The Subcellular Distribution of Early Endosomes Is Affected by the Annexin II_2p11_2 Complex

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Abstract. The tyrosine kinase substrate annexin II is a member of a multigene family of Ca^{2+} and lipid-binding proteins which have been implicated in a number of membrane-related events. We have analyzed the subcellular distribution of annexin II in relation to other cellular components in normal and specifically manipulated MDCK cells. In a polarized monolayer of MDCK cells annexin II and its cellular ligand p11 are restricted almost exclusively to the cortical regions of the cells which also contain peripheral early endosomes. Treatment of the polarized cells with low Ca^{2+} medium leads to a disintegration of the cortical cytoskeleton and a translocation of both, the annexin II_2p11_2 complex and early endosomes, to the cytoplasm. A similar translocation which is however specific for the annexin II_2p11_2 complex and early endosomes and does not affect other elements of the cell cortex is observed in cells expressing a *trans*-dominant annexin II–p11 mutant. This chimeric mutant protein causes the aggregation of endogenous annexin II and p11 and the simultaneous detachment of early endosomes from the cell periphery resulting in the binding of the early endosomes but no other components of the endocytotic or biosynthetic pathways to the annexin II/p11 aggregates. The specificity of this effect argues for the association of the annexin II_2p11_2 complex with early endosomes and suggests that this association contributes to establish the peripheral localization of early endosomal structures.

INTERACTIONS of cellular membranes with each other and with structural elements of the cell are required for both membrane traffic and the establishment and maintenance of cell topology. The annexins are a protein family thought to be involved in some of these multiple interactions (Creutz, 1992; Gruenberg and Emans, 1993). Of the 14 different annexins identified so far 10 are found in mammals. Unique members and homologues of mammalian annexins have been isolated from *Drosophila melanogaster*, *Hydra vulgaris*, higher plants, and *Dictyostelium discoideum*, respectively, indicating a broad distribution of annexins in eucaryotes (for review see Creutz, 1992). The association of annexins with cellular membranes most likely reflects itself in the biochemical hallmark of this protein family, the Ca^{2+}-dependent binding to phospholipids. The Ca^{2+}/lipid-binding sites of the annexins reside in a protease resistant core domain whose principal building block is a segment of 70–80 amino acids repeated four or eight times in the individual members. In the linear sequence the core is preceded by a protease sensitive NH_2-terminal region which is highly variable in length and sequence in the different annexins (for review see Moss, 1992).

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Annexin II, the member of the protein family described in this study, has attracted considerable interest since its NH_2-terminal domain of 30 amino acids has phosphorylation sites for several signal transducing protein kinases. Most notably, Tyr 23 and Ser 25 are phosphorylated by the src tyrosine kinase and protein kinase C, respectively (Glenney and Tack, 1985; Gould et al., 1986; Johnsson et al., 1986). In addition, the 14 NH_2-terminal amino acids including the NH_2-terminal acetyl group bind, upon forming an amphiphathic α-helix to a protein with an apparent molecular weight of 11 kD (Johnsson et al., 1988; Becker et al., 1990). This cellular ligand of annexin II, termed p11, belongs to the S100 family of EF hand type Ca^{2+}-binding proteins but has lost the ability to bind Ca^{2+} ions due to crucial amino acid deletions and substitutions in the two EF hand loops (Gerke and Weber, 1985*a*). p11 itself forms a tight dimer in which each polypeptide chain contains an annexin II-binding site (Weber, 1992). Complex formation thus leads to an annexin II_2p11_2 heterotetramer which is stabilized by hydrophobic interactions and has a dissociation constant of less than 30 nM (Johnsson et al., 1988). The annexin II_2p11_2 complex, which has an increased affinity for Ca^{2+} and phospholipid when compared to monomeric annexin II (Powell and Glenney, 1987), is associated with the cytoplasmic face of the plasma membrane and the submembranous cytoskeleton in cultured fibroblasts (Greenberg and Edelman, 1983; Zokas and Glenney, 1987; Osborn et al., 1988; Semich et al., 1989).

Monomeric annexin II, on the other hand, seems to be a cytosolic protein and is extracted from cells upon detergent treatment in the presence of Ca^{2+} indicating that the cytoskeletal association of annexin II depends on the formation of the annexin II_2pI1_2 heterotetramer (Zokas and Glenney, 1987; Thiel et al., 1992).

Despite the detailed knowledge of its biochemical and structural properties the actual biological role of annexin II remains unclear. Due to the ability of the annexin II_2pI1_2 complex to bind phospholipids and cytoskeletal elements in a Ca^{2+}-dependent manner a cross-linking function in the cell cortex has been postulated (Glenney et al., 1987; Gerke, 1989*a*). Moreover the complex has been implicated in regulated exocytosis as purified annexin II_2pI1_2 partially restores the Ca^{2+}-dependent secretory activity in digitonin permeabilized chromaffin cells (Ali et al., 1989; Sarafian et al., 1991). Recently, annexin II has also been linked to the endocytotic pathway since it is present on early endosomal membranes and is efficiently transferred from one endosomal membrane to another in an in vitro fusion assay (Emans et al., 1993). Two other annexins seem to be involved in endocytotic events as well although at different steps of the pathway. While annexin VI has been implicated in coated vesicle budding, i.e., very early in receptor-mediated endocytosis (Lin et al., 1992), annexin I, a substrate of the EGF receptor kinase (Fava and Cohen, 1984), is associated with multivesicular bodies, later intermediates of the endocytotic pathway (Futter et al., 1993). Based on their biochemical properties other functions have been proposed for different members of the annexin family. These include the regulation of protein kinase C, antiinflammatory, and anticoagulant activities, an enzymatic role in inositol metabolism, and the regulation and formation of ion channels (for review see Moss et al., 1991; Creutz, 1992).

Since no direct experimental evidence as to the precise role of annexins in intact living cells has been obtained so far, we have analyzed the function of annexin II by manipulating its intracellular distribution. Here we describe the impact of these manipulations on different cellular structures and the identification of early endosomes as the cellular elements specifically affected by an altered annexin II/pI1 distribution.

Materials and Methods

Cell Culture

MDCK I cells, kindly provided by Dr. Kai Simons (EMBL, Heidelberg), were grown in DME (GIBCO-BRL, Gaithersburg, MD) supplemented with 10% FCS (Boehringer Mannheim Biochemicals, Indianapolis, IN) at 37°C and 5% CO_2 in humidified atmosphere. Polarized monolayers were obtained by keeping the MDCK cells at a density of 4×10^5 cells /cm^2 on 0.3-μm pore size polycarbonate filters (Costar Corp., Cambridge, MA) for 3 to 5 d. 208F rat fibroblasts were grown in DME containing 10% FCS. For low-calcium medium experiments MDCK cells were kept for 3–5 d in DME plus 10% FCS on polycarbonate filters and subsequently transferred for 2 h into S minimal essential medium Jokliks modification (GIBCO-BRL) without serum (low-calcium medium, Ca^{2+}–concentration <5 μM).

DNA Constructs

Transient transfection experiments employed the mammalian expression vector pCMV5 which uses the strong cytomegalovirus promoter to drive expression of a foreign gene (Andersson et al., 1989). In addition, pLK neo (Hirt et al., 1992; kindly provided by Dr. Robert Hirt, University of Lausanne, Lausanne, Switzerland), which contains the dexamethasone inducible MMTV promoter and the neo^r gene in a SV-40 transcription unit, was used to generate G418 resistant cell lines stably carrying the plasmid DNA. The expression plasmid for the transferrin receptor was generated by inserting the human transferrin receptor (hTfR)[1] cDNA (Zerial et al., 1986; kindly provided by Dr. Marino Zerial, EMBL) into the unique EcoRI site of the pCMV5 polylinker (pCMV hTfR). Correct orientation of the recombinant plasmid was determined by restriction analysis.

To construct the chimeric annexin II-pI1 gene, suitable restriction sites were introduced into the cDNAs encoding human pI1 and human annexin II by site directed mutagenesis. A 581-bp EcoRI/HindIII fragment of the human pI1 cDNA containing the entire protein coding region plus the 5'- and most of the 3'-nontranslated sequences (Kube et al., 1991; Harder et al., 1992) was cloned into M13 mp18. A uracil containing ssDNA preparation of this clone served as template in a site-directed mutagenesis reaction (Kunkel et al., 1985) which used oligonucleotide XM3 (5'-CGTGTTCCA TTTGAGATGGATCCTTGGTGTGGTCCGTTG-3'). XM3 covers nucleotides (nt) 86-124 of the human pI1 cDNA but carries mutations introducing a unique BamHI site at nt positions 102-107 (underlined), i.e., at the site of the translational start codon. After mutagenesis and transformation, mutant cDNAs were identified by restriction analysis of the recombinant M13 clones with BamHI. An EcoRI/XbaI cDNA fragment covering the coding region plus most of the 5'- and 3'-nontranslated sequence of the human annexin II cDNA (Thiel et al., 1991) was used as template in a site-directed mutagenesis reaction with oligonucleotide XM4. XM4 (5'-GACCCATAT GCACTTAGATCTGTAGAGTGATCACC-3') covers nt 84-118 of the human annexin II cDNA and generates a unique BglII restriction site at nt positions 98-103 (underlined), i.e., after the sequence encoding the first 18 amino acids. The mutant cDNAs (annexin II and pI1, respectively) were digested with EcoRI/BglII or BamHI/HindIII, respectively, and the fragments encoding the NH_2-terminal 18 amino acids of annexin II (EcoRI/BglII) and the entire pI1 coding region (BamHI/HindIII), respectively, were isolated and ligated via their compatible BglII and BamHI overhangs. The resulting construct encodes a chimeric protein comprising the 18 NH_2-terminal amino acids of annexin II fused to the complete pI1 molecule with its starting methionine changed to an aspartate residue. The chimeric gene (referred to as XM) was sequenced and cloned into the expression vectors pCMV 5 (pCMV XM) and pLK neo (pLK XM), respectively, using standard procedures (Sambrook et al., 1989). For transfection the plasmid DNA was purified on Qiagen columns (Diagen, Düsseldorf, FRG) following the manufacturer's protocol.

Transfection and Generation of Stable Cell Lines

Approximately 2×10^6 early passage MDCK cells were trypsinized and washed once in 30 ml Hepes buffer (20 mM Hepes, pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM D-glucose). Subsequently, the cell pellet obtained by gentle centrifugation (6 min, 600 rpm, 20°C) was resuspended in 180 μl Hepes buffer. The DNA (20 μg of pCMV XM supercoiled plasmid DNA and/or 10 μg of pCMV hTfR DNA for transient expression experiments) was ethanol precipitated, thoroughly washed in 70% ethanol, solubilized in 50 μl Hepes buffer, and mixed with the cell suspension (final volume, 250 μl). Electroporation was performed in 0.4-cm cuvettes using a Bio-rad electroporation device at 250 μF and 330 V (Bio-Rad Laboratories, Cambridge, MA). After electroporation cells were kept for 10 min at 20°C, plated on 5-cm tissue culture dishes and allowed to recover for 2 h at 37°C in full medium. Subsequently, the surviving cells were plated on glass coverslips or on polycarbonate filters (Costar Corp.). 208 F rat fibroblasts were transfected with 20 μg of pCMV XM DNA following the Ca–phosphate technique (Graham and van der Eb, 1973).

To establish MDCK cell lines stably carrying the chimeric annexin II-pI1 gene, MDCK cells were transfected as described with 20 μg of XbaI-linearized pLK XM plasmid. 12 h after transfection the surviving cells were selected in full medium containing 1 mg/ml G418 (GIBCO-BRL). Resistant colonies were expanded and analyzed for inducible expression of the chimeric annexin II-pI1 protein after 16 h of cultivation in the presence of 10^{-6} M dexamethasone (Sigma Immunochemicals, St. Louis, MO). For Western blot analysis the dexamethasone-treated and -untreated control cells from a 5-cm tissue culture dish were lysed in 500 μl hot SDS sample buffer (Laemmli, 1970). The extracted polypeptides were separated in 16.5% tricine polyacrylamide gels (Schägger and von Jagow, 1987), trans-

1. *Abbreviations used in this paper:* hTfR, human transferrin receptor; nt, nucleotide; TfTx, Texas red-conjugated human transferrin.

ferred to nitrocellulose membranes, and probed with a mouse monoclonal p11 antibody (H21; Osborn et al., 1988), which recognizes both the endogenous canine p11 and the annexin II-p11 chimera. Incubation with the primary H21 mAb (10 μg/ml) was carried out in TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 0.1% Triton X-100 and 1% BSA for 1 h at room temperature and followed by treatment with peroxidase-coupled secondary goat anti-mouse antibodies (10 μg/ml in TBS, 0.1% Triton X-100, 1% BSA; 45 min at room temperature). After washing with TBS containing 0.1% Triton X-100, immunoreactive bands were detected using the ECL chemoluminescence system (Amersham Buchler, Braunschweig, FRG) following the manufacturer's instructions.

Antibodies

Annexin II was detected using an affinity-purified polyclonal rabbit antibody (at 50 μg/ml; Gerke and Weber, 1984) or a tissue culture supernatant of the mouse mAb H28 (Osborn et al., 1988). p11 was labeled with the mouse mAb H21 used as an undiluted tissue culture supernatant (Osborn et al., 1988). Affinity-purified polyclonal antibodies were used for staining of fodrin (Glenney et al., 1982) and actin (kindly provided by Dr. Mary Osborn, Max Planck Institute, Göttingen, Germany). The mouse mAb against the human transferrin receptor (B3/25) was purchased from Boehringer Mannheim GmbH (Mannheim, FRG). A polyclonal goat antibody directed against the large mannose 6-phosphate receptor (Causin et al., 1988) was obtained from Dr. Annette Hille (University of Göttingen). The rat mAb DECMA 1 reacting with uvomorulin/E cadherin was a gift from Dr. Rolf Kemler (Max Planck Institute, Freiburg, Germany). The ER was stained with a mouse mAb (1D3) directed against a synthetic peptide which contained the KDEL retention signal and corresponded to the COOH-terminal PDI tail (a gift from Dr. Stephen Fuller, EMBL, Heidelberg). A rabbit polyclonal antibody against galactosyltransferase was used as a Golgi marker (Hiller and Weber, 1982; kindly provided by Dr. Gerhard Hiller, Boehringer Mannheim GmbH). Immunofluorescence microscopy used FITC-coupled goat anti-mouse (Cappel Laboratories, Cochranville, PA) or rhodamine-coupled goat anti-mouse antibodies (Dianova, Hamburg, FRG) to stain primary mouse antibodies. Rabbit antibodies were decorated with FITC-coupled swine anti-rabbit (Orion, Helsinki, Finland) or rhodamine-coupled goat anti-rabbit antibodies (Dianova, Hamburg, FRG). Double immunofluorescence with goat and mouse antibodies was performed using Texas red-coupled donkey anti-mouse and DTAF-coupled donkey anti-goat antibodies (both Dianova, Hamburg, FRG). All secondary antibodies were tested in control experiments by omitting the primary antibody and showed neglectable fluorescence signals on the different cells studied (one representative example of these negative controls is given in Fig. 2 *C*).

Transferrin Labeling

Before labeling MDCK cells were incubated in serum-free medium at 37°C for 1 h. Subsequently, Texas red-coupled transferrin (Molecular Probes, Eugene, OR) was added at 20 μg/ml. MDCK cells growing in Costar filter chambers received the transferrin from the basolateral side. After 1 h of uptake the cells were washed twice in ice cold PBS, fixed for 10 min with 3.7% formaldehyde in PBS, and then analyzed by immunofluorescence.

Immunofluorescence and Confocal Laser Scanning Microscopy

Cells grown on glass coverslips were subjected to immunofluorescence analysis as described (Osborn et al., 1988). MDCK cells cultivated on Costar polycarbonate filters were washed briefly in PBS, fixed for 10 min with 3.7% formaldehyde in PBS, and subsequently permeabilized with ice cold methanol (−10°C). Small pieces of the polycarbonate filters were excised, placed on a microscope slide with the cells facing upwards, and incubated with 40 μl of the primary antibody (50 μg/ml) in a moist chamber at 37°C for 1 h. Subsequently the filters were washed 3 × 5 min in PBS, incubated with the respective secondary antibodies, washed again, placed between four drops of acrylic nail polish, and mounted in Moviol 4-88 (Hoechst, Frankfurt, FRG). Conventional microscopy was performed using a Zeiss Axiophot photomicroscope (Carl Zeiss, Oberkochen, Germany). Photography used a Kodak Tri X Pan 400 film (Eastman Kodak Co., Rochester, NY).

Confocal images of MDCK cells grown on polycarbonate filters were taken using a Zeiss laser scanning microscope. FITC and DTAF were exited using the argon laser 488-nm-long line whereas rhodamine and Texas red were exited at 514 nm. The emission signals were filtered with a Zeiss 520-565 nm filter (FITC and DTAF emission) or with a long pass 595 nm filter (rhodamine and Texas red signals).

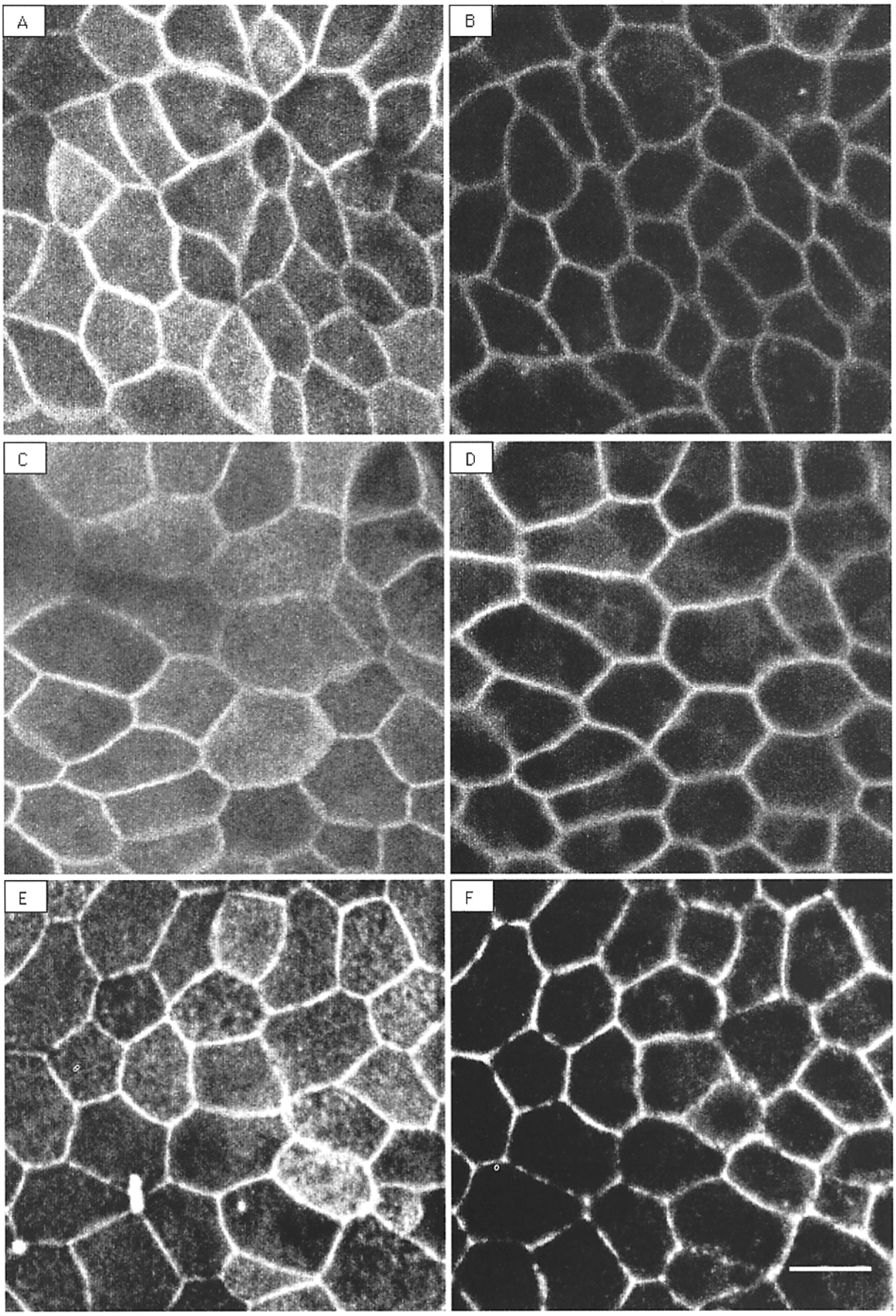
Results

Characterization of the Intracellular Distribution of Annexin II and p11 in MDCK Cells

We analyzed the intracellular distribution of annexin II and p11 in MDCK cells cultured under different conditions by employing monoclonal annexin II (H28) and p11 (H21) antibodies, respectively, in conventional immunofluorescence and confocal laser scanning microscopy. In all MDCK cell populations analyzed the staining pattern of annexin II is virtually identical to that of p11 indicating that formation of the annexin II-p11 complex is constitutive in the MDCK cell line. This is corraborated by differential extraction experiments. In buffers containing 0.5% Triton X-100 and 0.5 mM Ca^{2+} monomeric annexin II is solubilized and thus separated from the insoluble, p11-complexed form (Zokas and Glenney, 1987; Thiel et al., 1992). While in other cells, e.g., fibroblasts, up to 50% of annexin II can be in the monomeric form (Zokas and Glenney, 1987; Erikson et al., 1984), in MDCK cells >90% of annexin II and p11 are found in the heterotetrameric complex, i.e., the Triton-insoluble fraction (data not shown).

In fully polarized MDCK cells grown on microporous filter supports (Fuller et al., 1984) annexin II and p11 are restricted almost exclusively to the submembranous regions underneath the apical and basolateral membranes (Fig. 1, *A–D*), whereas nonpolarized or not completely polarized MDCK cells contain a significant proportion of annexin II and p11 in the cytoplasm (Fig. 2 *A*, see below). In fully polarized MDCK cells the annexin II/p11 distribution shows some resemblance to the actin pattern. While most actin is also found underneath the apical and basolateral membranes there is additional staining in the cytoplasm probably resulting from microfilament structures (Fig. 1, *D* and *E*). In contrast, fodrin, another protein of the cell cortex, is restricted to the basolateral region thus showing a distribution which argues against a direct association of annexin II with fodrin at least in the apical cortex (data not shown; Nelson and Veshnock, 1986).

Previous work has shown that the transferrin receptor resides in the basolateral plasma membrane of polarized MDCK cell and that the receptor cycles between early endosomes and the plasma membrane in most cells studied so far (for review see Parton, 1991). We made use of this transferrin receptor cycle first to visualize basolateral early endosomes in the polarized monolayers and then to compare their distribution to the annexin II/p11 labeling. Therefore we used transient transfection to overexpress a human transferrin receptor in MDCK and monitored its distribution either in immunofluorescence analysis with a monoclonal antibody or directly after incubating the cells with Texas red-conjugated human transferrin (TfTx). Both staining methods identify the transferrin receptors in the plasma membrane and the transferrin positive endosomes. In nonpolarized or not completely polarized MDCK cells, structures containing the transferrin receptor are often visible deeper in the cytoplasm (Fig. 2 *B*). Interestingly, these structures are frequently also stained with annexin II antibodies (Fig. 2 *A*). Polarized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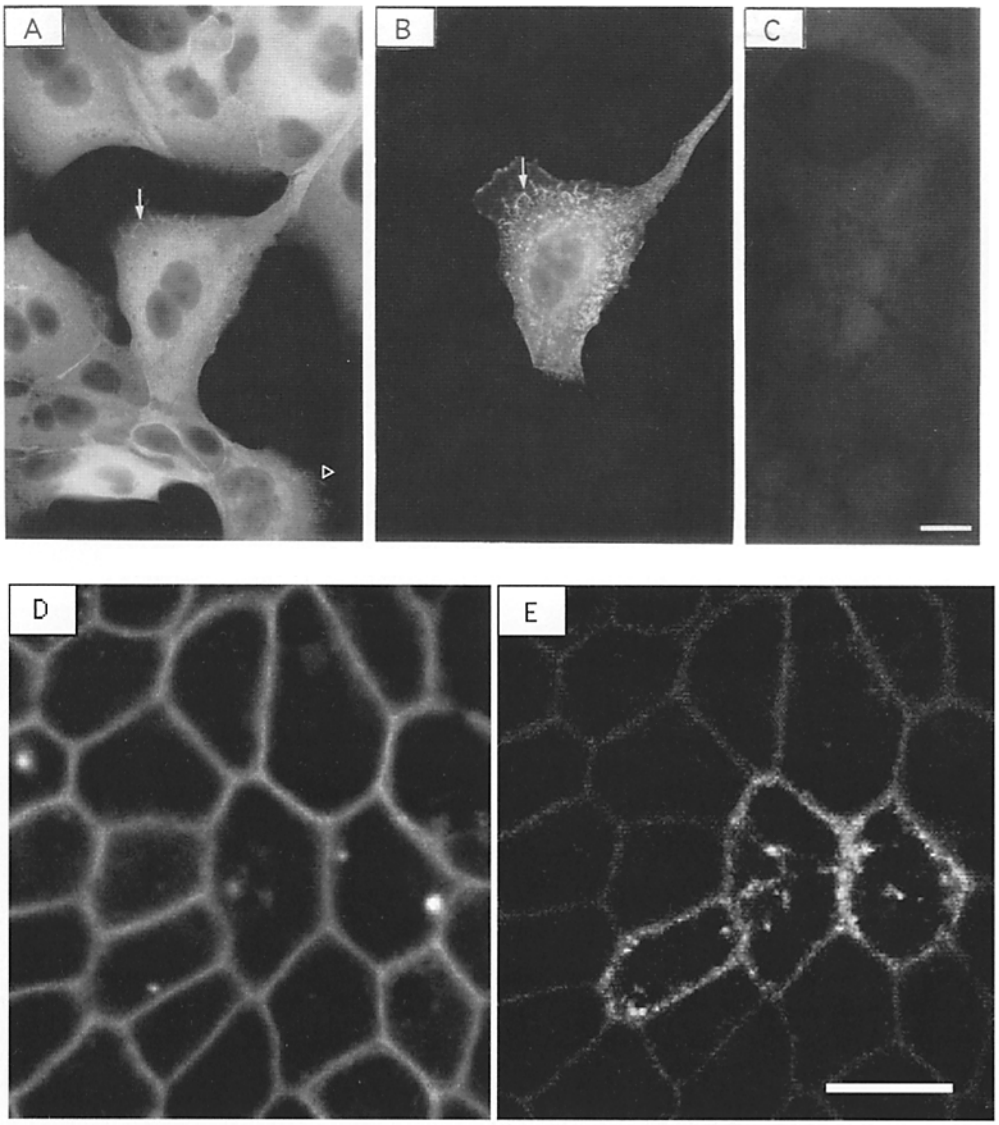

Figure 2. Co-localization of annexin II and transferrin-accessible endosomes in nonpolarized and polarized MDCK cells. Cells were transfected with an expression construct containing the human transferrin receptor (hTfR) cDNA. Early endosomes were visualized by incubation with 20 μg/ml Texas red–conjugated transferrin (TfTx; *B* and *E*) and annexin II was labeled using the mAb H28 (*A*) or an affinity-purified polyclonal rabbit antibody (*D*). The primary antibodies were decorated with the respective FITC-coupled secondary antibodies. A representative staining with the FITC-conjugated anti-mouse antibody alone, i.e., by omitting the primary antibody, is shown in *C*. Equivalent negative results were obtained with all other secondary antibodies used in this study (not shown). Transfected cells cultivated for 2 d on a glass coverslip, i.e., nonpolarized MDCK cells, are depicted in *A–C*. Annexin II (*A*) shows a submembranous localization and a relatively even cytoplasmic distribution which is not seen in the polarized cells. In addition, elongated endosomal structures, which are present in the cytoplasm and labeled with TfTx (*B*), are stained with the annexin II antibodies (*arrows*, *A* and *B*). Similar annexin II–positive structures are also visible in untransfected cells not overexpressing the human transferrin receptor (*arrowhead*, *A*). *D* and *E* show the distribution of annexin II and early endosomes, respectively, in MDCK cells which were allowed to polarize for 4 d after transfection. A confocal section was taken in a basolateral supranuclear plane showing three adjacent cells expressing the exogenous hTfR. Note that most of the intracellular TfTx is found in the submembranous region, thus co-localizing with the cortical annexin II. Some TfTx-accessible structures are visible deeper in the cytoplasm. A fraction of these structures is also labeled with the annexin II specific antibody. Bars: (*A–C*) 20 μm; and (*D* and *E*) 10 μm.

MDCK cells, on the other hand, contain most of their transferrin receptor-positive early endosomes in the submembranous region (Fig. 2 *E*) which also harbors the vast majority of the annexin II_2p11_2 complex (Figs. 1, *A–D* and 2 *D*). Some structures located deeper in the cytoplasm are also labeled with the Texas red–conjugated transferrin. A fraction of these intracellular structures also contains annexin II which is otherwise confined to the submembranous cortex of the fully polarized cells. This fraction is occasionally visible as dots in the MDCK monolayers shown in Fig. 1 and

Figure 1. Intracellular distribution of annexin II, p11, and actin in polarized MDCK cells. Confocal images of the immunofluorescence were obtained from a MDCK I monolayer allowed to polarize for 5 d. *A*, *C*, and *E* show an optical section through the apical plane whereas basolateral sections of the same cells are shown in *B*, *D*, and *F*. The fixed cells were stained with the annexin II–specific mAb H28 (*A* and *B*) and with mAb H21 which recognizes canine p11 (*C* and *D*), respectively. Actin was visualized with a polyclonal rabbit antibody (*E* and *F*). The primary antibodies were decorated with the respective rhodamine-coupled secondary antibodies. Pictures were taken with a near minimum pinhole size using excitation and emission filtering as described in Materials and Methods. Note that the annexin II and the p11 staining is restricted almost exclusively to the region underneath the apical and basolateral membranes and resembles the cortical actin distribution. Bar, 10 μm.

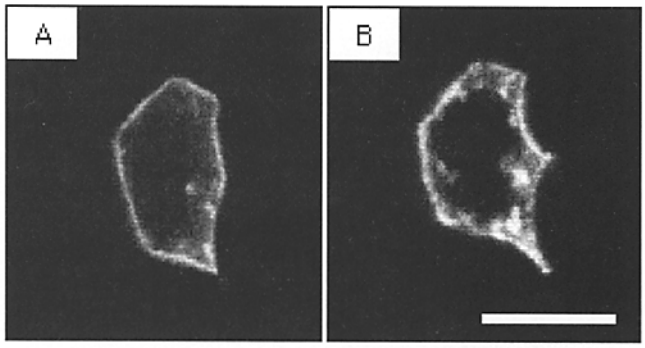

Figure 3. Distribution of Texas red–conjugated transferrin in polarized MDCK cells overexpressing the human transferrin receptor. The cells were transfected with the hTfR expression construct (pCMVhTfR) and then allowed to polarize for 4 d on filter support. Subsequently, the cells were allowed to internalize TfTx for 20 min. Confocal sections in the apical (*A*) and basolateral (*B*) plane were taken as described in Materials and Methods. Bar, 10 μm.

is also seen in the anti-annexin II/TfTx double-labeling experiment in Fig. 2 (*D* and *E*). Taken together these observations are in line with biochemical and immunoelectron microscopical results showing an association of annexin II with early endosomal membranes (Emans et al., 1993).

The TfTx labeling experiments (Fig. 2, *B* and *E*) were carried out with MDCK cells transiently overexpressing the human transferrin receptor. To verify that this overexpression does not alter the targeting of the transferrin receptor to the basolateral membrane we used confocal laser scanning microscopy and compared the TfTx labeling in the plane of the basolateral and the apical membrane. While the plasma membrane and early endosomes are strongly positive for TfTx in the section through the basolateral plane the apical membrane shows little if any TfTx staining (Fig. 3). This indicates that the specific localization of the transferrin receptors in the basolateral plasma membrane and in basolateral endosomes is not affected by the overexpression of the receptor.

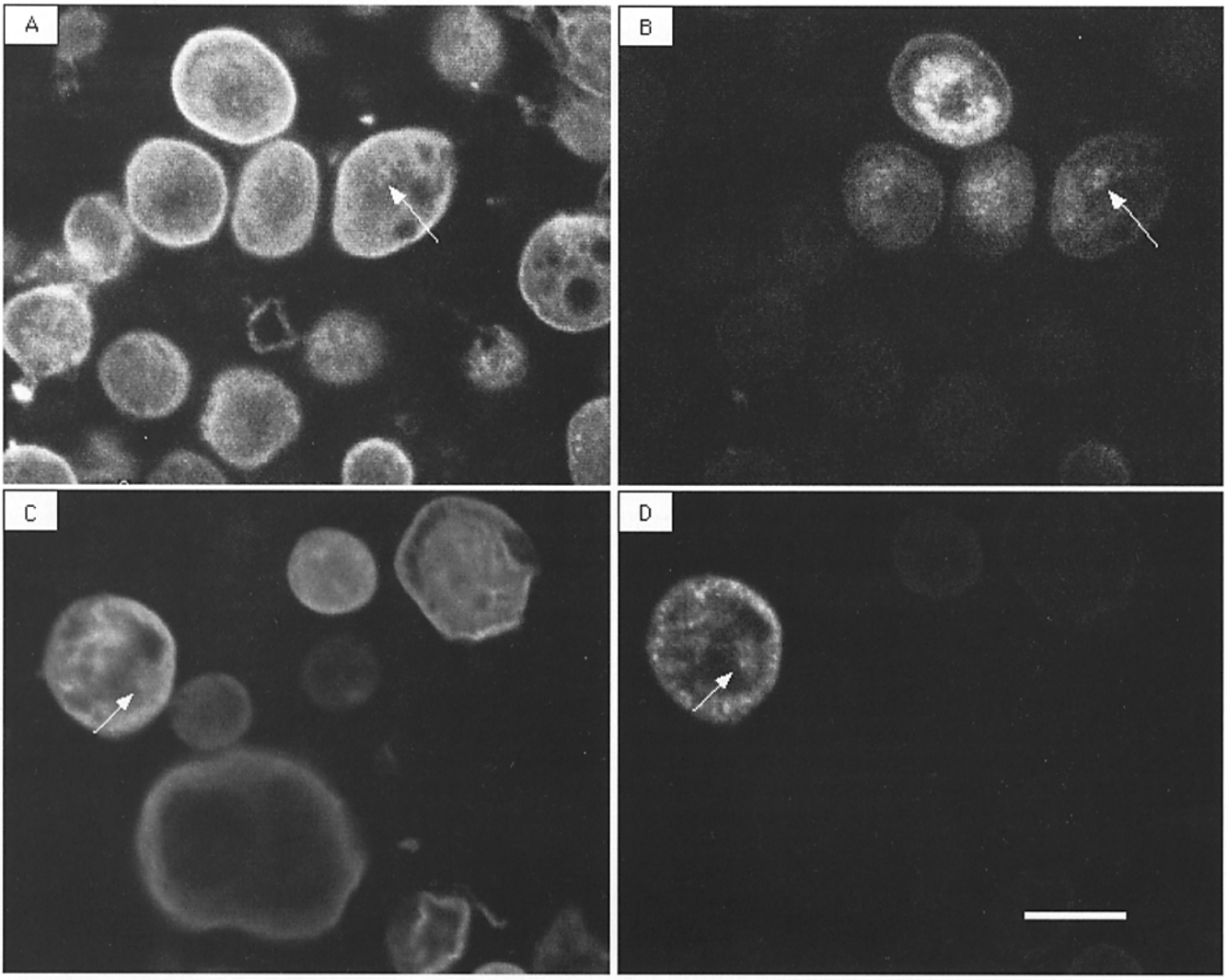

Figure 4. Alteration of the distribution of annexin II and TfTx labeled early endosomes upon treatment of polarized MDCK cells with low calcium medium. MDCK cells were transfected with an expression plasmid carrying the hTfR cDNA and grown for 3 d in standard medium to allow the formation of a polarized monolayer. Subsequently, the cells were transferred to S-MEM (low-calcium medium, Ca^{2+}-concentration below 5 μM) for 2 h and labeled with 20 μg/ml TfTx during the second hour. After fixation, the annexin II distribution was monitored by immunofluorescence using the annexin II specific mAb H28 as primary and FITC-coupled goat anti-mouse IgGs as

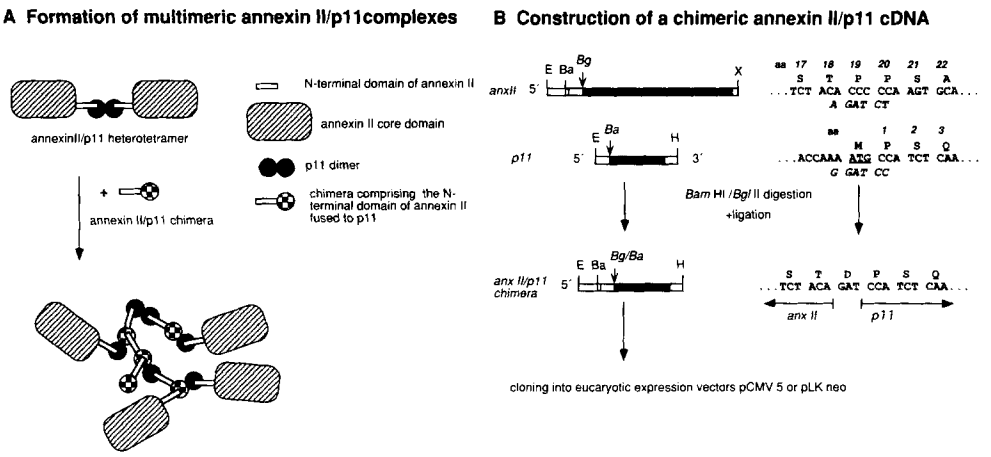

Figure 5. Construction of a chimeric annexin II–p11 protein acting as a *trans*-dominant mutant of the annexin II_2p11_2 complex. (*A*) Schematic representation of multimeric annexin II/p11 aggregates induced by a trivalent chimeric mutant. A chimera comprising the NH_2-terminal region of annexin II, i.e., the entire p11 binding domain, fused to a complete p11 protein should retain the capacity to dimerize and bind annexin II and should also display the annexin II-specific property of tightly binding additional p11 dimers via its NH_2 terminus. Expression of such a trivalent chimera in cells containing the annexin II_2p11_2 complex should therefore lead to the formation of aggregates containing endogenous annexin II and p11 in addition to the chimeric protein. (*B*) Construction of the chimeric annexin II–p11 cDNA. To fuse appropriate regions of the cDNAs encoding human annexin II and p11 suitable restriction sites were introduced by site directed mutagenesis. The positions of these sites are indicated by italicized letters in the restriction maps on the left and the nucleotide sequences on the right, respectively. The EcoRI/BglII cDNA fragment encoding the 18 NH_2-terminal amino acids of annexin II was fused in frame to the BamHI/HindIII cDNA fragment encoding the entire p11 protein using the compatible BglII and BamHI sticky ends. The resulting chimeric gene (XM) was cloned into the eucaryotic expression vectors pCMV 5 (pCMV XM) or pLK neo (pLK XM), respectively. This cloning strategy changes the starting methionine of p11 into an aspartic acid. Restriction sites are indicated as Ba: BamHI, Bg: BglII, E: EcoRI, H: HindIII, X: XbaI.

The Distribution of the Annexin II_2p11_2 Complex and of Early Endosomes Is Sensitive to Low Calcium Medium

Lowering the Ca^{2+} concentration in the culture medium of a MDCK monolayer to micromolar levels causes pleiomorphic effects such as loss of cell to cell contacts and a breakdown of the actin- and fodrin-based cell cortex (Nelson and Veshnock, 1987; Gumbiner et al., 1988). 2 h of low calcium treatment also results in a redistribution of annexin II and p11 from submembranous region into the cytoplasm (Fig. 4, *A* and *C*). Interestingly, basolateral early endosomes, which are labeled with TfTx, are also redistributed towards the cell center (Fig. 4, *B* and *D*). Detachment of the TfR-positive early endosomes from the cell periphery leads to a clear distinction of these structures from the transferrin receptors residing in the plasma membrane which now become visible as a sharp line in the fluorescence picture. A considerable portion of the TfTx-labeled intracellular structures co-localizes with the translocated annexin II/p11. Thus, low-calcium conditions do not interfere with a putative interaction of the annexin II_2p11_2 complex with early endosomes but affect the association of both elements with the cell periphery and/or the plasma membrane. Taken together these data indicate that early endosomes are associated with the cortical region in MDCK cells and that treatment with low-calcium medium seems to result in a parallel translocation of early endosomes and annexin II/p11 to the cytoplasm.

Alteration of the Intracellular Localization of Annexin II and p11 by Expression of a Chimeric Annexin II-p11 Mutant Protein

The p11 molecule forms a tight homodimer and the high-affinity binding site for this p11 dimer ($K_d < 30$ nM) resides in the NH_2-terminal 14 amino acids of annexin II (Gerke and Weber, 1985*b*; Johnsson et al., 1988). Consequently, a chimeric protein comprising the NH_2 terminal domain (residues 1-18) of annexin II fused to the NH_2 terminus of a p11 polypeptide would have two binding sites for p11 (in the NH_2-terminal annexin II domain and in the p11 chain) and one for annexin II (in the p11 chain). p11 dimers bound to such a trivalent chimera should in turn bind additional chimeras and wild-type p11 finally leading to the formation of large aggregates containing annexin II, p11 and the chimera (Fig. 5 *A*). The expression of the annexin II-p11 chimera in a given cell should therefore interfere with the normal annexin II/p11 distribution and the chimera should behave as a *trans*-dominant mutant of the annexin II_2p11_2 complex.

We constructed such a chimera on the cDNA level (referred to as XM or chimeric mutant; Fig. 5 *B*) and used the

secondary antibodies. Confocal images in a supranuclear plane were taken of the same cells in FITC filter settings to document the annexin II distribution (*A* and *C*) and in the Texas red setting to reveal the TfTx staining present on the plasma membrane and on early endosomes (*B* and *D*). Due to the high expression of the hTfR in the transfected cells the TfTx label is primarily incorporated into these cells and the nontransfected cells only show background TfTx staining. Note that the low-calcium treatment causes a translocation of annexin II from the submembranous region to the cytoplasm. Similarly, early endosomes have detached from the cell periphery resulting in a distinction from the plasma membrane associated TfTx now visible as a sharp line in most cells. Arrows indicate apparent costaining of the early endosomes and a fraction of the translocated annexin II. Bar, 10 μm.

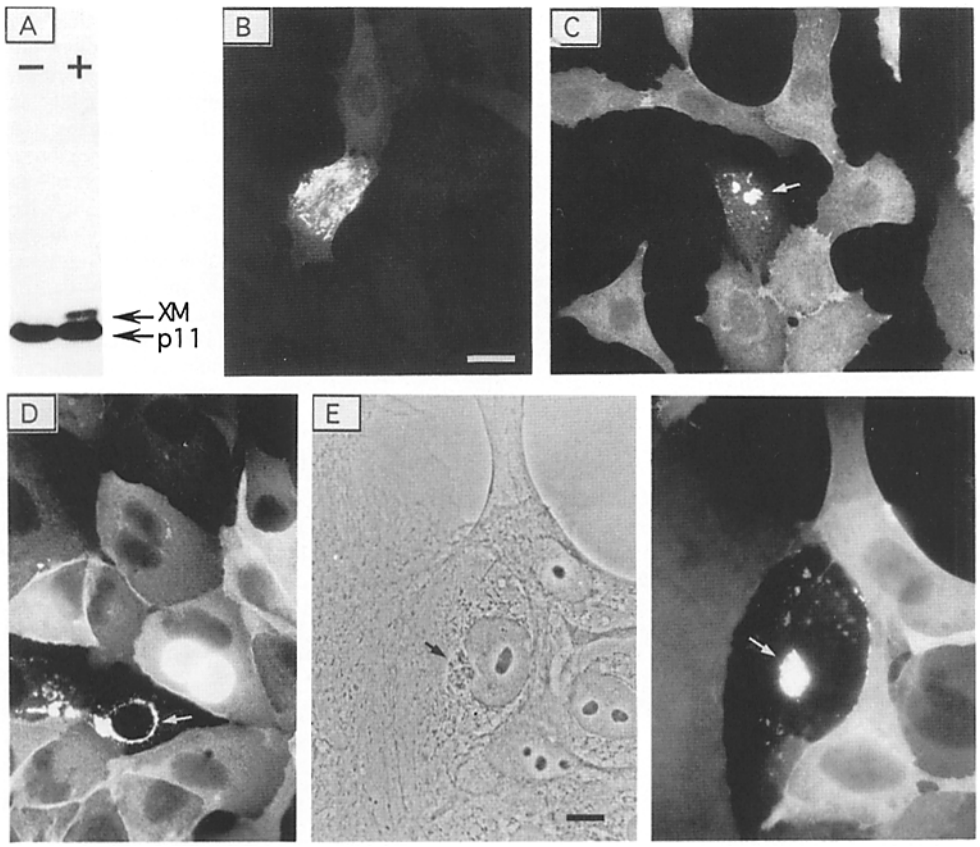

Figure 6. Expression of the chimeric annexin II-p11 protein as revealed by Western blotting and immunofluorescence. (*A*) Total protein of a MDCK cell line stably carrying pLK XM, i.e., the chimeric gene under the control of the dexamethasone inducible MMTV promoter, was subjected to Western blot analysis with the mAb H21 which recognizes the endogenous p11 and the chimera. The blot compares the results obtained for protein extracts of the same cell line grown in the absence (−) or presence (+) of dexamethasone. An additional band corresponding in size to the larger chimeric protein is decorated in extracts from cells treated with dexamethasone indicating that the chimeric gene is indeed expressed in a hormone-controlled fashion. *B* and *C* show immunofluorescence analyses of 208 F rat fibroblasts transiently transfected with pCMV XM. In *B* cells were stained with the mAb H21 which does not react with rat p11 but recognizes the human p11 component of the annexin II-p11 chimera and thus visualizes only the transfected cells. In *C* immunofluorescence employed mAb H42 which stains both, endogenous rat p11 and the chimera. Note that the chimeric protein is present in aggregates in the transfected cell seen in *B*. Such aggregates also incorporate endogenous p11 (*C*). *D–F* show MDCK cells transfected with pCMV XM and plated on glass coverslips for 36 h. Immunofluorescence (*D* and *F*) was carried out with mAb H28 which recognizes only the endogenous annexin II. Aggregates which completely incorporate the endogenous annexin II are present in approximately 20% of the transfected cells. *E* shows the corresponding phase contrast image of the cells stained for annexin II in *F*. Note that the aggregates are visible as phase-dense material (*arrow*, *E*) and that the morphology of the cell with a complete incorporation of the endogenous annexin II into the aggregates appears otherwise unaltered in comparison to nontransfected cells. Arrows mark the aggregates in the different cells. Bars: (*B–D*) 20 μm; and (*E* and *F*) 10 μm.

mammalian expression vectors pCMV5 (pCMV XM) and pLK neo (pLK XM) to drive the expression of the mutant gene in transfected cells. To verify that this approach indeed leads to the synthesis of the desired mutant protein, we generated MDCK cell lines stably carrying pLK XM, i.e., the chimeric gene under the control of the dexamethasone-inducible MMTV promoter. Fig. 6 *A* shows that these stably transfected cells indeed express a novel protein which is induced upon dexamethasone treatment. This polypeptide reacts with a p11-specific mAb and is ~2 kD larger than the wild-type molecule, thus corresponding in size to the product expected from the chimeric gene. Although we isolated several independent clones stably expressing the chimeric gene, dexamethasone induced synthesis of the chimera never reached more than ~10% of that of the endogenous p11. As this relatively low expression did not result in the formation of the predicted annexin II/p11 aggregates, we employed a different expression construct (pCMV XM) which used the strong CMV promoter in transient transfection experiments.

To characterize potential aggregates containing the chimera and to differentiate in immunofluorescence analyses endogenous p11 and the exogenous annexin II-p11 chimera we first chose 208 F rat fibroblasts as recipient cell line. This approach enabled us to use the monoclonal p11 antibody H21 (Osborn et al., 1988) which does not recognize the endogenous rat p11 but reacts with the human p11 part of the chimera and therefore only stains the transfected cells. When analyzed in immunofluorescence microscopy the transfected cells clearly contain patchy material which is specifically labeled with mAb H21 and most likely represents the postulated aggregates containing the product of the chimeric gene (Fig. 6 *B*). Similar aggregates are stained with the mAb H42 which recognizes both endogenous rat p11 and the p11 portion of the exogenous chimera. Moreover the transfected cells show a reduction of the general p11 staining indicating that endogenous p11 is bound by the chimera and trapped in the aggregates (Fig. 6 *C*).

An enrichment of endogenous p11 and annexin II in the aggregates present in cells expressing the chimeric gene is even more evident when MDCK cells are transfected transiently with the pCMV XM construct. Here, the aggregates formed react with the H21 monoclonal (which recognizes canine p11, not shown) and with poly- and monoclonal annexin II antibodies. In addition, there is a significant reduction of the

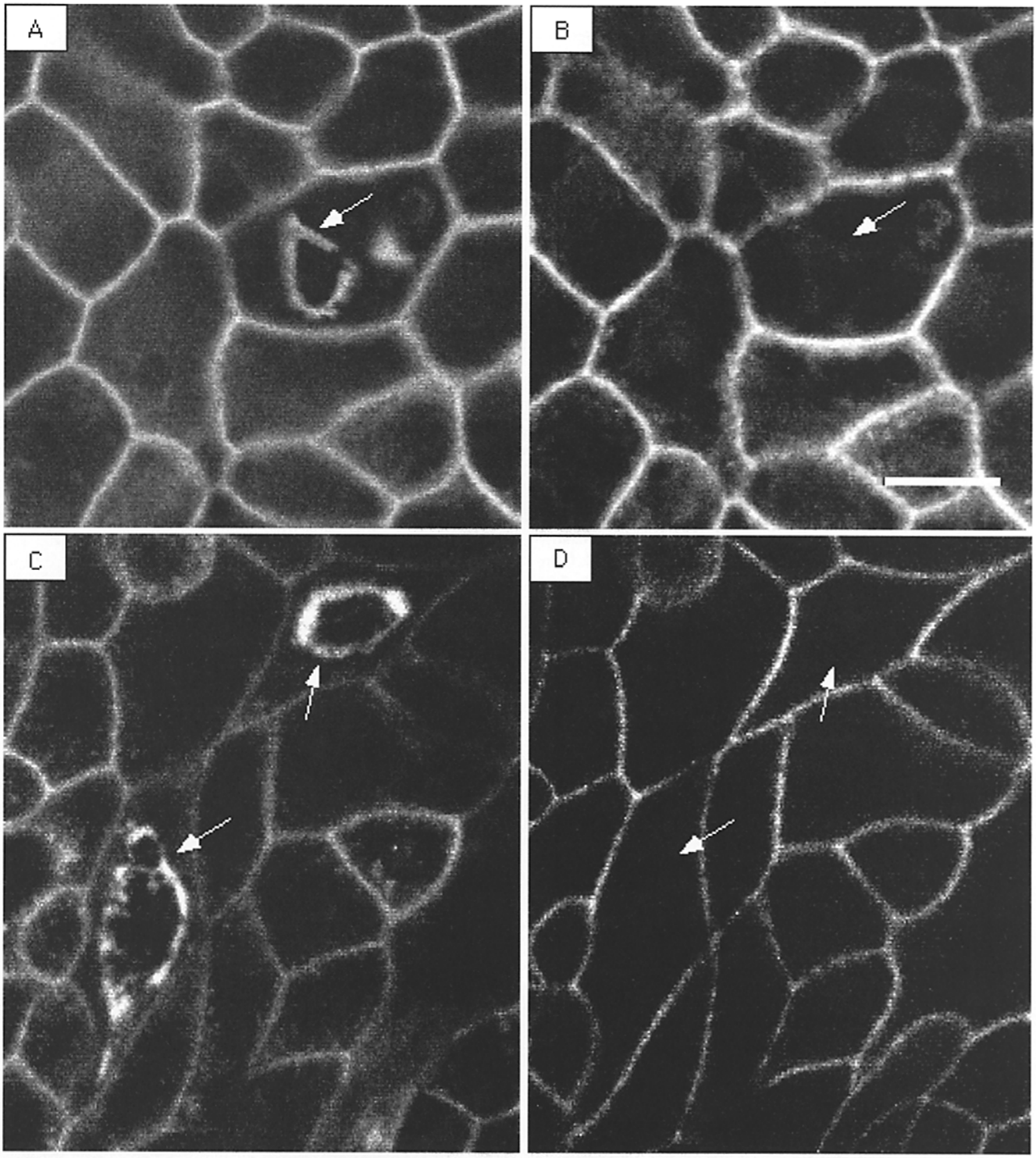

Figure 7. Annexin II/p11 aggregation leaves the distribution of cortical actin and uvomorulin unaffected. MDCK cells were transfected with pCMV XM and allowed to polarize for 3 d on filter supports. The cells were then fixed and double labeled with the annexin II–specific mouse mAB H28 (*A*) and a polyclonal rabbit antibody against actin (*B*). *C* and *D* show the double immunofluorescence with an affinity-purified polyclonal rabbit antibody against annexin II and the uvomorulin specific rat mAB DECMA1, respectively. Rhodamin-conjugated anti–rabbit and FITC-conjugated anti–mouse or anti–rat antibodies were used as secondary antibodies. Confocal sections were adjusted to the basolateral plane. Note that the distribution of cortical actin and uvomorulin appears unaltered in cells containing annexin II aggregates (*arrows*).

general annexin II and p11 staining. Approximately 20% of the transfected cells even show an incorporation of all or most of the endogenous annexin II into the aggregates (Fig. 6, *D* and *F*).

The Formation of Annexin II/p11 Aggregates Does Not Effect the Fodrin- and Actin-based Cell Cortex but Translocates Early Endosomes

MDCK cells containing the annexin II/p11 aggregates show a dramatic redistribution of the annexin II_2p11_2 complex but no gross change in morphology (Fig. 6 *E*). To analyze more specifically potential effects of the annexin II/p11 manipulation on other components of the cell we used double immunofluorescence in conjunction with confocal microscopy. In these studies we concentrated on elements of the cell cortex and the plasma membrane, i.e., the region of the cell which had been deprived of the complex. Cells with the aggregates containing annexin II (identified by staining with a polyclonal or monoclonal annexin II antibody) show no apparent changes in the localization of cortical actin (Fig. 7, *A* and *B*) and fodrin (not shown), two typical components of the submembranous cytoskeleton. Moreover, the distribution of uvomorulin, visualized with the specific mAb DECMA 1 (Vestweber and Kemler, 1985), appears indistinguishable from that of wild-type cells (Fig. 7, *C* and *D*). Consequently, the expression of the *trans*-dominant mutant of the annexin II_2p11_2 complex does not appear to alter the membrane cortex and the plasma membrane organization.

Since annexin II is associated with early endosomal membranes isolated from BHK cells (Emans et al., 1993) and since our results indicate that the annexin II_2p11_2 complex is present on early endosomes in MDCK cells, we tested whether aggregation of annexin II/p11 via the chimera alters the distribution of early endosomes. MDCK cells were cotransfected with expression plasmids carrying the hTfR cDNA and the chimeric gene and early endosomes and annexin II were visualized simultaneously by doublestaining the cells with an hTfR-specific mAb and polyclonal annexin II antibodies. Alternatively endosomes were labeled by uptake of TfTx and analyzed by fluorescence microscopy. Most of the hTfR-positive cells contain aggregates reactive with poly- and monoclonal annexin II antibodies, indicating that the vast majority of the cells received both plasmids. Even complete aggregation of annexin II does not abolish the capability of the transfected cells to take up TfTx. Thus, endocytosis occurs and endosomes apparently remain functional after annexin II aggregation. However, the endosomes stained with the hTfR-specific mAb are mislocalized in the polarized MDCK cells containing annexin II/p11 aggregates and become enriched at the sites of these aggregates (Fig. 8). Most notably, the hTfR-positive endosomes appear almost completely detached from the plasma membrane in the MDCK cells which show a complete aggregation of annexin II (Fig. 8). Thus the hTfR distribution in the MDCK cells containing annexin II/p11 aggregates is somewhat reminiscent of the situation observed in cells treated with low Ca^{2+} medium, i.e., the hTfR present on early endosomes is localized in the cytoplasm and can be clearly distinguished from the plasma membrane bound hTfR.

To unambiguously localize both early endosomes and the annexin II/p11 aggregates we labeled early endosomes of the cotransfected cells with the transferrin Texas red conjugate and subjected the cells to confocal laser scanning microscopy. This analysis clearly shows that the annexin II/p11 aggregates are covered with transferrin-labeled early endosomes (Fig. 9). To verify that the annexin II/p11 aggregates specifically contain early endosomes and no other compartments of the endocytotic pathway we used an antibody against the large mannose 6-phosphate receptor as marker for late endosomes. Double labeling with this antibody and Texas red–conjugated transferrin as marker for early endosomes did not reveal any differences in the late endosome distribution in cells containing annexin II aggregates and translocated early endosomes (Fig. 10). Markers of other membrane structures, e.g., ER and Golgi, or antibodies directed against cytoskeletal proteins like tubulin also showed no changes in the staining pattern in cells with annexin II/p11 aggregates (data not shown). These observations indicate that the effects are specific for early endosomes. Thus annexin II seems involved in the determination of the

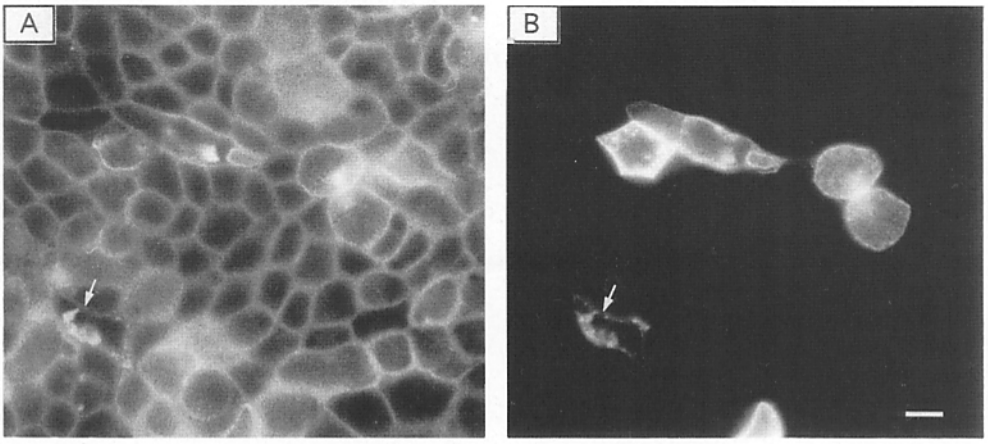

Figure 8. Colocalization of transferrin receptor positive early endosomes and annexin II/p11 containing aggregates. MDCK cells were cotransfected with pCMV XM and pCMV hTfR and subsequently allowed to polarize for 3 d on filters. After fixation the distribution of annexin II and the hTfR was monitored by double immunofluorescence using rabbit polyclonal antibodies against annexin II (*A*) and a mouse mAb (B3/25) against hTfR (*B*). TRITC-coupled goat anti-rabbit and FITC-coupled goat anti-mouse antibodies were used as secondary antibodies, respectively. Most cells expressing the human transferrin receptor also contain the typical aggregates of annexin II induced by the product of the chimeric annexin II-p11 gene. Note the colocalization of the annexin II/p11 aggregates and transferrin receptor positive endosomes. The arrow marks a cell which has suffered a complete depletion of annexin II from the cell cortex and in which all early endosomes appear detached from the plasma membrane. Bar, 10 μm.

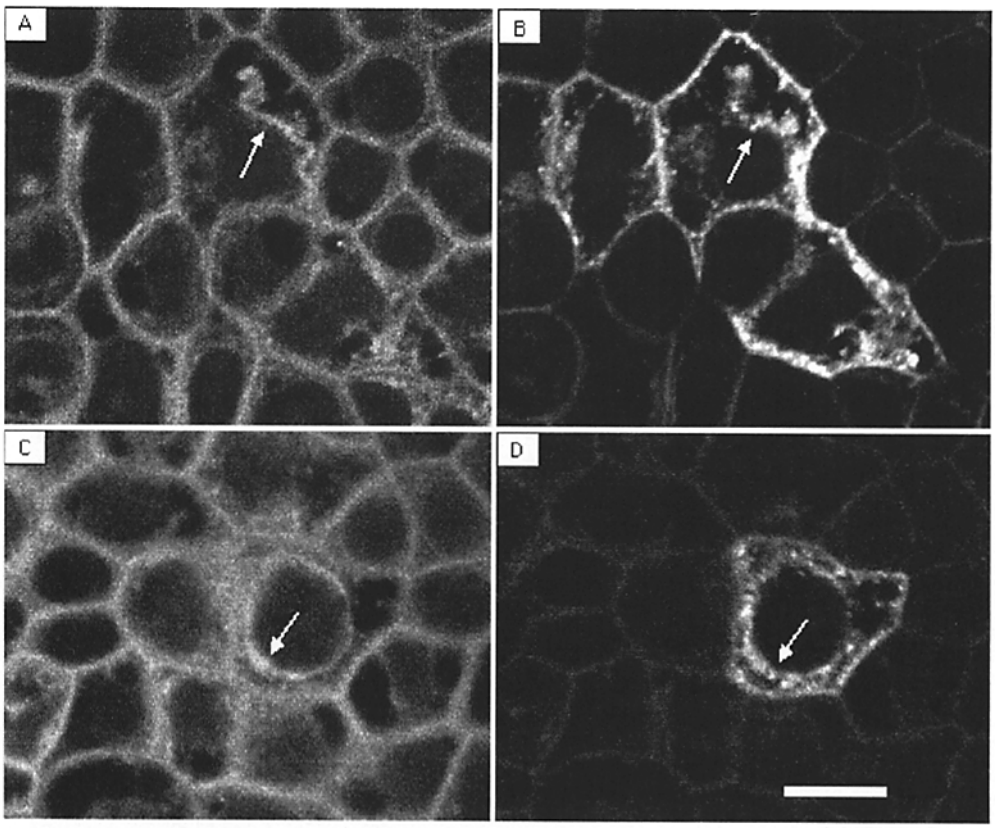

Figure 9. Aggregates of annexin II and p11 are covered with transferrin Texas red-labeled endosomes. MDCK cells were cotransfected with the pCMV XM and pCMV hTfR expression constructs and allowed to polarize for 3 d. Subsequently, annexin II was detected by immunofluorescence employing the mAb H28 and FITC-coupled goat anti-mouse IgGs (*A* and *C*) whereas early endosomes were visualized by incubation with a TfTx (*B* and *D*). Confocal sections were adjusted to the basolateral planes. Note that the annexin II/p11 aggregates are covered with early endosomes tagged by TfTx (*arrows*). Bar, 10 μm.

subcellular distribution of early endosomes, possibly by acting as a linker between early endosomes and the cortical cytoskeleton.

Discussion

The cortical localization of annexin II_2p11_2 in polarized MDCK cells most likely reflects its biochemical properties, i.e., the Ca^{2+}-dependent binding to membranes and elements of the submembranous cell cortex (Gerke and Weber, 1984; Glenney, 1986). The similarity of the actin and the annexin II_2p11_2 distribution raises the possibility that the actin-binding properties observed for monomeric and p11-complexed annexin II in vitro (Gerke and Weber, 1985*b*) could be responsible for anchoring the annexin II_2p11_2 complex in the cortical region of the cell. Whereas the submembranous localization of annexin II_2p11_2 is very evident in polarized MDCK cells, a significant portion of the heterotetramer resides in the cytoplasm of nonpolarized cells. The complete submembranous localization of annexin II and p11 therefore seems to require a fully established cortical cytoskeleton, a view also supported by the low-calcium experiments.

Treatment with low-calcium medium results in breakdown of the cell cortex of MDCK cells as indicated by the translocation of cortical cytoskeletal proteins like actin or fodrin to the cytoplasm (Nelson and Veshnock, 1987). As expected from their cortical localization annexin II and p11 also dissociate from the submembranous region upon low Ca^{2+} treatment. Interestingly, early endosomes which normally align along the basolateral membrane of polarized MDCK cells (Bomsel et al., 1989; Parton et al., 1989) are simultaneously translocated towards the cell center and then colocalize with a fraction of the cytoplasmic annexin II_2p11_2. This indicates: (*a*) that the structural elements to which peripheral early endosomes are associated are sensitive to low Ca^{2+} medium and possibly reside in or interact with the cell cortex; and (*b*) that the annexin II_2p11_2 complex could be involved in linking early endosomes to the periphery of polarized MDCK cells by simultaneously binding to the cortical cytoskeleton and to early endosomes. Such a structural role of annexin II_2p11_2 is in line with the fact that the complex is very abundant in most cells and tissues studied (in some cases up to 0.1% of the total protein), i.e., present at a concentration which argues against a purely regulatory function. Interestingly, some of the transferrin-containing endosomes that stain with annexin II antibodies are located deeper in the cytoplasm of the polarized cells (Fig. 2, *D* and *E*). At present we do not know why these structures are not linked to the cortical region but it remains possible that the peripheral localization of annexin II-containing early endosomes is regulated by posttranslational modifications on the annexin II molecule, e.g., the Ser- or Tyr-specific phosphorylations occurring in the NH_2-terminal domain (see Introduction for references).

Additional evidence for a possible function of the annexin II_2p11_2 complex in linking early endosomes to the cortical region of MDCK cells comes from experiments in which the annexin II/p11 distribution was artificially altered by the expression of a chimeric annexin II-p11 gene. The expression of this chimera leads to the formation of intracellular aggregates containing annexin II and p11 and the chimeric protein can thus be considered to be a *trans*-dominant mutant of the annexin II_2p11_2 complex. This aggregation does not

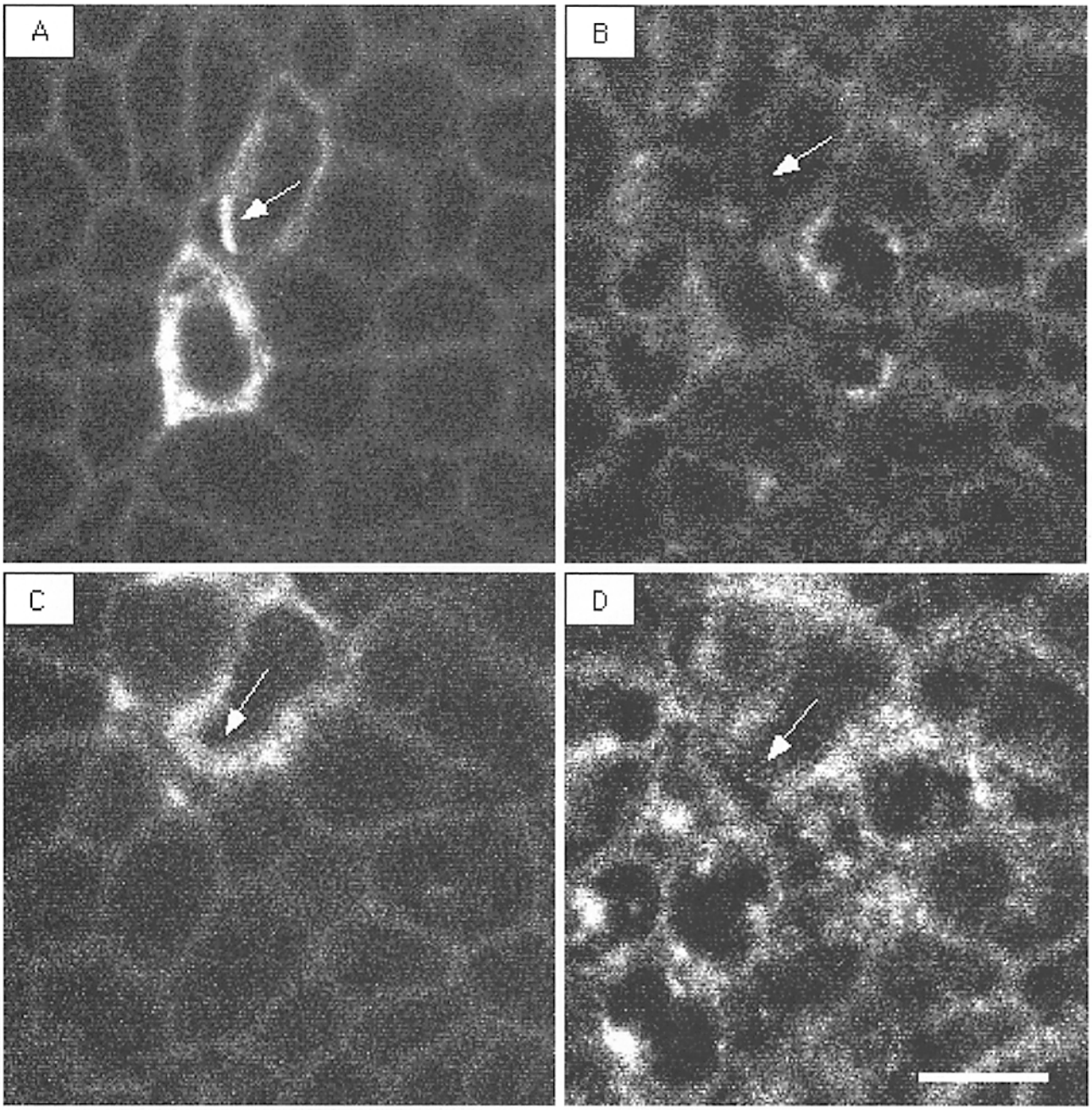

Figure 10. Translocation of the transferrin positive early endosomes to the annexin II/p11 aggregates does not affect mannose 6-phosphate receptor positive late endosomes. MDCK cells were cotransfected with the pCMV XM and pCMV hTfR expression constructs and allowed to polarize for 4 d. Subsequently, early endosomes were visualized by incubating the cells with TfTx at 20 μg/ml (*A* and *C*) whereas late endosomes were detected in the same cells by immunofluorescence using an affinity-purified polyclonal goat antibody against the large mannose 6-phosphate receptor and DTAF-labeled donkey anti-goat antibodies as secondary antibodies (*B* and *D*). To visualize simultaneously the annexin II/p11 aggregates and to obtain a weak staining of the nontransfected cells in *A* and *C* the fixed cells were also labeled with the polyclonal rabbit antibody against annexin II and Texas red-conjugated donkey anti-rabbit antibodies as secondary antibodies which were diluted 10-fold as compared to the standard procedures. This produces a weak annexin II pattern overlayed by a strong TfTx signal in the transfected cells seen in *A* and *C*. Confocal sections were adjusted to the basolateral plane. Two representative examples are shown in *A* and *B*, and *C* and *D*, respectively. Arrows mark typical annexin II aggregates that are covered with TfTx-labeled early endosomes (*A* and *C*). Note that the corresponding regions of the transfected cells are devoid of MPR staining (*arrows*, *B* and *D*) and that the MPR distribution in these transfected cells appears unaltered as compared to the surrounding non-transfected cells. Bar, 10 μm.

interfere with a normal appearance of the cortical cytoskeleton and the lateral plasma membrane suggesting that the formation of a normal cell cortex in MDCK cells does not require the annexin II_2p11_2 complex. While several other structures, e.g., the ER and late endosomes, also appear unaltered in cells expressing the *trans*-dominant gene, early endosomes represent a remarkable exception. The annexin II–p11 aggregates are covered with early endosomes which

still function properly since they contain internalized Texas red-labeled transferrin. Most notably, all early endosomes appear detached from the submembranous region and are translocated to the sites of the annexin II/p11 aggregates in cells showing a complete incorporation of the endogenous annexin II into the aggregates. Thus, early endosomes have a higher affinity for the improperly localized annexin II/p11 than for the annexin II-deprived cell periphery, indicating that the annexin II_2p11_2 complex normally enriched in the cell cortex of wild-type cells seems to participate in the peripheral localization of early endosomes. However, since early endosomes remain functional in cells expressing the *trans*-dominant gene it seems that annexin II_2p11_2 is not indispensable for endocytosis but rather has a supporting structural role in concentrating early endosomes in the cell cortex. The accumulation of early endosomes at sites of high annexin II/p11 concentration and the presence of annexin II on purified early endosomal membranes (Emans et al., 1993) support the view that the annexin II_2p11_2 complex is also involved in the homotypic association and possibly also in the fusion of early endosomes. Such a function would be in line with the finding that annexin II is efficiently transferred from a donor to an acceptor endosomal membrane in an in vitro fusion assay (Emans et al., 1993).

The peripheral localization of early endosomes reduces the transport distances in receptor recycling pathways such as the transferrin receptor cycle. Provided that the annexin II_2p11_2 complex is involved in this aspect of endocytosis by placing early endosomes close to the plasma membrane a high concentration of the complex at membranes actively engaged in endocytosis can be expected. This is indeed the case in enterocytes of the small intestine which in contrast to MDCK cells show a polar distribution of annexin II and p11 with a strong enrichment at the apical side of the cells (Gerke and Weber, 1984; Gould et al., 1984). The distribution of transferrin accessible endosomes in enterocyte-resembling CaCo-2 cells likewise shows an apical enrichment of early and recycling endosomal structures (Hughson and Hopkins, 1990) possibly caused by the highly polarized annexin II_2p11_2 distribution.

Similar to annexin II all mammalian annexins show a high degree of tissue specificity and are not expressed in all tissues or cell lines (for review see Moss et al., 1991). This property of annexins is already apparent in early metazoa as indicated by the finding that the two different annexins of the Cnidaria *Hydra vulgaris* are only expressed in specialized cell types (Schlaepfer et al., 1992). Thus, individual members of the annexin family have either overlapping functions in different cells or a specific activity of a certain annexin is not required in every cell type. It is therefore difficult to assign an indispensable function of a particular annexin to a process occurring in every mammalian cell. More likely, individual annexins seem to act specifically in certain differentiated cells which show a characteristic and cell type-specific architecture and topology of cellular membranes. It is tempting to speculate that the different annexins are required for this cell type-specific organization of the different intracellular membranes allowing, for instance, the formation of specialized membrane structures or a high efficiency in membrane transport, as probably in the case of annexin II and early endosomes. Another example for the association of a certain annexin with specific intracellular membranes is the interaction of annexin I with multivesicular bodies (Futter et al., 1993). Moreover, a recent immunoelectron microscopical analysis has revealed that an antibody directed against an annexin consensus peptide (Gerke, 1989*b*) stained all major intracellular membranes, thus providing additional evidence for a role of annexins as universal modulators of cellular membranes with the individual members acting on different targets (Gruenberg and Emans, 1993). Such a model still leaves many questions unanswered, e.g., the role of different isoforms of the individual annexins, the effects of phosphorylation and other posttranslational modifications, the possible physiological importance of a Ca^{2+} channel activity reported for several annexins in vitro (Burns et al., 1989; Rojas et al., 1990; Berendes et al., 1993), and a putative extracellular role of individual annexins as phospholipase A_2 inhibitors (Hirata et al., 1980; DiRosa et al., 1984; Pepinsky et al., 1986). However, the approach described here for annexin II, i.e., the manipulation of its intracellular localization and activity by the expression of a *trans*-dominant mutant, should also provide new information as to in vivo target membranes of other annexins and their possible roles on these membranes.

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