Annexin VI Stimulates Endocytosis and Is Involved in the Trafficking of Low Density Lipoprotein to the Prelysosomal Compartment*

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Annexins are calcium-binding proteins with a wide distribution in most polarized and nonpolarized cells that participate in a variety of membrane-membrane interactions. At the cell surface, annexin VI is thought to remodel the spectrin cytoskeleton to facilitate budding of coated pits. However, annexin VI is also found in late endocytic compartments in a number of cell types, indicating an additional important role at later stages of the endocytic pathway. Therefore overexpression of annexin VI in Chinese hamster ovary cells was used to investigate its possible role in endocytosis and intracellular trafficking of low density lipoprotein (LDL) and transferrin. While overexpression of annexin VI alone did not alter endocytosis and degradation of LDL, coexpression of annexin VI and LDL receptor resulted in an increase in LDL uptake with a concomitant increase of its degradation. Whereas annexin VI showed a wide intracellular distribution in resting Chinese hamster ovary cells, it was mainly found in the endocytic compartment and remained associated with LDL-containing vesicles even at later stages of the endocytic pathway. Thus, data presented in this study suggest that after stimulating endocytosis at the cell surface, annexin VI remains bound to endocytic vesicles to regulate entry of ligands into the prelysosomal compartment.

Annexins are a family of highly conserved proteins, which are characterized by their Ca$^{2+}$-dependent binding to phospholipids (1). Each annexin consists of a conserved core domain with four or eight repeats (70 amino acids) and a nonconserved, short, NH$_2$-terminal domain. More than 10 different family members, several of which exist as multiple isoforms, have been described in higher vertebrates (2). Since annexins are expressed in many tissues and are located in the same cellular compartments, the understanding of the distinct physiological role of each annexin still remains elusive (1, 3). In recent years, the involvement of annexins in membrane traffic has emerged as one of their predominant functions (1, 4). Several annexins including annexin I, II, IV, VI, VII, and XIIIb have been directly implicated in different steps of the intracellular trafficking pathways (5–14) and, despite some controversy, essentially due to the variety of cells and antibodies used, they are all associated with the endocytic compartment.

The enrichment of annexin VI in rat liver endosomes (12, 13), its polarized localization in the apical endosomes in rat hepatocytes (14) and WIF-B cells (15), and the colocalization with lgp120, a prelysosomal marker in normal rat kidney cells (15), indicate a potential role for annexin VI in the endocytic pathways of polarized and nonpolarized cells.

In support of this hypothesis, annexin VI has been found to bind β-spectrin at the cell surface, which in turn recruits and activates a calpain-like protease. This cascade of events seems to open the actin-cortical cytoskeleton to facilitate the initial steps of endocytosis (16, 17). The complexity of these interactions has recently been pointed out by Michaely and co-workers (18) and involves also some other cytoskeleton proteins such as ankyrin that associates with clathrin to participate in the annexin VI-dependent, clathrin-mediated endocytosis.

However, the possible involvement of annexin VI in fusion events in the late endocytic pathway is not completely understood (15, 19). The complex intracellular distribution of annexin VI in the various cell types analyzed (1–3, 12–15, 20) indicate an additional role of annexin VI, which may be responsible for the triggering of transient interactions with other structures that may regulate the entrance and the trafficking of different ligands.

In order to investigate the influence of annexin VI on endocytic processes, we studied the effect of overexpression of annexin VI in Chinese hamster ovary (CHO)$^1$ cells on LDL and transferrin (Tf) uptake and intracellular processing. Here we report that parallel overexpression of annexin VI and the LDL receptor increases the internalization and degradation of LDL. When cotransfected with the Tf receptor, annexin VI moderately stimulates endocytosis but has no effect on the recycling of Tf. In contrast, overexpression of annexin II does not facilitate a stimulatory effect on the endocytosis of both ligands. Cellular subfractionation and immunofluorescence analysis suggest that annexin VI enters the prelysosomal compartment while being associated with LDL-containing endocytic vesicles. These findings indicate that intracellular stores of annexin VI have the ability to respond to so far unknown signals (e.g.

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* This work was supported by the Deutsche Forschungsgemeinschaft in the form of a Clinical Research Group (Gr 258/10-2), by Deutsche Akademischer Austauschdienst Grant 314.AI-e-dr, and Akines Integradas Grant EA 98-0007 (to C. E. and S. J.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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$^1$ The abbreviations used are: CHO, Chinese hamster ovary; HRP, horseradish peroxidase; LDL, low density lipoprotein; LDLR, low density lipoprotein receptor; PBS, phosphate-buffered saline; Tf, transferrin; Dif, 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine; BSA, bovine serum albumin.
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**Ligands, cytokines, calcium** (21) and then concentrate in endosomal vesicles directed to the prelysosomal compartment.

**Experimental Procedures**

**Materials**—1,1’-Dioctadecyl-3,3’,3’‘-tetramethyldililorocarbocyanine (DiI) was purchased from Molecular Probes, Inc. (Leiden, The Netherlands). Ham’s F-12 medium, l-glutamine, PBS, fetal calf serum, trypsin, penicillin, and streptomycin were from Life Technologies, Inc. BSA, glycine, horseradish peroxidase (HRP), transferrin, parafomaldehyde were purchased from Sigma. [125I]Iodine was from Amersham Pharmacia Biotech, and heparin was from Roche Molecular Biochemicals. Mowi60 4–88 was purchased from Calbiochem. Low density lipoprotein (density 1.025–1.050 g/ml) was prepared from plasma of normal-lipidemic donors by two sequential density gradient ultracentrifugations in KBr gradients (22). Before experiments, LDL was dialyzed extensively against PBS and stored at 4 °C until use. Fluorescent labeled LDL was prepared by incorporation of DiI as described (23). Purified bovine liver annexin VI (24) was purchased from Biodies International.

**Antibodies**—Four different antibodies to annexin VI were used: the affinity-purified rabbit anti-annexin VI antibody (14), a rabbit anti-annexin VI antibody raised against glutathione S-transferase-annexin VI, a rabbit anti-annexin VI antibody (ICL203) (from Biozol, Munich) (25), and the affinity-purified sheep anti-annexin VI antibody (AB3718) raised against a synthetic peptide corresponding to amino acids 1–11 of the N terminus of rat annexin VI (MAKIQAAGMYR) (26) (Abimed, Darmstadt, Germany). Polyclonal anti-Rab5 and anti-Rab4 were from Santa Cruz Technology, Inc. (Santa Cruz, CA), anti-c-Myc antibody (9E10) was from Invitrogen (27), monoclonal anti-dynamin was from Transduction Laboratories, and anti-Lamp1 (UH1) was from the Developmental Studies Hybridoma Bank (University of Iowa). Monoclonal anti-transferrin receptor (B3/25) was from Roche Molecular Biochemicals. Monoclonal anti-annexin II (H28) (28) was kindly provided by Dr. V. Gerke (University of Münster). Rabbit anti-LDL receptor (LDLR) (29) was a gift from Dr. J. Herz (University of Texas, Dallas). Secondary antibodies (HRP or fluorescently labeled) were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, Hamburg, Germany).

**Cell Culture**—CHO cells were grown in Ham’s F-12 supplemented with 10% fetal calf serum, l-glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37 °C, 5% CO2. The annexin VI overexpressing CHO cell line CHOaxn6 was grown in the presence of 1 mg/ml G418. 48 h prior to incubation with radioactive ligands, 1 × 10⁶ cells/well were plated on 12-well plates. Cells were transfected after 24 h and with the various ligands the following day. Recombinant DNA—A rat annexin VI cDNA clone was isolated from a rat liver cDNA library (Fisher rats, Uni-Zap™ XR, Stratagene) and subcloned into pGEMT (Promega). The coding region was polymerase chain reaction-amplified and generated a 2.0-kilobase pair annexin VI cDNA fragment (26) that was cloned into a mammalian expression vector (pCMVSPORT, Invitrogen) to generate pcDNAaxn6. DNA sequence analysis using a Dye Terminator Cycle Sequencing reaction kit (PerkinElmer Life Sciences) together with T7, SP6 (Promega), and internal annexin VI primers confirmed the full-length and wild type rat annexin VI cDNA sequence in pcDNAaxn6. All cloning procedures were performed according to standard protocols (30). The expression vectors encoding the human LDL receptor (pCMVhLDLR), human Tf receptor (pCD-TFR) (21), and human Rab5 (mRab5p55) (32) were kindly provided by Dr. F. Schnieders and Dr. T. Jentsch (University of Hamburg). The expression vector for human annexin II (pCMV5-EX-AII) (33) was a gift from Dr. V. Gerke. β-Galactosidase encoding pCMVSPORT-βGal was obtained from Life Technologies, Inc.

**Transfections**—The DNA used for transfections was purified with the plasmid purification kit from Qiagen. 2–3 × 10⁵ cells were transfected with 1 μg of DNA and the FUGENE™ 6 Transfection Reagent (Roche Molecular Biochemicals) according to the instructions of the manufacturer. Control cells were transfected with pCMVSPORT-βGal. For cotransfections, 0.5 μg of each plasmid was used. In their experiments, LDLR- and Tf receptor-transfected cells were cotransfected with β-galactosidase-, annexin VI-, annexin II-, or Rab5-encoding plasmids. Expression vectors described in 10–20% of cells was confirmed by PCR. G418-resistant colonies were isolated and examined for expression of annexin VI by Western blotting and immunofluorescence.

**Immunoblot Analysis**—For Western blotting, cells were solubilized in 1% Triton X-100, 50 mM Tris, 2 mM CaCl₂, and 80 mM NaCl. 50 μg of cell protein or 500 ng of purified annexin VI were resolved by 10% SDS-PAGE and transferred to Protran® Nitrocellulose membranes (Schleicher and Schuell). Annexin VI was detected using polyclonal sheep (Abimed) and three different rabbit anti-annexin VI antibodies. Recombinant human LDL receptor expression was analyzed with the rabbit anti-LDLR antibody. Annexin II, Rab5 (data not shown), Rab4, and dynamin expression was confirmed by Western blotting.

**Internalization of Ligands and Fluid Phase Markers**—To determine the internalization of 125I-LDL (10 × 10⁵ cpm/ml) was added to every third sample prior to the incubation to determine non-specific uptake. For internalization, cells were washed three times with ice-cold PBS and subsequently with PBS/heparin (20 units/ml) to remove surface-bound LDL. Cells were lysed with 1 ml of 0.1 N NaOH, and the radioactivity was measured. An aliquot of the cell lysate was used to determine the cell protein concentration.

**For endocytosis of 125I-LDL and 125I-HRP, cells were washed with PBS and incubated in Ham’s F-12, 1% BSA for 30 min at 37 °C. Cells were chilled on ice, and 125I-LDL was added to 4–5 μg/ml (in triplicate). Cells were then incubated for 60 min or 24 h at 37 °C. 50-fold excess of unlabeled LDL was added to every third sample prior to the incubation to determine non-specific uptake of 125I-LDL. After internalization, cells were washed three times with ice-cold PBS and subsequently with PBS/heparin (20 units/ml) to remove surface-bound LDL. Cells were lysed with 1 ml of 0.1 N NaOH, and the radioactivity was measured. An aliquot of the cell lysate was used to determine the cell protein concentration.

**Analysis of 125I-LDL Degradation**—Cells were incubated with 125I-LDL as described above for 60 min at 37 °C. At each time point, 100 μl of medium was removed, and 3 μl cold trichloroacetic acid was added to a final concentration of 10%. The sample was vortexed vigorously and incubated for 10 min on ice. After the addition of 250 μl of 0.7 M AgNO₃ to remove free iodine (39), the sample was vortexed again and then centrifuged, and the amount of radioactivity in the remaining supernatant was determined. Cell lysates for protein concentration were prepared as described above.

**Analysis of 125I-LDL Recycling**—Cells were incubated with 125I-LDL for 60 min at 37 °C as described above. A 50-fold excess of unlabeled LDL was added to every third sample prior to the incubation. After incubation cells were washed on ice, the medium was removed, and cells were washed four times with ice-cold PBS. Cells were then incubated in Ham’s F-12, 1% BSA at 37 °C, and the radioactive labeled LDL remaining in the medium was collected at various times. Cells were lysed in 1 ml of 0.1 N NaOH to determine cell protein concentration and the amount of 125I-LDL remaining in the cells.

**Immunofluorescence**—1 × 10⁶ cells were grown on chamber slides (Nunc). 24 h after transfection, cells were washed with cold PBS and fixed in 4% paraformaldehyde. For DiI-LDL uptake experiments, the transfected cells were preincubated with DiI-LDL (5 μg/ml) for 30 min at 4 °C, washed with PBS and incubated for 5 min at 37 °C, fixed with 4% paraformaldehyde, and incubated with antibodies (15). To visualize the primary antibodies, we used immune adsorbed Cy3- or Cy2-conjugated F(ab‘)₂ fragments, Cy3- or Cy2-conjugated goat anti-rabbit (Dianova, Hamburg, Germany). 10-fold diluted secondary antibodies were used to determine the cell protein concentration (37).
RESULTS

Overexpression and Subcellular Characterization of Annexin VI in CHO Cells—To study the role of annexin VI in the endocytic pathways, rat annexin VI was overexpressed in CHO cells. These cells contain only low amounts of endogenous annexin VI as judged by reverse transcriptase-polymerase chain reaction (data not shown). Western blotting, and immunofluorescence with four different annexin VI antibodies. Fig. 1 shows a representative Western blot demonstrating increased annexin VI expression in transfected cells (lanes 2 and 5) compared with endogenous annexin VI levels in β-galactosidase-transfected controls (lanes 1 and 4; approximately 10–15-fold, compare lanes 4 and 5) using different antibodies. All four antibodies used positively recognize purified bovine liver annexin VI (Fig. 1, lane 3) and rat hepatocyte endosomal annexin VI (data not shown). In all experiments, the efficiency of transfection was approximately 10–25% of the cells. Transient or stable transfected annexin VI overexpressing cells showed morphological (actin-cytoskeleton, data not shown) and functional features (receptor-mediated endocytosis, see below) comparable with the wild type CHO cells.

Annexin VI Does Not Affect the Recycling of Transferrin—To...
investigate the role of annexin VI in the endocytotic pathway of Tf, we determined the uptake and recycling (Fig. 2A) of radiolabeled Tf in transiently annexin VI-overexpressing CHO cells. Rab5, a member of the Rab protein family that has been demonstrated to stimulate receptor-mediated endocytosis (32, 42), served as a positive control. Cells transfected only with annexin VI, annexin II, or Rab5 did not demonstrate a significant alteration of Tf internalization (data not shown). As expected (32, 42), cells coexpressing Rab5 together with the Tf receptor endocytosed Tf slightly faster than Tf receptor-expressing controls. Cotransfection of annexin II together with the Tf receptor did not alter endocytosis of transferrin compared with Tf receptor-transfected cells. Coexpression of annexin VI and the Tf receptor revealed a modest stimulatory effect on Tf internalization (1.5–1.8-fold) at the various time points (5–60 min) (data not shown).

Subsequently, the recycling of Tf in controls and annexin VI-transfected cells was examined. Overexpression of annexin VI, annexin II, and Rab5 did not affect Tf recycling (data not shown). As observed by others (32), more than 40% of internalized Tf was recycled in Tf receptor-overexpressing cells after 30 min (Fig. 2A). In cells cotransfected with annexin VI and Tf receptor, the recycling of Tf was not significantly increased compared with Tf receptor-transfected controls (p > 0.05 for t = 20, 30 min; Fig. 2A). Similar results were obtained with Rab5 and annexin II, indicating that none of those proteins significantly affected the rates of recycling of transferrin (Fig. 2A).

In order to identify the intracellular localization of annexin VI and Tf receptor, we then performed immunofluorescence experiments of cells cotransfected with annexin VI and the human transferrin receptor. As expected, overexpression of the Tf receptor shows some localization at the cell surface, as well as suggesting that annexin VI may have a role in the regulation of Tf internalization.
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Annexin VI Stimulates Internalization and Degradation of Low Density Lipoprotein—The effect of overexpression of annexin VI on the uptake (Fig. 3) and degradation (Fig. 4) of LDL was studied. We first determined the accumulation of radiolabeled LDL in transiently annexin VI-overexpressing CHO cells (data not shown). Rab5 served again as a positive control (32). Overexpression of annexin VI or Rab5 alone did not result in a significant accumulation of $^{125}$I-LDL compared with the control cells. Therefore, cotransfection of the above proteins together with the human LDL receptor was performed, and the accumulation of $^{125}$I-LDL after 24 h was compared with cells that were transfected with the LDL receptor alone. LDLR overexpression resulted in the 2.4-fold accumulation of LDL, whereas cotransfection of LDLR together with annexin VI or Rab5 resulted in a 2.7–2.8-fold increase of $^{125}$I-LDL accumulation compared with the $\beta$-galactosidase-expressing controls. We then compared the kinetics of $^{125}$I-LDL internalization for the control ($\beta$-galactosidase) and for cells transfected with the LDLR or cotransfected with annexin VI and LDL-R (Fig. 3A). These results demonstrate increased internalization of $^{125}$I-LDL in cells cotransfected with annexin VI and LDLR compared with LDLR-transfected controls. Furthermore, LDL endocytosis is stimulated in a dose-dependent manner (approximately 1.5–2.0-fold) in cells cotransfected with increasing amounts of annexin VI and constant amounts of LDL receptor (Fig. 3B). Subsequent Western blot analysis in transfected CHO cells demonstrated that equivalent amounts of LDL receptor were expressed in cells cotransfected with the LDLR and annexin VI compared with cells transfected with LDLR only (Fig. 3C). Taken together, these results suggest that annexin VI plays a stimulatory role in LDL receptor-mediated endocytosis.

In addition, overexpression of annexin VI and Rab5 alone did not result in a significant alteration of $^{125}$I-LDL degradation compared with $\beta$-galactosidase-transfected control (Fig. 4). After 24 h, LDLR overexpression increased degradation of LDL 1.8-fold compared with $\beta$-galactosidase-expressing control cells (Fig. 4). However, when annexin VI or Rab5 were coexpressed with the LDLR, degradation of $^{125}$I-LDL was stimulated 3–3.4-fold after 24 h compared with the control cells (Fig. 4). Taken together, these results indicate that the stimulation of LDL internalization (Fig. 3, A and B) and degradation (Fig. 4) reflects an increased internalization and intracellular processing of LDL, since LDLR expression levels are not elevated in cells cotransfected with annexin VI or Rab5 together with the LDLR compared with cells transfected with LDLR only.

In order to identify specific functions of annexins during the receptor-dependent internalization of ligands, we then compared internalization and degradation of LDL in annexin VI- and annexin II-overexpressing CHO cells. Similarly to the experiments described above annexin VI, annexin II, and Rab5 overexpression alone did not result in a significant alteration of $^{125}$I-LDL internalization (data not shown), which correlates with the observation that Rab5 induces internalization of Tf only when coexpressed with the Tf receptor (41). Although the effect of annexin VI on LDL uptake is modest considering the vast overexpression of annexin VI in the transfected cells (see Figs. 2, 5, and 7), coexpression of annexin VI and the LDL receptor significantly stimulated $^{125}$I-LDL internalization by...
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50% compared with cells expressing the LDL receptor alone (p < 0.0005) in seven independent experiments with triplicate samples. In contrast, when annexin II was coexpressed with the LDL receptor, no significant change in $^{125}$I-LDL endocytosis was observed. In these experiments, coexpression of annexin VI and the LDL receptor increased $^{125}$I-LDL degradation by 70% compared with LDL receptor-expressing cells (p < 0.003). Coexpression of annexin II together with the LDL receptor did not significantly increase the rate of $^{125}$I-LDL degradation compared with LDLR-transfected controls.

In order to analyze single transfected cells, we incubated CHO cells with DII-labeled LDL for 5 min at 37 °C that were transfected with either annexin VI, LDLR, or LDLR cotransfected with annexin VI (Fig. 5). Consistent with the previous findings annexin VI overexpressing cells did not reveal any enhanced DII-LDL uptake compared with the neighboring nontransfected cells (Fig. 5, a–c). Similar results were obtained with annexin II-overexpressing cells (data not shown). The expression of the LDLR (Fig. 5, d–f) alone as well as the overexpression of annexin VI together with the LDL receptor (Fig. 5, g–h) resulted in massive accumulation of DII-LDL in the transfected cells. These results confirm that LDLR activity was not affected in nontransfected neighboring cells.

Finally, in order to determine a possible stimulatory effect of annexin VI on fluid phase endocytosis cells transfected with annexin VI, annexin II, or Rab5 were incubated with radiolabeled HRP for 120 min at 37 °C. In three independent experiments with triplicate samples, the amount of internalized $^{125}$I-HRP was determined. In these experiments, overexpression of annexin VI (31.1 ± 16.7 ng of HRP/mg of cell protein), annexin II (30.3 ± 14.6 ng/mg) or Rab5 (30.7 ± 14.9 ng/mg) did not significantly affect the accumulation of $^{125}$I-HRP compared with β-galactosidase-transfected controls (32.2 ± 18.9 ng/mg). Furthermore, cells transfected with LDL receptor alone or cotransfected with annexin VI did not demonstrate increased HRP accumulation when incubated with $^{125}$I-HRP in lipoprotein-deficient medium (data not shown). These results indicate that the increased internalization of $^{125}$I-LDL or $^{125}$I-transferrin in annexin VI and LDLR- or transferrin receptor-cotransfected cells (Figs. 2–5) was not due to unspecific internalization mechanisms but rather reflects increased receptor-mediated endocytosis of ligands.

Annexin VI Is Involved in the Trafficking of LDL to the Prelysosomal Compartment—The increased degradation of $^{125}$I-LDL in annexin VI- and LDLR-overexpressing cells (Fig. 4) indicates a potential role of annexin VI at later stages of the endocytic pathway. Due to the bright diffuse staining throughout the cell, it was difficult to characterize the intracellular localization of annexin VI overexpression by immunofluorescence. Therefore, the intracellular localization of annexin VI was analyzed using subcellular fractionation by sucrose gradients.

Fig. 6A shows the distribution of annexin VI in fractions collected from a sucrose gradient (8–40%, w/v) designed to separate early and late endosomal fractions from plasma membrane and other membranes (40). Annexin VI was detected, by Western blotting, all along the gradient, being most abundant in the bottom region, where heavy membranes (e.g. plasma membranes) are found. Dynamin, a GTP-binding protein that is found at the plasma membrane and in clathrin-coated vesicles and that has recently been described to interact with annexin VI (20), is present predominantly in the bottom fractions of this gradient (Fig. 6A). In contrast, the early endosomal marker Rab4 peaks in the center fractions of the gradient (fractions 5–8). The prelysosomal content of the upper fractions (fractions 3 and 4) was confirmed by their increased β-hexosaminidase activity (Ref. 41; Fig. 6C).

To assess the possible involvement of annexin VI in the endocytic pathway, we induced endocytosis by $^{125}$I-LDL administration for 5 and 120 min (Fig. 6B). The identification of Rab4 and dynamin in the same fractions in control cells (0 min) and after 120-min LDL administration (Fig. 6A) as well as the almost identical profile of β-hexosaminidase activity throughout the sucrose gradient at all time points (Fig. 6C) confirmed the stability and reproducibility of this subcellular fractionation. After 5-min $^{125}$I-LDL administration, internalized $^{125}$I-LDL is mainly found in the early endosomal fractions of the gradient (Fig. 6B). Subsequently, 120 min after administration of radiolabeled LDL, $^{125}$I-LDL accumulated in the late endosomal fractions (Fig. 6B, fractions 3 and 4). In parallel, a shift of Annexin VI to these prelysosomal fractions (peak at 20–22% sucrose) was detected (Fig. 6A).

To confirm the biochemical results, we compared the distribution of annexin VI, by confocal microscopy, with Rab4, Rab5,
LDLR, and Lamp1 (Fig. 7) in stably transfected CHO cells overexpressing annexin VI. In control cells (0 min) and as mentioned above, the pattern of distribution of annexin VI in CHO cells was largely diffuse throughout the cell (Fig. 7, upper panel). However, after the administration of LDL (120 min), the staining becomes more punctate (vesicular) and more intense in the perinuclear region of the cells (Fig. 7, lower panel). In contrast, the intracellular distribution of the other proteins Rab4, Rab5, LDLR, and Lamp1 is not affected in such a manner after LDL administration (Fig. 7; compare upper and lower panels at 0 and 120 min). Thus, overexpression of annexin VI results in a ligand-induced trafficking of annexin VI as a consequence of uptake and trafficking of ligands that use the receptor-mediated endocytosis pathway.

**DISCUSSION**

In this study, we have demonstrated that overexpression of annexin VI together with the LDL receptor stimulates endocytosis of low density lipoproteins, whereas fluid phase endocytosis and the recycling of transferrin were not affected in annexin VI-overexpressing cells. After the internalization of LDL, the overall intracellular distribution of annexin VI proteins throughout the cell was shown to redistribute in order to concentrate in late endosomal fractions. These ligand-induced translocations of annexin VI correlate with recent biochemical studies in smooth muscle cells, demonstrating that annexin VI may undergo redistributions between different cellular compartments in a calcium and concentration-dependent manner (49).

Most of our knowledge on annexins in membrane traffic is based on its biochemical identification in isolated membrane fractions, cell-free membrane fusion assays, and colocalization with endocytic markers, but only a limited number of experiments have analyzed the function of annexins using transfection systems (1, 3, 4). Overexpression of annexin VI or Rab5 alone, which served as a positive control in our experiments, did not alter LDL internalization rates, possibly indicating that the low number of receptors on the cell surface are rate-limiting in these cells. The necessity for high receptor expression in this experimental approach was demonstrated in a number of experiments for Rab5, a member of the Ras-related family of small GTPases, which can stimulate Tf internalization when cotransfected with the Tf receptor (32, 42). Since annexin VI does not affect the fluid-phase endocytosis of HRP, it is unlikely that the increase of LDL internalization occurs via non-coated-pit-dependent mechanisms. Therefore, the stimulatory effect on ligand internalization rates in annexin VI- and LDL receptor-overexpressing cells is most likely due to increased internalization and budding of coated vesicles. These results correlate with the partial localization of annexin VI at the plasma membrane in transfected CHO cells, its co-purification with dynamin (20), and the stimulatory effect of purified annexin VI on the detachment of coated pits from purified plasma membranes in vitro (16, 17). Although Smythe et al. (44) have questioned a role of annexin VI in endocytosis in human A431 squamous carcinoma cells, these findings could be explained by the development of an annexin VI-independent mechanism to allow coated vesicle formation (17).

In contrast to the stimulatory effect of annexin VI on receptor-mediated endocytosis, annexin VI does not seem to participate in the regulation of Tf receptor recycling. Neither overexpression of annexin VI alone nor cotransfection with the Tf receptor significantly affected the rates of Tf recycling in CHO cells. Furthermore, immunofluorescence analysis demonstrates the absence of annexin VI in the Tf receptor recycling compartment in these cells. Similar negative results have also been described by Smythe et al. (44) and indicate that annexin VI rather plays a specific role in the internalization but is not actively involved in the recycling of endosomes to the cell surface. Consistent with these findings, only 2% of the annexin VI was found in tubular endocytic structures in Lowicryl sections of rat liver. When isolated fractions were examined by electron microscopy, most of annexin VI was identified in the vacuolar structures of the early/sorting endosomes (compartment of uncoupling of receptor and ligands, or CURL) or in the vesicles of receptor-recycling compartment but very little in the tubular extensions where receptors for recycling concentrate (14). In addition, in normal rat kidney cells little colocalization was detected between annexin VI and fluorescein isothiocyanate-transferrin 30 min after internalization (15).

Annexin II, the other member of the annexin family analyzed here, is located at the plasma membrane and in the early endocytic compartment in a number of cell types (1, 45, 46). It promotes the homotypic fusion of early endosomal membranes in vitro (7) and has therefore been implicated in early endocytic
events. However, upon transient transfection of annexin II constructs into CHO cells, we did not observe any stimulatory effect on the LDL or transferrin endocytosis, even when cotransfected with either the LDL or transferrin receptor. Since annexin II forms a stable heterotetrameric protein complex with p11, a protein of the S-100 family (47), limiting concentration of p11 protein in annexin II-transfected CHO cells could result in low amounts of active annexin II\textsubscript{p11}, complex.

When cotransfected with the LDL receptor, annexin VI also stimulates the degradation of LDL. Sucrose gradient and immunofluorescence analysis indicate that annexin VI is recruited to early endosomes after LDL internalization and most likely remains associated with LDL-containing vesicles entering the prelysosomal compartment. This ligand-induced shift of annexin VI into late endosomal fractions suggests that annexin VI-mediated interactions with cytoskeleton proteins not only participate in the budding of coated pits but also seem to play an important role during the delivery of ligands to lysosomes. In a different experimental approach, microinjection of a dominant negative annexin VI mutant not only reduced endocytosis of LDL but also resulted in the mislocalization of LDL-positive vesicles (17), indicating that transient interactions of annexin VI with the cytoskeleton guide endocytic vesicles along intracellular routes to the prelysosomal compartment. The partial localization of endogenous annexin VI in prelysosomes of normal rat kidney fibroblasts and polarized WIF-B hepatoma cells (15) further supports this observation. At the cell surface, annexin VI is thought to interact with membrane-bound spectrin and calpain to allow budding of clathrin-coated vesicles (17, 18, 48). In addition, annexin VI has recently been demonstrated to co-immunoprecipitate with dynamin, a GTPase essential for endocytic vesicles pinching off the plasma membrane, in endocytic and transferrin-positive vesicles (20). Therefore, similar annexin VI-dependent mechanisms could play a role in directing ligands from the cell surface to lysosomes.

Acknowledgments—We are grateful to W. Tauscher for excellent technical assistance. We thank Dr. V. Gerke, Dr. T. Jentsch, Dr. F. Schnieders, and Dr. Braulke for generously providing antibodies, recombinant plasmins, and technical advice.

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