RESULTADOS

Parte I

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Cholesterol Modulates the Membrane Binding and Intracellular Distribution of Annexin 6*

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Annexins are Ca^{2+}- and phospholipid-binding proteins that are widely expressed in mammalian tissues and that bind to different cellular membranes. In recent years its role in membrane traffic has emerged as one of its predominant functions, but the regulation of its intracellular distribution still remains unclear. We demonstrated that annexin 6 translocates to the late endocytic compartment in low density lipoprotein-loaded CHO cells. This prompted us to investigate whether cholesterol, one of the major constituents of low density lipoprotein, could influence the membrane binding affinity and intracellular distribution of annexin 6. Treatment of crude membranes or early and late endosomal fractions with digitonin, a cholesterol-sequestering agent, displayed a strong reduction in the binding affinity of a novel EDTA-resistant and cholesterol-sensitive pool of annexin 6 proteins. In addition, U18666A-induced accumulation of cholesterol in the late endosomal compartment resulted in a significant increase of annexin 6 in these vesicles *in vivo*. This translocation/recruitment correlates with an increased membrane binding affinity of GST-annexin 6 to late endosomes of U18666A-treated cells *in vitro*. In conclusion, the present study shows that changes in the intracellular distribution and concentration of cholesterol in different subcellular compartments participate in the reorganization of intracellular pools of Ca^{2+}-dependent and -independent annexin 6.

Annexins are a family of highly conserved proteins, which are characterized by their Ca^{2+}-dependent binding to phospholipids (1). They are widely expressed and have been demonstrated to reside in one or more membranous structures depending on the cell type or tissue analyzed. As any one cell might express as many as ten different annexins, the understanding of the distinct physiological role of each annexin still remains elusive (1, 2). In recent years several annexins, including annexins 2 and 6, have been found at the plasma membrane in endocytic and secretory vesicles and have directly been implicated in the regulation of different steps of endo- and/or exocytic trafficking pathways (1–4). In contrast to the defined roles of certain annexins in endocytic trafficking (5–7), the importance of annexin 6 in endocytosis is still unclear (for a recent review, see Ref. 8). Recently it was suggested that annexin 6 is involved in remodeling the spectrin cytoskeleton at the cell surface during receptor-mediated endocytosis (9, 10). Complex protein-protein interactions ultimately facilitate the release of clathrin-coated vesicles from the plasma membrane, although the involvement of annexin 6 is not instrumental in the budding event itself as previously hypothesized (9). In addition to the plasma membrane, annexin 6 is also found in other membrane structures of the endocytic and exocytic compartments (11–17). In previous studies we were able to demonstrate that annexin 6 is localized predominantly in the apical endosomes of rat hepatocytes (17), although it is also in the prelysosomal compartment of NRK[1] or WIF-B cells (18).

Little is known about how the cells control the topologic intracellular distribution of annexin 6. Although the majority of annexin 6 are most likely targeted to membranes via Ca^{2+}-dependent binding to negatively charged phospholipids, recent findings suggest that other components such as acidic pH and cholesterol could also stimulate the membrane binding affinity of annexin 6 (19, 20). Golzak *et al.* (19) demonstrated that acidic pH stimulates the binding of porcine liver annexin 6 to phospholipid bi- and monolayers in a Ca^{2+}-independent manner. Lowering the pH seems to induce conformational changes in annexin 6, leading to increased hydrophobicity and membrane binding affinity (21, 22). Most of the results obtained for the potential role of cholesterol affecting the membrane binding of annexin 6 are derived from studies on another member of the annexin family in the early endosomal compartment, annexin 2, which also binds to biological membranes in the absence of calcium (20, 23–28). Approximately 50% of annexin 2 is associated with endosomal membranes from BHK cells in a Ca^{2+}-independent manner (23–25). However, low concentrations of cholesterol-sequestering agents like filipin or digitonin quantitatively released annexin 2 from the membranes (23, 24). This is in agreement with the restoration of the Ca^{2+}-independent membrane association of annexin 2 to cholesterol-depleted chromaffin granule membranes after cholesterol replenishment (26). Furthermore, addition of cholesterol

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[1] The abbreviations used are: NRK, normal rat kidney; CHO, Chinese hamster ovary; GST, glutathione *S*-transferase; LDL, low density lipoprotein; LBPA, lysobisphosphatidic acid; PS, phosphatidylserine; U18666A, 3-β-[2-(diethylamino)ethoxy]androst-5-en-17-one; PNS, postnuclear supernatant; PBS, phosphate-buffered saline.

increased the membrane binding affinity of annexin 2 to phosphatidylserine (PS)-enriched liposomes (20, 26). Until recently it was believed that annexins do not interact directly with cholesterol, but Smart and co-workers (29) identified annexin 2 in a cytosolic complex together with cholesterol and caveolin. Therefore, cholesterol may directly or indirectly affect the membrane binding affinity of annexins, and eventually, their intracellular localization.

Annexin 6 is a very dynamic protein that undergoes dramatic changes in the intracellular distribution. Babiychuk *et al.* (30, 31) identified the Ca^{2+}-regulated and reversible association of annexin 6 with the membrane cytoskeleton in smooth muscle cells. The Ca^{2+}-dependent association of annexin 6 with lipid rafts was observed in synaptic plasma membranes of rat brain (32). Similarly, oxidative stress-induced changes in intracellular Ca^{2+} concentrations were accompanied by the translocation of annexin 6 from the plasma membrane to the cytoplasm (33). Besides these Ca^{2+}-induced changes, we recently described the ligand-induced translocation of annexin 6 in response to LDL uptake (34). In these experiments accumulation of LDL-containing vesicles was accompanied by an increased amount of annexin 6 in the late endosomal compartment (34). As cholesterol is the major lipid component of LDL, it could play a role in the increased association of annexin 6 in late endosomes of LDL-loaded cells. Up-to-date experimental evidence of cholesterol stimulating the membrane binding affinity of annexin 6 only comes from *in vitro* binding studies with artificial membranes (PS liposomes) (20). But essentially nothing is known about the role of cholesterol regarding the intracellular localization and binding affinity of annexin 6 to endosomal membranes. In the present study we determined that cholesterol sequestration of endosomal membranes reduces the membrane binding affinity of an EDTA-resistant pool of annexin 6. Furthermore, accumulation of cholesterol in late endosomes results in the rearrangement of the intracellular localization/distribution of annexin 6. Taken together these findings indicate that within the endosomal compartment not only phospholipids but also cholesterol participates in the modulation of annexin 6 localization and function.

EXPERIMENTAL PROCEDURES

Reagents—Ham's F-12 medium, Dulbecco's modified Eagle's medium, L-glutamine, PBS, fetal calf serum, trypsin, penicillin, and streptomycin were from Invitrogen. Bovine serum albumin, glycine, horseradish peroxidase, paraformaldehyde, and filipin were purchased from Sigma. Mowiol® 4-88 was from Calbiochem. Digitonin was purchased from Fluka, and U18666A was from Biomol. Two different antibodies to annexin 6 were used: a rabbit anti-annexin 6 antibody raised against GST-annexin 6 and an affinity-purified sheep anti-annexin 6 antibody (AB3718) raised against a synthetic peptide corresponding to the first 11 N-terminal amino acids of rat annexin 6 (MAKIAQGAMYR) (34). Polyclonal anti-caveolin was from BD Transduction Laboratories. Monoclonal anti-annexin 2 (H28) (35) and anti-LBPA (36) was kindly provided by Dr. V. Gerke (Münster, Germany) and Dr. J. Gruenberg (Geneva, Switzerland), respectively. Secondary antibodies (horseradish peroxidase or fluorescent labeled) were purchased from Jackson ImmunoResearch (Dianova, Hamburg, Germany).

Cell Culture—CHO cells were grown in Ham's F-12, and NRK cells were grown in in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, L-glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37 °C, 5% CO_2. The generation of the annexin 6-overexpressing CHO cell line CHOanx6 has been described (34). For the intracellular accumulation of cholesterol, cells were treated for 3–24 h with U18666A (2 μg/ml) as described (37).

Preparation of Recombinant Annexin 6—Recombinant annexin 6 was obtained as GST fusion protein. Full-length 2.0-kb annexin 6 cDNA was cloned into pGEX-KG (Amersham Biosciences) to generate the GST gene fusion vector GST-anx6. GST-anx6 was expressed in BL21 pLysE *Escherichia coli* strain and purified by glutathione-Sepharose chromatography as described (38).

Immunoblot Analysis—For Western blotting 5–20 μg of cell protein were resolved by 10–12% SDS-PAGE (39) and transferred to Protran^R nitrocellulose membranes (Schleicher and Schüll). Annexin 6 in the overexpressing CHOanx6 cell line and GST-annexin 6 were detected using the polyclonal sheep anti-annexin 6 antibody. Endogenous annexin 6 in wild-type CHO cells was detected with the polyclonal rabbit anti-annexin 6 antibody. Annexin 2 and caveolin proteins were detected with the antibodies described above. After incubation with peroxidase-conjugated secondary antibodies the reaction product was finally detected using the ECL system (Amersham Biosciences).

Immunofluorescence—CHO, CHOanx6, or NRK cells ($\sim 1 \times 10^5$) were grown on chamberslides (Nunc). 24 h after plating cells were washed with cold PBS and fixed for 10 min in 3.7% paraformaldehyde in 0.1 M phosphate buffer prior to permeabilization with 0.1% saponin in 0.5% bovine serum albumin, PBS for 15 min. Then cells were blocked with 2% bovine serum albumin, incubated for 1 h at 37 °C with primary antibodies, rinsed with PBS, and incubated for 45 min with Cy3-conjugated secondary antibodies anti-mouse $F(ab')_2$ or anti-sheep $F(ab')_2$ fragments (Jackson ImmunoResearch Laboratories). Cholesterol was stained with filipin (10–20 μg/ml) together with the secondary antibodies. Samples were washed extensively with PBS, and finally the chamberslides were mounted with Mowiol®. In some experiments, cells were treated with 2 μg/ml of U18666A for 4–24 h. After treatment cells were washed with PBS and fixed. Confocal images were collected using a Leica TCS NT equipped with a ×63 Leitz Plan-Apo objective (numerical aperture, 1.4). Deconvoluted images requiring UV analysis (filipin) were obtained with a Zeiss Axiovert 200 microscope equipped with a Cool SNAP-HQ (Photometrics) digital camera.

Crude Membrane Preparation and Subcellular Fractionation—For the preparation of crude membranes and endosomal membrane fractions, $4-6 \times 10^7$ CHOwt or CHOanx6 cells were lysed and separated on sucrose gradients as described (34). After cell lysis the homogenate was centrifuged and the postnuclear supernatant (PNS) served as a crude membrane extract. For the isolation of early and late endosomes the PNS was prepared according to the protocol of Gorvel *et al.* (40). PNS was brought to a final 40.2% sucrose (w/v) concentration by adding 62% sucrose (3 mM imidazole, pH 7.4) and 35% sucrose. 25% sucrose and finally homogenization buffer were poured stepwise on top of the PNS. The gradient was centrifuged for 90 min at 35,000 rpm, 4 °C in a swingout Beckman SW40 rotor. After centrifugation 1-ml fractions were collected from top to bottom, and the fractions representing early (4–6) and late (7–9) endosomes were pooled and analyzed. Aliquots of each fraction were assayed for β-hexosaminidase activity as described (34, 41).

Annexin 6 Membrane Binding Assays—For the characterization of the Ca^{2+}-dependent binding of annexin 6, 100 μl of PNS or early and late endosomes from 5×10^7 to 1×10^8 CHOwt or CHOanx6 cells were incubated in ± 5 mM EDTA for 30 min at 4 °C. Membranes were pelleted in a TL-100 Beckman ultracentrifuge at 45,000 rpm for 60 min at 4 °C. The membrane pellet was resuspended in 100–200 μl of HB buffer (250 mM sucrose, 3 mM imidazole, pH 7.4, and protease inhibitors), and aliquots of the supernatant and pellet were analyzed by Western blotting for the distribution of annexin 6, annexin 2, and caveolin. For the characterization of the cholesterol-sensitive binding of annexin 6, the membranes were pretreated with 5 mM EDTA for 30 min at 4 °C, pelleted as described above, resuspended in HB buffer, and incubated in ± 5 μg/ml digitonin for an additional 30 min at 4 °C. The membranes were pelleted again at 45,000 rpm for 60 min at 4 °C and resuspended in HB buffer. Aliquots of the membranes and the supernatant were analyzed for the distribution of annexins and caveolin. For the binding of GST-annexin 6 to ±U18666A-treated early and late endosomes, 3 μg of GST-annexin 6 were incubated in the presence of Ca^{2+} (0–50 μM) with 100 μl of late endosomal fraction for 30 min at 4 °C. Membranes were pelleted as described above, and aliquots of the membrane and unbound fraction were analyzed for distribution of GST-annexin 6.

Cholesterol Determination—The amount of cholesterol in early and late endosomes of cells incubated with ±U18666A was determined with the Amplex™ Red Cholesterol Assay kit (Molecular Probes). For the fluorometric quantification, 25 μl of endosomes and cholesterol standards (Precinorm™, Precipath™, Roche Molecular Biochemicals) were incubated in 24-well plates according to the instructions of the manufacturer, and fluorescence was detected using Fluorocount™ (Packard Instrument Co.).

RESULTS

The Membrane Binding Affinity of an EDTA-resistant Pool of Annexin 6 Is Sensitive to Cholesterol Depletion—To study the

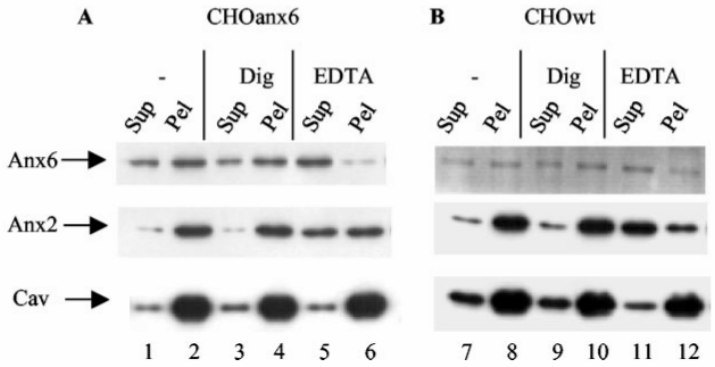

FIG. 1. **Calcium and cholesterol differentially affect the binding of annexins to crude CHO membranes.** PNS from $4-6 \times 10^7$ annexin 6-overexpressing CHO cells (*CHO-anx6*) (*A*) and CHOwt controls (*CHOwt*) (*B*) were prepared as described. 100 μl of PNS were left untreated (−) (*lanes 1*, *2*, *7*, and *8*) or were incubated with 5 μg/ml digitonin (*Dig*) (*lanes 3*, *4*, *9*, and *10*) or 5 mM EDTA (*lanes 5*, *6*, *11*, and *12*) for 30 min at 4 °C. The pelleted membranes were resuspended, and proteins of one-third of the membrane-bound (*Pel*) and one-half of the unbound fraction (*Sup*) were separated by 12.5% SDS-PAGE and analyzed by Western blotting for the distribution of annexin 6, annexin 2, and caveolin. Long exposure times were necessary to detect endogenous annexin 6 in CHOwt (*lanes 7–12*).

effect of cholesterol on the binding of annexin 6 to biological membranes, postnuclear crude membrane fractions from annexin 6-overexpressing cells (CHOanx6) (Fig. 1*A*) and CHOwt (Fig. 1*B*) were isolated and analyzed for their association with annexin 6 after cholesterol sequestration with digitonin. To control for the Ca^{2+}-dependent binding of annexin 6, membranes from CHOanx6 were first treated with or without EDTA, and the amount of annexin 6 in the membrane-bound (Fig 1, *Pel*, one-third of total) and unbound fractions (*Sup*, one-half of total) was determined. Annexin 2 and caveolin served as positive and negative controls, respectively. Whereas annexin 2 is known to bind to membranes in a Ca^{2+}- and cholesterol-dependent manner, caveolin is resistant to the incubation of membranes with either EDTA or digitonin (24, 25).

In untreated membranes of CHOanx6 cells, annexin 6, annexin 2, and caveolin were predominantly found in the membrane-bound fraction (with annexin 2 and caveolin being more abundant in the membrane-bound fraction) (Fig. 1*A*, compare *lanes 1* and *2*). As expected, incubation of membranes with 5 mM EDTA resulted in a strongly reduced binding of annexin 6 and annexin 2 (Fig. 1*A*, compare *lanes 1* and *2* with *lanes 5* and *6*). Similar results were obtained from crude membranes of CHO control cells (Fig. 1*B*, compare *lanes 7* and *8* with *lanes 11* and *12*), indicating that overexpression of annexin 6 did not alter annexin 2 and caveolin membrane binding properties. Treatment of membranes with low concentrations of digitonin (5 μg/ml) did not negatively affect the membrane binding affinity of annexin 6, annexin 2, or caveolin (Fig. 1*A*, compare *lanes 1* and *2* with *lanes 3* and *4*; Fig. 1*B*, compare *lanes 7* and *8* with *lanes 9* and *10*).

However, the treatment of crude membranes with EDTA did not remove all annexin 6 and annexin 2 proteins from cellular membranes. In five independent experiments the amount of EDTA-resistant annexin proteins represented approximately 20–30% total annexin 6 and 40–60% total annexin 2 (Fig. 1*A*, *lane 6*; Fig. 1*B*, *lane 12*). In order to study the effect of digitonin on the EDTA-resistant pool of annexin 6, the crude membrane extract of CHOanx6 cells (Fig. 2*A*) was pretreated with EDTA to remove the EDTA-sensitive pool of annexin proteins. These membranes were pelleted, resuspended, incubated with digitonin (Fig. 2*A*), and pelleted again. Then the distribution of annexin 6 was analyzed in the membrane-bound and the unbound fraction (50% each fraction). A second incubation of EDTA-pretreated membranes with EDTA did not affect the binding of the Ca^{2+}-insensitive annexin proteins (Fig. 2*A*, compare *lanes 1* and *2* with *lanes 3* and *4*). In contrast, after digitonin treatment we observed a significant reduction in the membrane binding of the annexin 6 and annexin 2 remaining after EDTA pretreatment (Fig. 2*A*, compare *lanes 1* and 2 with *lanes 5* and *6*). Incubation of EDTA together with digitonin did not lead to an additional loss of annexin 6 binding. But EDTA and digitonin strongly reduced the binding of annexin 2 (Fig. 2*A*, *lanes 7* and *8*) indicating a cooperative role for cholesterol in Ca^{2+}-dependent binding to annexin 2.

This digitonin-sensitive binding of EDTA-resistant annexin 6 in CHOanx6 could be the result of a partially reduced or altered Ca^{2+}-dependent binding affinity of a pool of annexin 6 proteins because of the high annexin 6 overexpression in CHOanx6 cells. Therefore, the effect of cholesterol sequestration on the EDTA-resistant pool of endogenous annexin 6 proteins in CHOwt cells was analyzed (Fig. 1*B*). Crude membrane extracts from CHO controls were pretreated with EDTA, pelleted, resuspended, and incubated again with EDTA or with digitonin. Then the distribution of endogenous annexins (6 and 2) was analyzed in the membrane-bound or the unbound fraction as described above. Similar to the results described in Fig. 2*A*, digitonin slightly reduced the membrane binding of EDTA-resistant annexin 2 (Fig. 2*B*, compare *lanes 1* and *2* with *lanes 3* and *4*). A second incubation of EDTA-pretreated membranes with EDTA did not affect the binding of the Ca^{2+}-insensitive endogenous annexin 6 proteins (Fig. 2*B*, *lanes 1* and *2*). Again, digitonin strongly reduced the membrane binding of endogenous annexin 6 remaining after EDTA pretreatment (Fig. 2*B*, compare *lanes 1* and *2* with *lanes 3* and *4*). Taken together, these experiments demonstrate that not only the annexin 6-overexpressing CHOanx6 cell line but also the CHOwt controls with low levels of endogenous annexin 6 contain a significant proportion of an EDTA-resistant pool of annexin 6.

Cholesterol Sequestration Reduces Annexin 6 Binding to Early and Late Endosomes—In CHO cells annexin 6 is predominantly found at the plasma membrane and in the endosomal compartment (34, 38). Therefore, early and late endosomes from CHOanx6 cells were analyzed to study the potential role of cholesterol on annexin 6 membrane binding in the endosomal compartment. Similar to the experiment described above, early and late endosomes from CHOanx6 cells were pretreated with 5 mM EDTA. These EDTA-pretreated endosomes were pelleted, resuspended, incubated with digitonin, and pelleted again to analyze the membrane-bound and unbound fraction. A second incubation of EDTA-pretreated membranes with EDTA confirmed the presence of EDTA-resistant annexin 6 (Fig. 3*A*, *lanes 1* and *2*). Incubation with digitonin strongly reduced the

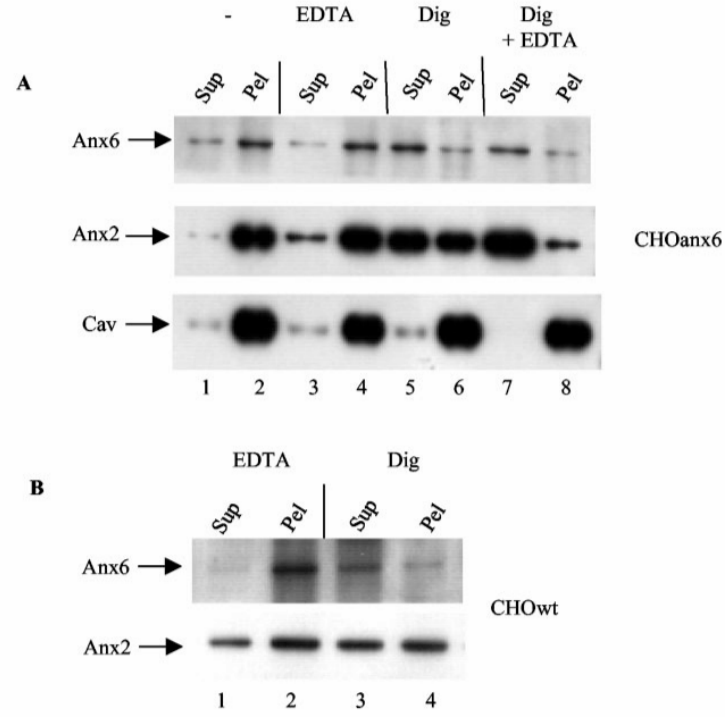

FIG. 2. **Digitonin reduces the binding of EDTA-resistant annexin 6 to crude membranes.** Crude cellular membranes from 4–6 × 10^7 CHOanx6 (*A*) and wild-type CHO (*CHOwt*) (*B*) cells were prepared as described and incubated with 5 mM EDTA for 30 min at 4 °C. EDTA-pretreated membranes were pelleted, resuspended in 100 μl of HB buffer, and were left either untreated (−) (*lanes 1* and *2* in *A*) or incubated again with 5 mM EDTA (*lanes 3* and *4* in *A*; *lanes 1* and *2* in *B*), 5 μg/ml digitonin (*Dig*) (*lanes 5* and *6* in *A*; *lanes 3* and *4* in *B*), or EDTA together with digitonin (*lanes 7* and *8* in *A*) for 30 min at 4 °C. Membranes were pelleted, and comparable aliquots (one-half) of the proteins of membrane-bound (*Pel*) and the unbound fraction (*Sup*) were separated on 12.5% SDS-PAGE and analyzed by Western blotting for the distribution of annexin 6, annexin 2, and caveolin. Long exposure times were necessary to detect endogenous annexin 6 in CHOwt (*lanes 1–4* in *B*).

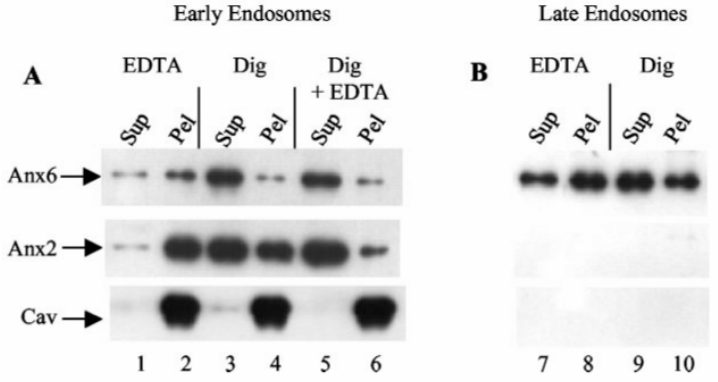

FIG. 3. **Digitonin reduces the binding of EDTA-resistent annexin 6 to early and late endosomes.** Early and late endosomes from postnuclear supernatants from 4–6 × 10^7 (*A*) and 1–2 × 10^8 (*B*) CHOanx6 were prepared as described. 100 μl of early or late endosomes were pretreated with 5 mM EDTA for 30 min at 4 °C and pelleted. The remaining membrane pellet was resuspended in 100 μl of HB buffer and incubated with 5 mM EDTA (*lanes 1*, *2*, *7*, and *8*), 5 μg/ml digitonin (*Dig*) (*lanes 3*, *4*, *9*, and *10*), and EDTA together with digitonin (*lanes 5* and *6*) for 30 min at 4 °C. After centrifugation the amount of annexin 6, annexin 2, and caveolin in comparable aliquots of the membrane-bound (*Pel*) and unbound fraction (*Sup*) was analyzed as indicated.

association of more than 90% EDTA-resistant annexin 6 to early endosomes (Fig. 3*A*, compare *lanes 1* and *2* with *lanes 3* and *4*). To remove the majority of EDTA-insensitive annexin 2 from the membranes, the cooperative effect of EDTA and digitonin was necessary (Fig. 3*A*, *lanes 5* and *6*). Caveolin associated with early endosomes was not affected by EDTA or digitonin treatment. Because of the low amounts and reduced membrane affinity of EDTA-resistant annexin 6 for late endosomes (compare the distribution of annexin 6 in Fig. 3*A*, *lanes 1* and *2* and Fig. 3*B*, *lanes 7* and *8*), four endosomal fractionations of each 5–10^7 CHOanx6 were pooled and analyzed (Fig. 3*B*). After cholesterol sequestration with digitonin the reduced membrane binding of Ca^{2+}-insensitive annexin 6 was significantly reproducible and represented ~30–50% of the membrane-bound and EDTA-resistant pool of annexin 6 proteins. Taken together these experiments indicated that alterations in the cholesterol distribution and content affect the membrane binding affinity of EDTA-resistant annexin 6 to membranes of the endosomal compartment.

Cholesterol Accumulation in Late Endosomes Leads to the Increased Binding of Annexin 6—Uptake of LDL is accompanied by a concomitant translocation of annexin 6 into the late endosomal/prelysosomal compartment (34). As cholesterol is a major constituent of LDL, which after internalization accumulates in the late endosomal compartment, we hypothesized that LDL-derived cholesterol could stimulate annexin 6 binding to late endosomes. To mimick increased levels of LDL cholesterol in late endosomes, CHOanx6 cells were incubated with U18666A, a pharmacological agent, that impairs the intracellular transport of internalized cholesterol and leads to the accumulation of cholesterol in late endosomes (37) (see also Figs. 4*A* and 5*B*). Early and late endosomes from CHOanx6 and CHOwt controls were isolated after incubation with U18666A (5 μg/ml) for 6–24 h and analyzed for their cholesterol content and annexin 6 distribution. In early endosomes from CHOanx6 the U18666A treatment did not significantly increase the cholesterol content (Fig. 4, *A* and *B*). In contrast, 6 h of incubation with U18666A was sufficient to induce a dramatic increase of cholesterol in late endosomes of CHOanx6 (Fig. 4, *A* and *B* and Fig. 5, *A* and *B*) and CHOwt controls (data not shown). These late endosomal fractions of U18666A-incubated cells were characterized by the presence of the late endosomal marker LBPA

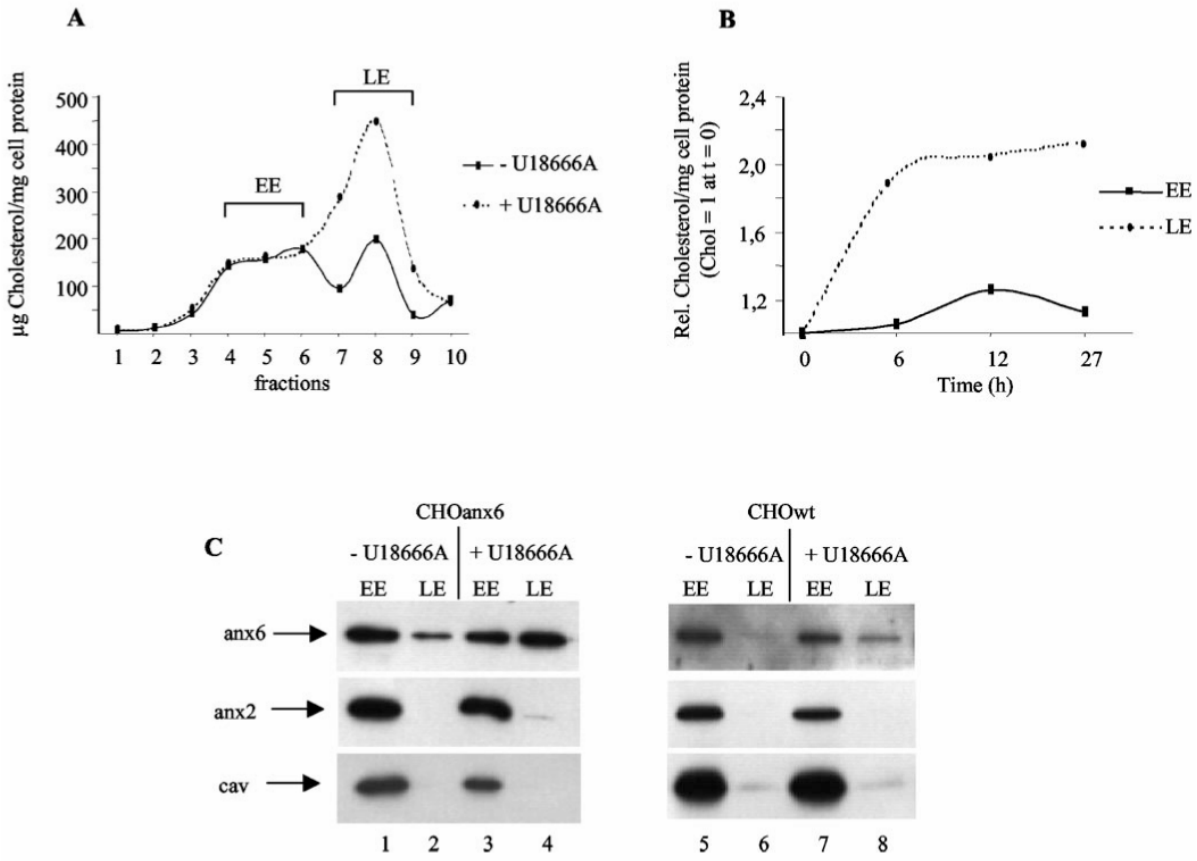

FIG. 4. **U18666A-induced cholesterol accumulation in late endosomes leads to a redistribution of annexin 6.** *A*, $4-6 \times 10^7$ CHOanx6 cells were incubated with (●) or without (■) 2 µg/ml U18666A for 24 h at 37 °C, harvested, and early (*EE*) and late (*LE*) endosomes were isolated as described. 50 µl of each fraction was used for the duplicate fluorometric determination of the cholesterol concentration. *B*, CHOanx6 cells were incubated with 2 µg/ml U18666A for 0, 6, 12, and 27 h, and the cholesterol content of early (■) and late (●) endosomes was determined as described above. *C*, 100 µl of early (*EE*) from $4-6 \times 10^7$ and late (*LE*) endosomes from $1-2 \times 10^8$ CHOanx6 (*lanes 1–4*) and CHOwt cells (*lanes 5–8*) incubated with (+) or without (−) U18666A (2 µg/ml), and the distribution of annexin 6, annexin 2, and caveolin was determined. To analyze the distribution of endogenous annexin 6 in endosomal EE and LE preparations of CHOwt controls (*lanes 5–8*), samples were concentrated ~10-fold, and long exposure times were necessary.

(36) (see Fig. 5*B*) and an approximate 25-fold increase in β-hexosaminidase activity compared with early endosomes (169.4 ± 27.8 milliunit/mg cell protein in early endosomes and 4231.3 ± 495.1 milliunit/mg cell protein in late endosomes), indicating that drug treatment did not result in the formation of new, yet non-characterized populations of endosomal vesicles. Whereas the distribution of annexin 2 and caveolin was not affected in early and late endosomes before and after U18666A treatment (Fig. 4*C*, compare *lanes 1* and *2* with *3* and *4*), the amount of annexin 6 in early endosomes of U18666A-incubated CHO cells was reduced (Fig. 4*C*, compare *lanes 1* and *3*). Most importantly late endosomes from U18666A-treated cells were characterized by increased amounts of annexin 6 (~3–5-fold, compare *lanes 2* and *4*).

As the overexpression of annexin 6 in CHOanx6 possibly saturated all physiologically relevant binding sites, leading to an intracellular localization that would normally not be found, the localization of endogenous annexin 6 in U18666A-treated CHOwt cells was analyzed (Fig. 4*C*, *lanes 5–8*). Similar to the results obtained from the overexpressing CHOanx6 cell line, the distribution of annexin 2 and caveolin was not altered in early and late endosomes of CHOwt controls before and after U18666A treatment (Fig. 4*C*, compare *lanes 5* and *6* with *7* and *8*). But most importantly increased amounts of endogenous annexin 6 were present in late endosomes of U18666A-treated CHOwt cells (Fig. 4*C*, compare *lanes 6* and *8*). Taken together these findings indicate that increased cholesterol concentrations in late endosomal membranes stimulate annexin 6 binding and lead to a redistribution of annexin 6 proteins within the cell.

Annexin 6 Co-localizes with Cholesterol-rich Late Endosomal Structures—To confirm that annexin 6 was recruited to late endocytic structures in response to cholesterol accumulation, we first performed immunocytochemical analysis of control and U18666A-treated CHOanx6 cells. Images were acquired either by a digital camera (Fig. 5, *A–F*) or by confocal microscopy (Fig. 5, *G* and *H*). Incubation of U18666A-incubated CHOanx6 cells with mouse monoclonal anti-LBPA (36) and filipin (0.2 mg/ml) confirmed the accumulation of cholesterol in late endosomes (Fig. 5, *A* and *B*). A similar experiment of representative fields of CHOanx6 with sheep anti-anx6 antibody and filipin demonstrated an altered distribution of annexin 6 after U18666A treatment (Fig. 5, *C–F*). Finally, confocal microscopy identified increased co-localization of annexin 6 and LBPA, indicating that U18666A-induced cholesterol accumulation leads to the translocation of annexin 6 to the late endosomal compartment (Fig. 5, *G* and *H*). This contrasted with the localization for annexin 2, which was characterized by a diffuse staining

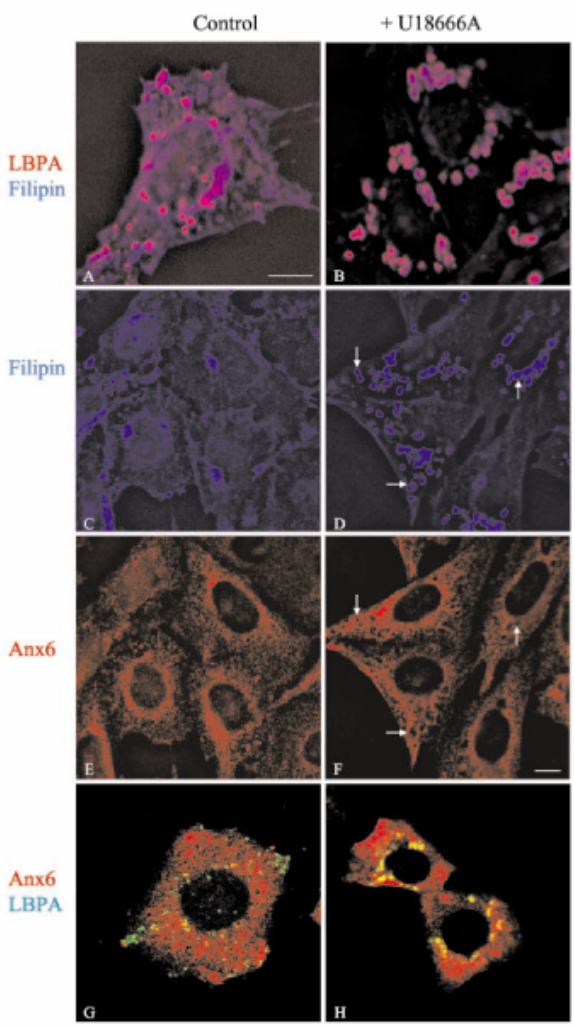

FIG. 5. **Annexin 6 co-localizes with cholesterol-rich late endosomal structures.** CHOanx6 cells were grown on chamberslides and incubated with and without (*Control*) U18666A (2 μg/ml) for 12 h at 37 °C as indicated. Cells were fixed, permeabilized, and immunolabeled with anti-LBPA or anti-annexin 6 together with filipin (2 μg/ml) (*panels A–F*). Double staining with filipin and anti-LBPA demonstrates the accumulation of cholesterol in late endosomes from cells treated with U18666A (*panels A* and *B*). Cells double-labeled with filipin (*panels C* and *D*) and annexin 6 (*panels E* and *F*) show a rearrangement of annexin 6 localization after U18666A treatment. *Arrows* point to annexin 6 staining surrounding cholesterol-rich structures labeled with filipin (*panels D* and *F*). Confocal microscopy reveals increased co-localization (*yellow*) of annexin 6 (*red*) and LBPA (*green*) in CHOanx6 cells after U18666A treatment. *Bars* are 5 μm.

throughout the cytoplasm which did not change significantly after the U18666A treatment (data not shown).

To exclude the possibility that heterologous expression of annexin 6 in the CHOanx6 cells could in part be responsible for the U18666A-induced translocation of annexin 6 to cholesterol-rich vesicles, similar experiments were performed with CHOwt controls to study the effect of U18666A treatment on annexin 6. Because of the low annexin 6 levels the endogenous annexin 6 could not be visualized by immunofluorescence analysis in CHO cells (data not shown). Therefore, digital and confocal microscopy of NRK cells that constitutively express annexin 6 at higher levels was performed. Similar to the results obtained from CHO cells, U18666A treatment resulted in a strong accumulation of cholesterol in LBPA positive, perinuclear vesicles (Fig. 6, compare *panels A* and *C* with *B* and *D*). In NRK cells annexin 6 is found in prelysosomal lgp120-positive structures that are distinct from M6PR-containing late endosomes (18, 38), which is in agreement with the minor co-localization of annexin 6 with the late endosomal marker LBPA (Fig. 7, *A*, *C*, and *E*). However, U18666A treatment was followed by an increased co-localization of annexin 6 with LBPA, indicating that cholesterol accumulation in late endosomes of NRK cells leads to an increased association of annexin 6 with these vesicles (Fig. 7, compare *panels E* and *F*). Therefore, cholesterol seems to have a general regulatory role for the membrane binding and intracellular location of annexin 6 in different cell types.

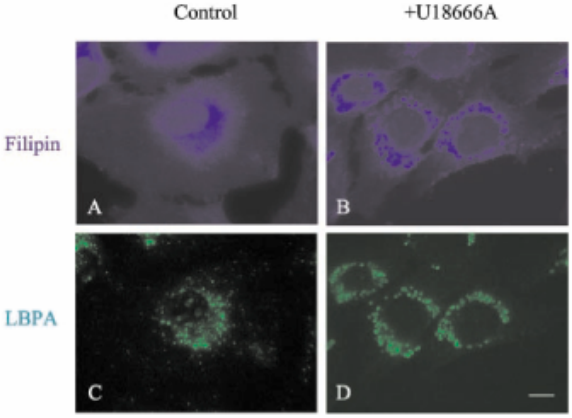

FIG. 6. **U18666A-induced cholesterol accumulation in late endosomes of NRK cells.** NRK cells were grown on chamberslides and incubated with (*panels B* and *D*) and without (*panels A* and *C*) U18666A (2 μg/ml) for 12 h at 37 °C as indicated. Cells were fixed, permeabilized, and immunolabeled with anti-LBPA (*panels C* and *D*) followed by a fluorescent-labeled secondary antibody together with filipin (2 μg/ml) (*panels A* and *B*). *Bar* is 5 μm.

U18666A Stimulates the Binding of GST-Annexin 6 to Late Endosomes—The elevated levels of annexin 6 in late endosomes of U18666A-treated CHOanx6 cells could be caused by an increased membrane binding affinity of annexin 6 for cholesterol-rich membranes. Therefore, early and late endosomes from CHOwt cells were incubated with U18666A, and the membrane binding affinity to GST-annexin 6 *in vitro* was analyzed (Fig. 8). Early endosomes from untreated CHO cells were characterized by their high binding affinity to GST-annexin 6 (Fig. 8, *lanes 1* and *2*). In contrast, the majority of GST-annexin 6 was found in the unbound fraction after incubation with late endosomes from untreated controls (*lanes 3* and *4*). Incubation of CHO cells with U18666A did not alter the binding affinity of GST-annexin 6 for early endosomes (*lanes 5* and *6*). In contrast, the binding of GST-annexin 6 to the late endosomal fraction of U18666A-incubated CHO cells was significantly increased (*lanes 7* and *8*), strongly indicating that increased cholesterol in late endosomes stimulates annexin 6 binding.

In a series of *in vitro* binding studies we determined that GST-annexin 6 requires the presence of Ca^{2+} for maximal binding to crude membranes. Moreover, when the Ca^{2+}-dependent binding of GST-annexin 6 was analyzed, we observed a higher binding affinity of GST-annexin 6 to late endosomes of U18666A-treated cells compared with late endosomal fractions from untreated controls (data not shown). Therefore, despite the EDTA-resistant membrane binding of some annexin 6 (Figs. 1–3), a potential role for Ca^{2+} in the U18666A-induced binding of GST-annexin 6 to late endosomes cannot be excluded.

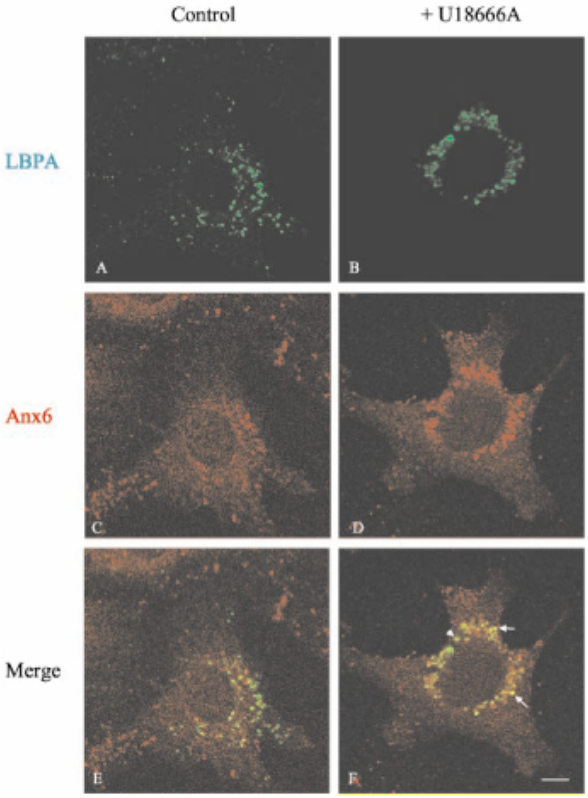

FIG. 7. **Increased co-localization of annexin 6 and LBPA in cholesterol-rich late endosomes of NRK cells.** NRK cells were grown on chamberslides and incubated with (*panels B, D, F*) and without (*panels A, C, E*) U18666A (2 μg/ml) for 12 h at 37 °C as indicated. For confocal microscopy, cells were fixed, permeabilized, and double immunolabeled with anti-LBPA (*panels A* and *B*) and anti-annexin 6 (*panels C* and *D*). The merged images are shown (*panels E* and *F*). Co-localization of annexin 6 and LBPA in perinuclear structures after U18666A treatment is indicated by *arrows* (*panel F*). The *arrowhead* points to a LBPA-positive structure (*green*) not stained with anti-annexin 6 (red). *Bar* is 5 μm.

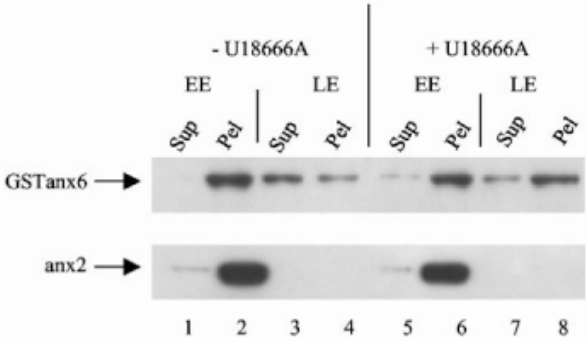

FIG. 8. **U18666A increases the binding of GST-annexin 6 to late endosomes.** CHOwt cells were incubated in ± 2 μg/ml U18666A for 24 h, and early (*EE*) and late endosomes (*LE*) were isolated as described. 100 μl of early and late endosomes were incubated with 3 μg of GST-annexin 6 for 30 min at 4 °C. Membranes were pelleted, and the amount of membrane-bound and unbound GST-annexin 6 and annexin 2 in the supernatant (*Sup*) and membrane pellet (*Pel*) was determined as described in Fig. 4.

DISCUSSION

In this study we have identified two different pools of annexin 6 in CHO cells. The majority (~70–80%) of annexin 6 binds to membranes in a Ca^{2+}-dependent manner, whereas the binding of an EDTA-resistant pool of annexin 6 to endosomal membranes is strongly influenced by the amount and distribution of cholesterol. Cholesterol sequestration reduces the membrane binding of Ca^{2+}-independent annexin 6 to early and late endosomes. Conversely, increased amounts of cholesterol in late endosomes stimulate the binding of annexin 6 to late endosomal membranes. Since this translocation of annexin 6 was also observed in drug-induced cholesterol-rich late endosomes of NRK fibroblasts, we propose that cholesterol is a general and additional modulator of annexin 6-membrane binding and intracellular distribution.

The determinants that confer the binding specificity for annexins for different intracellular compartments are not yet defined (for recent review, see Ref. 42). In the case of annexin 6, different experimental approaches have demonstrated that it is a highly dynamic protein that can undergo changes in its intracellular location according to Ca^{2+} mobilization (30–33), possibly changes in the pH (19, 21, 22), and also, as shown in the present study, by changes in the intracellular concentration and distribution of cholesterol.

Although annexin 2 has recently been found in a complex with caveolin-bound cholesterol (29), it is still believed that other annexins like annexin 6 do not directly interact with cholesterol. Whereas cholesterol associates preferentially with phosphatidylcholine rather than with PS (43), annexin 6 is thought to dominantly bind to PS (1, 2, 20). But annexin 6 has been identified in Triton X-insoluble, caveolin- and cholesterol-enriched membrane fractions (2, 8, 30–32). In addition the Ca^{2+}-induced translocation of annexin 6 to cholesterol-rich membranes was demonstrated in a number of different cell types (30–32). Therefore, the association of cholesterol and phosphatidylcholine in cholesterol-rich membrane microdomains might induce the formation of PS-rich domains in directly neighboring membrane microdomains to stimulate binding of annexin 6 to PS.

In previous studies we demonstrated that annexin 6 is predominantly associated with acidic, prelysosomal compartments of non-polarized as well as in polarized cells (18, 38). These findings could indicate a pH dependence of annexin 6-endosome binding as recently discussed by Golczak *et al.* (22). Interestingly, two pentapeptide KFERQ-like sequences, which are thought to target cytosolic proteins for the chaperone-mediated lysosomal pathway (44), are found within the annexin 6 (positions 81–85 in repeat 1 and positions 564–568 in repeat 7) (22, 45). These signal sequences might contribute to the preferential association of annexin 6 to late endosomal membranes in some cell types and indicate that phospholipids and cholesterol might not be the only determinants of annexin 6 localization. As full-length and degradation fragments of annexin 6 have been found in the lumen of late endocytic structures (45), annexin 6 could be able to sense the internal acidic pH as suggested for ARF1 (46) through an unknown molecule(s) to become associated with the endosomal membrane.

Since the potential KFERQ sequences of annexin 6 are located in α-helical regions buried in the core of the protein, the interaction of annexin 6 with the chaperone (hsc73) responsible for the transport of annexin 6 (anx6/hsc73 complex) to the lysosomal membrane must require some degree of annexin 6 unfolding (47, 48). Therefore, conformational changes of annexin 6 could possibly enhance or regulate the binding of annexin 6 to the cholesterol-enriched membranes of late endosomes after the U18666A treatment. Interaction of cholesterol with some phospholipids, such as PS, was demonstrated to increase the association for annexin 2 and 6. In fact, both of these two annexin members display the KFERQ-targeting se-

quence (45). However, the KFERQ-containing annexins may associate with the prelysosomal compartment for a reason other than degradation. In fact, a calcium-independent binding of certain annexins (1 and 5) to lipids has been described. Also, pH changes seem important in determining the calcium independence of annexin-phospholipid interaction (49, 50).

In summary we have demonstrated that alterations in the lipid composition in early and late endosomes could influence the membrane binding of annexin 6. These findings correlate with the Ca^{2+}-independent binding of annexin 2 to early endosomes (24–26), which has been attributed to the presence of cholesterol in the membrane (24, 25). Future experiments will have to clarify whether the targeting and incorporation of LDL-derived cholesterol into late endosomal membranes or the activation of annexin-binding chaperones during intracellular LDL processing are responsible for the ligand-induced translocation of annexin 6 to late endosomal structures.

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RESUMEN PARTE I:

CHOLESTEROL MODULATES THE MEMBRANE BINDING AND INTRACELLULAR DISTRIBUTION OF ANNEXIN 6

Antecedentes

La función de la anexina 6 en el sistema endocítico ha sido ampliamente descrita y del mismo modo se ha demostrado su funcionalidad en este compartimento. Por ejemplo, la anexina 6 (o A6) participa en la formación de vesículas de clatrina, y en el tráfico entre el compartimento endocítico tardío y los lisosomas.

De forma previa al presente trabajo se describió la redistribución, dentro del compartimento endocítico tardío, de anexina 6. Esta redistribución acontecía en paralelo a la internalización, mediada por receptor, de las lipoproteínas LDL, uno de cuyos componentes mayoritarios es el colesterol.

Si bien la principal característica de las anexinas es la unión a las bicapas lipídicas de forma dependiente de un incremento del calcio, ha sido descrita in vitro la capacidad de varias anexinas, entre ellas la A6, de unirse a las membranas de forma independiente de calcio y dependiente de colesterol. No obstante, no había sido descrita hasta la fecha la unión calcio independiente de anexina A6 a las membranas biológicas *in-vivo*.

Objetivos

1. Determinar si existe una población de anexina A6 unida a las membranas biológicas *in-vivo* de forma independiente de calcio.
2. Esclarecer la posible influencia del contenido de colesterol en dicha unión.
3. Estudiar los cambios de distribución de la anexina A6 en el compartimento endocítico debidos a la modificación del contenido de colesterol.

Resultados

Si bien la ausencia de calcio libera la mayor parte de anexina A6 unida a las membranas, se demuestra, en células CHO que sobreexpresan la anexina A6, que existe una subpoblación (20-30 %) de anexina A6 unida a las membranas en estas condiciones. La repetida incubación del extracto de membranas con agentes quelantes del calcio se muestra incapaz de solubilizar por completo esta anexina remanente. Por el contrario, el pretratamiento de estas membranas con agentes que alteran el contenido de colesterol solubiliza dicha subpoblación de anexina A6, indicando que el lípido contribuye a la unión a membranas de la anexina de forma significativa.

Al analizar dicho comportamiento en membranas aisladas del compartimento endosomal, se observa de modo similar como, tanto en membranas de endosomas tempranos como tardíos, la extracción del colesterol conlleva la disociación de una parte significativa de la anexina 6, cuya unión es además resistente a EDTA.

Mediante una estrategia alternativa consistente en la acumulación de colesterol en el compartimento endocítico tardío, al pretratar las células con U18666A, se observa que el incremento de colesterol en estas fracciones induce un reclutamiento de anexina A6.

El análisis de la distribución subcelular por immunomicroscopía de fluorescencia confirma la acumulación de colesterol y anexina en los endosomas tardíos de células CHO-AnxA6 tratadas con U18666A.
Además, idéntico comportamiento se observa para la anexina A6 endógena expresada por las células NRK, descartando la existencia de artefactos derivados de la sobreexpresión.

Finalmente, la incubación de membranas de endosomas, aisladas a partir de células pretratadas o no con U18666A, con anexina A6 añadida exógenamente, revela que las membranas de endosomas tardíos enriquecidas en colesterol reclutan mayor cantidad de anexina, comparado con las membranas de endosomas tardíos control, y descarta así que el efecto observado se deba a una interferencia de la droga en el tráfico intracelular de la anexina A6.