Subcellular localization of annexin V in human foreskin fibroblasts: nuclear localization depends on growth state

Jennifer L. Barwise, John H. Walker*

Department of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, UK

Received 10 July 1996; revised version received 9 August 1996

Abstract Annexin V is a major intracellular calcium-binding protein in human foreskin fibroblasts. Immunocytochemistry revealed that annexin V was localized in the nucleus and throughout the cytoplasm in human foreskin fibroblasts. The presence of annexin V in the nucleus was variable depending on the growth state. Nuclear staining was strongest in proliferating cells immediately after sub-culture, and decreased on prolonged culture without changing the culture medium. The cytoplasmic location of annexin V was not greatly affected by the same conditions. Refeeding cells with fresh serum restored annexin V to the nuclei of all cells within in 24 h indicating that nuclear localization of annexin V is dependent on serum factors.

Key words: Annexin V; Growth state; Nucleus; Fibroblasts

1. Introduction

Elevation of intracellular calcium is one of the most important signal transducing mechanisms available to eukaryotic cells [1]. Several major intracellular calcium-binding proteins have been identified as potential mediators of the calcium signal [2]. In particular, annexins represent evolutionarily conserved calcium-binding proteins present as major constituents of the cytoplasm of all cells studied to date with the exception of red blood cells [3]. Annexins are a family of at least 13 major cellular proteins which contain conserved sequences of ~70 amino acids, which are repeated four times in 35 kDa annexins and eight times in the 70 kDa annexin VI. These core domains are flanked by amino-terminal domains which are not conserved and may confer functional diversity on the individual proteins. Annexins have highly conserved calcium-binding sites in their core domains that are distinct from the 'E-F hand' calcium-binding sites found in many calcium-binding proteins including S-100 and calmodulin [4].

Annexins have been implicated in a number of important intracellular processes (reviewed in [3]) including signal transduction, calcium homeostasis [5], membrane trafficking [6,7] and the regulation of cytoskeleton-membrane interactions [8]. The 3-D structure of annexin V has been solved [9,10] and has led to proposals to account for its binding to phospholipids and ion-channel activity [11].

Annexin V is widely distributed in animal tissues [12] and is expressed at high levels within cells. In fibroblasts it can represent up to 2% of total cellular protein [13]. Furthermore, annexin V expression has been shown to vary as a function of cellular growth state in human foreskin fibroblasts and in a glioma cell line [13,14].

In this paper we have used immunocytochemistry to demonstrate that the nuclear localization of annexin V in human foreskin fibroblasts (HFF) varies depending on the cellular growth state.

2. Materials and methods

2.1. Materials

Rabbit antibodies to human placental annexin V were a gift from Dr. F. Seiler and Dr. J. Römisch. FITC-labelled goat anti-rabbit IgG was from Sigma. Tissue culture solutions were from Gibco BRL. All other reagents were obtained from Sigma or BDH chemicals, unless otherwise stated.

2.2. Primary tissue culture

Human skin samples were collected from Leeds General Infirmary or St. James's Hospital. All fat was removed from the sample and the tissue was cut into 3 mm strips and incubated in Dispase (2 mg/ml) in DMEM at 4°C overnight. The epidermis was subsequently removed from the dermis. The dermis was minced finely and incubated in collagenase (0.5 mg/ml in PBS (12.6 mM $Na_2HPO_4 \cdot 12H_2O$/1.9 mM $NaH_2PO_4 \cdot 2H_2O$/0.15 M NaCl) at 37°C overnight on an orbital shaker. The collagenase reaction was stopped by dilution with 2 volumes of PBS. The fibroblasts were washed in PBS and plated out in 25 cm^3 flasks containing DMEM supplemented with 10% calf serum, with subsequent changes in medium every 3 days. On reaching confluence, cells were exposed to trypsin (50 μg/ml) for 3–4 min at 37°C, taken up into media and transferred into a new flask.

For studies on prolonged cell culture, cells were plated either on 22 mm glass coverslips or in 35 mm dishes (day 0) at a density of 5×10^4 cells and cultured in DMEM supplemented with 10% calf serum. Subsequently cells were allowed to grow without changing the medium and fixed on days 1, 2, 5, 6 or 7 with 3.7% formaldehyde in neutral buffered saline (Sigma Catalogue No. HT50-1-128). Alternatively, cells were grown for 8 days without changing the medium and then on day 8 cells were supplied with fresh medium and then fixed on day 9. Fixed cells were processed and stained for annexin V as described below.

2.3. Immunofluorescence microscopy

Fibroblasts grown on coverslips were washed twice in PBS at 37°C before being fixed. Cells were fixed with 3.7% formaldehyde in neutral buffered saline for 5 min at 37°C. Following permeabilization with 0.5% Triton X-100 for 15 min, cells were reincubated in 3.7% formaldehyde. Subsequently, cells were washed 3 times in PBS and treated with $NaBH_4$ (1 mg/ml) 3×5 min to reduce autofluorescence. After 3 washes in PBS, cells were incubated with 5% goat serum for 3 h at room temperature to block non-specific sites. Cells were incubated overnight with antiserum against human annexin V (1:100) in PBS containing 5% goat serum plus 1 mM NaN_3, and subsequently incubated in FITC-labelled affinity-purified second antibody for 3 h. Following further washes with PBS the cells were mounted with Vectashield (Vector Laboratories). For controls, cells were also incubated with antiserum (5 μg) against human annexin V preadsorbed with pure human annexin V (20 μg). Immunofluorescence was observed using a Nikon Optiphot microscope and confocal microscopy was performed on the Leica laser scanning microscope.

2.4. Extraction of HFF protein

Cells were plated in 35 mm dishes (day 0) at a density of 5×10^4 cells and cultured in DMEM supplemented with 10% calf serum. Cells were allowed to grow without changing the medium and total cellular

*Corresponding author. Fax: (44) (113) 2333167.

PII S0014-5793(96)00950-7

protein was extracted on days 1, 2, 3, 6, 7 and 8. Alternatively, cells were grown for 8 days without changing the medium and then on day 8 cells were supplied with fresh medium and then protein was extracted on day 9. Cellular protein was extracted as described in [13]. Four dishes of cell extract were pooled for each time point and protein concentrations were determined using BCA reagent (Pierce, Rockford, IL) according to the manufacturer's instructions.

2.5. SDS-PAGE and Western blot analysis

Samples of cell extract (7 μg) were subjected to SDS-PAGE according to Laemmli et al. [15] using 10% (w/v) resolving gels. Proteins were transferred from 10% SDS-PAGE gels to nitrocellulose membrane according to Towbin et al. [16]. After staining with 0.1% (w/v) Ponceau S in 1% acetic acid, the nitrocellulose was incubated in TBS-Tween (0.15 M NaCl/10 mM Tris/0.1% Tween/HCl pH 7.4) containing 5% (w/v) powdered milk for 30 min. Nitrocellulose blots were incubated with antisera against annexin V diluted 1:1000. After washing, blots were incubated for 3 h with 1:1000 dilution horseradish peroxidase-conjugated goat anti-rabbit antisera in TBS-Tween/5% (w/v) powdered milk. Peroxidase activity was detected using Enhanced Chemical Luminescence (Amersham), according to the manufacturer's instructions. Densitometry was performed using NIH Image 1.52 to compare the levels of annexin V at each time point.

2.6. Vibratome sections of human skin samples

Samples of human skin were fixed by immersion in 3.7% formaldehyde in neutral buffered saline (Sigma Catalogue No. HT50-1-128) for 48 h. Vibratome sections (50 μm thick) were cut and subsequently processed for immunocytochemistry as described above.

3. Results

Previous studies have shown that annexin V expression varies as a function of cellular growth state [13,14]. Using immunocytochemistry, we investigated whether there was any change in the location of annexin V after prolonged cell culture. Cells were subcultured at 5×10^4/22 mm coverslip and allowed to grow for 8 days without changing the medium. Cells were fixed and stained for annexin V as described in Section 2 using an antiserum that has been characterized previously [18].

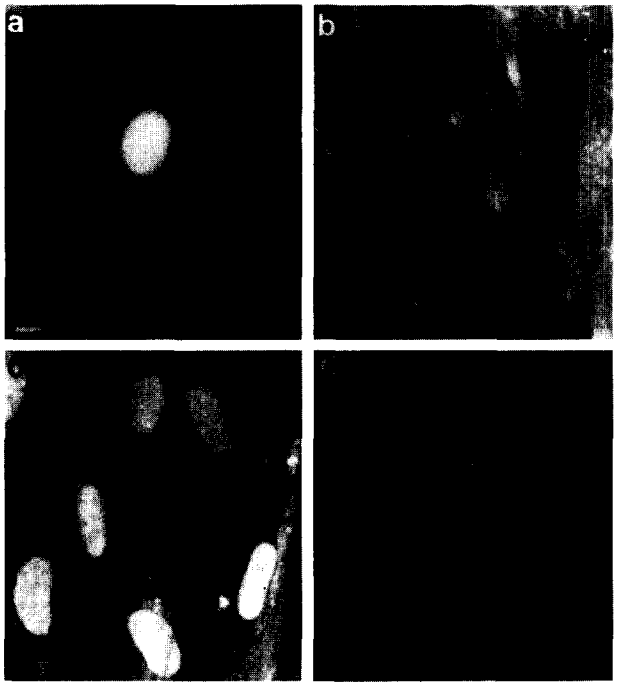

Fig. 1. Subcellular localization of annexin V in human foreskin fibroblasts with prolonged culture. Human foreskin fibroblasts were subcultured at 5×10^4 cells/22 mm coverslip (day 0), and allowed to grow for varying lengths of time without changes in media. Cells were fixed in formalin at day 1 (a and d) and day 7 (b). At day 8 the medium was changed and fixation was subsequently carried out on day 9 (c). Cells were permeabilized with Triton X-100 and stained for annexin V distribution using polyclonal anti-human annexin V antibody (a, b and c) or with antisera preadsorbed with pure annexin V (d) followed by FITC-labelled anti-rabbit. Scale bar, 10 μm.

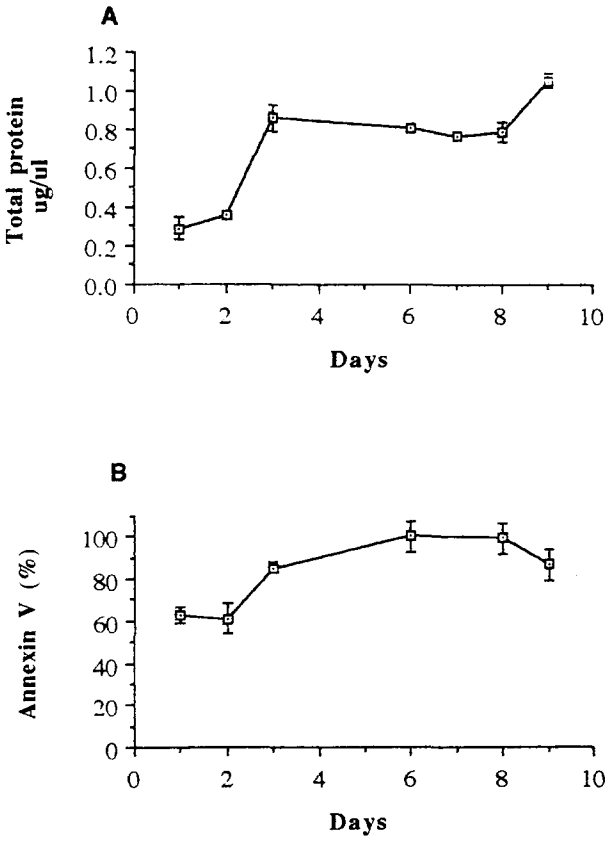

Fig. 2. Expression levels of annexin V in human foreskin fibroblasts during prolonged cell culture. Human foreskin fibroblasts were subcultured at 5×10^4 cell/35 mm dish (day 0) and allowed to grow without changes in medium. Total protein (μg/μl) was determined as described in Section 2 (A). Western blots were carried out on extracts containing equal amounts of protein. Intensity of the annexin V immunoreactive bands was measured using NIH image 1.52 and is expressed as a percentage of the maximum annexin V level (B). These results are representative of 2 separate experiments.

At day 1, annexin V was found diffusely in the cytoplasm, which is characteristic of cytosolic proteins, and in the nucleus at an elevated concentration compared to the surrounding cytoplasm (Fig. 1a). Seven days after subculture, cells had reached confluence and there was a loss of annexin V from the nuclei and the cytoplasmic staining appeared more structured with some co-localization with the Golgi apparatus (Fig. 1b). Interestingly, after addition of fresh medium to cells 8 days after subculture, nuclear staining for annexin V was restored in 24 h (Fig. 1c). No staining of cells was seen using antiserum preadsorbed with pure annexin V (Fig. 1d).

Variability in the level of annexin V expression was determined by immunoblot analysis. Extracts of total cell protein were obtained at the times used in the immunofluorescent localization study. Fig. 2A shows that total cell protein in-

creased dramatically up to day 4, due to rapid cell growth. After day 4 the total protein concentration remained constant indicating that the cells had become quiescent. This is in agreement with the work of Schlaepfer and co-workers [13] who showed that under identical conditions, human foreskin fibroblasts proliferated for the first 4 days after subculture and reached quiescence at day 6.

Renewing the medium at day 8 caused a further increase in total protein (Fig. 2A). Equal amounts of total cell protein extracts from cells at different times in culture were analyzed by immunoblotting. The results showed that annexin V levels were highest in quiescent cells and lowest in rapidly dividing cells (Fig. 2B, days 1 and 2). An increase of approximately 1.7-fold of annexin V was seen during this time. Following renewal of medium at day 8, there was no statistically significant change in annexin V content although the results suggest a decrease in annexin V content. Such a decrease would be in agreement with previous studies in which cell proliferation correlates with loss of expression of annexin V [13,14].

Quantitation of annexin V-positive nuclei reveals that during the period of cell proliferation, more than 95% of cells have positively stained nuclei (Fig. 3, days 1 and 2). As cells progress into a quiescent state the annexin V staining in the nucleus is lost (Fig. 3, days 6 and 7). These results indicate that the nuclear localization of annexin V depends on cellular growth state. Further evidence for a correlation between nuclear localization and proliferation is provided by refeeding quiescent cells (at day 8 post-plating) with DMEM supplemented with 10% FCS, which causes the reappearance of nuclear annexin V in greater than 95% of cells (Fig. 3, day 9). Thus it seems that stimulation of cell growth correlates with a high concentration of annexin V in the nucleus.

Further immunolocalization was carried out to investigate the location of annexin V in fibroblasts in the dermis of human skin samples. Vertical sections (50 μm) were cut through human skin using a vibratome and stained for annexin V as described above. Confocal sections through the dermis showed the presence of scarcely distributed fibroblasts. These cells were strongly stained for annexin V throughout the cytoplasm but were devoid of nuclear staining (Fig. 4a,b). This is particularly interesting since, as discussed above, fibroblasts grown in culture all show strong nuclear staining early on in culture (Figs. 4c and 1a) despite being derived from the same

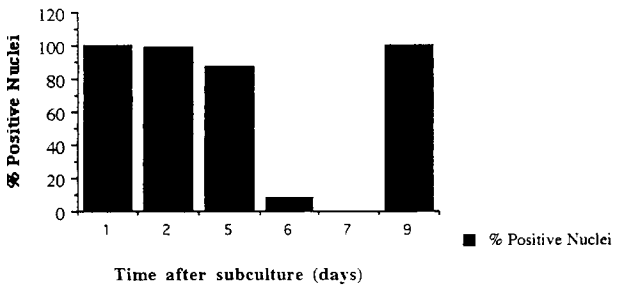

Fig. 3. Quantitation of nuclear localization of annexin V in human foreskin fibroblasts after prolonged cell culture. Human foreskin fibroblasts were subcultured at 5×10^4 cell/22 mm coverslip (day 0). Cells were allowed to grow for varying lengths of time without changes in media and counted on days indicated (day 1–7). On day 8 the medium was changed and cell counting was carried out on day 9. Detection of the nuclear localization of annexin V was carried out by immunocytochemistry as described in the legend to Fig. 2. Standard deviations were < 1.

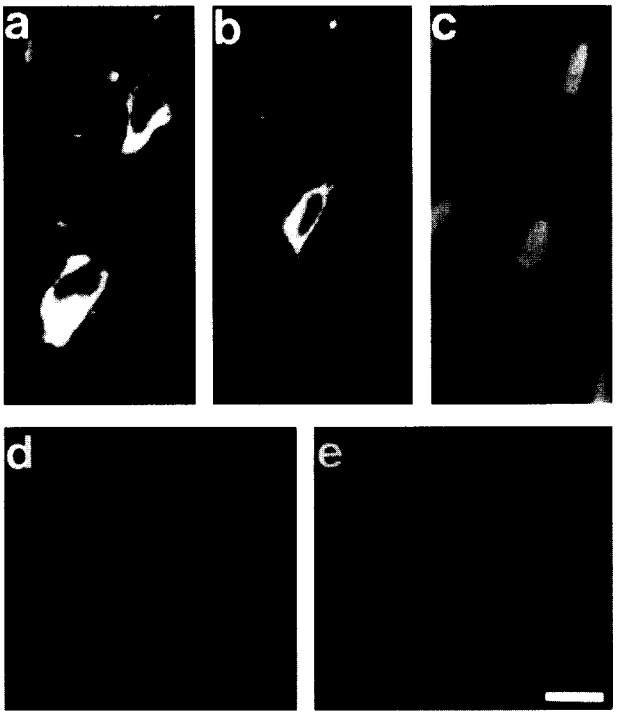

Fig. 4. Localization of annexin V in vibratome sections of human skin dermis. Samples of human skin were fixed in formaldehyde. Vibratome sections (50 μm) were permeabilized with Triton X-100 and stained for annexin V distribution using polyclonal anti-human annexin V antibody followed by FITC-labelled anti-rabbit IgG (a and b). Controls were incubated with preimmune serum followed by FITC-labelled anti-rabbit IgG (d) or with FITC-labelled anti-rabbit IgG antibody alone (e). Optical sections obtained by confocal microscopy were then used to locate annexin V in the dermis. The location of annexin V in human foreskin fibroblasts in culture after isolation from the dermis is shown in c. Scale bar, 20 μm.

tissue shown in Fig. 4a,b. Fibroblasts are responsible for the architectural framework of the dermis, secreting collagen to form the extracellular matrix. Within the dermis fibroblasts are generally in a quiescent state and only proliferate when tissue damage occurs [17]. The absence of annexin V in the nucleus of dermal fibroblasts is in agreement with the staining patterns observed for fibroblasts in prolonged culture (Fig. 1b) and further indicates a role for annexin V in the nucleus of proliferating cells.

4. Discussion

Annexin V is a structurally well characterized calcium-binding protein although its biological function remains unclear. We have shown that annexin V is present in the cytoplasm and has a variable nuclear location. Previous workers also demonstrated the presence of annexin V in the nucleus [19,20]. In relation to nuclear variability, Koster and co-workers reported that a nuclear location for annexin V in chick embryo fibroblasts was dependent on cell culture conditions [19]. Both biochemical and immunocytochemical studies have revealed nuclear association of other members of the annexin family: annexin II (monomer) has been described as a subunit of the primer recognition protein (PRP), a co-factor of DNA polymerase-α, and was shown to have a nuclear location in HeLa cells [23] although we only see plasma membrane asso-

ciation of annexin II [18]. Also annexin I is found in the nucleus of cultured endothelial cells [24]. In addition, we find that annexin IV is also present in the nucleus of human foreskin fibroblasts [18] but, in contrast to annexin V, it remains in the nucleus throughout different stages of cellular growth (not shown).

Another member of the annexin family which has a nuclear location is annexin XI [21]. Recent studies have shown that nuclear targeting of annexin XI in 3Y1 cells requires a sequence located in the N-terminus of the protein [22]. Interestingly, annexin XI has been reported to have a variable nuclear localization [25] with variability depending on the developmental stage of cells. No nuclear staining is obtained in adult rat tissues which are in a quiescent state. In contrast to our results with annexin V, when 3Y1 cells are forced into a quiescent state, the positive staining for annexin XI in the nucleus remains unchanged.

In conclusion we have shown that nuclear location of annexin V is variable depending on the cellular growth state: proliferating cells show strong nuclear staining which is reduced as cells progress into a quiescent phase. Future work will concentrate on identifying which factors regulate expression of annexin V in the nucleus.

Acknowledgements: This work was supported by the BBSRC. We would like to thank Dr. F. Seiler and Dr. J. Römisch for the gift of polyclonal antiserum against annexin V.

References

[1] Berridge, M.J. (1993) Nature 361, 315–325.
[2] Smith, V.L., Kaetzel, M.A. and Dedman, J.R. (1990) Cell Regul. 1, 165–172.
[3] Raynal, P. and Pollard, H.B. (1994) Biochim. Biophys. Acta 1197, 63–93.
[4] Kretsinger, R.H. (1980) CRC Crit. Rev. Biochem. 8, 119–174.
[5] Döring, V., Veretout, F., Albrecht, R., Mühlbauer, B., Schlatterer, C., Schleicher, M. and Noegel, A.A. (1995) J. Cell Sci. 108, 2065–2076.
[6] Creutz, C.E. (1992) Science 258, 924–931.
[7] Burgoyne, R.D. and Clague, M.J. (1994) Trends Biochem. Sci. 19, 231–232.
[8] Gerke, V. and Weber, K. (1984) EMBO J. 3, 227–233.
[9] Bewley, M.C., Boustead, C.M., Walker, J.H. and Waller, D.A. (1993) Biochemistry-USA 32, 3923–3929.
[10] Brisson, A., Mosser, G. and Huber, R. (1991) J. Mol. Biol. 220, 199–203.
[11] Rojas, E., Pollard, H.B., Haigler, H.T., Parra, C. and Burns, A.L. (1990) J. Biol. Chem. 265, 21207–21215.
[12] Giambanco, I., Pula, G., Ceccarelli, P., Bianchi, R. and Donato, R. (1991) J. Histochem. Cytochem. 39, 1189–1198.
[13] Schlaepfer, D.D. and Haigler, H.T. (1990) J. Cell Biol. 111, 229–238.
[14] Giambanco, I., Sorci, G., Antonioli, S., Rambotti, M.G., Spreca, A., Bocchini, V. and Donato, R. (1993) FEBS Lett. 323, 45–50.
[15] Laemmli, U.K. (1970) Nature 227, 680–685.
[16] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[17] Morgan, C.J. and Pledger, W.J. (1992) Wound Healing – Biochemical and Clinical Aspects (Cohen, Diegelmann, and Lingblad, Eds.), pp. 63–76.
[18] Barwise, J.L. and Walker, J.H. (1996) J. Cell Sci. 109, 247–255.
[19] Koster, J.J., Boustead, C.M., Middleton, C.A. and Walker, J.H. (1993) Biochem. J. 291, 595–600.
[20] Sun, J., Salem, H.H. and Bird, P. (1992) FEBS Lett. 314, 425–429.
[21] Mizutani, A., Usuda, N., Tokumitsu, H., Minami, H., Yasui, K., Kobayashi, R. and Hidaka, H. (1992) J. Biol. Chem. 267, 13498–13504.
[22] Mizutani, A., Watanabe, N., Kitao, T., Tokumitsu, H. and Hidaka, H. (1995) Arch. Biochem. Biophys. 318, 157–165.
[23] Kumble, K.D., Iversen, P.L. and Vishwanatha, J.K. (1992) J. Cell Sci. 101, 35–41.
[24] Raynal, P., van Bergen en Henegouwen, P.M., Hullin, F., Ragab, T.J., Fauvel, J., Verkleij, A. and Chap, H. (1992) Biochem. Biophys. Res. Commun. 186, 432–439.
[25] Mamiya, N., Iino, S., Mizutani, A., Kobayashi, S. and Hidaka, H. (1994) Biochem. Biophys. Res. Commun. 202, 403–409.