# Endocytosis Occurs Independently of Annexin VI in Human A431 Cells

**Elizabeth Smythe,* Paul D. Smith, Sara M. Jacob, Jeremy Theobald, and Stephen E. Moss**

Department of Physiology, University College London, London WC1E 6BT, United Kingdom; and *Biochemistry Department, Dundee University, Dundee DD1 4HN, Scotland, United Kingdom

*Abstract.* Annexin VI is one of a family of calcium-dependent phospholipid-binding proteins. Although the function of this protein is not known, various physiological roles have been proposed, including a role in the budding of clathrin-coated pits (Lin et al., 1992. *Cell.* 70:283–291.). In this study we have investigated a possible endocytotic role for annexin VI in intact cells, using the human squamous carcinoma cell line A431, and report that these cells do not express endogenous annexin VI, as judged by Western and Northern blotting and PCR/Southern blotting. To examine whether endocytosis might in some way be either facilitated or inhibited by the presence of annexin VI, a series of A431 clones were isolated in which annexin VI expression was achieved by stable transfection. These cells expressed annexin VI at similar levels to other human cell types. Using assays for endocytosis and recycling of the transferrin receptor, we report that each of these cellular processes occurs with identical kinetics in both transfected and wild-type A431 cells. In addition, purified annexin VI failed to support the scission of coated pits in permeabilized A431 cells. We conclude that annexin VI is not an essential component of the endocytic pathway, and that in A431 cells, annexin VI fails to exert any influence on internalization and recycling of the transferrin receptor.

THE annexins are a major class of calcium-binding proteins, structurally defined by the presence of a conserved 70–amino acid domain that occurs as a tetrad repeat in most members of the family, but as an octad repeat in annexin VI (Crompton et al., 1988*a*, *b*). The mammalian family includes at least 10 unique gene products, several of which exist as multiple isoforms that arise as a consequence of alternative splicing (Moss and Crumpton, 1990; Magendzo et al., 1991; Towle et al., 1992). The annexins are broadly expressed with representatives of the family present in virtually all cell types, sometimes constituting more than 1% of total cell protein. Their conservation of structure (annexins typically share ∼40–50% sequence identity with one another) is restricted to the protein core, which in most annexins comprises almost the entire structure. The repeated domains have been shown by both x-ray crystallography and mutagenesis, to harbor the $Ca^{2+}$-binding sites (Huber et al., 1990; Jost et al., 1992).

In contrast, the $NH_2$-terminal domains have little in common between annexins and have therefore been suggested to confer functional individuality (Crompton et al., 1988*b*). There is good evidence to support this, in that the $NH_2$-terminus of annexin II contains both the binding site for p11 in addition to sites phosphorylated by $pp60^{v\text{-}src}$ and protein kinase C. The $NH_2$-terminus of annexin I similarly contains sites for phosphorylation by the epidermal growth factor receptor and protein kinase C. Of all the other annexins, only annexins VI and XI are known to be phosphorylated in whole cells (Moss et al., 1992; Mizutani et al., 1993), although the sites of phosphorylation are not known. Several functions have been proposed for annexin VI, largely based on observations from in vitro-derived data. Like all annexins, annexin VI is a potent in vitro inhibitor of phospholipase $A_2$ (Edwards and Crumpton, 1991), and it also exhibits anticoagulant activity (Yoshizaki et al., 1992), although both phenomena can be explained by $Ca^{2+}$-dependent sequestration of phospholipid by annexin VI, removing the substrate in the former case, and the interactive matrix in the latter. Other functions attributed to annexin VI include inhibition of protein kinase C (Shibata et al., 1992) and modulation of the activity of sarcoplasmic reticulum $Ca^{2+}$-channels (Diaz-Munoz et al., 1990).

Recently it was reported that annexin VI was required for the budding of coated pits from plasma membranes isolated from fibroblasts (Lin et al., 1992). The early stages of receptor-mediated endocytosis involve a dynamic series of events culminating in the formation of a coated vesicle from a deeply invaginated coated pit. Ligands bind to specific transmembrane receptors which are either already clustered, or become clustered as a result of ligand binding, into coated pits. Rearrangement of the coat proteins, clathrin and adap-

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Address all correspondence to Dr. Stephen E. Moss, Department of Physiology, University College London, Gower Street, London WC1E 6BT, U.K.

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tors, results in increased invagination of the pit (for reviews see Pearse and Robinson, 1990; Smythe and Warren, 1991). Scission of the deeply invaginated coated pit to form a coated vesicle has an absolute requirement for ATP (Schmid and Carter, 1990). Endocytosis in A431 cells has been extensively studied both morphologically and biochemically and the uptake and recycling of transferrin has been very well-characterized (Hopkins and Trowbridge, 1983; Hopkins, 1985). It has also been possible to use this cell line to reconstitute the early steps of endocytosis, from coated pit formation to coated vesicle budding, in a permeabilized cell system (Smythe et al., 1989; Schmid and Smythe, 1991). Morphological verification of the in vitro assay demonstrated that ligand was sequestered into coated pits and internalized into coated vesicles which were indistinguishable from those seen in intact cells. We now report that annexin VI is not expressed in A431 cells and that in these cells endocytosis must therefore occur via a mechanism independent of this protein.

# *Materials and Methods*

## *Cell Culture*

Human A431 squamous carcinoma cells were routinely maintained in DME containing 5% fetal calf serum. 100 U/ml penicillin, and 100 μg/ml streptomycin. Stock dishes were subcultured to 1:20 at $\sim$90% confluence.

## *Transfection of A431 Cells with Annexin VI*

The construct for transfection, namely pRC/CMV.anxVI contained a full-length human annexin VI cDNA, created by the ligation of partial length clones A2 and 10.6 (Crompton et al., 1988*a*). The full-length cDNA was directionally cloned into HindIII/XbaI cut pRC/CMV (Invitrogen Corp., U.K.). This vector offers high-level constitutive expression driven by the cytomegalovirus promoter. On the day before transfection, exponentially growing A431 cells were subcultured at $\sim 10^4$ cells/cm$^2$ on 90-mm dishes and cultured for an additional 24 h. Transfection was achieved using the calcium phosphate method essentially as described by Graham and Van der Eb (1973). Neomycin-resistant colonies were isolated after two weeks and examined for expression of annexin VI by Western blotting. Control transfectants were generated using identical procedures but with wild-type vector.

## *Immunoblot and Protein Analyses of Annexin VI Transfectants*

Wild-type (wt)[1] A431 cells and control and annexin VI transfectants were routinely cultured as described above. For Western blotting, whole cell lysates of known cell numbers (refer to Figures for details) were resolved by SDS-PAGE (Laemmli, 1970) and transferred to Immobilon-P overnight at 0.2 A. Annexin VI was detected using a purified IgG fraction of the extensively characterized rabbit polyclonal antibody MC2 (Crompton et al., 1988*a*; Moss et al., 1988; Moss and Crumpton, 1990; Clark et al., 1991). After incubation in goat anti-rabbit IgG conjugated to alkaline phosphatase, color development was achieved using Western blue (Promega, U.K.).

## *Northern Blotting, Southern Blotting, and PCR*

Total cellular RNA was prepared from Jurkat leukemia cells and wild-type and transfected A431 cells as described (Chomcyzymski and Sachi, 1987). Blots were probed with the A2 human annexin VI cDNA and where indicated, stripped and reprobed with a glyceraldehyde-3-phosphate dehydrogenase probe as positive control.

Annexin VI PCR products were generated from cytoplasmic RNA isolated from Jurkat cells, wtA431 cells, and annexin VI$^+$ transfected A431 cells. The 5′ and 3′ oligonucleotide primers (5′-TTCAACCCTGACGCAGAT-3′ and 5′-TTCTTGATGGTGTGCTCC-3′, respectively) were designed to amplify the region between nucleotides 1,186 and 1,840 in the cDNA sequence, and are therefore predicted to yield a fragment of 654 bp. PCR reactions were performed using Taq DNA polymerase (Promega, U.K.) following first strand cDNA synthesis using Superscript RNase H$^-$ reverse transcriptase (GIBCO-BRL, U.K.) with conditions as recommended by the manufacturers. Reaction products were resolved on a 1% (wt/vol) agarose gel and visualized by staining in ethidium bromide. Southern blot analysis of the PCR products was as described (Maniatis et al., 1982) using the A2 annexin VI cDNA probe.

## *Iodinated Transferrin*

Iodinated transferrin was prepared according to the iodogen method essentially as described (Woodman and Warren, 1988) except that 1 mCi of Na$^{125}$I was used to label 500 μg of transferrin and the final concentration of $^{125}$I-transferrin was 250 μg/ml.

## *Endocytosis Assays*

*Internalization Assays.* Endocytosis of $^{125}$I-transferrin was performed essentially as described by Hopkins and Trowbridge (1983). Briefly, A431 cell lines, grown in 35-mm Corning dishes to 90% confluency, were preincubated with serum-free medium (SFM: DME containing 25 mM Hepes, pH 7.4, and 1 mg/ml BSA) for 30 min at 37°C. They were then incubated with $^{125}$I-transferrin (1 μg/ml) in SFM for 90 min at 4°C. The cells were washed three times with dPBS containing 1 mg/ml BSA to remove unbound ligand. For internalization to occur, the cells were then incubated at 30°C for various times in prewarmed SFM containing 10 μg/ml unlabeled transferrin. To stop internalization, the cells were chilled on ice and the media were removed. Surface $^{125}$I-transferrin was stripped by two 10 min washes with 0.5 ml acetic acid/saline (0.2 M acetic acid, pH 2.5, 0.5 M NaCl). Internalized $^{125}$I-transferrin was assessed by measuring the radioactivity after solubilizing the cells in 1 M NaOH. For each time point, the amount of $^{125}$I-transferrin internalized was expressed as a percentage of the total ligand associated with the cells at zero time.

*Recycling Assays.* To measure recycling in A431 cell lines, cells were grown to 90% confluency on 35-mm Corning dishes. The cells were loaded with $^{125}$I-transferrin (4 μg/ml) in SFM for 10 min at 30°C. After chilling on ice, the medium was removed and the surface label removed by mild acid washing. The cells were rinsed in 20 mM citrate, pH 5.0, 0.15 M NaCl. They were then incubated on ice for 15 min in 20 mM citrate, pH 5.0, 0.15 M NaCl containing 50 μM desferal (Ciba Laboratories, Horsham, West Sussex, U.K.). This was followed by a 5-min incubation in dPBS/BSA. The cells were then rewarmed for various times at 30°C in SFM containing 10 μg/ml unlabeled transferrin. The medium was removed and after solubilization of the cells with 1 M NaOH, the amount of cell-associated $^{125}$I-transferrin was measured and expressed as a percentage of that associated with cells that were not rewarmed.

*In Vitro Assay of Coated Vesicle Budding.* The assay used to measure coated vesicle budding was the MesNa resistance assay which was carried out as described previously (Smythe et al., 1992; Carter et al., 1993) with the modification that the anti-transferrin antibody used to coat the ELISA plates was an IgG fraction prepared from sheep anti-transferrin antibody. This antibody was a generous gift from Scottish Antibody Production Unit (Carluke, Scotland). Annexin VI was purified from human placenta as described previously (Edwards and Crumpton, 1990).

# *Results*

Lin et al., (1992) have recently reported that annexin VI is required for the budding of clathrin-coated pits from isolated plasma membranes from sonicated fibroblasts. In this study, the aim was to determine whether or not budding of clathrin-coated pits required the presence of annexin VI in whole cells. Human A431 cells, which have been extensively used as a model system for the study of endocytosis were therefore examined with respect to expression of annexin VI.

## *Annexin VI Is Not Expressed in A431 Cells*

wtA431 cells were examined for expression of annexin VI by Western blotting, Northern blotting, and PCR/Southern

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[1] *Abbreviations used in this paper*: SFM, serum-free medium; wtA431, wild-type A431.

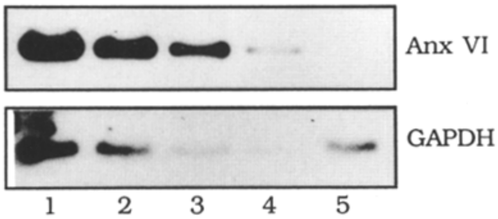

*Figure 1.* Northern blot of annexin VI in A431 and Jurkat cells. Cytoplasmic RNA was isolated from Jurkat cells and A431 cells and probed for annexin VI (*Anx VI*) by Northern blotting, using the $A_2$ probe described in Crompton et al., (1988*a*). The blot was stripped and reprobed for GAPDH as an internal control. Lanes *1–4* contained 20, 10, 5, and 1 $\mu$g Jurkat RNA respectively and lane *5* contained 20 $\mu$g A431 RNA.

blotting. With all of these techniques, annexin VI was undetectable in A431 cells. Annexin VI mRNA could be readily detected in Jurkat cells (Fig. 1, lanes *1–4*) even using as little as 1 $\mu$g total RNA, whereas in A431 cells, no signal was observed with 20 $\mu$g RNA (Fig. 1, lane *5*). Reprobing of the stripped blot with GAPDH testified to the integrity of all the samples. Analysis of A431 cell RNA by PCR using annexin VI-specific primers similarly failed to yield a clear product (Fig. 2 *A*, lane *2*), whereas RNA from both Jurkat cells and A431 cells stably transfected with annexin VI (Fig. 2 *A*, lanes *1* and *3*) both yielded a single product of the correct predicted size. To further confirm that this product was indeed annexin VI in derivation, the same gel was Southern blotted and probed with the A2 annexin VI cDNA. Bands corresponding to the PCR products from the Jurkat cells and the transfected A431 cells (Fig. 2 *B*, lanes *1* and *3*) both gave a positive signal, whereas no signal was observed in the wtA431 cells (Fig. 2 *B*, lane 2).

### *Stable Expression of Annexin VI in A431 Cells*

Following transfection of A431 cells with pRC/CMV.anxVI, a series of stable neomycin-resistant lines was isolated and cultured for several months with constant monitoring for expression of annexin VI (judged by Western blotting). For the purposes of these experiments, two expressing clones (C3 and CK), one nonexpressing clone (C7), and wtA431 cells were selected. Full analyses of annexin VI expression levels and growth characteristics of these clones will be reported elsewhere (Theobald, J., P. D. Smith, S. M. Jacob, and S. E. Moss, manuscript in preparation). A representative Western blot is shown in Fig. 3, illustrating annexin VI in a whole cell lysate of C3 but undetectable in wtA431. These results, together with the Northern blotting and PCR/Southern blotting data, provide convincing evidence that annexin VI is not expressed in wtA431 cells.

### *Endocytosis of Transferrin Receptors in wtA431 and Transfectants*

***Endocytosis and Recycling of Transferrin Receptors in wtA431 Cells and Transfectants.*** The results of Lin et al. (1992) suggest that annexin VI has a role in coated-vesicle budding. Since A431 cells do not normally express annexin VI, it was of interest to examine whether the presence of annexin VI in these cells affected the rates of endocytosis. The internalization kinetics of $^{125}$I-transferrin were examined in wtA431 cells and in the transfected cell lines. Fig. 4 shows that all the cell lines tested show essentially the same rates and extent of internalization. We next examined the rates of recycling in both wild-type and transfected cells. Fig. 5 shows that the rates of recycling are unchanged in the transfected cells.

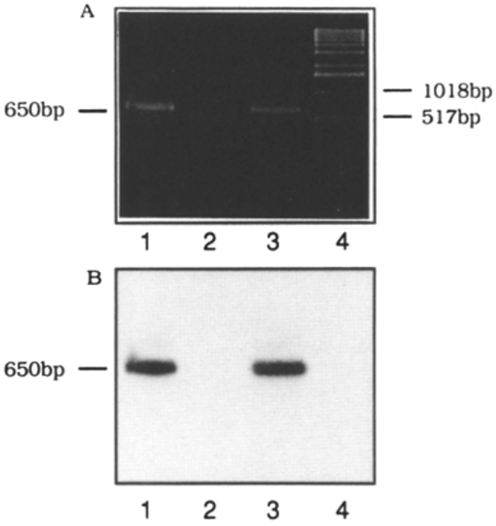

*Figure 2.* PCR analysis of annexin VI in A431, C3, and Jurkat cells. (*A*) Annexin VI PCR products were generated from cytoplasmic RNA isolated from Jurkat cells (lane *1*), A431 cells (lane *2*), and C3 (A431 cells transfected with annexin VI) (lane *3*). DNA size markers are shown in lane *4*. The 5′ and 3′ oligonucleotide primers were designed to amplify the region between nucleotides 1,186 and 1,840 in the cDNA sequence, and are therefore predicted to yield a fragment of ~654 bp. PCR products were visualized by ethidium bromide staining and photographed under UV light. (*B*) Southern blot of the same gel using a random-primed probe derived from nucleotides 1,260–1,600 in the human annexin VI cDNA. Bands of the correct predicted size are observed in lanes *1* and *3*.

***Annexin VI Does Not Support Coated Vesicle Budding in Permeabilized A431 Cells.*** The formation of coated vesicles in permeabilized A431 cells has been reconstituted (Schmid and Smythe, 1991). In order to measure budding, transferrin which has been biotinylated via a cleavable disulphide linkage (BSST) is used as a reporter molecule. The acquisition of BSST resistance to the small reducing agent, MesNa, provides a measure of transferrin internalization into bona fide coated vesicles. Morphological studies confirmed that BSST was being internalized into coated vesicles identical to those found in intact cells (Smythe et al., 1989; Schmid and Smythe, 1991). Scission of deeply invaginated coated pits to form coated vesicles requires both cytosol and ATP.

We investigated the possibility that annexin VI might fulfill some or all of the cytosolic requirement for this event. Titration of annexin VI over a range of concentrations from 12.5–100 $\mu$g/ml revealed that purified annexin VI is unable to stimulate coated vesicle budding in permeabilized A431 cells either in the absence or presence of low levels of cytosol (Fig. 6 *a*). Treatment of bovine brain cytosol with 10 mM calcium results in the formation of aggregates of annexins which may be separated from cytosol by centrifugation. Studies by Lin et al., (1992) showed that cytosol treated in

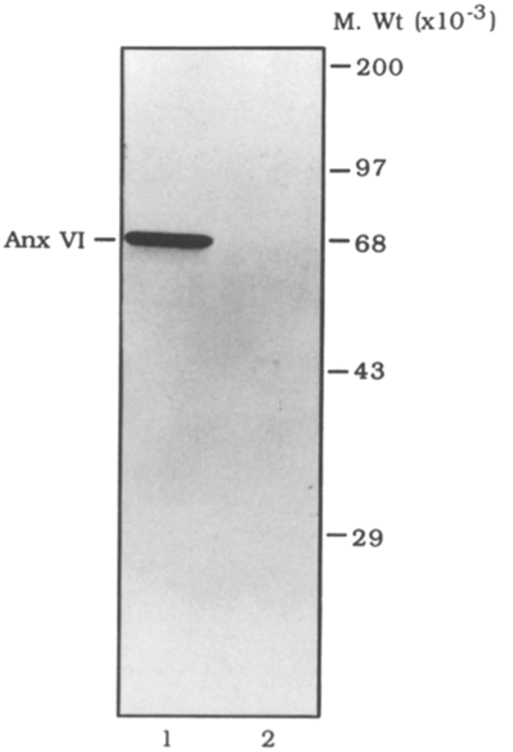

*Figure 3.* Western blot of annexin VI in C3 and wtA431 cells. Samples of whole cell lysates containing ~5 × $10^6$ cells were separated by SDS-PAGE and transferred to Immobilon-P. The first antibody was a polyclonal rabbit anti-(annexin VI) serum and the second was a goat anti-(rabbit IgG)-alkaline phosphatase conjugate. Color development was achieved using Western blue. Lane *1*, C3. Lane 2, wtA431. The positions of annexin VI (*Anx VI*) and molecular weight markers are indicated.

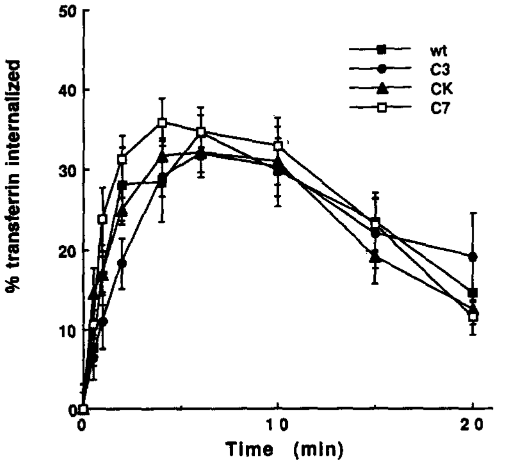

*Figure 4.* Endocytosis of $^{125}$I-transferrin in wild-type and transfected A431 cells. $^{125}$I-Transferrin (1 μg/ml) was bound at 4°C to the cell surface of near confluent A431 cell lines (wt, annexin VI transfected clones, C3 and CK, and mock-transfected clone C7). Following removal of unbound ligand, the cells were warmed at 30°C for various times. After chilling on ice, surface $^{125}$I-transferrin was removed by acid stripping as described in Materials and Methods. The amount of internalized $^{125}$I-transferrin was determined after solubilization of the cells using 1 M NaOH. Values are expressed as the mean of three separate experiments, ±SEM, each performed in duplicate.

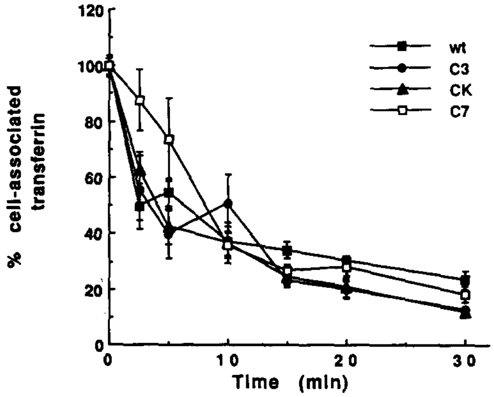

*Figure 5.* Recycling of $^{125}$I-transferrin in wild-type and transfected A431 cells. Wild-type and transfected A431 cells (*wt, C3, C7, CK*) were loaded with $^{125}$I-transferrin for 10 min at 30°C. Cell-surface ligand was removed at 0°C using conditions of mild acid stripping as described in Materials and Methods. The cells were then rewarmed at 30°C for various times and the amount of cell-associated $^{125}$I-transferrin determined after solubilizing the cells in 1 M NaOH. Values are expressed as the mean of three separate experiments, ±SEM, each performed in duplicate.

this manner was reduced in its ability to stimulate clathrin loss from isolated fibroblast plasma membranes. Similarly we also observed that calcium-treated cytosol shows a loss in ability to support coated-vesicle budding in permeabilized A431 cells. Bovine brain cytosol (10 mg/ml) was treated with 10 mM $CaCl_2$ on ice for 15 min and then centrifuged at 100,000 *g* for 1 h. The supernatant was dialyzed extensively against assay buffer and then assayed for coated-vesicle budding activity (Figure 6 *b*). The depleted cytosol shows reduced activity in the assay. Purified annexin VI, added back at protein concentrations corresponding to 1% of the cytosolic protein, was unable to restore activity to the calcium-treated cytosol.

Efforts to reconstitute activity from the pellet obtained after calcium treatment were unsuccessful (data not shown), suggesting that some component essential for budding had been irreversibly precipitated/inactivated. SDS-PAGE and Western blotting analysis of the pellet and supernatant after centrifugation revealed that, in addition to precipitation of ≥95% of annexin VI, calcium precipitation resulted in precipitation of a number of other proteins (data not shown). These proteins may be essential components of the scission machinery. Therefore, we conclude from these results that annexin VI has no apparent role in the budding of coated vesicles in permeabilized A431 cells.

## Discussion

Members of the annexin family have been attributed with a variety of proposed functions including phospholipase $A_2$ inhibition (Russo-Marie, 1992), inhibition of blood coagulation (Yoshizaki et al., 1992), exocytosis (Creutz, 1992), $Ca^{2+}$-channel activity (Huber et al., 1990), inositol phosphate metabolism (Ross et al., 1990), and most recently, steps in the endocytic pathway including budding of clathrin-coated pits (Lin et al., 1992), endosome fusion (Emans et al., 1993), and lysosomal targetting (Futter et al., 1993). It

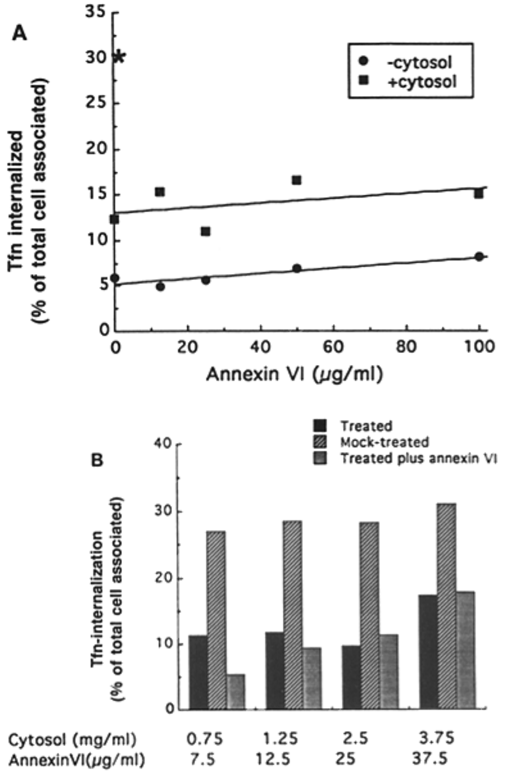

*Figure 6.* Coated-vesicle budding in permeabilized A431 cells occurs independently of annexin VI. (*A*) Purified annexin VI (12.5–100 μg/ml final concentration) was added to permeabilized A431 cells in the presence or absence of low levels of cytosol (1.25 mg/ml). The extent of coated vesicle budding was assayed using the MesNa resistance assay as described in the Materials and Methods. The asterisk indicates the maximum extent of coated vesicle budding under conditions of saturating cytosol observed in this experiment. (*B*) Cytosol was treated with 10 mM $CaCl_2$, centrifuged at 100,000 *g*, and dialyzed extensively to remove $Ca^{2+}$. It was then assayed for its ability to support coated-vesicle budding as measured by the MesNa resistance assay compared with mock-treated cytosol. Purified annexin VI was added back to the calcium-treated cytosol at concentrations corresponding to 1% of the cytosolic protein in each case.

is interesting to note that individual annexins are now suggested to function in both endocytic and exocytic pathways.

We have shown here by a variety of techniques that human A431 squamous carcinoma cells do not express annexin VI. The protein was undetectable by Western blotting, and the mRNA was undetectable by either Northern blotting or PCR/Southern blotting. Positive controls in each case testified to the efficacy of the techniques. A431 cells have been extensively used in numerous laboratories for the study of endocytosis and the budding of clathrin-coated pits. Using an assay for the internalization of transferrin, which is known to occur via the budding of clathrin-coated pits in these cells, we have demonstrated that A431 cells effectively perform this function without a requirement for annexin VI. Furthermore, stable expression of annexin VI in these cells had no effect on the endocytosis of transferrin receptors. Our results clearly challenge the conclusions of Lin et al. (1992), who reported that annexin VI is required for the budding of clathrin-coated pits. How may one account for this apparent discrepancy?

One possible explanation is functional redundancy within the annexin family. There are precedents for this, exemplified by the observations that annexins exhibit similar activities as both anticoagulants and phospholipase $A_2$ inhibitors in vitro. However, this is unlikely in this case since Lin et al. (1992) demonstrated that budding was stimulated specifically by annexin VI, although not all annexins were tested. In addition, the detection methods used in this study, Western blotting, Northern blotting, and PCR/Southern blotting, collectively failed to detect any other protein in A431 cells, the implication being that if A431 cells express a functional homolog, then it is unlikely to be structurally related to annexin VI. A second more plausible explanation is that annexin VI, like other members of the family, is capable of multiple functions in vitro, and that the cell-free assay conditions of Lin et al. (1992) invoke unusual properties in annexin VI. A third possibility is that annexin VI forms part of the budding machinery in certain cell types but not in others. This argument precludes annexin VI from part of a universal mechanism of endocytosis and this would be surprising given the high degree of conservation of the components of the endocytic pathway throughout eukaryotic evolution.

Stable transfection of annexin VI in A431 cells had no effect on the endocytosis of transferrin receptors. An effect on the kinetics of endocytosis might only be observed after transfection if the protein was rate limiting on the endocytic pathway. The small GTP-binding proteins rab4 and rab5 appear to be rate limiting on the early endocytic pathway and cells where they are overexpressed show altered rates of recycling and internalization, respectively (Bucci et al., 1992; van der Sluijs et al., 1992). In contrast, although rab2 is involved in transport from the ER to the Golgi complex, overexpression of the protein does not accelerate the rate of transport at this step (Tisdale et al., 1992). However, since annexin VI is normally absent from A431 cells, one would predict that its presence would affect the rates and extent of internalization if it were an essential component of the budding machinery.

In addition, we were unable to detect a role for purified annexin VI in an assay for coated-vesicle budding in permeabilized A431 cells. Annexin VI, either alone or in the presence of cytosol, was unable to support coated vesicle budding in permeabilized A431 cells. Depletion of annexin VI and other components from cytosol by precipitation with calcium resulted in a reduced ability of the cytosol to support coated vesicle budding but this loss in activity could not be rescued by addition of purified annexin VI, suggesting that some other essential component(s) was removed/inactivated by this treatment. Although it is possible that the purified annexin VI had lost some essential but uncharacterized activity during its preparation, this is unlikely given that there was no evidence of proteolysis or loss of ability to bind calcium dependently to phospholipids (results not shown). These data, in combination with the results using the transfected cells, lead us to conclude that annexin VI is not essential for the budding of clathrin-coated pits, since A431 cells lack annexin VI yet perform this function.

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