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In adrenocortical tissue, annexins II and VI are attached to clathrin coated vesicles in a calcium-independent manner [1]

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Abstract

We have previously characterized three populations of clathrin coated vesicles (CCVs) isolated from bovine adrenocortical tissue and designated them as large, medium and small coated vesicles, i.e., LCV, MCV and SCV, respectively. Here, we show that annexins II and VI, two of the annexins involved in membrane traffic, are present in the three populations of CCVs but with different distributions between coat proteins (CP) and lipidic vesicle membrane. Annexin VI is only associated with the membrane, whatever the CCV population. In contrast, annexin II is differently distributed between coat and membrane, depending on the CCV population. Both annexins are bound to membranes in a calcium-independent manner and solubilization studies in Triton X114 (TX114) suggest that they interact poorly with lipids by hydrophobic interactions. Ligand blotting experiments show that both annexins bind to CCV proteins: annexin II to a 200-kDa component in all CCVs and annexin VI to a 100-kDa component in LCV and SCV identified as dynamin, a GTPase essential for endocytic CCV pinching off. Dynamin is tightly associated to annexin VI only in LCVs, the endocytic [transferrin (Tf) positive] vesicles. Our data suggest that annexins II and VI could define specific protein–lipid interaction microdomains that could play a role in the different functions of the CCVs. © 1998 Elsevier Science B.V.

Keywords: Adrenal cortex; Endocytosis; Membrane traffic; Annexin II; Annexin VI

Abbreviations: CCV, clathrin coated vesicles; LCV, large coated vesicles; MCV, medium coated vesicles; SCV, small coated vesicles; LDL-R, low-density lipoprotein receptor; Tf, transferrin; Tf-R, transferrin receptor; CI-MP/IGF-II R, cation independent-mannose-6-phosphate/insulin-like growth factor II receptor; TGN, trans Golgi network; PM, plasma membrane; TX100, triton X100; TX114, triton X114; OG, octyl-β-D-glucopyranoside; PMSF, phenyl–methyl-sulfonylfluoride

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[1] **In memoriam**: This last work as well as my scientific accomplishment are dedicated to the memory of Jeffries Wyman who was my best teacher and the most humane of all scientists I have ever met. Annette Alfsen.

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1. Introduction

Clathrin coated vesicles (CCVs) are the very first organelles initiating the intracellular traffic from the compartment membranes, i.e., plasma membrane (PM), trans Golgi network (TGN) and early endosomes [1–3]. Recently, CCVs have also been proposed to mediate transport from lysosome to endosomes [4]. CCVs are well characterized by their clathrin coat proteins (CP) surrounding the vesicle membrane. They can be easily isolated as intact organelles carrying trafficking proteins as well as proteins involved in the trafficking machinery. They are thus a good model for studying molecules involved in pinching off, routing and docking, signalling and fusion.

We have previously characterized three populations of CCVs isolated from bovine adrenocortical tissue [5]. They all carry the low-density lipoprotein receptor (LDL-R) but differ in their density and their size; we designated them as large, medium and small coated vesicles, LCV, MCV and SCV, respectively. The three populations differ qualitatively and quantitatively in their lipid and protein components and in their organization. Furthermore, LCV and SCV contain transferrin (Tf), α adaptin, the 96-kDa dynamin isoform and the 110-kDa PI3 kinase subunit, and MCV do not carry Tf, the 96-kDa dynamin isoform and the 110-kDa PI3 kinase subunit, and contain larger amounts of γ adaptin [6]. We, therefore, proposed the involvement of LCV and SCV in the endocytic/recycling pathway and of MCV in the biosynthetic pathway.

Annexins belong to a family of calcium and phospholipid binding proteins whose functions are not yet clearly established. At the present day, more than 10 mammalian annexins have been described [7–10]. They are formed of four or eight (in the case of annexin VI) highly conserved sequence repeats and a unique N terminal domain which carries the phosphorylation and proteolytic sites, as well as the site of interaction with other proteins [11,12]. They share common properties carried by the core of repeats, i.e., they bind to negatively charged phospholipids in the presence of micromolar amounts of calcium [13,14]. They can be found either as cytosolic or membrane-associated proteins. The biochemistry of these proteins has been studied extensively, yet despite numerous suggested functions, the exact cellular role of annexins is still a matter of debate. Recent works showed that some annexins namely annexins II, VI and XIIIb, are involved in vesicular traffic [11,15–24]. Their role is, however, far from being well understood.

In order to better characterize the function(s) of these annexins, we investigated whether some annexins were associated differentially to the three populations of CCVs that we have described [5,6]. Here, we show that annexins II and VI are present in the three populations of CCVs but with different distributions between CP and vesicle membrane. Annexin VI is only associated with the vesicle membrane, whatever the CCV population. In contrast, annexin II is differently distributed between coat and membrane, depending on the CCV population. Both annexins are bound to CCV membranes in a calcium-independent manner. Annexins II and VI partitioned mostly in the aqueous phase following Triton X114 (TX114) solubilization suggesting that these annexins do not interact with the lipid bilayer via hydrophobic interactions. Ligand blotting experiments show that both annexins can bind to CCV proteins: annexin II to a 200-kDa component in all CCVs and annexin VI to a 100-kDa component in LCV and SCV. After immunoprecipitation with an anti-annexin VI antibody, we identified the 100-kDa component as dynamin, a GTPase essential for endocytic CCV pinching off [25]. Dynamin is tightly associated to annexin VI only in LCVs, the endocytic (Tf positive) vesicles. On cell lines, we found a partial colocalization of dynamin and annexin VI. Our data suggest that annexins II and VI could define specific protein–lipid interaction microdomains that could play a role in the different functions of the CCVs.

2. Materials and methods

2.1. Materials, cells, proteins, antibodies and buffers

All reagents were of analytical grade.

HeLa cells (human cervix derived cells) or COS cells (kidney fibroblast-like cells transformed with SV40) were used for immunofluorescence experiments. Annexins were isolated from human placenta and purified as already described by us [26,27]. Final

separation of annexins was performed on either mono-Q (annexin VI) or mono-S (annexin II) columns (Pharmacia, France). The purity of proteins was above 98%.

Polyclonal antibodies against annexins were raised in rabbits using the lymph node injection route [28]. The anti-annexin VI antibody (1/500 for WB) was immunoaffinity purified before use as we described in Ref. [29]. The anti-annexin II antibody (1/250 for WB) was highly specific and did not cross react with other annexins (F. Russo-Marie, unpublished results). Anti-annexin II polyclonal antibody LC2 [30], and anti-annexins II and VI monoclonal antibodies (Zymed, South San Francisco, CA) were used for immunofluorescence experiments. The anti-p11 monoclonal antibody was obtained from Dr. V. Gerke (Max Plank Institute, Goettingen, and University of Münster, Germany). The rabbit anti-clathrin serum was obtained from Dr. P. Mangeat et al. [31]. The anti-clathrin monoclonal antibody X22 was from Dr. F. Brodsky [32]. The monoclonal antibody 'Hudy 2' (1/10 000 for WB) recognizes the neuronal dynamin 1 and the non-neuronal dynamin 2, and was obtained from Dr. S. Schmid. Hudy 2 is not efficient for immunoprecipitating the non-neuronal dynamin 2 (Dr. S. Schmid, personal communication).

Buffers used for vesicle preparation were prepared as follows:

Buffer M: 100 mM MES, pH 6.5, 1 mM EGTA, 0.5 mM $MgCl_2$, 200 mM NaCl, 3 mM NaN_3 and 0.1 mM phenyl–methyl-sulfonylfluoride (PMSF);

Buffer T: 100 mM K_2 tartrate, 10 mM HEPES, pH 7.0, 3 mM NaN_3 and 0.5 mM $MgCl_2$.

2.2. Clathrin coated vesicle preparation

The three populations of CCVs were prepared from bovine adrenal cortex as previously described [5]. The final purification was achieved by two rounds of sucrose/D_20 step gradient. The three CCV populations collected at the 1.175/1.210, 1.125/1.155 and 1.155/1.175 density interfaces, respectively, were designated as large (LCV), medium (MCV) and small (SCV) according to their size. Their purity and homogeneity were routinely controlled by electron microscopy after negative staining. In the preparations used, empty clathrin coat or uncoated membranes were less than 1% and CCV diameter distribution was homogeneous as previously described [5]. It should be noted that CCVs are 0.1% of all membrane proteins in a post-nuclear supernatant.

2.3. Dissociation of the CCV CP and membrane

Uncoated vesicles (UV) and CP were obtained as follows: CCVs were suspended in buffer M, dialyzed overnight against 10 mM Tris–HCl, pH 8.5 supplemented with 2 M urea and centrifuged at 105 000 $\times g$ for 1 h. Proteins in the supernatant were termed CP. The pellet termed UV, was resuspended in buffer M and both fractions were dialyzed overnight against buffer M to remove urea. Proteins were quantified according to Peterson [33]. Before treatment with EGTA and ATP, UVs were dialyzed extensively against 10 mM Tris–HCl, pH 8.0. UV suspension was then separated into four fractions which were further dialyzed against the same buffer supplemented with: (i) nothing, (ii) 5 mM EGTA, (iii) 5 mM ATP, 2 mM $MgCl_2$, (iv) 5 mM EGTA, 5 mM ATP, 2 mM $MgCl_2$. After centrifugation at 105 000 $\times g$ for 45 min, pellets were solubilized in 10 mM Tris–HCl, pH 7.4, 2 mM EGTA, 1% Triton X100 (TX100). Sample buffer was added for SDS-PAGE and Western blotting decorated with anti-annexin II and VI antibodies.

2.4. SDS-PAGE and Western blotting

SDS-PAGE was performed according to Laemmli [34] with β-mercaptoethanol as a reducing agent and Western blotting was performed according to Burnette [35]. Goat anti-rabbit IgG antibodies (Amersham, France, 1:5000) and rabbit anti-mouse IgG antibodies (Amersham, 1:10 000), both conjugated with horseradish peroxidase, were used as secondary antibodies; detection was performed using either chemiluminescence (ECL, Amersham) or ^{125}I-protein A binding. Quantification of the immunoblots was performed by densitometric scanning using a personal densitometer or by phosphorimager analysis (Molecular Dynamics, France). Similar ratio of annexins between the different CCV populations were obtained with both quantifications.

2.5. Solubilization of CCVs with non-ionic detergents

2.5.1. Solubilization with TX100 and octyl-β-D-glucopyranoside (OG)

CCVs were resuspended in buffer T with a protease inhibitor cocktail (0.1 mM PMSF, 10 μg/ml E64, 10 μg/ml leupeptin and 1 μg/ml pepstatin A) and solubilized with 2% TX100, 25 mM OG. After magnetic stirring at 4°C for 20 min, insoluble and soluble fractions were separated by centrifugation at 105 000 × *g* for 45 min at 4°C. Both fractions were solubilized in sample buffer, resolved by SDS-PAGE and analyzed by Western blotting.

2.5.2. Temperature-induced phase separation of TX114

Hydrophobic proteins were separated from hydrophilic proteins by the method of Bordier [36]. Briefly, UV and CP fractions were solubilized with 1% TX114 and incubated on ice for 15 min. Insoluble material was removed by centrifugation at 15 000 × *g* for 15 min at 4°C. The solubilized fractions were then incubated at 31°C for 10 min and phase separation was achieved by centrifugation at 3000 × *g* for 3 min. Proteins from both aqueous and detergent rich phases were precipitated with 10 vol. acetone at −20°C, and the resulting pellet solubilized in sample buffer and analyzed by Western blotting.

2.6. Ligand blotting

CCVs solubilized in sample buffer with β-mercaptoethanol were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane. Further experiments were performed in 10 mM Tris–HCl pH 7.4, 150 mM NaCl with 2 mM $CaCl_2$, supplemented with either 5% wt./vol. non-fat dry milk for blocking non-specific binding sites or 1% wt./vol. BSA for ligand binding and immunoblotting; 0.05% Tween 20 was added while washing the blots. For annexin binding, blots were incubated with annexin II (20 μg/ml) or annexin VI (10 μg/ml) overnight at 4°C. Immunoblotting with anti-annexins II and VI antibodies was performed as described above.

2.7. Immunoprecipitation

CCVs were solubilized in buffer T, TX100 + OG as described above. Immunoprecipitation steps were performed in buffer T, 1 mM $CaCl_2$, 0.5% TX100, 25 mM OG, 0.5 mM $MgCl_2$, 0.5% BSA in the absence or the presence of purified Annexin VI (20 μg/ml). To avoid non-specific binding, the protein solution was precleared twice with Sepharose CL4B (Pharmacia). The supernatant was incubated with anti-annexin VI antibody (1/100) overnight at 4°C, protein A Sepharose suspension was added and the mixture was further incubated for 2 h at room temperature. Beads were washed three times in buffer T with 1 mM $CaCl_2$, 0.5% TX100 and once in buffer T alone. The immunoprecipitate was first eluted in buffer T with 5 mM EGTA at 4°C for 10 min (fractions e). In a second step, to release annexins precipitated by a divalent-cation independent mechanism, beads were boiled in sample buffer containing β-mercaptoethanol (fractions sb). Fractions were analyzed by Western blotting probed with anti-dynamin antibody.

2.8. Fluorescence confocal microscopy analysis

HeLa and COS cells grown on coverslips were processed for indirect immunofluorescence. For annexins and clathrin dual detection, rabbit anti-clathrin (1:200) and mAb anti-annexin II or VI (8 μg/ml) were used as primary antibodies. Alternatively, when HeLa and COS cells were stained with mAB anti-clathrin (X22, 5 μg/ml) and rabbit anti-AII and AVI as primary antibodies, similar stainings were obtained (not shown). Annexin antibodies were revealed using FITC labeled goat anti mouse (Jackson, CA, USA; 8 μg/ml), and clathrin staining was revealed using goat anti-rabbit (Biosys, CA, USA; 1:150) followed by Texas Red labeled Streptavidin (Pierce, Rockford, IL, USA; 1:300). For annexins and dynamin dual detection, rabbit anti-annexins (AII: LC2, 1:100 and AVI: affinity purified, 10 μg/ml) and mAb anti-dynamin (Hudy 2, 10 μg/ml) were used. Annexin antibodies were revealed using FITC labeled goat anti-rabbit (Jackson, CA, USA; 1:100), and dynamin staining was revealed using biotinylated goat anti-mouse (Biosys, CA, USA; 1:150) followed by Texas Red labeled Streptavidin (Pierce, 1:300). Coverslips were mounted in Mowiol and observed by confocal microscopy (Bio-Rad 1024 confocal microscope equipped with an Argon/Krypton laser and the Laser Sharp software). Scanning was performed with a

×60 optique (Nikon). Consecutive sections were 0.2 μm apart. Pinhole aperture was devised so that the thickness of the optical section was of 0.6 μm. Fluorescence bleed-through from FITC to Texas Red channel was negligible. Images were processed using the double labeling Laser Sharp software. Using this programme, colocalizations appeared as yellow pixels after merging two sections recorded at the same *xz* level in each channel. Further analyses of the colocalization were performed by comparing red to green pixel distribution on a line taken randomly across a section; the intensity histograms obtained for each channel along the chosen line were then compared point to point and presented as a double entry fluorogram (or histogram) using the Elite software (Coulter France). Using this method of calculation, overlapping pixels are found in the upper right quadrat of the fluorogram.

3. Results

3.1. Annexins II and VI are present in the three populations of CCVs

We have previously shown [37] that annexins I to VI are present in adrenocortical tissue at a concentration of approximately 1 μg/mg of cortex tissue. All annexins contributed to 0.2% post-nuclear supernatant proteins and were distributed between cytosolic and membrane associated forms. The latter could be separated in two pools, membrane bound (70% recovered with EGTA and 9% further recovered by increasing ionic strength up to 500 mM NaCl) or membrane inserted (21% extractable with detergents).

In the present work, the three types of CCVs were probed for annexins I to VI. Only annexins II and VI were detected, the largest amount being in MCV and the smallest one in LCV (Fig. 1A). CCVs represented 0.01% of membrane proteins and enrichment of annexins II and VI as compared to total cellular membranes were 2.5 and 1, respectively, as determined from blot quantification. As shown in Fig. 1A, annexin II is present as a monomer (p36) in all CCVs but interestingly, no p11 could be detected. The absence of p11 from the CCVs but its presence in the whole tissue may suggest that in CCVs annexin II is not associated with p11. Along this line, Jost et al. [19] report that, in early endosomes, the calcium-independent membrane bound form of annexin II is independent of p11 binding. Finally and as expected, clathrin that characterizes CCVs was detected in all types of CCVs (Fig. 1A).

To further confirm our data observed on whole tissue on which it is difficult to perform immunocytochemical analysis, colocalization studies of annexins II and VI with clathrin were performed in two different cell lines (HeLa and Cos cells) by indirect immunofluorescence and confocal microscopy analysis. These morphological studies showed that both annexins colocalized to a large extent with clathrin (Fig. 1B).

3.2. The distribution of annexins II and VI between CP and lipid bilayer is different for the three CCVs

CCVs are formed of a protein coat composed of clathrin and adaptors interacting with a lipid bilayer embedding proteins. We, therefore, addressed the localization of annexins II and VI in these two fractions.

CCV coat can be dissociated from the vesicle membrane upon 2-M urea treatment in a pH sensitive manner [5]. After uncoating of each CCV population as described in Section 2, proteins in coat fractions (CP) and in UV were resolved by SDS-PAGE and immunostained with anti-annexin II and anti-annexin VI antibodies (Fig. 2A). Annexins II and VI were distributed differently: annexin VI was only found associated with the lipid bilayer whatever the CCV population while annexin II was found associated to both UV and CP. Annexin II distribution between UV and CP was dependent on the CCV population. In SCV, annexin II was only found in UV; in MCV, annexin II was equally distributed between UV and CP; in LCV, annexin II was mostly found in CP. Experiments with surface biotinylated CCVs have shown that both annexins were located at the cytoplasmic side of the coated vesicle and not in the lumen (not shown).

As shown above, most of the annexin molecules are associated to the membrane of CCVs. We, therefore, asked how annexins II and VI could interact with the lipid bilayer.

3.3. Association of annexins II and VI with UV cannot be disrupted by EGTA and ATP

Annexin binding to membranes was originally characterized as a Ca^{2+} and phospholipid-dependent process. However, recent studies reported that some annexins, and among them Annexin II, interacted with membranes in a calcium-independent manner [17–19]. In the present work, CCVs purification was performed in buffer M containing 1 mM EGTA. In these conditions, the annexins bound to membranes via calcium bridging should have been disrupted [38]. Consequently, the annexins II and VI associated to the CCVs (Fig. 1) are most likely attached via calcium-independent interactions. To formally address whether annexins II and VI binding to CCV lipid bilayer involved calcium, UV fractions were treated with EGTA. The experiment was performed on MCV, the population most enriched in acidic phospholipids, without and with ATP because Ca^{2+} requirement could be significantly lower in the presence of the nucleotide [39]. Treatment with 5 mM EGTA or 5 mM ATP or with both agents did not change the amount of annexins II and VI associated with the membrane as compared to untreated UV (Fig. 2B).

Since annexin association to CCV lipid bilayer is not dependent on calcium or ATP, it could depend on hydrophobic interactions. Therefore, our next question was whether annexin binding to CCV membrane involved hydrophobic interactions.

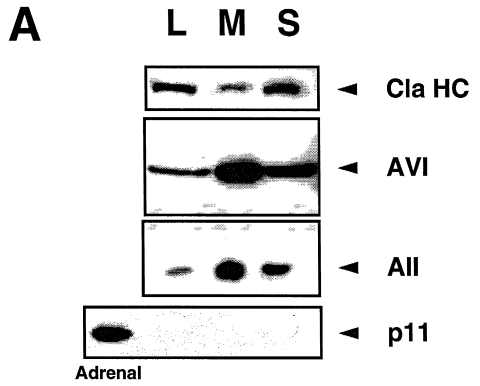
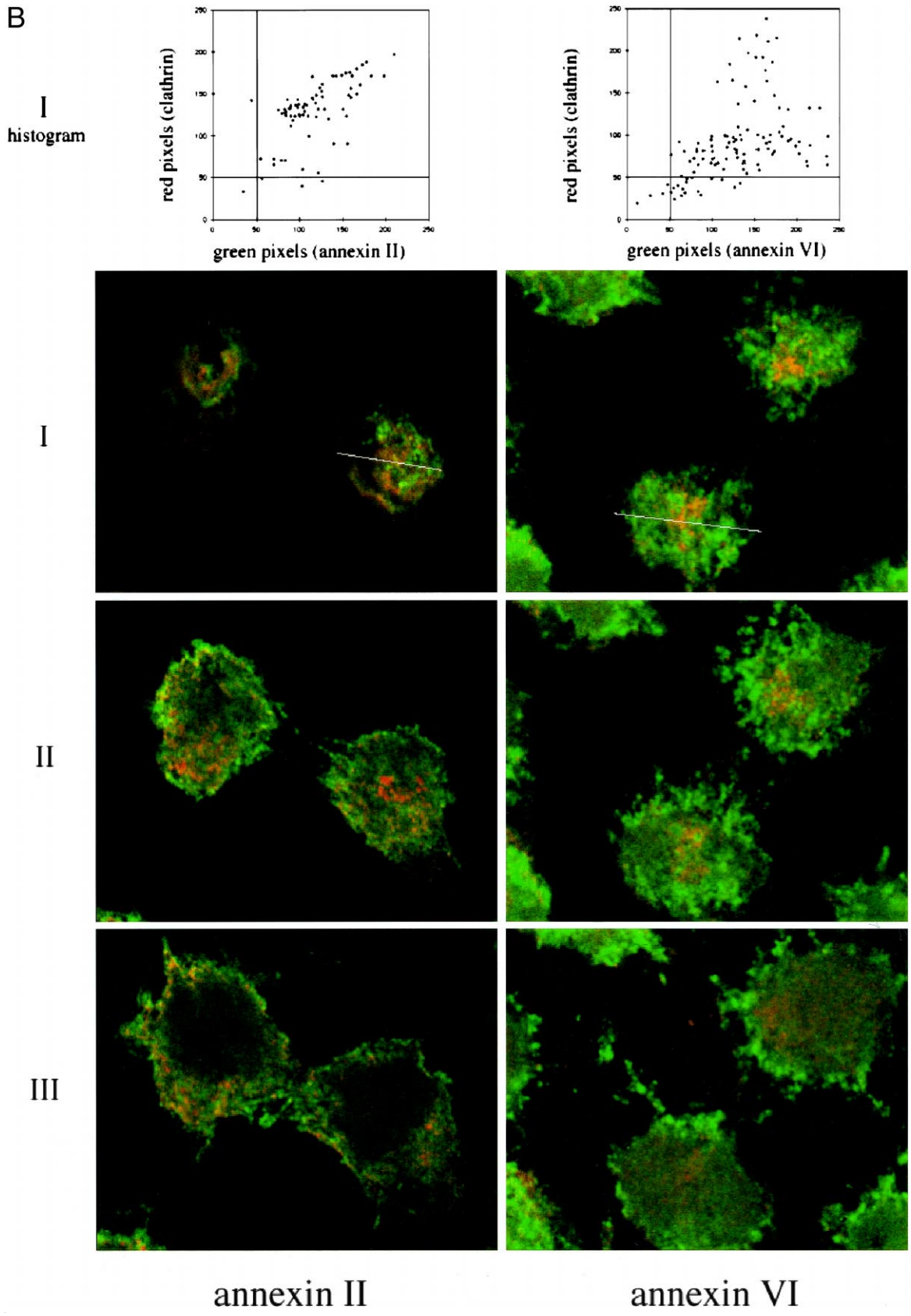


Fig. 1. (A) Annexins II and VI are in the three CCV populations. The three populations of CCVs were prepared from bovine adrenal cortex. The final purification was achieved by two rounds of sucrose/D_2O step gradient. The three CCV populations collected at different density interfaces were respectively designated as large (LCV), medium (MCV) and small (SCV) according to their size. Purification and homogeneity of the vesicles were routinely controlled after negative staining using electron microscopy. Equal amounts of proteins (25 μg) from each population of CCV were then solubilized in sample buffer and their components resolved by SDS-PAGE and immunostained with anti-annexin II, anti-annexin VI polyclonal antibodies, anti-clathrin polyclonal antibody and a monoclonal antibody against p11 (in the latter case, a positive control was given by the use of adrenal tissue). (B) Partial colocalization of annexins II and VI with clathrin. COS cells were doubly stained for clathrin (red) and annexins (green) II (left panels) or VI (right panels) and analyzed in a *z*-series by confocal microscopy. Three merged focal planes are shown that are representative of three cell levels; i.e., at the PM above the nucleus (I), at the nucleus level (II), at the cell coverslip contact zone (III). Colocalizations appeared as yellow pixels. To emphasize the extent or overlapping, pixel intensity histograms (fluorograms) taken for each chromophore along the white line drawn on focal plane I were generated. Cytofluorograms are shown on their respective top panel. Pixels located on the upper right quadrant colocalized.

3.4. CCV membrane-associated annexins II and VI partition predominantly into aqueous TX114 phase

Separation of hydrophobic and hydrophilic forms of membrane-associated proteins can be performed by phase partitioning with TX114 as described by Bordier [36]. Partitioning of annexins II and VI was carried out on UV from the three CCV populations. After the temperature induced phase separation, annexin II partitioned into aqueous (A) and detergent (D) rich phases in the three populations but with different ratio (Table 1). In LUV, annexin II was mostly found in the D phase while in MUV and SUV it was mostly in the A phase. On the contrary, annexin VI was found only in the A phase of LUV and SUV while in MUV it was found mostly (85%) in the A phase but also slightly in the D phase (15%). It should be noted that in LCV and MCV, annexin II was found also in CP (Fig. 2A), indicating a predominance of hydrophilic exposed amino-acids. Taken together, these data indicated that in CCVs few annexin molecules exhibit a configuration with hydrophobic characteristics that may contribute to their anchoring in the lipid bilayer.

These experiments were performed on the protein fractions soluble in non-ionic detergent after having discarded insoluble material before heating for parti-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red pixels (clathrin)
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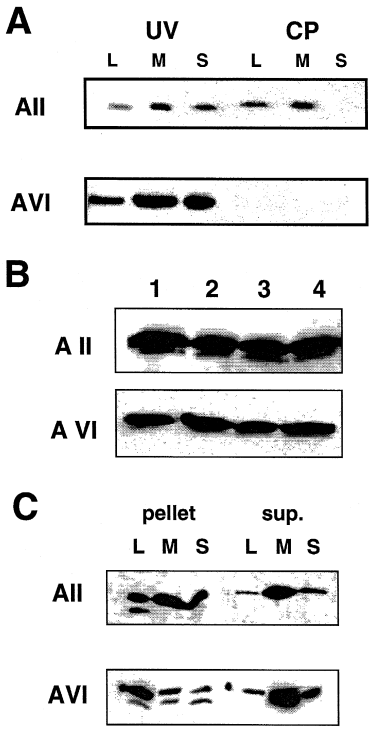


Fig. 2. (A) Annexins II and VI are differently distributed in CP and UV. CP and UV were prepared from the three CCV populations as described in Section 2. The same amount of proteins were solubilized in sample buffer, separated on SDS-PAGE and analyzed by Western blotting immunostained with anti-annexin II and anti-annexin VI antibodies. CCV populations: L: LCV; M: MCV and S: SCV. (B) Association of annexins II and VI with CCV membrane can not be disrupted by EGTA and ATP. UV were prepared in 10 mM Tris–HCl, pH 8.0 from MCV and dialyzed against the same buffer supplemented with nothing (lanes 1), 5 mM EGTA (lanes 2), 5 mM ATP, 2 mM $MgCl_2$ (lanes 3) or 5 mM EGTA, 5 mM ATP, 2 mM $MgCl_2$ (lanes 4). UV were solubilized in sample buffer and analyzed by Western blotting probed with anti-annexin II and anti-annexin VI antibodies. (C) Annexins II and VI exist under two forms in CCVs: non-ionic detergent-soluble and -insoluble. An equal amount of proteins from the three populations of CCVs were solubilized in buffer T containing 2% TX100, 25 mM OG. After magnetic stirring at 4°C, soluble (Sup) and insoluble (pellet) material was separated by centrifugation at $105\,000 \times g$ for 45 min. Each fraction was solubilized in sample buffer and analyzed by Western blotting probed with anti-annexin II and anti-annexin VI antibodies. L, M, S as referred to in Fig. 1.

tioning. We therefore investigated whether annexins II and VI molecules could also be found in the material insoluble in non-ionic detergent.

3.5. Two forms of annexins II and VI, soluble and insoluble, are extracted from CCVs by non-ionic detergents

When solubilizing CCVs with 2% TX100 + 25 mM OG, heterocomplexes containing LDL-R associated with the α or γ adaptor subunit were found in the soluble fraction, while the β and β' adaptor subunits and clathrin that form the periphery of the coat were found in the pellet [6]. Under the same solubilization conditions, annexins II and VI distributed into soluble and insoluble fractions (Fig. 2C). Annexins II and VI were present as a doublet in the pellet or insoluble fraction. Only the higher molecular weight isoform of both annexins was found in the detergent-soluble fractions. In LCV, both annexins II and VI were mostly present in the pellet (detergent-insoluble forms). In MCV and SCV, annexin VI was mostly found in the soluble fraction while annexin II was equally distributed between the detergent-soluble and -insoluble forms. Therefore, in MCV and SCV, annexin VI is in a different configuration than in LCV. It should be mentioned that the soluble forms of both annexins were not found associated with the heterocomplexes containing the LDL-R/adaptins (not shown). Annexins found in the insoluble fraction are likely associated with the cytoskeleton [7]. Annexin VI is known to exist under two isoforms, due to an

Table 1
Annexins II and VI associated with UV partition into TX114 detergent and aqueous phases

		UV	
		TX114 D (%)	Partition A (%)
LCV	AII	85	15
	AVI	0	100
MCV	AII	30	70
	AVI	15	85
SCV	AII	30	70
	AVI	0	100

UV fractions prepared from the three CCV populations were solubilized in 1% TX114 and partitioning was performed as described in Section 2. Proteins from aqueous (A) and detergent (D) rich phases were solubilized in sample buffer and analyzed by Western blotting decorated with anti-annexin II and anti-annexin VI antibodies, (AII and AVI, respectively). Quantification of the immunoblots were performed by densitometric scanning (Molecular Dynamics). For each fraction, results are presented as percent of the total A + D phases. Standard deviations were less than 5% with $n = 3$.

alternative splicing adding six amino-acids, namely VAAEIL, in the seventh domain in one isoform, as reported by Moss and Crumpton [40]. Our results seem to indicate that both isoforms are associated to CCVs, but only the spliced higher isoform of annexin VI would be associated with the detergent soluble fraction. This detergent insolubility may suggest that annexin VI is associated to cytoskeleton as it has already been shown for annexin II [7]. The presence of a lower form of annexin II only in the pellet of LCVs may be related to the enrichment in cholesterol of this fraction [17]. The nature of the two isoforms of annexin II is not elucidated, the upper form could be a phosphorylated form that would prevent p11 binding [7,20,41].

Taken together, these data suggest that different types of interactions contribute to the annexin binding to the CCV membranes.

Since there was no contribution of calcium and few of hydrophobic interactions in the association of annexins II and VI with the vesicle bilayer, we investigated whether annexins could bind to CCV protein component(s).

3.6. Annexins II and VI can bind to CCV proteins

To identify potential ligands in CCVs, ligand blotting experiments were performed with purified annexins II and VI as described in Section 2. Annexin II binds to a 200-kDa polypeptide in the three types of CCVs (Fig. 3A, left panel). The important and almost equal intensity of the bands of annexin II in the ligand blotting experiments (when compared to the pattern presented in Fig. 1A) suggests that, in our experimental conditions (i.e., 2 mM $CaCl_2$), annexin II itself binds even better to annexin II than to its 200-kDa ligand and may suggest that annexin II could form oligomers even in the absence of p11. In an attempt to identify this annexin II binding protein, co-immunoprecipitation of annexin II was performed from each CCVs with or without addition of exogenous annexin II. The precipitate was resolved by SDS-PAGE, electrotransferred and blotted for clathrin. No clathrin could be detected whereas a clear band could be revealed when a total CCV extract was loaded on the same SDS-PAGE (not shown). These experiments indicate that the 200-kDa polypeptide to which annexin II binds is not the clathrin heavy chain; this band remains to be identified.

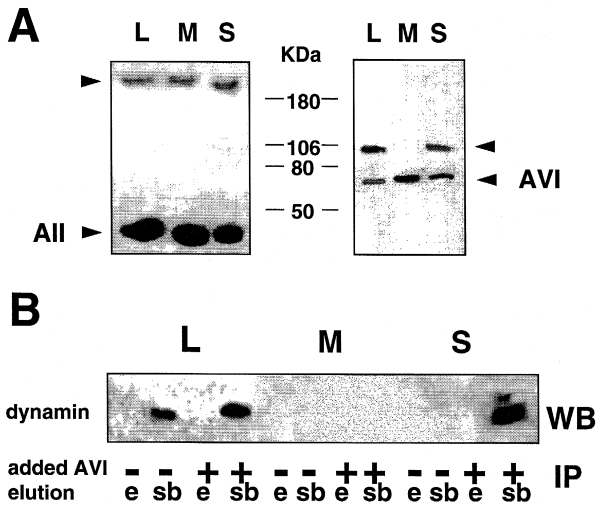
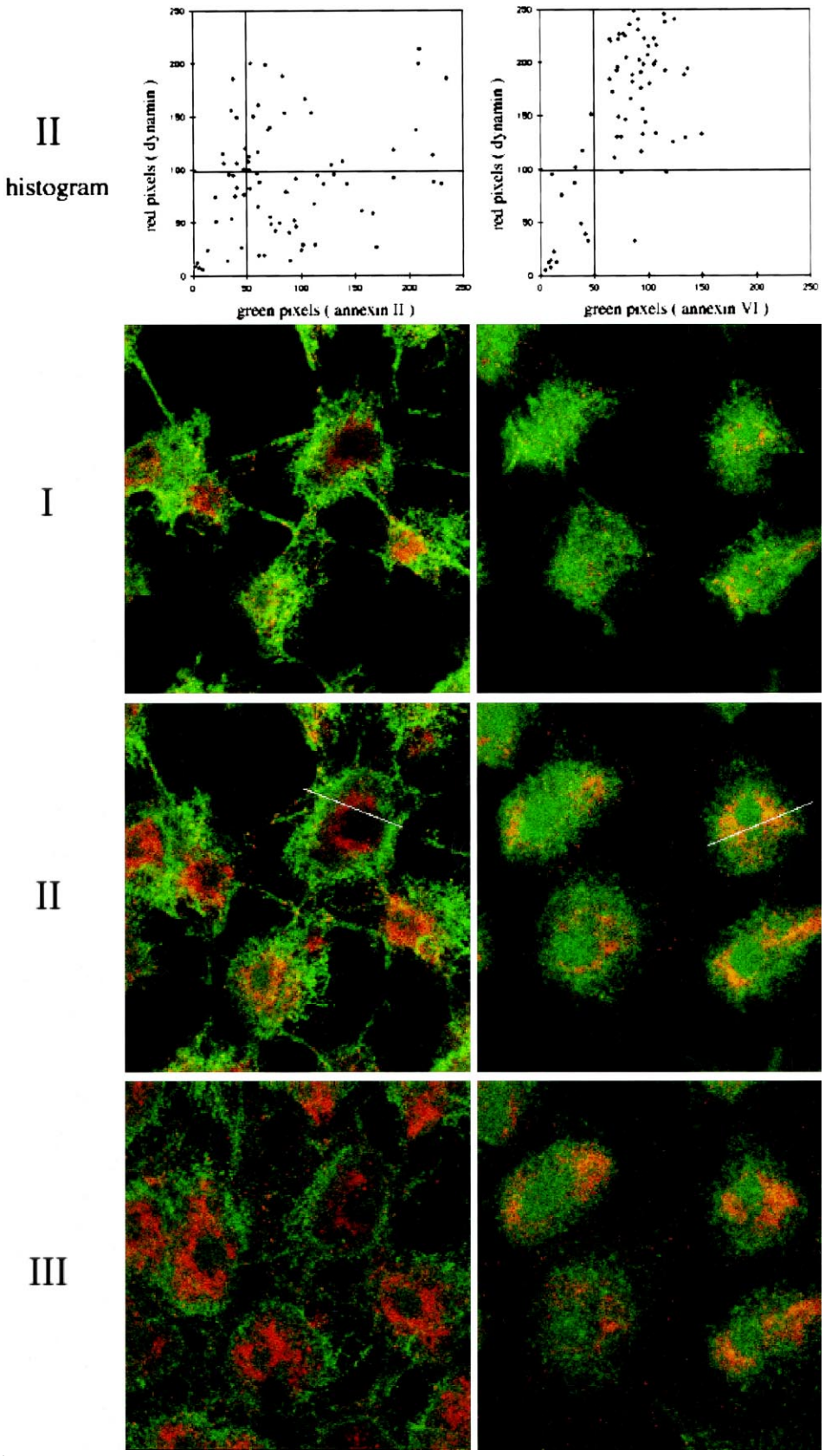


Fig. 3. (A) Annexins II and VI ligand blotting on CCV protein components. An equal amount of proteins from the three populations of CCVs were solubilized in sample buffer with β-mercaptoethanol, resolved by SDS-PAGE and electrotransferred onto nitrocellulose membranes. Blots were incubated with annexin II (20 μg/ml) or annexin VI (10 μg/ml) and immunostained with anti-annexin II and anti-annexin VI antibodies, respectively. L, M, S as referred to in Fig. 1. (B) The 100-kDa ligand polypeptide is dynamin; in LCV annexin VI is tightly associated to dynamin. The three populations of CCVs were solubilized in buffer T containing TX100 + OG. Immunoprecipitation steps were performed on the soluble fraction in the absence or presence of purified Annexin VI (20 μg/ml). The supernatant was then incubated with anti-annexin VI antibody. The immunoprecipitate was first eluted with 5 mM EGTA to release annexins attached by a calcium-dependent mechanism (fractions e). In a second step, to release annexins precipitated by a calcium-independent mechanism, beads were boiled in sample buffer containing β-mercaptoethanol (fractions sb). Fractions were analyzed by Western blotting probed with anti-dynamin antibody.

Annexin VI binds to a 100-kDa polypeptide only on LCV and SCV (Fig. 3A). We have previously shown that LCV and SCV but not MCV contain dynamin [6], a 100-kDa GTP binding protein that appears to be essential for CCV budding from PM [25] and endosome-like intermediate [42,43]. Our previously reported results imply that LCV and SCV participate in the endocytic/recycling pathway [6]. As reported by Lin et al. [22], annexin VI could be involved in coated vesicle budding from PM. We, therefore, investigated whether dynamin could be the 100-kDa annexin VI ligand.


II
histogram
red pixels (dynamin)
green pixels (annexin II)
red pixels (dynamin)
green pixels (annexin VI)
I
II
III

3.7. Annexin VI is tightly associated to dynamin in LCV

Anti-annexin VI co-immunoprecipitation experiments were performed with CCV components solubilized in TX100, OG in the absence or presence of exogenous added annexin VI. Results are presented in Fig. 3B. Neither dynamin nor annexin VI could be eluted with EGTA. When precipitating with anti-annexin VI antibody without adding exogenous annexin VI, dynamin was co-precipitated only in LCV. In these vesicles part of dynamin was not bound to annexin VI since a larger amount of dynamin was co-precipitated when adding annexin VI before immunoprecipitation. In SCV, dynamin which is present was not associated to endogenous annexin VI but could bind to exogenous added annexin VI. Co-immunoprecipitation of dynamin from LCV with anti-annexin VI antibody could not be due to a cross reactivity of the antibody to dynamin since the latter was not precipitated from SCV where it is present but not strongly bound to endogenous annexin VI. Furthermore, as shown in Fig. 1A dynamin was not probed with anti-annexin VI antibody and immunofluorescent labelling with this antibody was suppressed with purified annexin VI. We previously showed that MCV do not contain the ubiquitous dynamin isoform [6]; therefore, they were our inner negative control for co-precipitating with annexin VI. These results demonstrated that only in LCV, the endocytic Tf positive vesicles, dynamin appeared tightly associated with annexin VI. Interestingly, as reported for dynamin [44], annexin VI was not enriched in CCVs (see above) pointing to a possible interference in the function of the two molecules. Colocalization of annexin VI and dynamin was then analyzed by double immunofluorescence in cell lines.

3.8. Partial colocalization of annexin VI and dynamin in cells

In addition to the biochemical analyses performed on whole adrenocortical tissue extract, morphological analyses were conducted on cell lines. HeLa cells grown on coverslips were doubly stained for dynamin (red pattern) and annexin (green pattern) II (Fig. 4, left panels) or VI (Fig. 4, right panels). Double fluorescence stainings were analyzed by confocal microscopy. The colocalization pattern of dynamin with annexin II and VI were very different as confirmed in the fluorogram (histogram) generated from a line drawn on focal plane II. The annexin VI (right panels) overlapped the dynamin pattern in all sections whereas annexin II (left panels) did not colocalize with dynamin. Similar staining patterns were observed with COS cells (not shown). These morphological studies are in good agreement with the tight association of dynamin with annexin VI as previously detected biochemically.

In entire cells, a very small pool of annexin VI binds to dynamin as compared to the total cellular amount of annexin VI that is largely cytosolic. Furthermore, this interaction occurred in the absence of calcium. In order to detect specifically this small membrane-bound annexin VI pool that is somewhat obscured by the larger cytosolic and calcium-dependent other annexin VI pool, we permeabilized the cells before fixation. An EGTA-containing buffer was used to allow cytosol and calcium-dependent membrane bound proteins wash out. Similarly a calcium-containing buffer was used to deplete cytosolic proteins. Unfortunately in both cases, the resulting images were not interpretable, either due to a low level of detection of annexins in EGTA conditions or to an overall disorganization of the cell in calcium

Fig. 4. Partial colocalization of annexin VI with dynamin in cells. HeLa cells were doubly immunostained for dynamin (red) and annexins (green) II (left panels) or VI (right panels) and analyzed in a *z*-series by confocal microscopy. Three merged focal planes are shown that are representative of three cell levels, i.e., at the PM level above the nucleus (I), at the nucleus level (II) at the cell–coverslip contact zone (III). Colocalizations appeared as yellow pixels. To emphasize the extent of overlapping, pixel intensity histograms taken for each chromophore along the white line drawn on focal plane II were generated. Resulting cytofluorograms are shown on their respective top panel. Pixels located on the upper right quadrant are colocalized.

conditions. These data indicate that during the 1 min permeabilization period, the membrane intracellular traffic proceeds modifying the vesicle membrane composition.

4. Discussion

The present paper investigating the presence of annexins in the three types of CCVs has demonstrated that annexins II and VI were the only annexins present whereas the bovine adrenocortical tissue contains all annexins I to VI [37].

The presence of only annexins II and VI in CCVs is in agreement with the postulated involvement of these annexins in vesicular traffic [15]. They are located at the cytoplasmic side of CCVs in interaction with the lipid bilayer. Their amount is strikingly correlated with the enrichment in negatively charged phospholipids, namely PS + PI and PE [6]. It should be noted that both the lipid/protein ratio and the negatively charged phospholipid/total lipid ratio increase from LCV to SCV to MCV [5], but the organization of the lipid species in each leaflet of the membrane is not known precisely. If, however, enrichment in negatively charged phospholipids is occurring mainly at the cytoplasmic leaflet during vesicle formation, then the affinity of the membranes for annexins may increase dramatically at some time during vesicle formation in the presence of calcium.

The major part of both annexins remained associated with the vesicle membrane after the dissociation of CP. Annexin association to CCV membrane cannot be disrupted by EGTA and/or ATP, this nucleotide being described to lower the calcium concentration required for annexin VI binding to the rat liver membrane fraction [39]. Therefore, calcium is no longer involved in the binding of annexins II and VI to CCV membranes. CCVs were isolated in the presence of EGTA, conditions that have restricted the analysis to the calcium-independent interactions by dissociating from the CCVs all molecules bound through calcium ions. Annexins are usually described as bound to membrane in a calcium-dependent manner. Since only two of the six tested were found associated to CCVs, this rules out an artefactual co-purification of annexins with the vesicles.

How could annexins interact with CCVs? Several annexins have already been described to specifically bind to membranes in a calcium-independent manner [17,19,38,44]. This could be compatible with a first transient binding of annexins to phospholipids through calcium ions as suggested above, followed by a stabilisation through calcium-independent binding to other membrane components. Such a process has already been suggested by Tagoe et al. [39] in the binding of annexin VI to rat liver membranes and for annexin II in early endosomes [19]. Both annexins might have been present during the membrane reorganization involving lipids, cargo proteins [LDL-R, cation independent-mannose-6-phosphate/insulin-like growth factor II receptor (CI-MP/IGF-II R), Tf receptor (Tf-R)], adaptors (AP1, AP2), before clathrin coating occurred. The coat achievement would participate in the stabilization of annexin association with the vesicle membrane.

Interactions of α/γ adaptin with the cytoplasmic tail of cargo proteins are thought to account for the specific targeting of these cargo proteins to CCVs [45–47]. In the three populations of CCVs, annexins II and VI were never associated with the LDL-R/adaptin heterocomplexes solubilized with non-ionic detergents (not shown). Furthermore, both annexins II and VI were found associated with the three populations of CCVs involved in different intracellular pathways, i.e., endocytic/recycling (containing Tf) for LCV and SCV, and biosynthesis (devoid of Tf) for MCV. Taken together, these data suggest that both annexins could be part of a more general machinery necessary for budding and/or fusion and common to all vesicles, but not in the specific targeting machinery.

The descriptions outlined above suggest the existence of annexin-membrane interactions independent of calcium during at least part of the membrane reorganizations occurring in vesicular traffic. If annexins were bound to lipids (membrane-inserted), interactions may be largely hydrophobic. Detergent solubility and TX114 partitioning experiments indicated a low contribution of hydrophobic interactions mainly in LCV and SCV, thus annexin–protein interactions were most likely responsible for annexin association with membrane in CCVs. Indeed, ligand blotting experiments showed potential partner proteins for both annexins in relation to their possible role in CCVs.

In LCV and SCV, two CCV populations involved in the endocytic/recycling pathway, dynamin is a potential ligand for annexin VI, although it is only found actually associated in LCV. Dynamin that belongs to a novel GTPase superfamily, is also a calcium-sensitive, phospholipid-binding protein [48]. Recently DeCamilli et al. [49] suggested the existence of Ca^{2+} sensors in endocytosis that are activated with different kinetics and that produce opposite effects. These sensors might include annexin VI and dynamin. Their attachment to membranes through calcium bridges or pleckstrin hemology (PH) domains, might help them to come into close vicinity, then interact in a calcium-independent manner and cooperate in CCV budding from PM. Dynamin has been proposed to be responsible for the constriction of clathrin coated pits and the detachment of CCVs from PM [25]. Warnock and Schmid [50] proposed as ''a reasonable paradigm that dynamin function involves nucleotide-regulated interactions with upstream and downstream partners''. Since dynamin binds to the α-adaptin subunit of AP2 complexes in vitro [51], they suggested that ''while AP2 complexes may be involved in the initial targeting to coated pits, other factors could act to stabilize dynamin-membrane interactions''. Our data suggest that annexin VI, which has been proposed by Lin et al. [22] to be involved in CCV budding, could be one of these factors.

Dynamin–annexin VI interaction is different in LCV and SCV. Immunoprecipitation experiments indicate that this interaction occurs actually only in LCV's while in both types of vesicles, dynamin and annexin VI are present and capable of interacting, as demonstrated by ligand blotting. Indeed, adding annexin VI before co-immunoprecipitation from SCV restored the dynamin–annexin VI interaction indicating that dynamin is present in excess and still capable of recognizing annexin VI in this population. Taken together, this set of results indicate that although annexin VI and dynamin are both present in LCV and SCV, they only interact in LCVs. Multiple isoforms of dynamin and/or annexins may explain these differences but this is not likely since interaction occurs normally after adding exogenous annexin VI. More likely, since the lipid composition differs from LCVs to SCVs [5], the cytoplasmic leaflet from LCVs could be the only one capable of maintaining a specific relevant interaction between dynamin and annexin VI after the dynamin conformational changes triggered by GTP binding and hydrolysis [52]. Alternatively, another non-mutually exclusive possibility could be that only in LCVs, another yet unidentified third protein would bridge annexin VI and dynamin to form an active heterocomplex. This binding partner could be an SH3 domain-containing protein as proposed by Shpetner et al. [53]. In nerve terminals, amphiphysin has been shown to colocalize with dynamin [54] and could play this role in synaptic vesicle endocytosis.

MCV, which we suggested to bud from TGN, contain the largest amount of annexin VI that exhibits more hydrophobic characteristics. In this case, annexin VI could also participate in the budding either alone or in association with another partner, either lipid or protein, that we could not detect in our experimental conditions. It is reminiscent of yeast in which a dynamin-like protein is required for vesicular transport from the Golgi complex [55]. Although, our results do indicate a possible role of annexin VI during the early phase of endocytosis, one should be aware that endocytosis may exist in the absence of annexin VI, as reported by Smythe et al. [23] in the human A 431 cell model. These cells that overexpress the EGF receptor possess a form of annexin I which seems associated with internal vesicles involved in the endocytotic degradation of the receptor [9]. In this cellular model, annexin I may have replaced annexin VI although this has not been demonstrated yet.

According to the recent models, vesicles originating from either PM or TGN migrate to early endosomes and fuse after uncoating of clathrin and associated proteins [3,56,57]. There is evidence that annexin II is involved as a heterotetramer $p36_2$,$p11_2$ in the fusion of endosomes [58]. However, it is also reported that in the early endocytotic pathway, annexin II is found associated with membranes in a calcium-independent manner and that p11 is not necessary for this interaction [19]. As presented here, annexin II is present in CCVs before uncoating but whatever its location on vesicle membrane or CP, it is as a monomer without p11. As suggested above, annexin II could bind to membrane during its reorganization through calcium bridges and then stabilize in a calcium-independent manner through binding to a

not yet identified 200-kDa component and/or other protein or lipid factors. Due to these co-factors, the evenly distributed 200-kDa component might either bind or not bind annexin II in the different CCVs. Whatever their other partners, we propose that in CCVs annexin II monomer is under an inactive fusogenic form ready to bind p11 after clathrin depolymerization. It could then become fusogenic in a minimal amount of time when necessary for the early endosome formation. In further support of our hypothesis, Jost et al. [19] have proposed a model of interaction of annexin II with the endosomal membrane that is calcium-independent and would implicate a putative receptor. As far as their calcium-dependent association is concerned, annexins II and VI could be mutually exclusive in their membrane location as reported by Creutz [11].

5. Conclusion

Annexins II and VI are found associated with CCVs in both the endocytic/recycling pathway and the biosynthetic pathway. We propose that they fulfil their functions in a two-step process. The first step would be calcium-dependent and would depend on the membrane reorganization involving lipid and protein movements occurring before clathrin coating. The second step is calcium-independent but protein- and lipid-dependent, and would define the annexins II and VI specificity. Annexin II in CCVs, located in different lipid environments as shown by its partitioning between aqueous and detergent fractions, and potentially bound to a 200-kDa component, is in an inactive fusogenic form but ready to become fusogenic after clathrin uncoating. Annexin VI could regulate and target dynamin assembly during the constriction of clathrin coated pits and the budding of CCVs from PM. Taken altogether, our data suggest that annexins II and VI could define specific protein–lipid interaction microdomains that could play a role in the different functions of the CCVs.

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