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The sub-cellular localization of annexin V in cultured chick-embryo fibroblasts

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The Ca^{2+}- and phospholipid-binding protein, annexin V, has been shown by an immune assay to represent 0.4% of total cell protein in cultured chick-embryo fibroblasts. Immunofluorescent localization studies indicate that in primary cultures the protein is abundant in the cytoplasm of the cells and also extends into the nucleus. Nuclear staining is no longer detectable, however, in approx. 25% of the cells following sub-culture. Sub-populations of annexin V are associated with cytoskeletal structures and with the inner face of the plasma membrane in a Ca^{2+}-independent manner. In addition, we report results indicating the secretion of annexin V from this cell type.

INTRODUCTION

The annexins [1] are a group of at least 13 major cellular proteins, related and conserved through evolution, but whose precise functions remain, as yet, unknown. They represent a new class of intracellular Ca^{2+}-binding proteins, being structurally distinct from calmodulin, parvalbumin, S100 and other small proteins involved in the response to the Ca^{2+} signal and containing the 'EF-hand' motif for Ca^{2+} binding [2]. The annexin family includes calelectrin from the electric organ of the ray *Torpedo marmorata* [3], which was one of the first annexins to be identified, as well as a number of mammalian and avian proteins (see [4–7] for recent reviews) which can each form up to 1% of total cell protein [8,9]. Recently novel family members have been identified in plants [10–12] and *Drosophila melanogaster* [13].

The relationship between the annexin proteins is characterized by the presence of a common 70-amino-acid domain within the sequence, repeated four times in annexins of M_r approx. 35000 and eight times in the larger annexin VI (M_r 68000). This has led to the suggestion that annexins have evolved by gene duplication from a common precursor [4,14]. In addition, all of the annexins so far studied bind to acidic phospholipids at micromolar Ca^{2+} concentrations and inhibit phospholipase A_2 activity *in vitro* [15,16].

Annexin V is an annexin of M_r 32500, which binds reversibly to membranes in a Ca^{2+}-dependent manner. It has been described as an anti-coagulant protein [17,18], and as an inhibitor of phospholipase A_2 [16]. The anti-coagulant and anti-inflammatory properties *in vitro* have been intensively investigated [19]. The determination of the three-dimensional structure of annexin V by X-ray crystallography [20] has shown that the protein has some structural features of channel-forming integral membrane proteins and, although true integration into the membrane is unlikely to occur due to the molecule's polar surface, annexin V has been shown to be capable of transporting both monovalent and bivalent cations across lipid bilayers [21].

Current immunohistochemical data suggest that the protein is present in both secretory and non-secretory cells and is abundant in the cytoplasm [22]. In this paper we have used a highly specific polyclonal antiserum to study the sub-cellular localization of annexin V in cultured fibroblasts with the aim of relating this to a possible cellular function.

EXPERIMENTAL

Gel electrophoresis and immunoblotting

SDS/PAGE was performed according to Laemmli [23] using a modified running buffer (188 mM Tris/188 mM glycine/0.1% SDS) and 10% (w/v) polyacrylamide gels. Gels were stained with Coomassie Brilliant Blue. Relative molecular mass markers were obtained from Sigma. To extract total fibroblast protein, cells were grown to confluence in 16-mm-diam. wells (see below for details). Using a disposable scraper (Costar), cells were scraped from the plastic into 100 μl of sample buffer [10% (w/v) glycerol/5% (v/v) 2-mercaptoethanol/3% (w/v) SDS/0.0625 M Tris/HCl, pH 6.8], 20 μl aliquots of which were loaded on to polyacrylamide gels. After separation by SDS/PAGE, proteins were electrophoretically transferred to nitrocellulose [24]. Immunoblotting was performed essentially as described previously [25] using a 1:200 dilution of rabbit antiserum to chicken annexin V and a 1:500 dilution of horseradish peroxidase-conjugated anti-(rabbit IgG) antibody prepared in goat (Sigma A9169). Peroxidase activity was detected with either 0.06% (w/v) 4-chloronaphthol and 0.03% (v/v) H_2O_2 in 50 mM Tris/HCl (pH 7.4), or 0.05% (w/v) 3,3'-diaminobenzidine and 0.01% (v/v) H_2O_2 in 50 mM Tris/HCl (pH 7.4).

Purification of annexin V for use in quantification

Annexin V was isolated using the method of Boustead et al. [26]. Frozen chicken liver (50 g) was homogenized in a Waring blender with 250 ml of 0.15 M NaCl/5 mM Hepes/5 mM EGTA, pH 7.4, and centrifuged at 40000 *g* for 30 min. The supernatant was collected and $CaCl_2$ added to a final concentration of 6 mM (1 mM excess). After 15 min on ice, the fraction was centrifuged again (40000 *g*, 30 min). The pellet obtained was washed by resuspension/recentrifugation, first in 100 ml of 0.15 M NaCl/10 mM Hepes/1 mM $CaCl_2$, pH 7.4, and then in 100 ml of 10 mM Hepes/1 mM $CaCl_2$, pH 7.4. The final pellet was resuspended in 15 ml of 10 mM Hepes/5 mM EGTA, pH 7.4,

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; FITC, fluorescein isothiocyanate; HBS, Hepes-buffered saline.

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and centrifuged at 100000 *g* for 1 h. All steps were performed at 4 °C in the presence of 0.25 mM phenylmethanesulphonyl fluoride (PMSF).

Annexin V was purified from the annexin-containing final supernatant by chromatography on a 1.0 cm × 5.0 cm column of DEAE-cellulose (DE52, Whatman) following dialysis against 20 mM Hepes, pH 7.4, and column equilibration in the same buffer. After elution of unbound material, a convex exponential salt gradient was applied (40 ml of 20 mM Hepes, pH 7.4, in the mixing vessel with 0.6 M NaCl/20 mM Hepes, pH 7.4, as the limiting buffer [27]). The column was run at 12 ml/h and 1 ml fractions were collected. Samples which were found to contain uncontaminated annexin V, as determined by SDS/PAGE, were pooled to form the annexin V standard.

Immunoquantification

Five samples of purified annexin V (16, 32, 64, 125 and 250 ng) were separated by SDS/10% (w/v) PAGE together with three identical samples (25 μg) of total fibroblast protein. Protein from the unstained gel was electrophoretically transferred to nitrocellulose [24] and immunoblotting performed using antiserum to annexin V as described above. After staining, the blot was washed, dried, and the visualized bands were individually scanned (LKB Ultroscan XL laser densitometer). Peaks were analysed to produce a standard curve of peak area versus the amount of purified annexin V loaded, from which the amount of annexin V in the fibroblast samples could be estimated.

Cell culture

The medium used throughout consisted of Dulbecco's modified Eagle's medium (DMEM) plus Ham's F-12 medium (1:1) supplemented with 10% (w/v) foetal-calf serum and containing 100 units/ml penicillin and 100 μg/ml streptomycin (all from Gibco Ltd.). Cultures were maintained at 37 °C in an atmosphere of 5% CO_2 in air.

Suspensions of primary chick-heart fibroblasts were obtained from the ventricles of 8-day-old chick embryos as described by Middleton et al. [28]. These were used as described under the appropriate sections below. Where secondary chick-heart fibroblasts were required, primary cells were plated out [1×10^6 cells per 25 cm^2 flask (Falcon)] and grown to confluence (for approx. 1 week) with changes of medium after 24 h and 5 days. Cultures were harvested with 0.25% (w/v) trypsin (Sigma Ltd.) in Ca^{2+}- and Mg^{2+}-free Earle's saline (Gibco Ltd.) and the cells resuspended in the medium described above before plating out.

Immunofluorescence microscopy

Aliquots (1 ml) of primary or secondary suspensions (2.5×10^5 cells/ml) of fibroblasts were plated out on to 13-mm-diam. cover slips contained in 16-mm-diam. tissue culture wells. After 24 h, the cultures were washed with PBS (Dulbecco's PBS) at 37 °C, before fixation at −20 °C in acetone/methanol (1:1) or 0.25% (v/v) glutaraldehyde in PBS for 5 min. Glutaraldehyde-fixed cells were permeabilized for 15 min with either 0.5% (v/v) Triton X-100 in PBS, or 0.02% (v/v) saponin in PBS, post-fixed for 5 min in 0.25% (v/v) glutaraldehyde in PBS and incubated for 5 min in each of three changes of sodium borohydride (1 mg/ml) in PBS. Cells were treated sequentially with (1) 5% (v/v) normal sheep serum for 20 min at 37 °C to block non-specific binding, (2) 1% (v/v) rabbit anti-(chicken annexin V) antibody for 1 h at 37 °C and (3) 0.4% (v/v) fluorescein isothiocyanate (FITC)-conjugated sheep anti-(rabbit IgG) antibody (affinity purified, Sigma Ltd.) for 1 h at 37 °C. Between steps (2) and (3) and again before mounting in a polyvinyl alcohol medium, cultures were washed with five changes of PBS containing 0.05% (v/v) Tween 20. Antibody dilutions were carried out in PBS/0.05% (v/v) Tween 20 supplemented with 5% (v/v) normal sheep serum. After 24 h at room temperature to allow the mountant to harden, samples were viewed using a light (Nikon Optiphot) or confocal scanning (Leitz) microscope. In some experiments, cells were disrupted by using nitrocellulose to tear the unfixed cells apart [29].

Determination of the Ca^{2+}-dependency of association of annexin V with membranes and cytoskeleton

Primary cultures of chick-embryo fibroblasts were seeded in 25 cm^2 flasks (Falcon) at a concentration of 1×10^6 cells per flask and grown to confluence (approx. 5 days). The medium was removed, the cultures washed in Ca^{2+}- and Mg^{2+}-free Earle's saline at 37 °C and the cells from individual flasks were scraped (disposable cell scraper, Costar) into 2 ml of one of the following buffers to obtain four samples, each containing equivalent amounts of total fibroblast protein. Two samples of cells were scraped into 0.15 M NaCl/10 mM Hepes, pH 7.4, containing either 5 mM EGTA or 1 mM $CaCl_2$. In order to preserve membrane structure this pair of buffers contained no detergent. The two remaining flasks were harvested into 100 mM KCl/1 mM NaN_3/0.5 mM dithiothreitol/1% (v/v) Triton-X-100/10 mM imidazole/HCl, pH 7.4, also containing either 5 mM EGTA or 1 mM $CaCl_2$, and designed to maintain actin filaments while solubilizing cellular membranes. The resulting cell suspensions were frozen in liquid nitrogen, thawed and centrifuged at 100000 *g* for 1 h. Following removal of the supernatants, the pellets were washed once in the appropriate buffer and resuspended in 200 μl of sample buffer. Samples of pellet and supernatant proteins were analysed by immunoblotting as described above.

Annexin V secretion

Primary cultures of chick-heart-ventricle fibroblasts were seeded into 32-mm-diam. wells (Falcon) at a final concentration of 2.5×10^5 cells per well in a total volume of 4 ml, and grown to confluence. The medium was changed after 24 h to remove contaminating blood cells and cell debris. After 5 days, the tissue-culture supernatant was removed and retained for further analysis. The cell monolayer remaining was washed once in Hepes-buffered saline (HBS, 0.15 M NaCl/10 mM Hepes, pH 7.4) containing 1 mM $CaCl_2$, then twice in HBS containing 5 mM EGTA in order to release any annexin V associated with the extracellular surface of the plasma membrane in a Ca^{2+}-dependent manner. The cells were then frozen and thawed in HBS containing 5 mM EGTA before treatment with HBS/1% (v/v) Triton X-100/5 mM EGTA. The final supernatant was removed and remaining cell material taken up in 0.2 ml of SDS sample buffer. All buffers were kept at 37 °C. Wash volumes were 2 ml. At each stage, the supernatant from above the cells was removed and centrifuged at 100000 *g* for 1 h to remove any cell fragments released from the substratum during EGTA-containing washes. Samples of the tissue-culture supernatant, each wash supernatant, and any cell material remaining after the treatments were analysed by SDS/PAGE and immunoblotting as described above.

RESULTS

Antibody specificity and immunoquantification

A rabbit antiserum was raised to annexin V purified from chicken liver according to the method of Boustead et al. [26]. In liver the antiserum is specific to annexin V and does not cross-react with any other chicken annexins [29a]. When tested on immunoblots of total fibroblast protein, the antiserum was found to be specific to a protein of M_r 32500 (Figure 1). The antiserum was used to quantify annexin V in primary cultures of fibroblasts. The results (not shown) demonstrated that annexin V represents $0.41 \pm 0.04\%$ (mean $\pm$ S.D., $n = 6$) of total fibroblast protein.

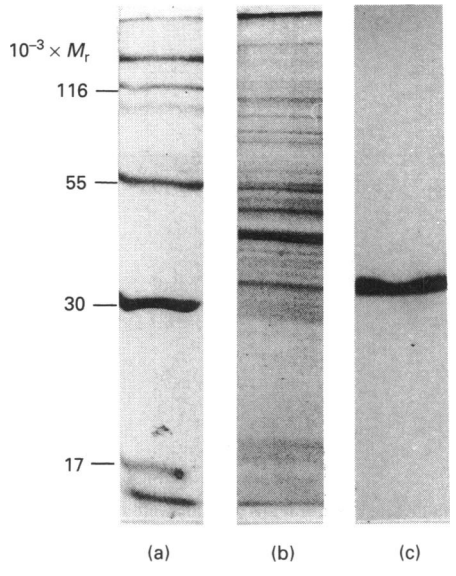

Figure 1 PAGE and immunoblotting

SDS/10% (w/v) polyacrylamide gel of (**a**) M_r standards, and (**b**) total fibroblast protein, stained with Coomassie Brilliant Blue. (**c**) Immunoblot of a fraction similar to (**b**) using antiserum to chicken annexin V.

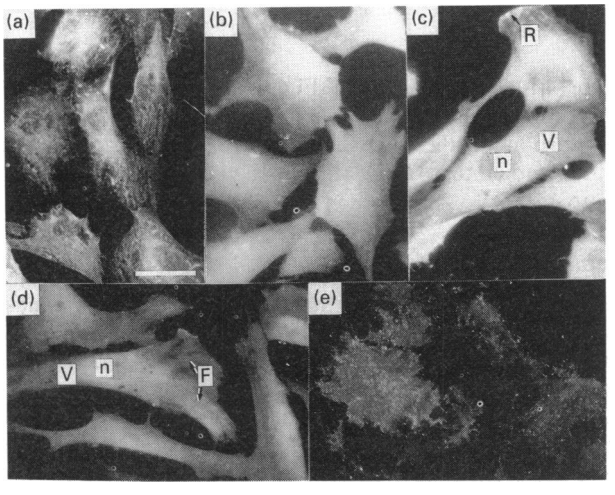

Figure 2 Localization of annexin V in primary chick-embryo fibroblasts by immunofluorescent light microscopy

Cells were fixed with acetone/methanol at −20 °C (**a**) or glutaraldehyde (**b–e**) and permeabilized with Triton X-100 (**b** and **d**) or saponin (**c**). Image (**e**) shows lower cell membrane fragments remaining after nitrocellulose-induced cell lysis (see the Experimental section). Cytoplasmic inclusions (V), ruffles (R), filamentous structures (F) and a nucleus with visible nucleoli (n) are indicated. Samples treated with pre-immune serum and antigen-adsorbed antiserum were negative (results not shown). Scale bar, 50 μm.

Immunofluorescent localization of annexin V in primary cells

The polyclonal antiserum to annexin V was used to determine the localization of annexin V in cultured primary chick-embryo fibroblasts (Figure 2). As can be seen from Figure 2(a–c), the apparent localization of annexin V is dependent on the method of fixation used. Cells stained after acetone/methanol fixation (Figure 2a) have a fibrous or reticular staining similar to that seen for antigens associated with the plasma membrane cytoskeleton. Glutaraldehyde fixation followed by permeabilization with Triton X-100 gave stronger staining of cells, with staining increasing from the cell periphery to the cell centre. The improved staining after glutaraldehyde fixation is probably due to improved preservation of cellular structure. It has been shown that annexins can, in fact, be purified from acetone powders of tissues [30], and it is therefore possible that a significant proportion of annexin V is lost from acetone-fixed cells. In glutaraldehyde-fixed cells (Figures 2b and 2d), annexin V can be seen to extend throughout the cytoplasm and into the nucleus. In most cells, small unstained areas (V) of the cytoplasm are present which probably correspond to lipid droplets [31]. Fine filopodia-like projections from fibroblasts are positive, and membrane ruffles are brightly stained (Figure 2b). A number of cells also appear to show an association of the protein with filamentous structures (F) in peripheral regions of the cytoplasm (Figures 2b and 2d).

Permeabilization of glutaraldehyde-fixed cells with saponin, which is known not to render nuclear membranes permeable to antibody molecules [32], eliminated the nuclear staining observed in Triton-permeabilized cells, but left that of the surrounding cytoplasm unaffected (Figure 2c).

In some experiments, cells were disrupted before fixation by pressing moistened nitrocellulose against their upper surfaces [29]. Subsequent removal of the nitrocellulose causes the loss of upper cell membrane fragments and cytosol, leaving the lower membranes of the cells still attached to the substratum. These were then fixed and processed in the same manner as for whole cells. These fragments were also positive for annexin V (Figure 2e).

Comparison of annexin V localization in primary and secondary cells

In order to confirm the results obtained with saponin, primary and secondary fibroblasts stained with antiserum to annexin V were examined by confocal scanning microscopy. A series of optical sections 0.4 μm thick were taken through the cells starting nearest the substratum and moving gradually towards the upper surface of the cell. Figures 3(a)–3(c) are the second, fourth and sixth sections from a series of eight such images and show that in 24 h cultures of primary chick-heart fibroblasts, the antigenic response extends throughout the cell including the nucleus, with the possible exception of nucleoli. After 7 days in culture, nuclear staining was lost from a small proportion of the primary fibroblasts ($< 5\%$), although the cytoplasm was still strongly positive. However, this proportion of unstained nuclei was increased to 25% in secondary fibroblasts 24 h after being subcultured from 5-day-old primary cultures. Figures 3(d) and 3(e) are illustrative sections (fourth and sixth from a series of 10) of secondary fibroblasts showing a binucleate cell from which nuclear staining is absent.

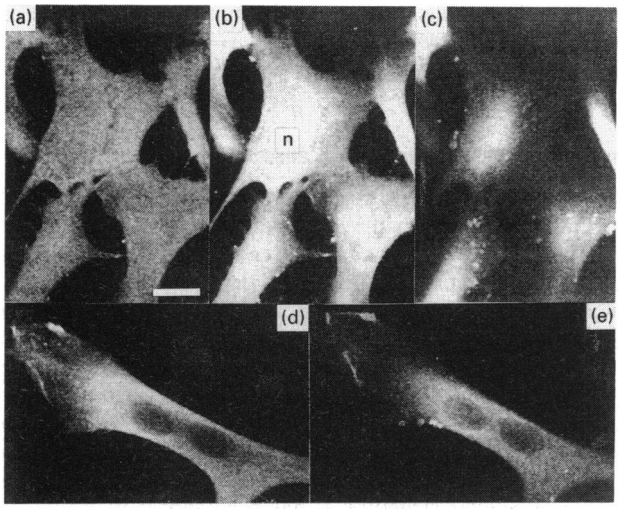

Figure 3 Developmental study of annexin V localization in primary and secondary chick-embryo fibroblasts viewed by immunofluorescent confocal scanning microscopy

Cells were fixed with glutaraldehyde, permeabilized with Triton X-100 and stained with antiserum to chicken annexin V, followed by FITC-labelled second antibody. Studies of primary (**a**–**c**) and secondary (**d** and **e**) cells are included. The images are individual 0.4 μm sections. Controls using pre-immune serum and antigen-adsorbed antiserum (results not shown) were negative. Scale bar, 10 μm.

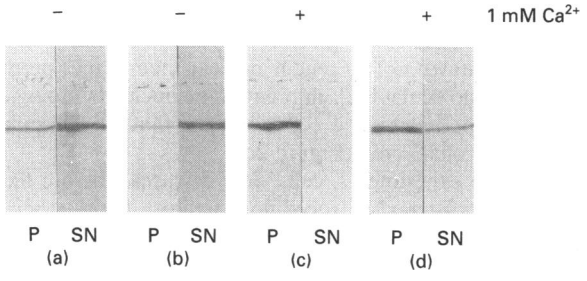

Figure 4 The Ca^{2+}-dependency of association of annexin V with membranes and the cytoskeleton

Confluent cultures of primary fibroblasts were harvested into one of two buffers designed to stabilize either membranes (**a** and **c**) or the cytoskeleton (**b** and **d**) in the presence of 5 mM EGTA (**a** and **b**) or 1 mM Ca^{2+} (**c** and **d**). Following centrifugation, supernatants (SN) and pellets (P) were separated by SDS/PAGE, transferred to nitrocellulose, and the blots probed with antiserum to chicken annexin V.

Ca^{2+}-dependency of membrane and cytoskeletal annexin V association

Figure 4 shows the results from experiments designed to provide biochemical data to complement the immunolocalization studies. Figures 4(a) and 4(c) show the association of annexin V with the membrane-containing pellet (P) or the supernatant (SN) following sub-fractionation of the cells in a detergent-free buffer. Although some membrane association is seen in the presence of 5 mM EGTA (Figure 4a), the addition of 1 mM Ca^{2+} induces all previously soluble annexin V to become associated with the particulate fraction of the cell (Figure 4c). Cells were also extracted with buffer containing 5 mM EGTA and 100 mM KCl to stabilize actin filaments, and detergent to solubilize membranes. In this case, a similar distribution between pellet and supernatant to that observed in the absence of detergent is seen [Figure 4(b), compared with Figure 4(a)]. However, in this instance, when the EGTA in the buffer is replaced by 1 mM Ca^{2+}, a proportion of the annexin V remains in the supernatant fraction, rather than associating with the 'cytoskeletal' pellet (Figure 4d).

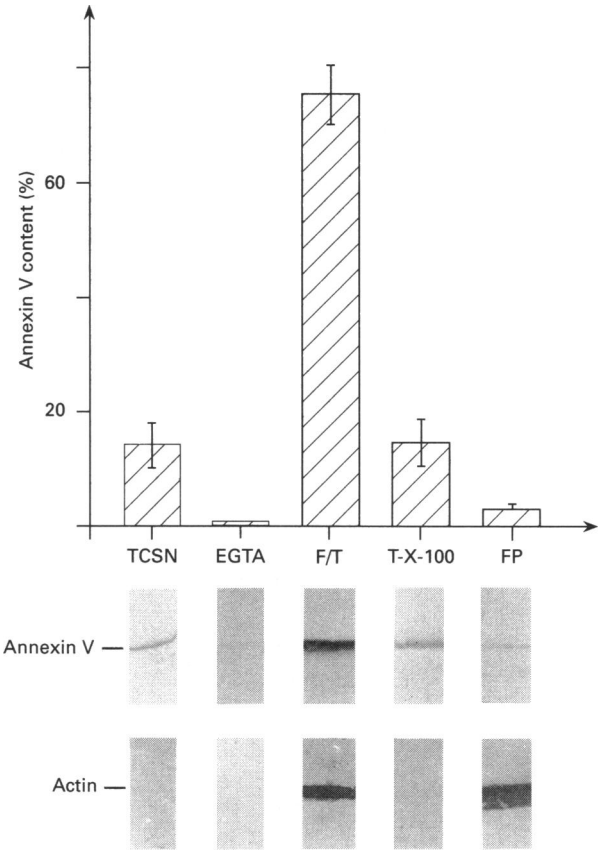

Figure 5 Study of annexin V secretion from chick-embryo fibroblasts

Following removal of the tissue-culture supernatant (TCSN), confluent primary cultures were washed in EGTA-containing buffer (EGTA), freeze-thawed (F/T) and washed in Triton X-100-containing buffer (T-X-100). Any remaining cell fragments forming the final pellet were taken up into sample buffer (FP). All samples were separated by SDS/PAGE, transferred to nitrocellulose, and the blots treated with antiserum to chicken annexin V (upper panel of immunoblots) or monoclonal antibody to actin (lower panel of immunoblots). In order to quantify results, annexin V immunoblots were scanned using an LKB densitometer. Results are displayed as the percentage of total annexin V, both intra- and extra-cellular, represented by each fraction.

Annexin V secretion

Recent studies have suggested that some members of the annexin family can be detected in extracellular locations and that their secretion may be occurring via a previously undescribed mechanism [33]. Figure 5 shows results from experiments to establish whether annexin V is secreted from growing fibroblasts. Figure 5 (TCSN) shows that annexin V can be detected in tissue-culture supernatants isolated from 5-day-old cultures of confluent primary fibroblasts. In control experiments, antiserum raised to chicken annexin V gave no response on immunoblots of the foetal-calf serum used in tissue culture (results not shown). Samples of tissue-culture supernatant were also tested for actin (lower panel) as a marker for the release of cytosol from lysed cells. There have been no reports of actin being secreted and it was not detected in the culture supernatants. Control experiments (results not shown) demonstrated that actin was not proteolysed

by the tissue-culture medium. Subsequent washing of the cells with EGTA-containing buffer released negligible amounts of annexin V and actin (Figure 5, EGTA). Cells were then treated by freeze–thawing in the absence of EGTA to release cytosolic proteins. The major proportion of cellular annexin V and over 50% of cellular actin can be detected in this fraction (Figure 5, F/T). Further annexin V is released by Triton-X-100 treatment (Figure 5, T-X-100). A significant proportion of annexin V remains in association with the final pellet of residual cell structure following these treatments (Figure 5, FP). The remaining 50% of cellular actin is also detected in this fraction. All steps except the initial wash of the intact cells were performed in the presence of EGTA. The associations observed between annexin V and cellular structure may therefore be independent of Ca^{2+} and mimic the situation in unstimulated fibroblasts.

DISCUSSION

Immunoquantification

We have shown by an immunoassay that annexin V represents 0.4% of total fibroblast protein. This figure is similar to values reported for other members of the annexin family in various tissues and cell types. In normal cultured diploid human foreskin fibroblasts, where levels of annexin expression have been shown to change in a growth-dependent manner, annexin I has been shown to represent 0.5%–2.0% of total cell protein, annexin II 1% of total cell protein and annexin IV 0.08%–0.13% of total cell protein [8]. Annexin VI has been shown to represent 0.24% of total cell protein in rat liver (J. J. Koster, unpublished work) and 0.3% of total cell protein in pig brain [9]. The high levels of annexin expression observed in a number of different tissues and cell types suggest that these proteins have a fundamental role in cell structure and/or function.

Immunofluorescent localization of annexin V in cultured cells

Our studies demonstrate that, in primary chick-embryo fibroblasts, annexin V is located throughout the cytoplasm and also extends into the nucleus. Other annexins have also been located in cells in culture. Annexin I has been located in cytoplasm and nuclei [34,35], while the immunofluorescent staining pattern of annexin II in cells has been shown to increase from the cell periphery towards the nucleus, with some authors reporting intense perinuclear fluorescence [36–38]. In one case unstained vacuoles were seen in the cytoplasm [36], which is similar to our findings with annexin V, and in other studies annexin II was detected in the nuclei of chick-embryo fibroblasts [39], and HeLa cells [40]. Immunohistochemistry on sections of rat tissues has demonstrated the presence of annexin V in the cytoplasm of a range of cell types [22].

When primary cells are examined after being maintained in culture for approx. 1 week before fixation, nuclear staining is absent from a very small number ($< 5\%$) of cells. If after 5 or 6 days, however, cells are sub-cultured, within 24 h annexin V cannot be detected in the nuclei of over 25% of the secondary cell population. Proteins specifically targeted to the nucleus normally possess a characteristic sequence [41] which has not been observed in any of the annexins. However, studies using labelled dextrans have shown that the channel size of a resting nuclear pore is sufficient to permit the free diffusion of molecules under approx. 40000-M_r, while excluding larger cytoplasmic proteins [42,43]. Thus the smaller annexins of 32000–38000-M_r, such as annexin V and monomeric annexin II, could gain access to the nucleus without containing a specific nuclear targeting sequence. However, it is not clear why, following subculture, annexin V should become excluded from the nucleus in a subpopulation of secondary cells. It will be important to determine whether, over longer periods of time, nuclear staining is lost by all cells and to assess how these observations relate to the physiological role of annexin V.

Results from immunofluorescence also indicate a possible association of the protein with cytoskeletal structures and with the inner face of the plasma membrane. Annexins I and II have been shown to bind F-actin *in vitro* [44], the latter in its tetrameric form when complexed with p10. Annexin V has also been demonstrated to bind F-actin at high Ca^{2+} concentrations *in vitro* [45]. However, our preliminary cell extraction experiments (not presented) have not shown an extensive association of annexin V with actin filaments, although a residual population of annexin V does remain following solubilization of cells with non-ionic detergents under conditions which preserve the F-actin-rich cytoskeleton. A sub-plasmalemmal association has been reported for annexins II, IV and VI [9,46,47]. The positive response seen on membranes from cells disrupted in Ca^{2+}-free buffer by nitrocellulose cell stripping therefore suggests the presence of a Ca^{2+}-independent or low-Ca^{2+}-requiring form of membrane-associated annexin V, which would only be extractable by detergent treatment. A similar result has previously been reported for annexin II in its monomeric form [48].

Ca^{2+}-dependency of annexin V association with membranes and the cytoskeleton

At physiological Ca^{2+} concentrations we find that the bulk of annexin V in fibroblasts can be extracted from cells. Even up to 100 μM Ca^{2+} concentrations most of the annexin V is released by simple disruption of the cell membranes by freeze–thawing. In addition, the biochemical studies provide further evidence to support a Ca^{2+}-independent association of annexin V with both membrane and cytoskeletal components of the cell, since even in the presence of EGTA a significant proportion of annexin V remains in the pelletable fraction after centrifugation. A Ca^{2+} concentration of 1 mM induces complete association of cellular annexin V with the 'membrane' but not the 'cytoskeletal' fraction of the cell. This suggests that while all the annexin V has the ability to bind to membranes *in vitro*, there may be a pool which cannot become associated with the cytoskeleton, even at high Ca^{2+} concentrations.

Annexin V secretion

The presence of annexin V, but absence of actin, in a tissue-culture supernatant isolated from growing cells provides evidence to support the secretion of this protein. If annexin V was present as a result of cell lysis, a response to actin would also be seen since the anti-(actin) antibody detects both polymerized (F) and unpolymerized (G) forms of the protein, and there are significant amounts of the latter present in the cytoplasm of these cells. Annexins I and V have recently been shown to account collectively for 1.3% of total protein in human prostate fluid and 0.2% of total protein in seminal plasma [33]. The source of the annexins appears to be the epithelial cells lining the prostate gland. Immunofluorescent studies have shown extensive co-localization of annexins I and IV in this cell type, but annexin IV does not appear in prostate secretions, supporting the hypothesis that the appearance of annexins I and VI in extracellular fluids is a specific process and does not arise from cell degradation or lysis. Since annexins do not possess hydrophobic signal sequences

and have a highly polar outer surface, which would prohibit total incorporation into the lipid bilayer of the plasma membrane, their presence in extracellular fluids cannot be explained by current theories of secretion and may therefore be occurring via a novel secretory mechanism.

In conclusion, annexin V exists within fibroblasts in several sub-cellular locations. Of particular interest is the existence of a major pool of cytosolic annexin V which shows no Ca^{2+}-dependent binding to membranes at physiological Ca^{2+} levels. Our future work will centre on identifying the biochemical basis for the heterogeneity of annexin V in chick-heart fibroblasts.

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