Vol. 186, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1992 Pages 432-439

MORPHOLOGICAL AND BIOCHEMICAL EVIDENCE FOR PARTIAL NUCLEAR LOCALIZATION OF ANNEXIN 1 IN ENDOTHELIAL CELLS

Patrick Raynal[1], Paul M.P. van Bergen en Henegouwen[2], Francoise Hullin[1], Jeannie M.F. Ragab-Thomas[1], Josette Fauvel[1], Arie Verkleij[2], and Hugues Chap[1,*]

[1]*INSERM Unité 326, Phospholipides Membranaires, Signalisation Cellulaire et Lipoprotéines, Hôpital Purpan, 31059 Toulouse, France*

[2]*Department of Molecular Cell Biology, University of Utrecht, 3584 CH Utrecht, The Netherlands*

Received May 22, 1992

Using immunofluorescence, an affinity-purified anti-annexin-1 polyclonal antibody showed both cytoplasmic and nuclear staining, whereas antibodies against annexins 2, 5 and 6 labelled almost exclusively the cytoplasm of cultured endothelial cells. This was further confirmed by immunogold labelling and electron microscopy using a monoclonal antibody, annexin 1 being detected close to the plasma membrane, in the cytoplasm, as well as inside the nucleus. Finally, using immunoblotting, purified nuclei were shown to contain annexin 1, which was not removed by EDTA treatment. These data open some new perspectives in the understanding of annexin function, including possible involvement in nucleoskeleton dynamics and regulation of proliferation through cell signalling.

Annexins are products of a multigenic family characterized by their ability to bind to anionic phospholipids in the presence of Ca^{2+}, which is responsible for their antiphospholipase, anticoagulant and antiinflammatory activities (for a Review see (1)). Furthermore, annexins could also be involved in membrane-cytoskeleton interactions (1) and secretory processes (2), or display membrane channel activity (3,4). Among this family, annexin 1 was described as a cellular substrate of the protein tyrosine kinase associated with the epidermal growth-factor receptor (5) and its expression is modulated by cell proliferation (6) or cell differentiation (7,8). But despite many functional possibilities, the cellular role of annexin 1 and of annexins in general still remains obscure. In order to understand its function *in vivo*, it would be important to know where annexin 1 is localized in the cell. Up to now annexins were thought to be present near the plasma membrane or in close contact with the cytoskeleton, owing to their ability to interact with phospholipids and with actin filaments and fodrin (1). Interestingly, monomeric annexin 2 (p36) and annexin 1 display a cytoplasmic

* To whom correspondence should be addressed.

Abbreviations used: HUVE cells, human umbilical vein endothelial cells; DMEM, Dubelcco's modified essential medium; PBS, phosphate buffered saline; FITC, fluorescein isothiocyanate.

0006-291X/92 $4.00

localization, whereas calpactin 1 (the heterotetramer of p36 and p11) is bound to the membrane (9). In the present study, we have used immunoblotting of purified nuclei, immunofluorescence and immunoelectron microscopy to determine in more detail the distribution of annexin 1 in cultured endothelial cells.

MATERIALS AND METHODS

Proteins and antibodies. Annexins were purified and used to prepare specific polyclonal antibodies as described in (10). An antiserum raised against annexin 1 was purified by affinity essentially as described in (11) using annexin 1 from human placenta adsorbed onto Hybond-C membrane (Amersham, UK). Anti-annexin-1 monoclonal antibody was from Zymed, San Francisco, USA. Goat antibody against von Willebrand factor was from ICN, Costa Mesa, CA, USA, and monoclonal anti-vimentin antibody was from Sigma (Saint-Louis, MO, USA).

Cells and culture conditions. Human umbilical vein endothelial cells (HUVE cells) were cultured as previously described (10) on fibronectin-coated supports (1 $\mu g/cm^2$, Sigma). Endothelial cells from fetal bovine aorta (ATCC CRL 1395) were kindly provided by Peter Meyer (Hubrecht Laboratory, Institute for Developmental Biology, Utrecht, The Netherlands) and were grown in DMEM containing 7.5 % (v/v) of fetal calf serum.

Preparation of nuclei from endothelial cells. This was achieved according to (12), using a hypotonic lysis buffer lacking Ca^{2+} (10 mM Tris, pH 7.8, 10 mM KCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin). Briefly, cells were passed 30 times through a 25-gauge needle and the homogenate was centrifuged at 4 °C for 10 min at 1,000 g. The supernatant was then removed and the nuclei-containing pellet was washed 4 times in the lysis buffer.

Protein electrophoresis and immunoblot experiments. This was performed as described previously (10), including prior digestion of nuclei with RNase and DNase. Immunodetection was achieved using ^{125}I-labelled second antibody (1 μCi per immunoblot, Amersham).

Immunofluorescence microscopy. Endothelial cells from bovine or human origin were grown, respectively, on glass coverslips or fibronectin-coated 8 chamber plastic slides (ICN). Cells were rinsed with PBS (137 mM NaCl, 8 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4, pH 7.4, which was used all along the procedure as vehicle) and fixed for 15 min at room temperature with 3 % (w/v) paraformaldehyde. Then, cells were permeabilized during 2 min with 0.2 % (v/v) Triton X-100, washed twice and incubated with 50 mM glycine for 10 min. Fish skin gelatin (0.2 %, w/v, in PBS, Sigma) was added during 30 min. Subsequently, cells were labelled overnight at 4 °C with anti-annexin or anti-von Willebrand factor antibodies, washed and incubated for 1 h at room temperature with FITC-conjugated anti-immunoglobulins (goat anti-rabbit preadsorbed with human serum proteins, from Sigma; goat anti-mouse from Jackson Immunoresearch Laboratories, Avondale, PA, USA; rabbit anti-goat from ICN). Slides were viewed on a Nikon fluorescence microscope and pictures were taken with Kodak T-Max 400 films.

Immunoelectron microscopy. Immunogold labelling of cryosections was performed according to (13). Sections were labelled overnight at 4 °C with anti-annexin 1 antibodies, then washed and incubated at room temperature during 1 h with gold-conjugated anti-rabbit or mouse immunoglobulins (1 nm particle size, Janssen Life Science Products, Beerse, Belgium). Gold particles were amplified up to 10 nm by the silver enhancement procedure according to (14). Sections were examined in a CM-10 Philips electron microscope operated at 100 kV.

RESULTS

Immunofluorescent staining of annexins in HUVE cells. As shown in Fig. 1, a most striking observation was the appearance of a strong nuclear staining of HUVE cells incubated with anti-annexin-1 antiserum (Fig. 1B), which contrasted with the intense cytoplasmic labelling detected with a primary antibody directed against von Willebrand factor (Fig. 1A). At variance with annexin 1, antibodies raised against annexin 2, 5 and 6 showed weaker and diffuse cell staining, with a very discrete fluorescence signal in the nucleus (Fig. 1C-E). A similar pattern of annexin-1 distribution was observed using affinity-purified anti-annexin-1 immunoglobulins (Fig. 2A), the fluorescent signal being suppressed upon prior incubation of immunoglobulins with an excess of pure annexin 1 (Fig. 2B).

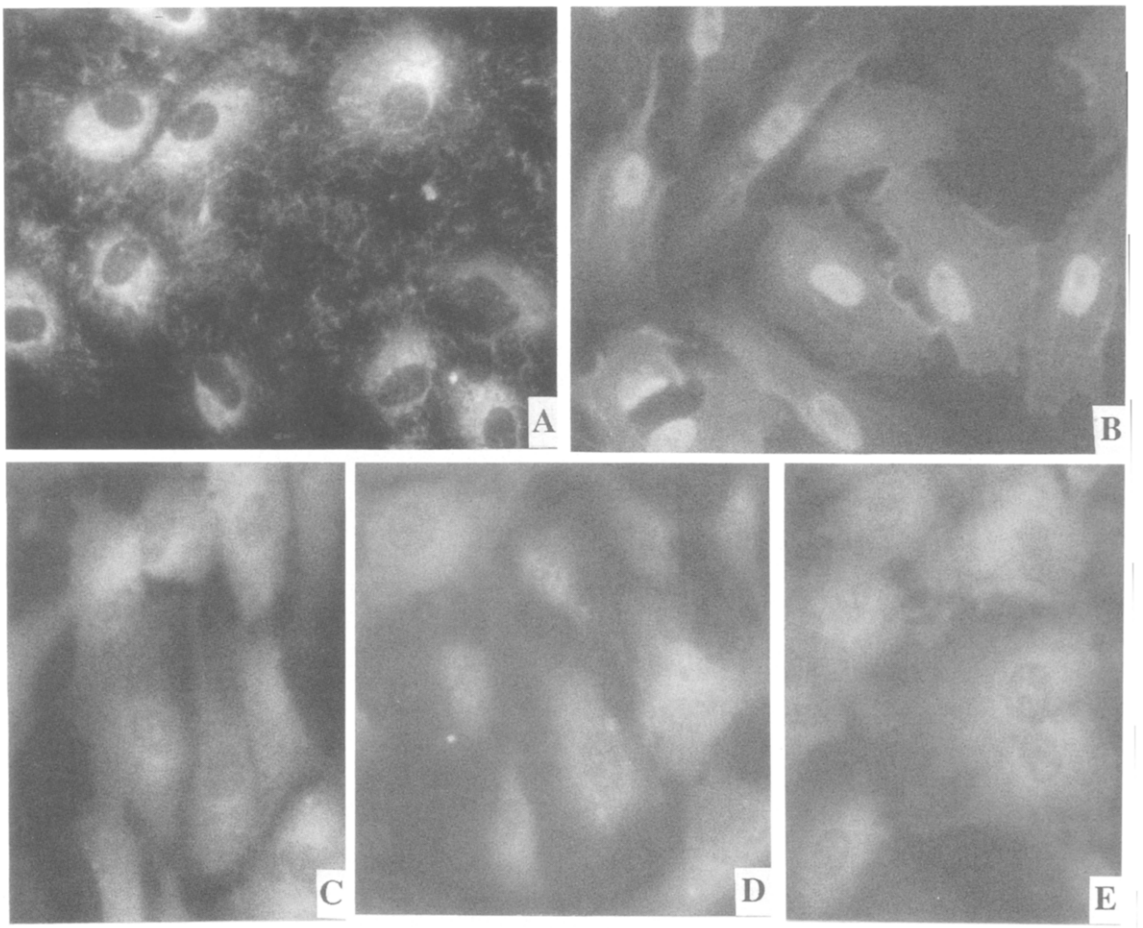

Fig. 1. Immunofluorescent staining of von Willebrand factor and annexins 1, 2, 5 and 6 in HUVE cells. Cultured human endothelial cells were fixed, permeabilized and incubated with polyclonal antibodies (1/100 dilution) directed against von Willebrand factor (A) and annexins 1 (B), 2 (C), 5 (D), 6 (E). After washing, adsorbed immunoglobulins were revealed with an FITC-labelled anti-immunoglobulin antibody and slides were viewed on a fluorescence microscope (magnification x 340).

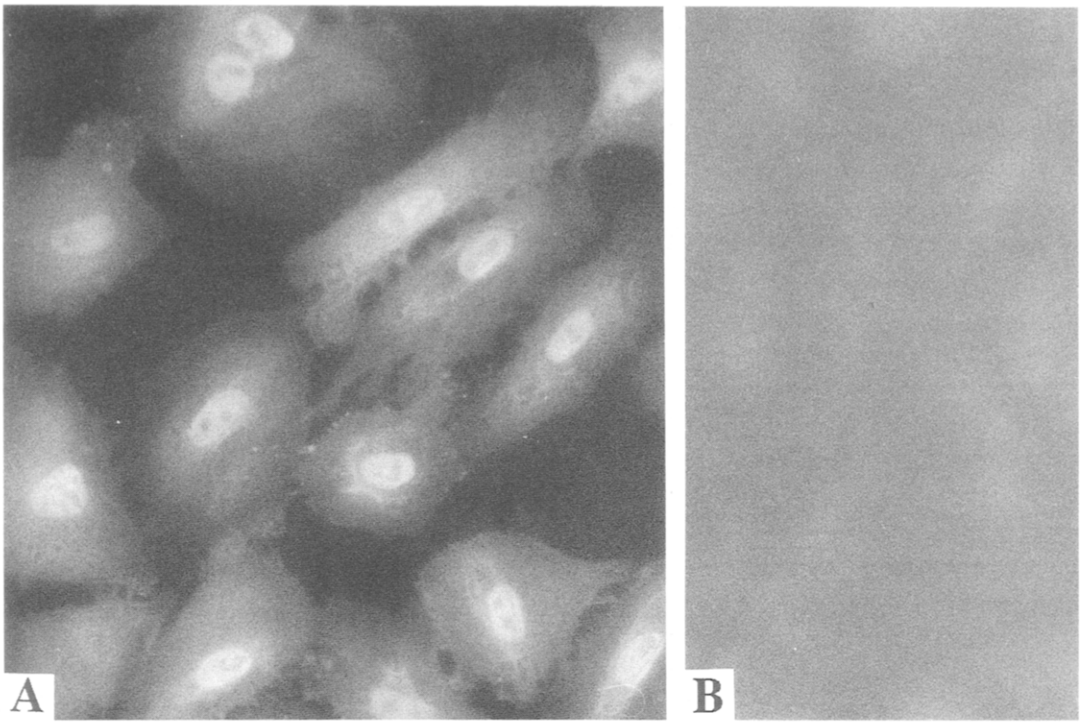

Fig. 2. Immunofluorescent staining of HUVE cells with the affinity-purified anti-annexin-1 polyclonal antibody. The rabbit antiserum raised against annexin 1 was affinity purified as described under "Materials and Methods". The affinity-purified antibody was applied onto human endothelial cells either directly (A) or after a 3 h preincubation with 25 μg of pure annexin 1 (B). Exposure time for pictures: 10 s (A), 50 s (B); magnification x 380.

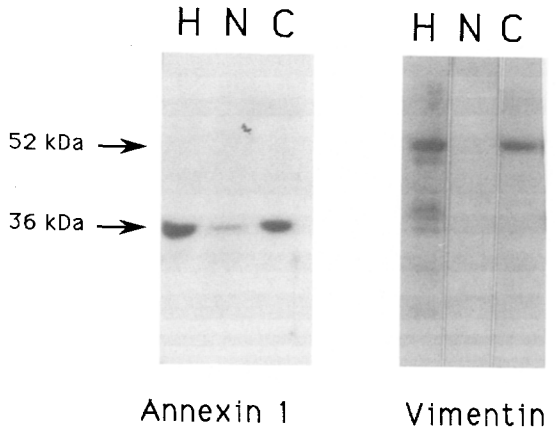

Fig. 3. Immunodetection of annexin 1 in nuclear fraction from bovine endothelial cells. Nuclei were isolated as described under "Materials and Methods". Proteins (30 μg) from the homogenate (H), the nuclear fraction (N) or the cytosolic/cytoskeletal fraction (C) were then immunoblotted, after polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, using the polyclonal anti-annexin-1 antibody (left panel) or a monoclonal anti-vimentin antibody (right panel). Proteins immunodetected were revealed using ^{125}I-labelled second antibodies and autoradiography.

Association of annexin 1 with the nuclear fraction of bovine endothelial cells. When applied to cultured endothelial cells, the method of Tessler and Neufeld (12) allowed us to obtain nuclei with an excellent degree of purity, as shown by the disappearance of vimentin in the nuclear pellet (Fig. 3, right panel). This differs from other methods using Triton X-100, where vimentin is present in higher amounts in the final preparation (not shown). By immunoblotting with the anti-annexin-1 antibody, a single band with an apparent molecular mass of 35 kDa was observed in significant amounts in the nuclear fraction (Fig. 3, left panel). However, annexin 1 was also detected in still higher amounts both in whole homogenates and in supernatants. It is important to note that nuclei were prepared and washed 4 times in a buffer lacking Ca^{2+} and containing 1 mM EDTA, which should have removed any trace of annexin 1 adsorbed onto the nuclear membrane.

Ultrastructural localization of annexin 1 by immunogold labelling. The nuclear localization of annexin 1 in bovine endothelial cells was further confirmed by immunoelectron microscopy. As shown in Fig. 4A, many gold particles were detected in the cells with a monoclonal anti-annexin 1 antibody, confirming the high expression level of this protein (10). An identical pattern was observed using affinity-purified anti-annexin-1 polyclonal antibody. Meanwhile, there was no labelling when cryosections were incubated with gold-conjugated anti-mouse immunoglobulins only (Fig. 4B). In the nuclear compartment, annexin 1 did not appear associated with the membrane, but was distributed all over the nucleus. Outside the nucleus, the labelling was scattered without any obvious association with cell organelles like mitochondria, endoplasmic reticulum or the Golgi system. However, annexin 1 was clearly associated with the plasma membrane (Fig. 4A).

DISCUSSION

The present data support the surprising conclusion that annexin 1 is partly localized in the nucleus of endothelial cells. Such an unexpected finding is actually based on three different morphological or biochemical methods using either an affinity-purified polyclonal antibody or a monoclonal antibody directed against annexin 1. The specificity of the detection was clearly demonstrated, since the fluorescence disappeared when the affinity-purified antibody was preincubated with pure annexin 1 (see Fig. 2), and non specific adsorption of the antibody was in all cases avoided by the use of an excess of gelatin. In addition, we could rule out the possibility that nuclear staining was due to annexin 2, the reactivity of the antibody against the latter being 5 % compared to annexin 1 (not shown). It remains also important to underline that annexin 1 was not extracted from nuclear fraction in the presence of EDTA, ruling out the possibility that its presence in this fraction would be due to some contaminating membranes.

A main question about annexins concerns their ability to cross lipid bilayers. Such a possibility is suggested from electrophysiological studies indicating they are able to display ionic channel activity in artificial membranes (3,4), although X-ray cristallography or electron image analysis revealed that annexin 5 is simply adsorbed onto phospholipids (15-17).

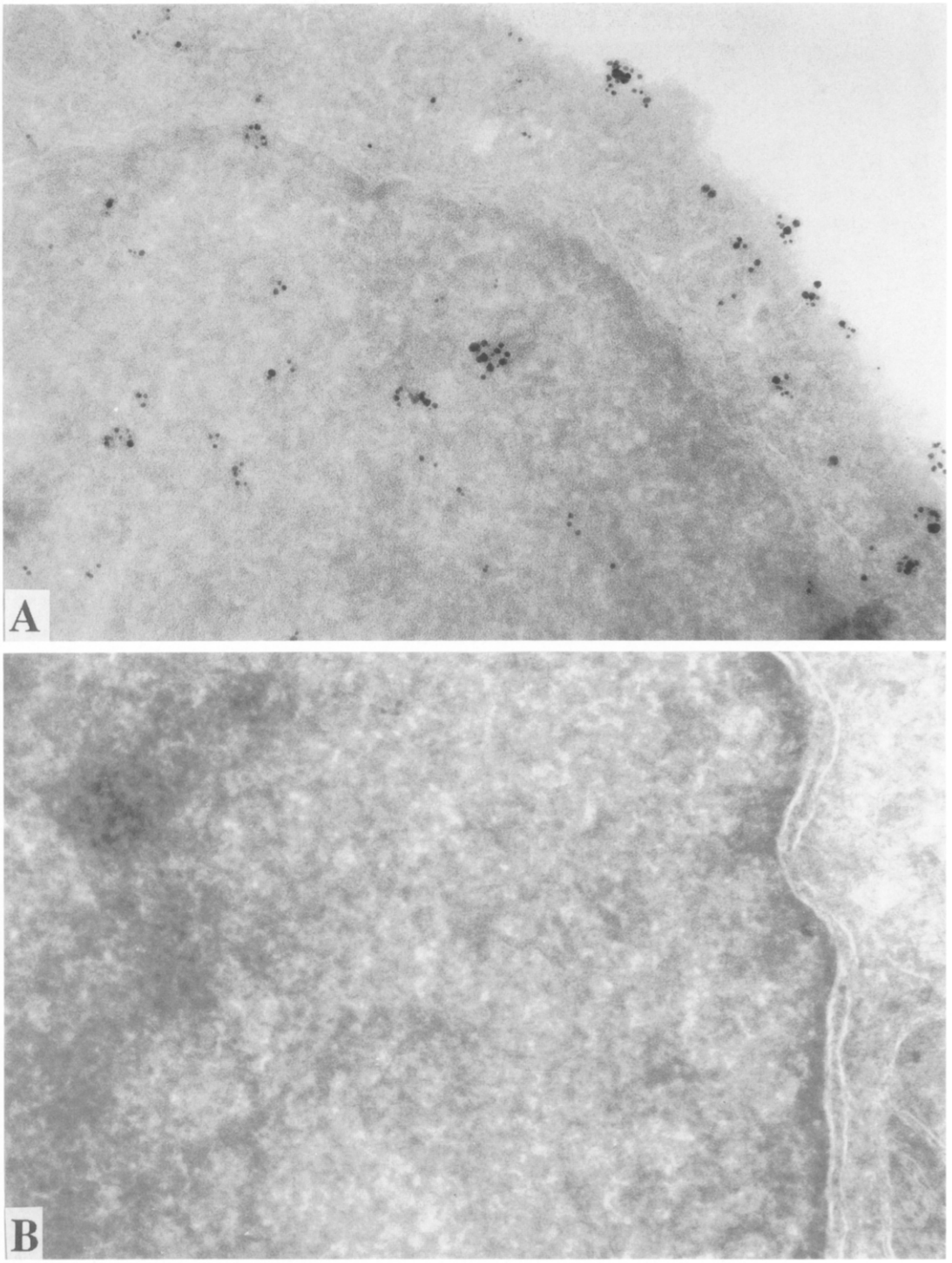

Fig. 4. Immunogold labelling of annexin 1 in bovine endothelial cells. Ultrathin sections were adsorbed on grids, blocked with gelatin, incubated with anti-annexin-1 monoclonal antibody (A) or buffer only (B), and processed as described under "Materials and Methods". Then, sections were treated with 1 nm gold-conjugated anti-immunoglobulins which were subsequently amplified. Finally, the preparations were stained with uranyl acetate in 1.8 % methocel and examined on an electron microscope (magnification x 36,000 (A), x 30,000 (B)).

However, one study showed an active secretion of annexins 1 and 5 from prostatic epithelial cells, despite the lack of signal peptide in their amino acid sequence (18). There is also good evidence for the presence of annexin 6 in the lumen of sarcoplasmic reticulum vesicles (19). One can thus suggest a similar, albeit unknown, mechanism being responsible for the translocation of annexin 1 across the nuclear membrane. Another possibility would be the presence of a cryptic nuclear localization signal allowing its active transport through nuclear pores (20).

Another question raised by the present study concerns the biological significance of annexin-1 localization in cell nucleus. A similar observation was recently made for annexin 2, although at a lower level (21), in agreement with our data obtained with anti-annexin-2 antibody, which showed some discrete nuclear fluorescence (see Fig. 1). In the latter case, annexin 2 and phosphoglycerate kinase are associated with the nuclear matrix, and they are considered as "primer recognition proteins" (PRP) involved in the replication of the lagging strand of DNA by polymerase α (22,23). A similar evidence is still lacking for annexin 1, but the present observation should stimulate further studies to identify some specific interactions of annexin 1 with other nuclear macromolecules.

Considering the ability of various annexins to interact with cytoskeleton, it is interesting to note that several cytoskeletal proteins such as actin, α-spectrin, myosin light chain kinase, caldesmon, calmodulin, and protein 4.1 were recently found to display a partial nuclear localization, which might vary between proliferating and quiescent cells (24-26). Annexin 1 can thus be added to the list of cytoskeletal proteins potentially involved in the dynamics of nucleoskeleton. But further studies are still needed to precise the possible role of each of these proteins in the nucleus.

Finally, annexins are primarily considered as proteins interacting with phospholipids. In this context, there is growing evidence for the presence in cell nuclei of various enzymes activated by lipid metabolites or involved in phospholipid metabolism such as protein kinase C (27-33), phospholipase A_2 (34), phosphoinositide-specific phospholipase C and various lipid kinases (35,36). Noteworthy, these enzymes seem to be associated with nuclear matrices (and not with nuclear membrane) and a possible involvement of phosphoinositide metabolism in cell nucleus in relation to signal transduction promoted by insulin-like growth factor-1 (IGF-1) has even been recently shown (37,38). The present study adds another candidate potentially involved in the regulation of this metabolism, as it also opens a new way to investigate the biological role of annexin 1.

REFERENCES

1. Klee, C.B. (1988) *Biochemistry* **27**, 6646-6653.
2. Ali, S.M., Geisow, M.J., and Burgoyne, R.D. (1989) *Nature* **340**, 313-315.
3. Burns, A.L., Magendzo, K., Shirvan, A., Srivastava, M., Rojas, E., Alijani, M.R., and Pollard, H.B. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 3798-3802.
4. Rojas, E., Pollard, H.B., Haigler, H.T., Parra, C., and Burns, A.L. (1990) *J. Biol. Chem.* **265**, 21207-21215.
5. Sawyer, S.T., and Cohen, S. (1985) *J. Biol. Chem.* **260**, 8233-8236.
6. Schlaepfer, D.D., and Haigler, H.T. (1990) *J. Cell Biol.* **111**, 229-238.

7. William, F., Mroczkowski, B., Cohen, S., and Kraft, A.S. (1988) *J. Cell. Physiol.* **137**, 402-410.
8. Isacke, C.M., Lindberg, R.A., and Hunter, T. (1989) *Mol. Cell. Biol.* **9**, 232-240.
9. Zokas, L., and Glenney, J.R., Jr (1987) *J. Cell Biol.* **105**, 2111-2121.
10. Hullin, F., Raynal, P., Ragab-Thomas, J.M.F., Fauvel, J., and Chap, H. (1989) *J. Biol. Chem.* **264**, 3506-3513.
11. Olmsted, J.B. (1981) *J. Biol. Chem.* **256**, 11955-11957.
12. Tessler, S., and Neufeld, G. (1990) *J. Cell. Physiol.* **145**, 310-317.
13. Van Bergen en Henegouwen, P. (1989) In *Colloidal Gold: Principles, Methods and Applications* (Hayat, M.A. ed) Vol. I, pp. 191-216, Academic Press, New York.
14. Danscher, G., and Norgaard, J.O. (1983) *J. Histochem. Cytochem.* **31**, 1394-1398.
15. Huber, R., Römisch, J., and Pâques, E.P. (1990) *EMBO J.* **9**, 3867-3874.
16. Huber, R., Schneider, M., Mayr, I., Römisch, J., and Pâques, E.P. (1990) *FEBS Lett.* **275**, 15-21.
17. Mosser, G., Ravanat, C., Freyssinet, J.M., and Brisson, A. (1991) *J. Mol. Biol.* **217**, 241-245.
18. Christmas, P., Callaway, J., Fallon, J., Jones, J., and Haigler, H.T. (1991) *J. Biol. Chem.* **266**, 2499-2507.
19. Diaz-Munoz, M., Hamilton, S.L., Kaetzel, M.A., Hazarika, P., and Dedman, J. (1990) *J. Biol. Chem.* **265**, 15894-15899.
20. Burke, B. (1990) *Curr. Opin. Cell Biol.* **2**, 514-520.
21. Kumble, K.D., and Vishwanatha, J.K. (1991) *J. Cell Sci.* **99**, 751-758.
22. Vishwanata, J.K., Jindal, H.K., and Davis, R.G. (1992) *J. Cell Sci.* **101**, 25-34.
23. Kumble, K.D., Iversen, P., and Vishwanata, J.K. (1992) *J. Cell Sci.* **101**, 35-41.
24. Pujol, M.J., Soriano, M., Aligué, R., Carafoli, E., and Bachs, O. (1989) *J. Biol. Chem.* **264**, 18863-18865.
25. Bachs, O., Lanini, L., Serratosa, J., Coll, M.J., Bastos, R., Aligué, R., Rius, E., and Carafoli, E. (1990) *J. Biol. Chem.* **265**, 18595-18600.
26. Correas, I. (1991) *Biochem. J.* **279**, 581-585.
27. Kiss, Z., Deli, E., and Kuo, J.F. (1988) *FEBS Lett.* **231**, 41-46.
28. Thomas, T.P., Talwar, H.S., and Anderson, W.B. (1988) *Cancer Res.* **48**, 1910-1919.
29. Masmoudi, A., Labourdette, G., Mersel, M., Huang, F.L., Huang K-P., Vincendon, G., and Malviya, A.N. (1989) *J. Biol. Chem.* **264**, 1172-1179.
30. Leach, K.L., Powers, E.A., Ruff, V.A., Jaken, S., and Kaufmann, S. (1989) *J. Cell Biol.* **109**, 685-695.
31. Berry, N., Ase, K., Kikkawa, U., Kishimoto, A., and Nishizuka, Y. (1989) *J. Immunol.* **143**, 1407-1413.
32. Fields, A.P., Tyler, G., Kraft, A.S., and May, W.S. (1990) *J. Cell Sci.* **96**, 107-114.
33. Martelli, A.M., Neri, L.M., Gilmour, R.S., Barker, P.J., Huskisson, N.S., Manzoli, F.A., and Cocco, L. (1991) *Biochem. Biophys. Res. Commun.* **177**, 480-487.
34. Tamiya-Koizumi, K., Umekawa, H., Yoshida, S., Ishihara, H., and Kojima, K. (1989) *Biochim. Biophys. Acta* **1002**, 182-188.
35. Cocco, L., Gilmour, R.S., Ognibene, A., Letcher, A.J., Manzoli, F.A., and Irvine, R.F. (1987) *Biochem. J.* **248**, 765-770.
36. Payrastre, B., Nievers, M., Boonstra, J., Breton, M., Verkleij, A., and van Bergen en Henegouwen, P.M.P. (1992) *J. Biol. Chem.* **267**, 5078-5084.
37. Cocco, L., Martelli, A.M., Gilmour, R.S., Ognibene, A., Manzoli, F.A., and Irvine, R.F. (1989) *Biochem. Biophys. Res. Commun.* **159**, 720-725.
38. Divecha, N., Banfic, H., and Irvine, R.F. (1991) *EMBO J.* **10**, 3207-3214.