proteins were dissolved in equal volumes of SDS gel sample buffer and analyzed by SDS gel electrophoresis followed by Western blotting with the HH7 antibody.

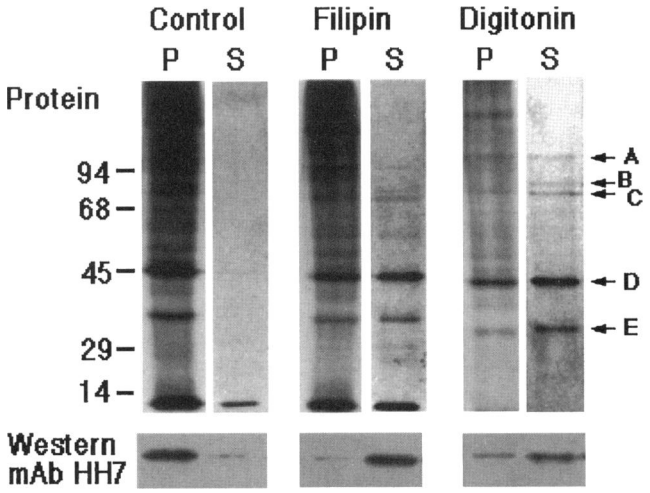

Figure 3. Sequestration of membrane cholesterol releases annexin II from the membranes. Cells were metabolically labeled to equilibrium with 0.5 mCi/ml [^{35}S]methionine and [^{35}S]cysteine and fractionated as in Figure 1. ^{35}S-labeled fraction 2 (50,000 cpm) was mixed with 10 μg of unlabeled fraction 2, and then the mixture was treated under mock conditions (control) or extracted with filipin or digitonin. Membrane-associated (P) and soluble (S) proteins were separated by high-speed centrifugation. Soluble proteins were then precipitated in chloroform/methanol. All samples were solubilized in the same volume of gel sample buffer, and equal volumes of P and S were analyzed by gel electrophoresis. Gels were either processed for autoradiography (upper panel) or transferred to nitrocellulose and analyzed by Western blotting using the HH7 antibody (lower panel). Molecular weight markers are indicated. Five major polypeptides were extracted (upper panel) and are indicated by arrows at apparent molecular weights of 100,000 (A), 80,000 (B and C doublets), 45,000 (D), and 36,000 (E). Annexin II itself (protein E, see Figures 4 and 5 and text) is efficiently extracted by both filipin and digitonin (lower panel).

Sequestration of Membrane Cholesterol Specifically Releases Cortical Cytoskeletal Proteins

An SDS gel analysis of metabolically labeled membrane proteins released by either one of the two cho-

lesterol-sequestering drugs showed a surprisingly simple and very similar pattern (Figure 3). Whereas no protein could be detected in the supernatants of mock-treated membranes, both digitonin and filipin extracts contained predominant polypeptides (in order of apparent abundance), at M_r 45,000 (D), 36,000 (E), a doublet around 80,000 (B and C), and a 100,000 polypeptide (A) (see arrows in Figure 3). To facilitate identification, the same experiments were repeated using high resolution 2D gels (Bravo, 1984) after extraction with digitonin (our unpublished observations) and filipin (Figure 4). Actin (Figure 4D, *, 45 kDa) and annexin II (Figure 4D, anx II, 36 kDa) could easily be identified by their well-established migration properties in the 2D gel system used (Emans *et al.*, 1993) as the most abundant proteins that were extracted (Figure 4). Two additional prominent polypeptides migrated at the known positions of moesin (Figure 4D, m) and ezrin (Figure 4D, e; Julio Celis 2D databases are available on internet at http://biobase.dk/cgi-bin/celis). In these high-resolution 2D gels, several less abundant proteins, which remain to be identified, were also detected after extraction with filipin. (The most efficiently extracted lower abundance polypeptides are marked by arrowheads in Figure 4.)

Proteins released by the cholesterol-acting drugs could be unambigously identified after microsequencing and Western blotting using specific antibodies. N-terminal sequencing confirmed that band C (lower band of the M_r 80,000 doublet, Figure 3) was indeed moesin, a member of the ERM family of proteins, which was proposed to play a role in the linkage of actin filaments to the plasma membrane (Algrain *et al.*, 1993; Berryman *et al.*, 1993). This observation was confirmed by Western blotting of digitonin and filipin extracts using an anti-moesin antiserum (Figure 5). The anti-moesin antibody showed some cross-reactivity with band B (Figure 5, upper band of the M_r 80,000 doublet). Since this anti-moesin antiserum is known to slightly cross-react with ezrin, we concluded that band B may well correspond to ezrin. Using an anti-ezrin specific antiserum, we unambiguously confirmed that protein B was indeed ezrin, another member of the ERM protein family. Since two actin-binding proteins were identified among the extracted proteins, we speculated that band A (M_r 100,000 polypeptide) may correspond to the actin-bundling protein α-actinin frequently found at regions of membrane actin attachment sites (Bretscher, 1991). This was indeed confirmed using a monoclonal antibody against α-actinin. We, therefore, identified a small subset of proteins, which are all related to the cortical actin cytoskeleton and extracted by cholesterol-clustering drugs (Figure 3), namely, actin (band D, 45 kDa), annexin II (band E, 36 kDa), ezrin and moesin (bands B and C, doublet at 80 kDa), and, finally, α-actinin (band A, 100 kDa). The sensitivity of each of these proteins to cholesterol aggregation by filipin varied, since moesin and ezrin were most efficiently extracted, whereas annexin II and α-actinin appeared to be less sensitive. These observations indicate that cholesterol sequestration does not release these five polypeptides in a stoichiometric complex.

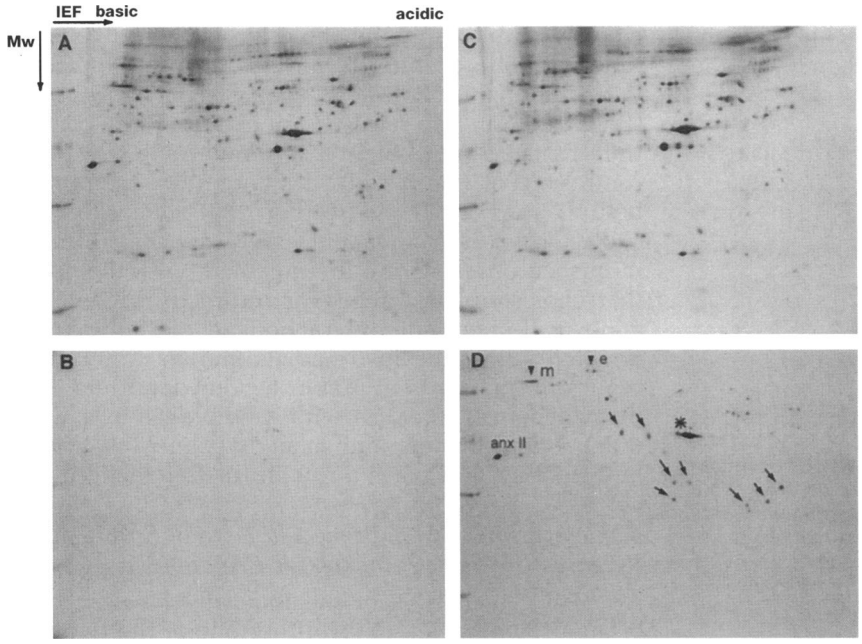

Figure 4. 2D gel analysis of proteins released after cholesterol sequestration. The same experiments as described in Figure 3 were repeated using 500,000 cpm ^{35}S-labeled fraction 2 (5 μg of protein) and 75 μg of unlabeled fraction 2. Membranes were either extracted with filipin or mock treated, and then soluble and membrane-associated proteins were separated by centrifugation. All samples were then solubilized in the same volume of sample buffer, and equal volumes were analyzed in high-resolution 2D gels; membrane-associated (A and C) and filipin-extracted (B and D) proteins from mock- (A and B) and filipin-treated (C and D) membranes. Actin (*) at 45 kDa and annexin II (anx II) at 36 kDa were both unambiguously identified because of their well-established migration in these gels (Emans *et al.*, 1993). By comparison with the existing database (internet address: http://biobase.dk/cgi-bin/celis), proteins labeled with m and e show the mobility and migration pattern of moesin and ezrin, respectively. A few less abundant proteins also appeared to be extracted and are indicated by arrows. Molecular weight markers are 14,000, 29,000, 45,000, 68,000, 94,000, and 200,000.

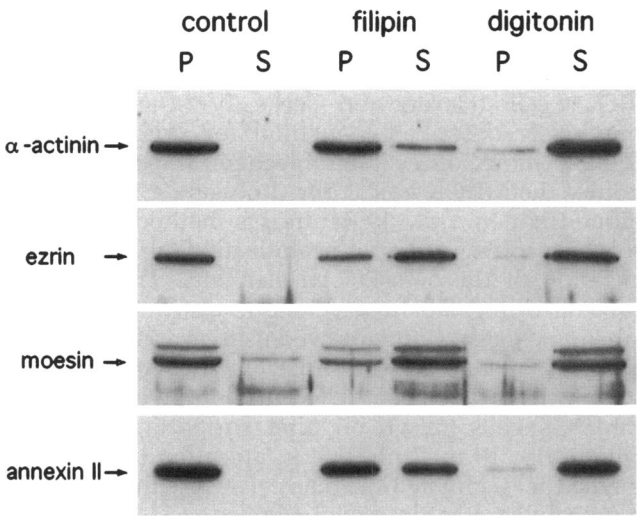

Figure 5. Identification of proteins released after cholesterol sequestration. The experiments described in Figures 3 and 4 were repeated using 100 μg of protein of fraction 2, except that cells were not metabolically labeled. Membrane-associated (P) and soluble-extracted proteins (S) were analyzed by Western blotting using antibodies against α-actinin, moesin, ezrin, or annexin II. The anti-moesin antiserum exhibits some cross-reactivity with ezrin, which runs at a slightly higher molecular weight (band B of the M_r 80,000 doublet).

Immunoprecipitation of Annexin II Complexed to Cytoskeletal Proteins

The five proteins we have identified share both functional properties in the organization of the cortical actin cytoskeleton and a unique sensitivity toward cholesterol-clustering agents. We, therefore, investigated whether they may also be physically associated. As a first step, we compared the solubilization properties of several detergents with those of filipin and digitonin, and analyzed the extracts by rapid micro-high-performance liquid chromatography gel filtration on a Superose 12 column. Annexin II could be quantitatively released from membranes in the presence of NP40 (Figure 2), and a significant proportion of the protein was then eluted in the column flow through (M_r 300,000) in fractions corresponding to molecular weights much higher than those of the monomer or heterotetramer (Figure 6A). (Monomer and heterotetramer could not be resolved under these conditions.) These annexin II-containing complexes did not appear to behave as a single molecular species, but smeared over several fractions, reflecting the relative instability of the complexes in the detergent (our unpublished results) and possibly differences in the complex stoichiometry. Most strikingly, however, annexin II was eluted exclusively as a free molecule after solubilization with NP40 in the presence of a sub-CMC concentration of digitonin (Figure 6A, 0.2% NP40 and 0.05% digitonin). These experiments show that annexin II-containing complexes were not simply formed by artificial aggregation in detergent and that digitonin released annexin II from high molecular weight complexes.

Next, membranes obtained from metabolically labeled cells were solubilized in detergents and immediately diluted to a submicellar NP40 concentration to ensure higher stability of the extracts. The samples

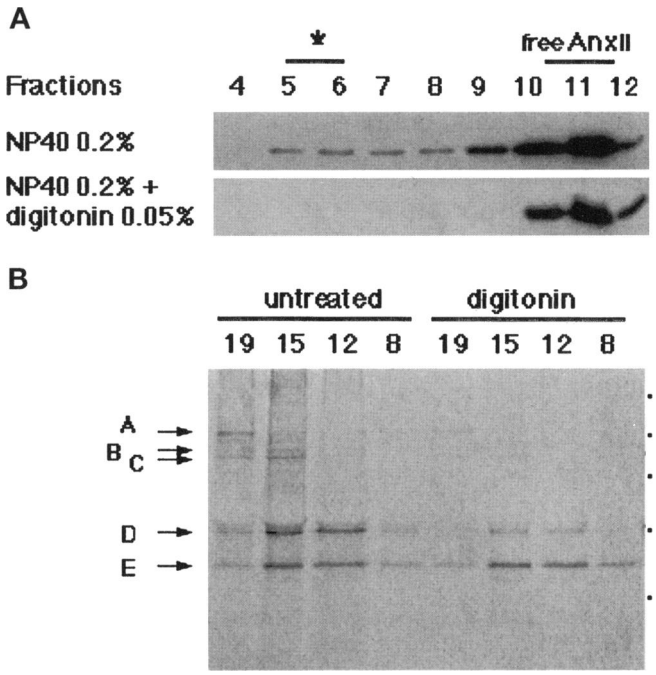

Figure 6. Coimmunoprecipitation of annexin II and cytoskeletal proteins. (A) After solubilization with 0.2% NP40 with or without 0.05% digitonin, proteins present in fraction 2 were separated by micro-FPLC using a Superose 12 column. Fractions were precipitated and analyzed by SDS gel electrophoresis followed by Western blotting using the HH7 antibody. A significant proportion of annexin II was recovered in high molecular weight fractions (up to more than M_r 300,000), whereas the rest was eluted in fractions 10–12, corresponding to the position of the free protein. (The elution profiles of monomeric and heterotetrameric annexin II, which cannot be resolved under the conditions used, were established using the purified proteins.) Cosolubilization by 0.2% NP40 and 0.05% digitonin caused annexin II to be eluted exclusively as a free protein. *, void volume of the column after calibration with thyroglobulin, M_r 400,000. (B) Cells were metabolically labeled to equilibrium with 0.5 mCi/ml [^{35}S]methionine and [^{35}S]cysteine and fractionated as in Figure 1. Then, fraction 2 was collected and extracted with NP40, as in Figure 2. The sample was rapidly diluted to decrease the detergent concentration, and the extracted protein complexes were separated on a linear sucrose gradient. Gradient fractions containing annexin II (8, 12, 15, and 19% sucrose) were divided into two equal samples: one was then treated with 0.05% digitonin and the other left untreated. Both samples were then subjected to immunoprecipitation using anti-annexin II antibodies. Equal amounts of annexin II (36 kDa, band E) were recovered from untreated or digitonin-treated samples. In the absence of digitonin, α-actinin (A, 100 kDa), moesin and ezrin (B and C doublets, 80 kDa), and actin (D, 45 kDa) are coimmunoprecipitated with annexin II, but not when digitonin was present.

were then analyzed by equilibrium centrifugation on a linear sucrose gradient. Autoradiography of SDS gels showed that fractions with sucrose concentrations ranging from approximately 10 to 20% contained polypeptides with migration properties reminiscent of annexin II, actin, α-actinin, and the two ERM proteins. These fractions were thus subjected to immunoprecipitation using anti-annexin II antibodies. As shown in Figure 6B, a small group of proteins was coimmunoprecipitated with annexin II, and its pattern was identical to that of the proteins extracted by either cholesterol-acting drug (compare with Figure 3, bands A–E). These experiments demonstrate that annexin II could be immunoprecipitated as part of protein/lipid complexes, in agreement with our gel filtration data, and that these complexes contain actin, α-actinin, and the two ERM proteins.

The complex immunoprecipitated from the different gradient fractions exhibited roughly the same ratio of annexin II to actin but different amounts of moesin, ezrin, and α-actinin. These differences are unlikely to reflect differences in antigenic properties, since similar subcomplexes were already separated on the gradient. The fact that different subcomplexes containing varying amounts of α-actinin or the two ERM proteins were resolved in these experiments can explain the differences we have observed in both the sizes of the complexes resolved by gel chromatography and the sensitivity of these proteins to filipin extraction (Figure 5). One may, thus, speculate that these differences also reflect the existence of different types of complexes on the membranes in vivo.

The same experiments were repeated after treating the gradient fractions with the sub-CMC concentration of digitonin (0.05%). Then, coimmunoprecipitation of the cortical cytoskeletal proteins with annexin II was significantly reduced, although immunoprecipitation of annexin II itself was not affected. These data establish the specificity of our immunoprecipitation observations, demonstrate that the stability of the complexes is indeed sensitive to cholesterol-acting drugs, and explain our observation that digitonin released annexin II as a free molecule. Whether the other protein partners identified in the complex were also released as free molecules could not be tested by chromatography because of their relatively large size and tendency to oligomerize (Berryman *et al.*, 1995). These data, thus, indicate that cholesterol is required for both annexin II membrane association and the stability of annexin II-containing complexes.

Localization of Annexin II on Membranes

We have also shown that annexin II can be isolated as a physical complex along with elements of the cortical cytoskeleton. In an attempt to visualize potential cytoskeletal interactions of annexin II on the endosomal membrane, we investigated the precise ultrastructural distribution of annexin II in permeabilized MDCK cells. Annexin II has been characterized functionally in MDCK cells (Harder and Gerke, 1993), and we have previously described a method for preservation of cellular morphology after mechanical permeabilization of the MDCK apical domain using a rip-off technique (Ikonen *et al.*, 1996). In this method, release of soluble proteins after permeabilization facilitates visualization of the cytoskeletal elements. The use of a preembedding labeling technique also increased the labeling efficiency and allowed us to examine whether annexin II was uniformly distributed over the endosomal membrane or enriched in discrete domains.

MDCK cells grown on filter supports were incubated with 10 mg/ml HRP in apical and basolateral media for 10 min at 37°C. The cells were then washed, permeabilized as described (Ikonen *et al.*, 1996), and further incubated at 4°C to allow cytosol to leak out of the cells. Pieces of filter were then fixed and incubated with antibodies to annexin II followed by 10 nm of protein A-gold. Dense labeling for annexin II was found to be associated with the surface of early endosomes labeled with internalized HRP. The gold labeling was not uniform over the entire membrane but was found to be concentrated in discrete regions (Figure 7). The annexin II-enriched domains were often visible as patches of three (Figure 7, B and D) or more (Figure 7C) gold particles with little labeling associated with the intervening membrane. Filamentous material was often observed to be associated with the endosomal membrane and in some cases these filaments colocalized with the annexin II labeling at the endosomal membrane (Figure 7, A, B, and D). Although we have been unable to perform double labeling experiments with the available anti-actin and anti-annexin II antibodies, single labeling experiments showed a concentration of similar actin-labeled elements around the early endosomes (Parton, unpublished results).

DISCUSSION

Membrane association of annexins is thought to be mediated via the well-characterized Ca^{2+}-dependent binding of the protease-resistant C-terminal core domain of these proteins to negatively charged phospholipids (Moss, 1992). Here, we report that approximately half of the total cellular annexin II in BHK cells is extremely tightly associated with its target cellular membranes in the presence of Ca^{2+}-chelating agents, the remainder being soluble under our conditions. Membrane binding, thus, does not only depend on Ca^{2+} lipid binding mediated by the core of the protein. Although the extent to which Ca^{2+}-dependent binding may also occur was not addressed in our studies, one may expect membrane binding of annexin

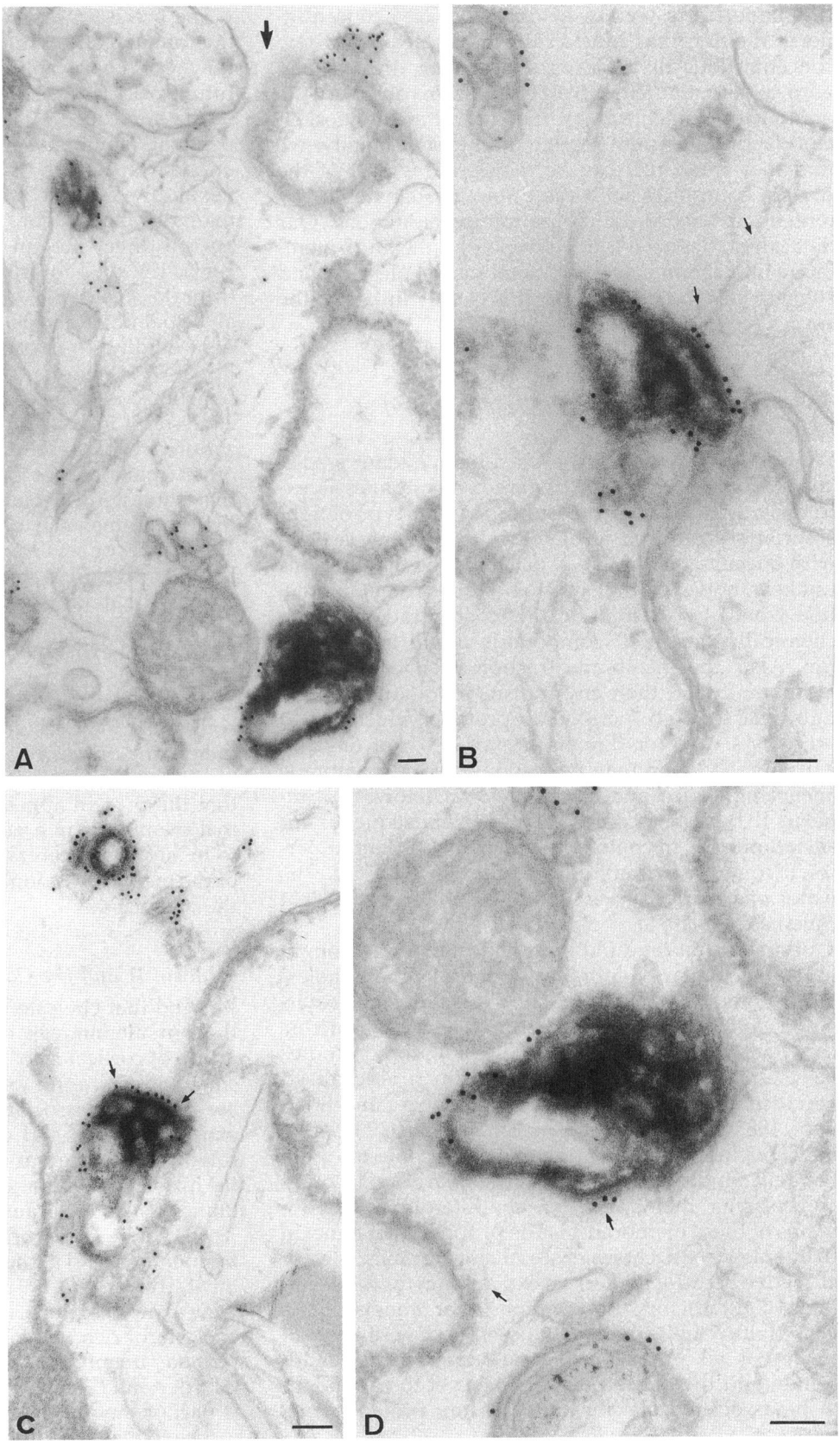

Figure 7. Immunolocalization of annexin II in perforated MDCK cells. MDCK cells grown on a filter support were incubated with HRP for 10 min at 37°C and then mechanically permeabilized using nitrocellulose. The perforated cells were incubated with antibodies to annexin II followed by 10 nm of protein A-gold. A shows a low-power overview of the permeabilized cells (D shows a higher magnification of the lower region). The early endosomes containing the electron-dense reaction product are labeled for annexin II. Mitochondria, endoplasmic reticulum, and the bulk of the plasma membrane are unlabeled. The open apical surface of the cell is indicated by the arrow. The labeling for annexin II is not uniform around the endosome and in some cases extends for some distance from the early endosomal surface (see upper endosome in A). Examples of this annexin II labeling are shown at higher magnification in B–D. Note the patches of endosome labeling which may consist of two to three gold particles (as shown in B and D) or more (see row of eight gold particles between arrows in C). A common observation was the association of filamentous material with the early endosome. These filaments were often in close proximity to annexin II as indicated by the arrows in B and D. Bars, 100 nm.

II to be tuned in vivo by cytoplasmic Ca^{2+} concentrations. We find that annexin II can be released by low concentrations of cholesterol-clustering drugs along with a very limited set of proteins involved in the organization/anchoring of the cortical actin cytoskeleton (actin, α-actinin, moesin, and ezrin). Finally, we find that annexin II can be recovered as part of the protein complexes with these four proteins after detergent extraction and immunoprecipitation. Our data, thus, describe a novel type of annexin II-membrane interaction involving membrane cholesterol and suggest that in vivo annexin II is closely linked to the cortical actin cytoskeleton.

Annexin II-Membrane Interactions: Role of Cholesterol

We have observed that the Ca^{2+}-independent association of annexin II with its target membranes is extremely tight. One may speculate that this type of tight association could be mediated by strong interactions with specific membrane-associated proteins. It seems unlikely, however, that a pure protein-protein interaction would be sensitive to low concentrations of the chemically unrelated compounds filipin and digitonin. Also, the membrane fraction used contains far more annexin II than any transmembrane protein or tightly associated peripheral protein, which could serve as a receptor (Emans *et al.*, 1993). For annexin XIII, myristylation may be responsible for membrane anchoring (Wice and Gordon, 1992). However, annexin II does not contain a consensus sequence for acylation, and, in contrast to annexin XIII (Lafont personal communication), partitions exclusively into the water phase after phase separation with Triton X-114 (Emans *et al.*, 1993).

Plasma membrane and early endosomes are thought to be the cellular membranes with the highest cholesterol content (Dickson *et al.*, 1983; Liscum and Underwood, 1995), and, indeed, immunoelectron microscopy and immunofluorescence microscopic studies have established that in vivo annexin II is localized specifically to these membranes (Greenberg and Edelman, 1983; Osborn *et al.*, 1988; Emans *et al.*, 1993). One may conclude that the mechanisms of cholesterol-dependent binding is shared by both early endosomes and plasma membrane (or cholesterol-rich subdomains of these membranes). The proposal that annexin II interacts with cholesterol-rich membranes is very attractive because it offers an explanation for the seemingly contradictory observations that annexin II is very tightly and specifically associated with a small subset of all intracellular membranes, early endosomes, and the plasma membranes, yet too abundant to be associated specifically with any proteinaceous receptor.

The precise mechanism responsible for the interaction of annexin II with cholesterol-rich membranes is, however, not established, and will be the subject of future work. Jost *et al.* (1997) have shown that deletion of the annexin II NH_2 terminus abolishes Ca^{2+}-independent membrane association in vivo. At present, one may speculate that the N-terminal domain of annexin II interacts directly with cholesterol-rich domains of the bilayer, perhaps with cholesterol itself. Such a direct protein/cholesterol interaction was recently revealed for membrane protein VIP21-caveolin (Murata *et al.*, 1995). Until now, however, we were not able to mimic cholesterol-dependent, Ca^{2+}-independent binding to liposomes (our unpublished results). Possibly the mechanism of annexin II-membrane interactions is more complex than direct annexin II-cholesterol interaction. The formation and/or stabilization of cholesterol-rich domains are likely to require other lipids and/or proteins. One may also envision that cholesterol-dependent membrane binding of individual annexin II molecules is stabilized by lateral association of several annexin II molecules to form a lattice onto cholesterol-rich membranes or membrane domains. Lateral association between annexins has, in fact, been observed for Ca^{2+}-dependent interaction of several annexins with phospholipid bilayers (Zaks and Creutz, 1991). Whether similar interactions may occur in the absence of Ca^{2+} is unknown. Such a lattice could also interact with, and be stabilized by, transmembrane proteins and the cortical cytoskeleton. This view is consistent with our microscopy observations that the protein appears to distribute on the endosomal membrane in a nonrandom manner and appears to be highly concentrated in some specialized regions of the membrane connected to filamentous elements of the cytoplasm.

Annexin II and the Cortical Cytoskeleton

We find that cholesterol aggregation releases annexin II from membranes in combination with a specific group of cortical cytoskeletal proteins (α-actinin, moesin, ezrin, and actin itself), and that the same proteins are physically associated with annexin II in detergent extracts. Moesin and ezrin are both known to play a role in the linkage of actin to specific subdomains of the plasma membrane such as microvilli and membrane ruffles (Algrain *et al.*, 1993; Berryman *et al.*, 1993), whereas α-actinin is an actin-bundling protein frequently found at the sites of actin membrane attachment (Bretscher, 1991). These findings support the view that annexin II is part of the interface between cholesterol-rich membranes such as endosomes/plasma membrane and the cortical cytoskeleton (Harder and Gerke, 1993), and suggest that annexin II is part of the molecular system anchoring cortical cytoskeletal elements to cellular membranes.

The differences we have observed in size and apparent stoichiometry of complexes containing annexin II and cortical cytoskeletal proteins may be due to partial dissociation of large multimeric complexes in detergent. However, it is also possible that these differences reflect, at least in part, the existence of subcomplexes containing varying amounts of cytoskeletal proteins, perhaps indicating that different subcomplexes may coexist on membranes, according to the needs, at different sites within the cell. Our observation that cholesterol aggregation releases annexin II and cortical cytoskeletal elements raises the interesting possibility that annexin II anchors actin elements of the cytoskeleton to the recently described cholesterol- and glycosphingolipid-rich membrane subdomains [termed detergent-insoluble glycosphingolipid-enriched complexes or DIGs by Parton and Simons (1995)]. Indeed, annexin II has been described to be a component of such a detergent-insoluble membrane fraction (Sargiacomo *et al.*, 1993; Harder and Gerke, 1994). This has been interpreted as indicative of a caveolar localization (Sargiacomo *et al.*, 1993, but see also Kurzchalia *et al.*, 1995), but it is is tempting to speculate that membrane ruffles and microvilli containing ezrin and/or moesin (Berryman *et al.*, 1993) as well as subdomains of the early endosome also represent such cholesterol- and glycosphingolipid-rich microdomains on the plasma membranes.

A similar actin-related mechanism, perhaps containing some of the same components or functional homologues of the same components, is likely to operate on the early endosomal membrane. This link among annexin II, actin cytoskeleton, and early endosomes also contributes to explain our previous observations that annexin II itself is involved in early endosome structure and dynamics (Emans *et al.*, 1993; Harder and Gerke, 1993). That the actin cytoskeleton itself plays a role in endocytic membrane traffic was observed in recent in vivo (Durrbach *et al.*, 1996) and in vitro studies (Huber, Gagescu, Hunziker, Boeck, Sachs, Way, Bähler, and Gruenberg, unpublished data) showing that the transferrin cycle is facilitated by the presence of polymerized actin filaments. Finally, our electron microscopic studies show that filamentous structures appear to be associated with lattice-like annexin II clusters on early endosomes. It is, therefore, tempting to speculate that cholesterol-rich rafts or subdomains are present on early endosomal membranes and correspond to the regions intensely labeled with the annexin II antibodies. Involvement of membrane rafts in endocytic events has recently been suggested, particularly in nonclathrin-mediated uptake and caveolae internalization. Interestingly, endocytosis utilizing these pathways is inhibited by perturbation of actin cytoskeleton as well as by clustering of membrane cholesterol (Parton *et al.*, 1994; Schnitzer *et al.*, 1994; Deckert *et al.*, 1996). One may also speculate that a similar mechanism accounts for the reported role of annexin II in the regulated release of catecholamine by chromaffin cells, since regulated exocytosis has been shown to critically depend on the cortical actin underlying the plasma membrane of cells undergoing secretion (Muallem *et al.*, 1995).

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