Vol. 202, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1994 Pages 403-409

Development-Related and Cell-Type Specific Nuclear Localization of Annexin XI: Immunolocalization Analysis in Rat Tissues

Naoto Mamiya, Satoshi Iino#, Akihiro Mizutani, Shigeru Kobayashi#, and Hiroyoshi Hidaka*

Department of Pharmacology and #Department of Anatomy, Nagoya University School of Medicine, Showa-ku, Nagoya 466, Japan

Received May 25, 1994

Subcellular distribution of a novel annexin, annexin XI, was examined in rat tissues. As we have previously reported, annexin XI mainly localizes in nuclei of 3Y1, the rat embryonic fibroblast cell line; however, immunoperoxidase staining was not particularly nuclear in most of the tissues examined in adult rats. Nuclear localization of annexin XI was uncommon in adult rat tissues. By contrast, in the day-14 rat embryo, the undifferentiated mesenchymal cells were labeled mainly in the nuclei. The connective tissues of 18-day fetus, however, did not show predominantly nuclear staining any longer. In addition, the developing gray matter of the embryonic rat spinal cord exhibited primarily nuclear localization of annexin XI while the annexin XI immunoreactivity diminished and became absent from the nuclei in the adult spinal cord. On the other hand, the endodermal cells never displayed nuclear annexin XI at any developemental stages examined. All these findings suggested that subcellular localization of annexin XI is regulated depending on developmental stages and the cell types.

Annexin XI is a newly identified member of the annexin family, which binds to anionic phospholipids in a calcium-dependent fashion (1, 2). Increasing numbers of annexin proteins have been discovered and are mostly localized in the cytosol or on the plasma menbrane. To date, a wide variety of functions have been advocated; involvement in the cell trafficking events including exocytosis (3), anti-inflammatory effect, and anticoagulant activity etc. At the carboxyl terminal region, annexin XI has four imperfect internal annexin repeats, which are highly homologous in all annexin proteins. The amino terminal region of annexin XI is composed of 202 amino acids and is the longest of all known annexins. It is relatively hydrophobic and thought to be implicated in its possible, yet unidentified functions. At this unique amino terminal domain, annexin XI binds in the presence of calcium to a EF-hand protein, calcyclin, a cell proliferation or differentiation-related gene product (4, 5). Presumably, these proteins play important roles in the control

*To whom correspondence should be addressed. Fax: 052-733-4774.

0006-291X/94 $5.00

of cell proliferation. We have raised specific polyclonal antibodies against bovine lung annexin XI, and these antibodies proved to immunoreact only with a single band of 50 kDa in almost all the rat tissues tested on immunoblot analysis. Western blots have revealed ubiquitous distribution of annexin XI in rat tissues (6). Immunocytological study and subcellular fractionation of 3Y1 cells have demonstrated the unique nuclear localization of annexin XI (6). In the src-infected 3Y1 cells, the coincidence of its phosphorylation with relocalization from nucleus to cytoplasm, has been shown (2). Therefore, translocation of annexin XI possibly regulates cell functions. We sought to investigate the subcellular localization of annexin XI in normal rat tissues, which may provide insights into the understanding of the physiological function of annexin XI.

Materials and Methods

Animals and tissue processsing for immunohistochemistry. Male Wister rats (250-300g) were anesthetized with diethylether. After perfusing Ringer's solution through the left ventricle of the heart with a cut in the right atrium, Zamboni's fixative (7) was circulated. The tissues were excised and washed in PBS, and were further fixed in Zamboni's fixative overnight. Then the tissues were washed in ice cold PBS and immersed in 30 % sucrose in PBS for a night. Embeded in OCT, the tissues were frozen and cut by cryostat.

Immunostaining. Avidin-biotinylated complex staining was performed according to a stadard procedure. The anti-annexin XI antiserum diluted 1: 500 was used. Endogeneous biotin was blocked by sequential incubations with avidin and biotin (Vector, Burlingame, California).As we have previously presented (6), the polyclonal antibodies we have raised against bovine lung annexin XI, recognized a single band of 50 kDa in Western blot analysis of rat tissues. When the antibodies were immunodepleted by preincubation with an excess amount of antigens, immunoreactivity disappeared, which shows the reaction was specific.

Partial hepatectomy. The livers were induced to regenerate by partial hepatectomy according to the method of Higgins and Anderson (8).

Results and Discussion

In contrast to the conspicuous nuclear annexin XI in 3Y1 cells, immunolabeling of adult rat tissues revealed that nuclear staining was uncommon. For instance, immunolocalization of annexin XI in liver parenchyma showed homogeneously scattered reactivity of the moderate intensity in the cytoplasm of the hepatocytes. The plasma membrane labeling was denser than the cytoplasm (Fig. 1). Similarly, immunolocalization of annexin XI in the smooth muscle showed dense staining in the cytoplasm, while fibroblasts in various rat tissues showed only weak staining with no evidence of predominantly nuclear localization (not shown).

Annexin XI has been detected in almost all tissues examined by immunoblotting. Its widespread distribution suggests a fundamental role in the cell function. Several cell lines showed primarily nuclear localization of annexin XI. However, they were analysed in the log phase. On the other hand, most cells in adult rat tissues are in their G_0 phase. Calcyclin, the binding protein of annexin XI, has been shown to be regulated in a cell-cycle dependent fashion; its expression is greatly enhanced in cycling cells as compared with quiescent cells (4, 9). The amounts of annexin XI were relatively constant at G_0/G_1

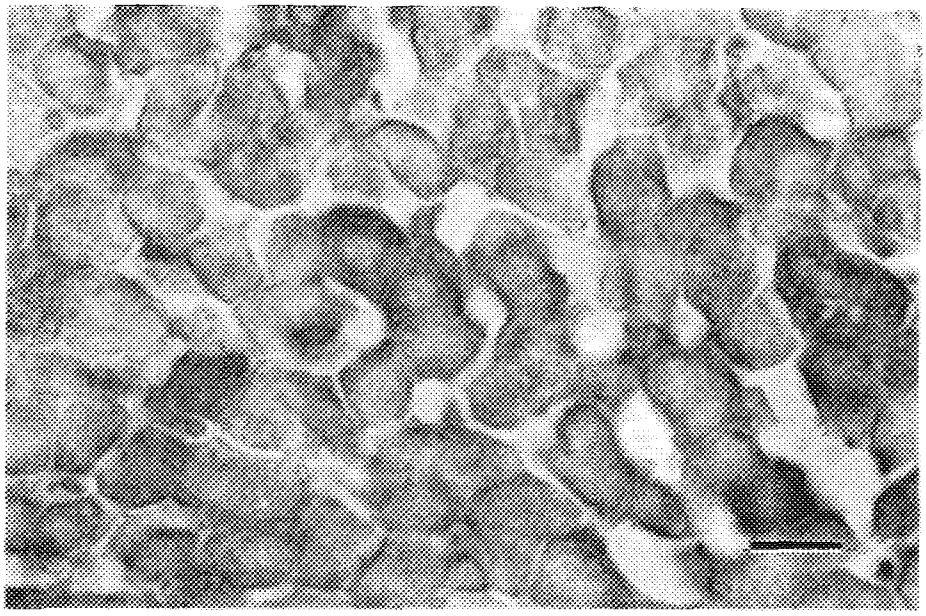

Fig. 1. Immunohistochemical staining of annexin XI in the liver of an adult rat (6w). The hepatocytes are stained in the cytoplasm, and the plasma membrane appears more densely stained. The nucleus is devoid of immune reaction. Bar = 20 μm.

interphase in 3Y1 cells (unpublished data), however, it is possible that the subcellular distribution of annexin XI changes as cells start to proliferate. Thus, we also examined livers from partially hepatectomized rats to determine whether the nuclear localization of annexin XI is simply a consequence of proliferation. At 26 hours after 70% partial hepatectomy, when the hepatocytes enter the cell cycle and synchronous DNA replication was on its course (10), sections of the remnant liver were examined. Immunoreactivity with annexin XI was detected mainly in the cytosol and at the cell periphery of the proliferating hepatocytes as well as in the quiescent hepatocytes (not shown). Contrarily, several proteins such as calmodulin, myosin light chain kinase and α-spectrin have been demonstrated to translocate from the cytosol to the nucleus in rat hepatocytes after proliferative activation was induced by partial hepatectomy (11). The nuclear or cytoplasmic localization of different calcium binding proteins must be differentially regulated. Additionally, when 3Y1 cells were rendered quiecent by serum deprivation, annexin XI still remained in nuclei (unpublished data). These observations would argue that there is not a correspondence between nuclear localization and proliferation.

Then we proceeded to the analysis in rat embryos. As demonstrated in Fig. 2A, the undifferentiated mesenchymal cells of rat embryo at 2w were labeled predominantly in nuclei, just like the case with 3Y1 cells. This photomicrograph shows regions immediately ventral to the developing lumbar vertebral body. The corresponding regions

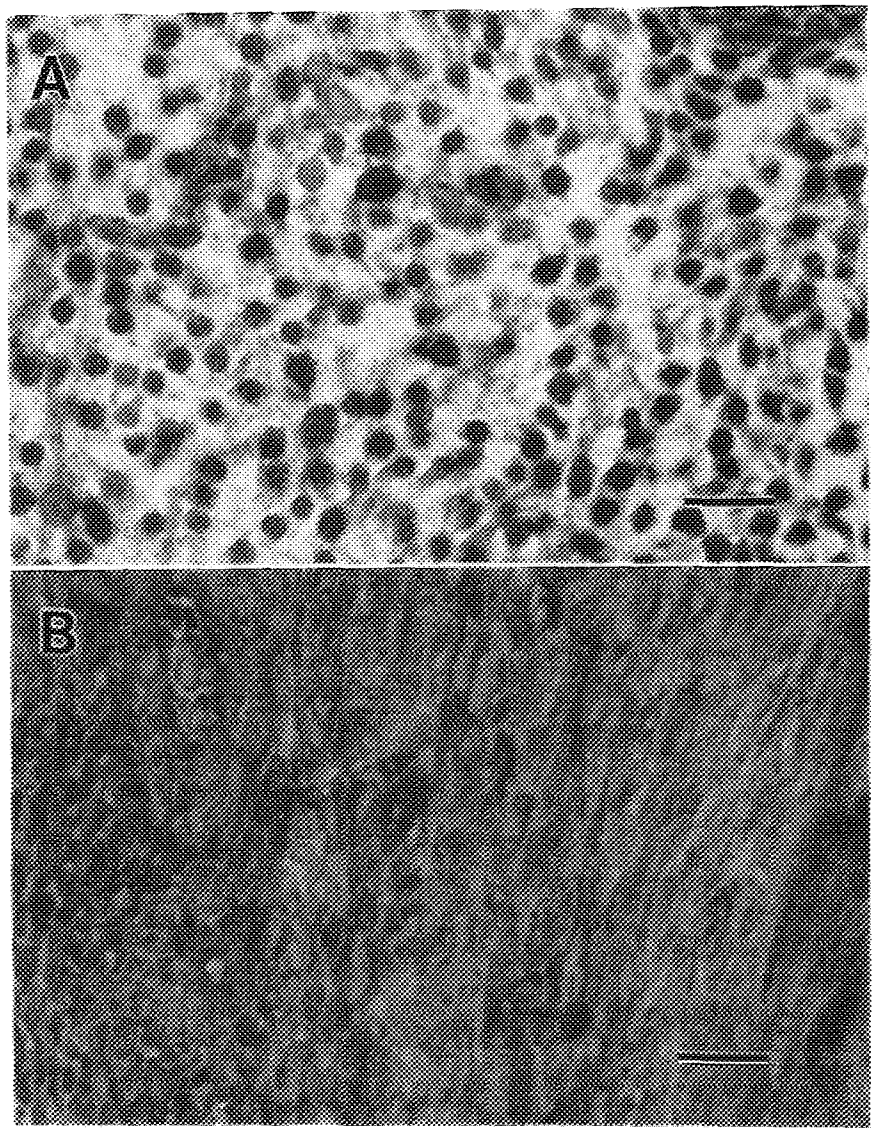

Fig. 2. (A) Undifferentiated mesenchymal cells in a day-14 rat embryo stained for annexin XI. Apparent nuclear staining is demonstrated in the mesenchymal cells immediately ventral to the developing lumbar vertebral body, which give origin to the connective tissues and the musculature. Bar = 20 μm.
(B) Distribution of annexin XI in the connective tissue corresponding to (A) in a day-18 rat fetus. The pattern of staining appears homoeneous throughout the cells at this light-microscope level. The immunostaining in the nuclei is not particularly denser than that in the cytoplasm.; Bar = 20 μm.

in 18-day rat fetus were next analyzed. Labeling appeared lighter and the nuclei no longer stained more strongly than the cytoplasm (Fig. 2B).

We have previoulsy reported that the central nervous systems have much lower levels of annexin XI than other tissues in the adult rat on the basis of Western analysis (6). Fig.

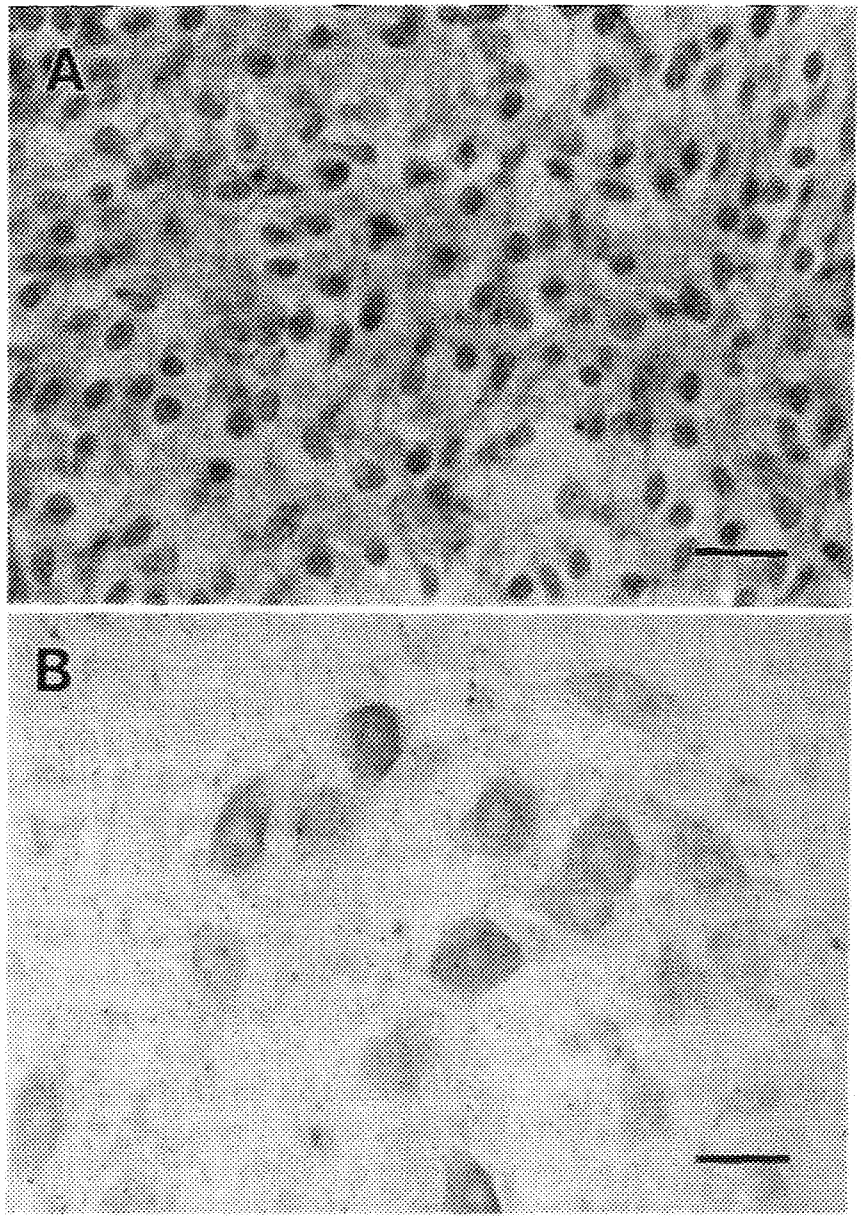

Fig. 3. (A) The cervical cord gray matter in a day-14 embryo. Annexin XI-positive cells can be found throughout the developing gray matter. Annexin XI immunoreactivity is mainly localized in the nuclei of the immature neurons. Bar = 40 μm.
(B) Annexin XI-immunolabeling in the adult rat cervical cord. The dorsal horn is not labeled. Some cells in the ventral horn showed moderate staining which is mainly associated with the plasma membrane or with the perinuclear structures. Bar = 20 μm.

3B shows the annexin XI immunostaining of the adult rat spinal cord. The immunoreactivity was confined to a subpopulation of cells in the anterior horn. And the immunolabeling was dense at the plasma membrane or the perinuclear regions, but the

nuclei was devoid of staining. We also studied the developing spinal cord. In the day-14 rat embryo, the distribution of annexin XI differed markedly from that observed in the adult. As can be seen in Fig. 3A, annexin XI immunoreactivity was evident throughout the developing gray matter, and it was localized primarily in nuclei of the immature cells.

3Y1 cells clearly showed primarily nuclear peroxidase staining in spite of the limited resolution of the present experimental procedure (not shown). When nuclear labeling is obviously denser than cytoplasmic staining, it is ascertained that the nuclear staining is positive at the light microscopic level. Thus, the threshold to detect nuclear staining was set at a relatively high level. Our main objective was to demonstrate the physiological conditions in which nuclear localization of annexin XI was as evident as in 3Y1 cells, hence the present procedure was adequate. It can be stated that the level of nuclear staining in Fig. 2B is significantly lower than that detected in Fig. 2A or in the case of 3Y1 cells. All these findings make it likely that nuclear localization of annexin XI is primarily involved in differentiation or deveolement rather than proliferation.

In the hepatocytes of rat embryos as well as of adult rats, immunostaining of annexin XI was dense on the plasma membrane with apparent cytosolic staining (not shown). Furthermore, the endodermal cells did not display primarily nuclear localization of annexin XI at any developmental stage examined (unpublished data), whereas annexin XI was predominantly nuclear in the undifferentiated mesenchymal cells and in the developing gray matter of the embryonic spinal cord. Accordingly, nuclear localization of annexin XI seemes to be cell-type specific as well as developmental stage-dependent.

Annexin XI shows wide-spreading subcellular distributions. In the like manner, protein 4.1 that localizes at the plasma membrane of MDCK cells, has proved to be present in the nucleus as high-molecular-mass isoforms (12). Therefore, different isoforms might locate in the different compartments since annexin XI isoforms generated by alternative splicing have been predicted (13). The other possibilities are that variations in post-translational modifications or in postulated effector molecules of annexin XI, are responsible for the distinct subcellular distribution in different situations.

The different annexins have been shown to have different tissue and cell distributions. And the expressions of some annexins have been found to be regulated as a function of developmental stage, differentiation, maturation or cell growth (14-17). However, no annexin has thus far been indicated to be regulated concerning its subcellular localization. We present here that annexin XI alters its nucleocytoplasmic compartmentalization according to the developmental stage in a cell-type specific manner. Although the members of the annexin family are generally localized at the plasma membrane, in the cytoplasm or in association of the cytoskeletons (18-24), annexin I, II, V, and VII have been described to be localized at least partially in the nucleus (25-27). While annexin II has recently been characterized regarding the nuclear function (28), the functions of other members remain obscure. Since these nuclear annexins lack typical nuclear location sequences, the mechanism of nuclear targeting is elusive, and the physiological importance is far from general understanding. The investigation to clarify the mechanism

by which annexin XI shows dynamic translocation is under way now, which is expected to provide further informations as to its possible functions.

References

1. Tokumitsu, H., Mizutani, A., Minami, H., Kobayashi, R., and Hidaka, H. (1992) J. Biol. Chem. 267, 8919-8924.
2. Mizutani, A., Tokumitsu, H., Kobayashi, R., and Hidaka, H. (1993) J. Biol. Chem. 268, 15517-15522.
3. Creutz, C. E. (1992) Science 258, 924-931.
4. Calabretta, B., Battini, R., Kaczmarek, L., de Riel, J. K., and Baserga, R. (1986) J. Biol. Chem. 261, 12628-12632.
5. Tokumitsu, H., Mizutani, A., and Hidaka, H. (1993) Arch. Biochem. Biophys. 303, 302-306.
6. Mizutani, A., Usuda N., Tokumitsu, H., Minami, H., Yasui, K., Kobayashi, R., and Hidaka, H. (1992) J. Biol. Chem. 267, 13498-13504.
7. Stefanini, M., De Martino, C., and Zamboni, L. (1967) Nature 216, 173-174.
8. Higgins, G., and Anderson, R. M. (1931) Arch. Pathol. 12, 186-202.
9. Hirschhorn, R. R., Aller, P., Yuan, Z., Gibson, C. W., and Baserga, R. (1984) Proc. Natl. Acad. Sci. USA 81, 6004-6008.
10. Fausto, N. (1990) In Hepatology (Zakim, D., and Boyer, T. D., Eds.), W. B. Saunders Company, Phil. pp.49-65.
11. 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.
12. Correas, I. (1991) Biochem. J. 279, 581-585.
13. Towle, C. A., Weissbach, L., and Treadwell, B. V. (1992) Biochim. Biophys. Acta 1131, 223-226.
14. Carter, V. C., Howlett, A. R., Martin, G. S., and Bissell, M. J. (1986) J. Cell Biol. 103, 2017-2024.
15. Giambanco, I., Verizini, M., and Donato, R. (1993) Biochem. Biophys. Res. Commun. 196, 1221-1226.
16. Schlaepfer, D. D. and Haigler, H. T. (1990) J. Cell Biol. 111, 229-238.
17. Giambanco, I., Sorci, G., Antonioli, S., Rambotti, M. G., Spreca, A., Bocchini, V., and Donato, R. (1993) FEBS Lett. 323, 45-50.
18. Chailley, B. and Pradel, L. A. (1992) Biol. Cell. 75, 45-54.
19. Clark, D. M., Moss, S. E., Wright, N. A., and Crumpton, M. J. (1991) Histochemistry 96, 405-412.
20. Feinberg, J. M., Rainteau, D. P., Kaetzel, M. A., Dacheux, J. L., Dedman, J. R., and Weiman, S. J. (1991) J. Histochem. Cytochem. 39, 955-963.
21. Giambanco, I., Pula, G., Ceccarelli, P., Bianchi, R., and Donato, R. (1992) J. Cell. Physiol. 152, 587-598.
22. Massey, D., Traverso, V., and Maroux, S. (1991) J, Biol. Chem. 266, 3125-3130.
23. Nakata, T., Sobue, K., and Hirokawa, N. (1990) J. Cell. Biol. 11, 313-323.
24. Gould, K. L., Cooper, J. A., and Hunter, T. (1984) J. Cell Biol. 98, 487-497.
25. Raynal, P., Van Bergen en Henegouwen, P.M.P., Hullin, F., Ragab-Thomas, J. M. F.,Fauvel, J., Verkleij, A., and Chap, H. (1992) Biochem. Biophys. Res. Commun. 186, 432-439.
26. Sun, J., Salem, H. H., and Bird, P.(1992) FEBS Lett. 314, 425-429.
27. Kuijpers, G. A., Lee, G., and Pollard, H. B. (1993) Cell Tissue Res. 269, 323-330.
28. Jindal, H. K., Chaney, W. G., Anderson, C. W., Davis, R. G.,and Vishwanata, J. K. (1991) J. Biol. Chem. 266, 5169-5179.