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Nucleolar and cytoplasmic localization of annexin V

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The subcellular localization of annexin V in cultured human umbilical vein endothelial cells, epithelial cells and fibroblasts was examined. Indirect immunofluorescence and immunoblotting studies using affinity-purified anti-annexin V antibodies revealed that annexin V is located within the cytoplasm and nucleus of these cells. Further examination and direct binding studies showed that annexin V within the nucleus is associated with the nucleolus. These findings suggest that annexin V may play a role in a nucleolar function, such as ribosome assembly and transport.

Annexin V; Nucleolus; Endothelial cell

1. INTRODUCTION

Annexin V belongs to a large protein family the members of which exhibit calcium-dependent binding to phospholipid. This characteristic is the basis for the potent anti-phospholipase and anticoagulant functions of the annexins [1]. Because of its therapeutic potential as an anticoagulant or anti-inflammatory agent, the physical properties of annexin V have been studied in some detail. Like most annexins, it has an internal 4-fold repeat of a hydrophobic 70 amino acid domain which is flanked by short amino- and carboxy-terminal regions [2]. Analysis of the 3D structure of the protein has shown that it is a symmetrical molecule that binds 5 atoms of calcium and has characteristics of membrane channel-forming proteins [3,4]. Despite detailed investigation of its properties and structure, the true function of annexin V is unknown.

Annexin V is widely distributed. Very low levels of annexin V have been detected in plasma, however, the protein is abundant in cultured endothelial cells, in soluble extracts of many human tissues, and appears to be ubiquitous [5,6]. This type of distribution raises the possibility that the protein functions at a cellular level. Analysis of the subcellular localization of annexin V may therefore help to elucidate its physiological function. We have studied the subcellular localization of annexin V in primary human endothelial cells, as well as in the HES, HeLa and A549 cell lines. The protein is located within the cell and appears in the cytoplasm and nucleolus.

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2. MATERIALS AND METHODS

2.1. *Preparation of monospecific anti-annexin V antibody*

Annexin V was purified to homogeneity from human placenta as described [7]. The preparation contained a single 35 kDa protein, as assessed by SDS-PAGE followed by silver staining (Bio-Rad). Iodination [8] of the preparation followed by SDS-PAGE and autoradiography yielded a single 35 kDa band. The protein was confirmed as annexin V by partial amino acid sequence of cyanogen bromide-cleaved fragments. Polyclonal antiserum to native annexin V was raised in rabbits using standard procedures [8]. Monospecific antibody was prepared by annexin V affinity chromatography followed by plasma protein affinity chromatography. 10 ml of annexin V affinity resin (1 mg protein/ml Bio-Rad Affigel 15 resin) was equilibrated with 20 mM Tris (pH 7.4), 0.15 M NaCl. 50 ml of antiserum was applied to the column and after extensive washing with the equilibration buffer, bound antibody was eluted using 0.1 M glycine (pH 2.5). The eluate was immediately neutralized with a 0.2 vol. of 1 M K_2HPO_4, and dialyzed overnight against 20 mM Tris (pH 7.4), 0.15 M NaCl. To ensure elimination of small amounts of anti-albumin present in the antiserum, the eluate was then applied to a column containing normal plasma protein coupled to Affigel 15 (5 mg protein/ml resin). This was performed essentially as described for the annexin V affinity step. The flow through was collected and concentrated by ultrafiltration.

Control IgG was prepared from the flow through of the annexin V Affigel affinity column. 10 ml of this material was applied to a 5 ml protein G-Sepharose column (Pharmacia). After extensive washing with the equilibration buffer, the IgG was eluted using 0.1 M glycine (pH 2.5), immediately neutralized with 1 M K_2HPO_4 and dialyzed as described above. The material was concentrated to 2 mg/ml by ultrafiltration.

2.2. *Cells*

Materials for cell culture were from Flow Laboratories. Human endothelial cells were isolated from umbilical veins as described [4]. Endothelial cells were grown on gelatinized (0.1%) plates in M199 medium supplemented with 10% heat-inactivated fetal calf serum. The human fibroblast cell line, HES, was purchased from Commonwealth Serum Laboratories (Australia) and the HeLa cell line was a gift from Dr. P. Gleeson (Department of Immunology and Pathology, Monash Medical School, Alfred Hospital). A549 cells were purchased from ATCC. All cell lines were grown in Dulbecco's modified Eagle's medium and fetal calf serum (10% v/v).

2.3. *Fractionation of cells*

Cytoplasmic and nuclear extracts were obtained by sequential extraction in detergent-containing buffers [10]. Using this procedure, leakage of nuclear components into cytoplasmic fractions is minimal [10]. Briefly, washed monolayers were disrupted in situ with a buffer containing 0.5% Triton X-100 in order to release cytoplasmic components. The resulting solution (cytoplasmic extract) was collected, nuclei remaining on the dish were washed, then lysed in a buffer containing 0.5% Nonidet P-40. Following centrifugation at 13,000 × *g* to remove debris, the supernatant (nuclear extract) was collected.

2.4. *Immunoassays*

Indirect immunofluorescence was essentially as described [11]. Cells grown on coverslips were fixed in 3.7% formaldehyde and permeabilized using 0.1% Triton X-100. In experiments to demonstrate annexin V in isolated nuclei, cells were treated first with 0.1% Triton X-100 and then fixed. For double staining, samples were first incubated with anti-annexin V antibody (1 μg/ml) for 60 min, then washed and incubated with a sheep anti-rabbit fluorescein isothiocyanate (FITC)-conjugated second antibody (Silenus, Australia). This was followed by a second 60 min incubation in a 1:200 dilution of an autoimmune human serum known to contain anti-nucleolar antibodies (a gift from Dr. J. Rolland, Department of Immunology and Pathology, Monash University), washing and incubation with sheep anti-human rhodamine isothiocyanate (RITC)-conjugated second antibody (Silenus, Australia). Immunoblotting followed standard procedures [8] using a horseradish peroxidase-conjugated anti-rabbit second antibody and a 4-chloro-1-naphthol development system (Bio-Rad).

2.5. *Fluorescent labeling and direct binding of annexin V*

200 μg of purified annexin V was labeled with FITC as described [8]. Cells were fixed in 3.7% formaldehyde and permeabilized using 0.1% Triton X-100 as described above. In experiments to demonstrate binding of annexin V to nuclei, cells were treated first with 0.1% Triton X-100 and then fixed. The FITC-labeled annexin V (1 μg/ml in 20 mM Tris (pH 7.4), 0.15 M NaCl, 1 mM EGTA, 0.1% Triton X-100) was incubated with the cells at 37°C overnight. Cells were washed at room temperature 3 times (10 min each) in the same solution lacking annexin V, and then 3 times in a modified solution lacking annexin V and containing 2 M NaCl.

3. RESULTS

3.1. *Characterization of a monospecific annexin V antibody*

A monospecific anti-annexin V antibody was prepared by chromatography of rabbit antiserum on a column containing immobilized, pure annexin V. Western blotting was used to determine the specificity of the anti-annexin V antibody. The crude antiserum recognised two proteins in a partially pure preparation of annexin V known to contain albumin, and several proteins in plasma (Fig. 1). On the other hand, the antibody eluted from the annexin V affinity column reacted with only one protein in the semi-purified annexin V preparation, and showed no detectable reactivity with proteins in plasma (Fig. 1). Further analysis revealed that the antibody reacted with a single 35 kDa protein in total protein extracts prepared from endothelial cells and fibroblasts (data not shown). Although annexin V is structurally similar to other members of the annexin family, these results show that the purified antibodies do not recognize other annexins present in these cells or in plasma.

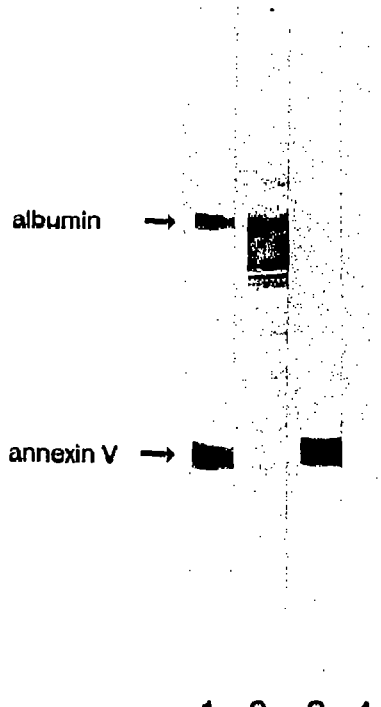

Fig. 1. Monospecificity of anti-annexin V antibodies. Samples were separated on 10% SDS-PAGE gels and transferred to nitrocellulose. Membranes were probed with a 1:200 dilution of the crude rabbit anti-annexin antiserum (lanes 1 and 2) or 2 μg/ml affinity-purified anti-annexin antibodies (lanes 3 and 4). Target antigens were 10 μg partially purified annexin V (lanes 1 and 3) and 100 μg normal human plasma protein (lanes 2 and 4).

3.2. *Cellular localization of annexin V*

The distribution of annexin V in human endothelial cells was studied using the monospecific antibody in an indirect immunofluorescence assay. Annexin V was not detected on the endothelial cell surface (Fig. 2a), however, when the cells were permeabilized with 0.1% Triton X-100, positive staining was observed in the cytoplasm and nucleus (Fig. 2b). No staining was observed using a control antibody (data not shown). Similar results were obtained using human HES, HeLa and A549 cells, as well as with several mouse and simian lines (data not shown).

The bright cytoplasmic fluorescence obscured nuclear details and made it difficult to accurately determine the nuclear distribution of annexin V. To overcome this problem, endothelial cells attached to coverslips were treated with lysis buffer, gently washed, then fixed. This served to lyse the cells without affecting nuclear structures. Nuclei prepared in this way and treated with the anti-annexin V antibody revealed a nucleolar pattern of staining (Fig. 2c). To confirm this nucleolar distribution a double-staining procedure was used in which nuclei were treated first with the anti-annexin V antibody and a fluorescein-conjugated second antibody (Fig. 2c), and then with antiserum con-

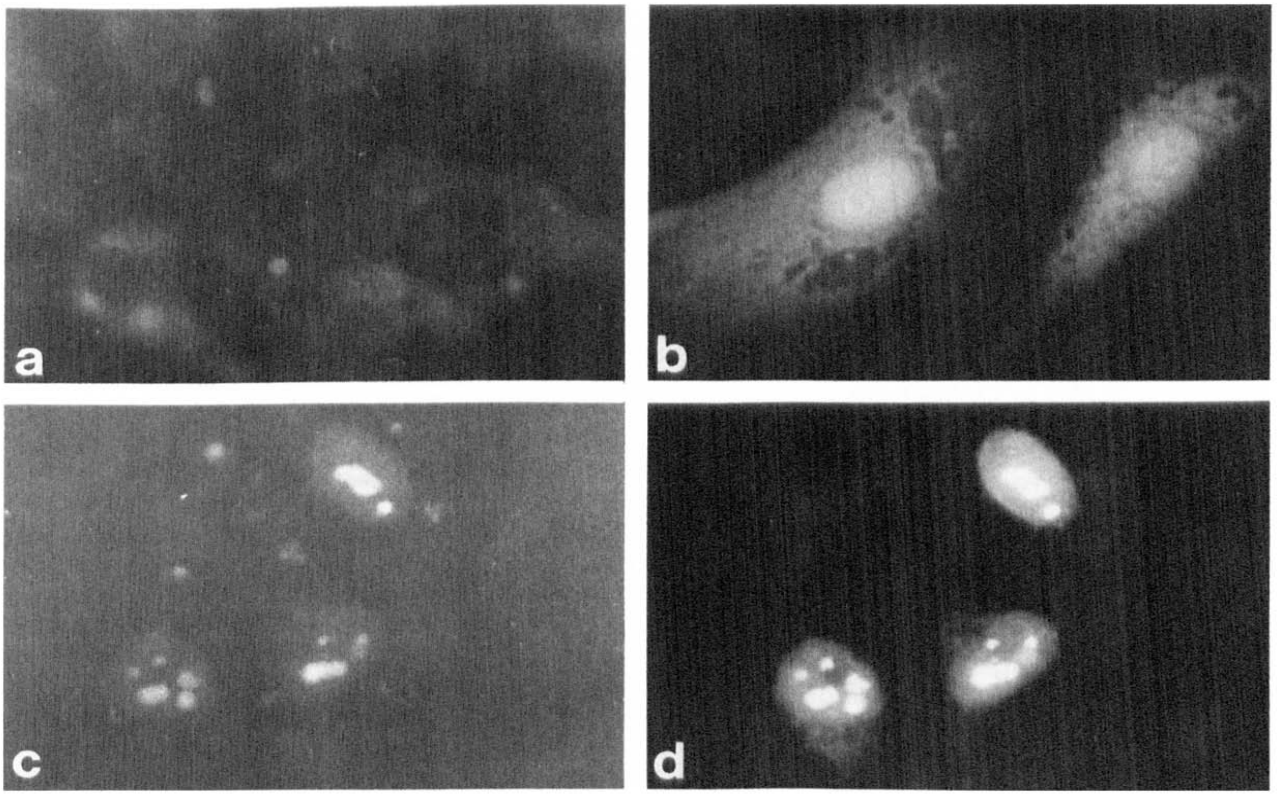

Fig. 2. Intracellular localization of annexin V. Intact (a) or permeabilized (b) endothelial cells were stained using 1 μg/ml anti-annexin V as the first antibody and a 1:200 dilution of a FITC-conjugated anti-rabbit IgG as the second antibody. Endothelial cell nuclei (c,d) were first stained as described for a and b, and then were stained with a 1:200 dilution of human anti-nucleolar antiserum and a 1:200 dilution of a RITC-conjugated anti-human IgG. Panel c shows nuclei marked with FITC (annexin), and panel d shows the same nuclei marked with RITC (nucleolar). There was no cross-reaction observed between the antibodies (not shown).

taining anti-nucleolar antibodies and a rhodamine-conjugated second antibody (Fig. 2d). Co-localization of the annexin V and nucleolar antibodies was observed, confirming the nucleolar distribution of annexin V.

The subcellular distribution of annexin V was also studied by preparing crude cytoplasmic and nuclear extracts from endothelial and HES cells and immunoblotting using the monospecific antibody (Fig. 3). A 35 kDa protein was present in all of the extracts, which is consistent with the distribution of annexin V observed using indirect immunofluorescence.

3.3. *Binding of labeled annexin V to fixed cells*

To confirm the intracellular distribution of annexin V and to assess the role of calcium in the compartmentalization of annexin V, direct binding experiments were carried out. Fluorescently labeled annexin V was incubated with fixed and permeabilized cells or nuclei in a solution lacking calcium and containing EGTA (Fig. 4). A similar staining pattern to that obtained using anti-annexin V antibodies was observed. Addition of 2.5 mM calcium to the binding solution did not significantly alter the distribution of annexin V, whereas the inclusion of unlabeled annexin V in 100-fold excess abrogated staining (data not shown).

4. DISCUSSION

The annexin gene superfamily presently contains over 13 members, most of which show restricted species or tissue distribution. Although the annexins share the property of calcium-dependent phospholipid binding, specific physiological functions have been ascribed to only three. These are annexin II, which participates in calcium-dependent exocytosis [12]; annexin III, which is an inositol 1,2-cyclic phosphate 2-phosphohydrolase [13]; and annexin VI, which is required for the budding of clathrin-coated endocytic pits [14].

The structure, function and tissue distribution of annexin V has been studied in some detail, yet its physiological role is unclear. Our studies, using both anti-annexin V antibodies and a direct binding approach, show that annexin V can be found in the cytoplasm. Due to the limitations of the immunofluorescence and fractionation techniques, at present we cannot rule out an association of annexin V with the cytoskeleton or with cytoplasmic organelles such as mitochondria. Annexin V is, however, clearly present in the nucleolus. Although it is small enough to diffuse into the nucleus, the retention of annexin V in the nucleolus probably requires a specific mechanism. For example, it may re-

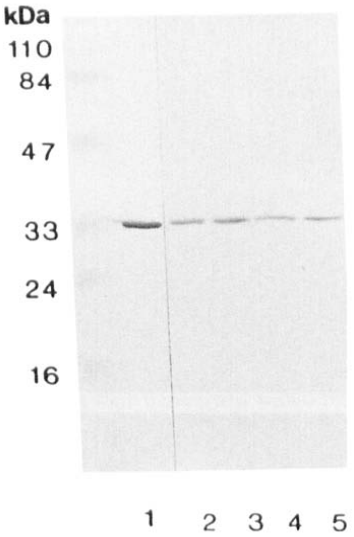

Fig. 3. Annexin V in cytosolic and nuclear fractions from endothelial cells and fibroblasts. Extracts containing 50 μg of protein were separated on 10% SDS-PAGE gels and transferred to nitrocellulose. The membrane was probed using the monospecific anti-annexin V antibody (2 μg/ml) and a HRP-conjugated second antibody (1:400). Lane 1, annexin V, 0.2 μg; lane 2, endothelial cell cytosolic extract; lane 3, cytosolic extract from HES cells; lane 4, endothelial cell nuclear extract; lane 5, nuclear extract from HES cells.

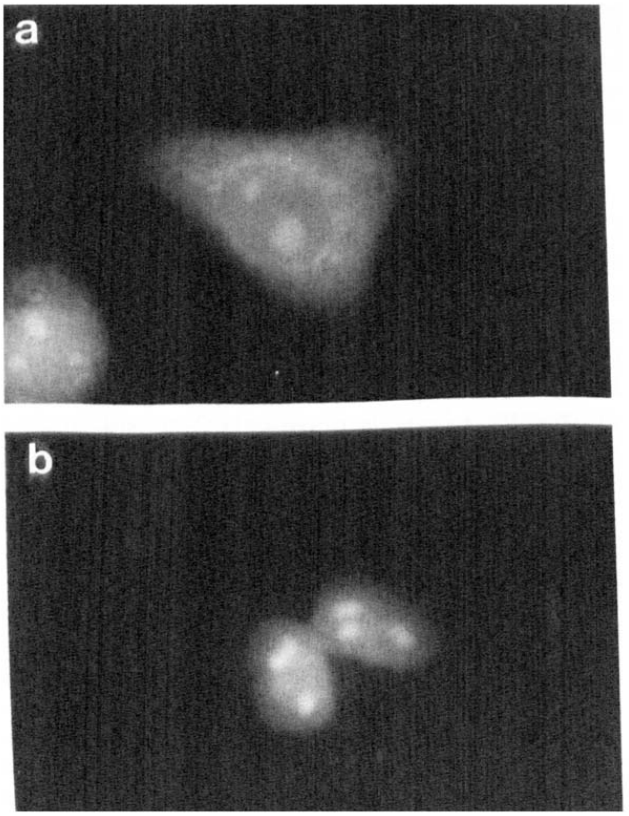

Fig. 4. Binding of annexin V to cells and nuclei. Permeabilized A549 cells (a) or nuclei (b) were stained with 1 μg/ml FITC-conjugated annexin V in the absence of calcium.

quire nucleic acid binding ability, as recently demonstrated for the nucleolar transcription factor, mUBF [15]. Interestingly, we have observed that annexin V can bind to DNA (unpublished results).

Recently, Mizutani et al. have reported that a new 50 kDa annexin (CAP 50) localizes in the nuclei (but not nucleoli) of 3Y1 fibroblasts in a calcium-dependent manner [16]. The distribution of annexin V we observed occurs in the presence of detergent and in the absence of calcium, suggesting that the compartmentalization of annexin V between cytoplasm and nucleus probably does not depend on binding to phospholipid.

The demonstration that two different annexins localize to the nucleus raises the prospect that annexins may contribute to processes that are fundamental for cell growth or division. Annexin V is found in the nucleolus, which is the most obvious structure contained in the nucleus of a non-mitotic cell. As the only known nucleolar function is the synthesis of ribosomal RNA and the assembly of ribosomes, it is possible that annexin V is involved in either the establishment and maintenance of nucleolar structure or in ribosome synthesis, assembly, transport and function. Either role would probably be important for cell growth and survival, but our finding that annexin V is also present in the cytoplasm is more consistent with a contribution to ribosome biosynthesis or function.

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