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Immunocytochemical analyses of annexin V (CaBP33) in a human-derived glioma cell line

Expression of annexin V depends on cellular growth state

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The subcellular distribution of annexin V, a calcium-dependent phospholipid- and membrane-binding protein, in a human-derived cell line, GL15, was investigated by immunocytochemistry at light and electron microscope levels. Annexin V was found diffusely in the cytoplasm and associated with plasma membranes, membranes delimiting cytoplasmic vacuoles, membranes of the endoplasmic reticulum, and filamentous structures the identity of which remains to be established. By immunocytochemistry at the light microscope level and immunochemistry, the expression of annexin V in these cells was found to depend on cellular growth state, being maximal soon after plating and progressively declining thereafter. However, re-expression of annexin V was observed whenever cell proliferation slowed down or arrested. These findings suggest that annexin V in glioma cells is mostly expressed in connection with cell differentiation. Also, the present ultrastructural data suggest that plasma membranes, membranes of the endoplasmic reticulum and the cytoskeleton are prominent sites of action of annexin V in vivo, thus lending support to the possibility that this protein might have a role in the regulation of cytoskeleton elements and/or of the structural organization of membranes.

Annexin V (CaBP33); Glioma cell, Immunocytochemistry; Electron microscope; Cell growth

1. INTRODUCTION

In recent years, the search for intracellular proteins potentially involved in the regulation of stimulus–response coupling led to the identification of a novel family of Ca^{2+}-binding proteins, the annexins, structurally unrelated to Ca^{2+}-binding proteins of the EF-hand type (for reviews see [1–5]). The annexin family comprises at least ten members in vertebrates, each of which has been given different names by investigators in the laboratories where individual proteins have been isolated and characterized [6]. The annexins, which have been suggested to play a role in the regulation of exocytosis, cell growth, the structural organization of membranes, blood coagulation, and the inflammatory response, share the ability to bind to acidic phospholipids and natural membranes in a Ca^{2+}-dependent way.

Two proteins, CaBP33 and CaBP37, purified from bovine brain by Ca^{2+}-dependent affinity chromatography on phenyl-Sepharose [7], were shown to be annexins [8]. Amino acid sequence analysis revealed that CaBP33 is the brain form of annexin V, whereas CaBP37 is an isoform of annexin V [9], which has been detected only in bovine tissues thus far [8,10,11]. Although annexin V has been suggested to have Ca^{2+}-channel activities [12,13], and demonstrated to be a potent anticoagulant [14–16] and to inhibit protein kinase C [17], its biological role(s) is (are) not known. Annexin V has been shown to be widely distributed in animal tissues by both immunochemistry and immunocytochemistry [18–21]. Yet, it is not ubiquitous. Following the observation that in the central and peripheral nervous system annexin V is restricted to glial cells [20–22], we analyzed the expression of this protein in a human-derived glioma cell line, GL15 [23]. Our data indicate that: (i) GL15 glioma cells express annexin V (CaBP33), but not its isoform, CaBP37; (ii) annexin V in these cells is found diffusely in the cytoplasm as well as associated with plasma membranes, membranes of the endoplasmic reticulum, and filamentous (cytoskeletal) structures; and (iii) the expression of the protein in these cells changes as a function of growth state, being high whenever the mitotic index is low, e.g. during the first post-plating day or following serum deprivation.

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2. MATERIALS AND METHODS

2.1. *Protein purification and characterization of the anti-annexin V antiserum*

The CaBP33/CaBP37 mixture was purified from bovine brain as in [7]. Annexin V (CaBP33) was purified from porcine heart as in [10].

Annexin V was used to immunize rabbits as in [8]. The anti-annexin V antiserum used in the present study was shown to be specific to annexin V when tested with cytosolic fractions or with EGTA- or Triton X-100-extracts of membranes from rat and porcine organs by immunoblotting [8,10,20] With bovine tissues, this antiserum also recognized the annexin V isoform, CaBP37, but no immunological cross reactivity was observed between annexin V isoforms and other annexins [8,10]

2.2. *Immunochemistry*

GL15 glioma cells were plated on 35-mm plastic Petri dishes at a density of 2×10^5 cells/dish and cultured for one day in DMEM supplemented with 20% heat-inactivated fetal calf serum and 100 IU of penicillin and 0.1 mg of streptomycin/ml. Cells were washed free of the culture medium with 20 mM Tris-HCl, pH 7.5, containing 0.9% NaCl (TBS) and fixed with 3% glutaraldehyde in TBS for 10 min at room temperature. Cells were washed free of the fixative with TBS, dehydrated in a series of alcohols and propylene dioxide, and embedded in Epon-Araldite. Thin sections were mounted onto nickel grids and sequentially incubated for 5 min in 3% bovine serum albumin (BSA) in TBS (buffer A) containing 20 mM NaN_3 (buffer B), and for 10 min in 20 mM glycine in buffer B. This step was followed by incubation with the anti-annexin V antiserum (1:100 in buffer B, 1 h at room temperature and then overnight at 4°C, in a humid chamber). Sections were washed with buffer B containing 1% Tween-20 (five times, 1 min each), and incubated for 3 h at room temperature with a goat-anti-rabbit IgG antibody labelled with 15 nm gold particles (Biocell Research Laboratories, Cardiff, UK) (1:100 in buffer B). Sections were washed with buffer B minus BSA (buffer C) (once for 1 min), post-fixed for 3 min with 1% glutaraldehyde in buffer C, and counterstained with uranyl acetate and lead citrate Sections were viewed in a Philips TEM 400 electron microscope. In control experiments, the primary antiserum was either omitted, substituted for by the preimmune serum, or absorbed with annexin V before use. Identical results were obtained, irrespective of the procedure used, i.e. no immune reaction was detected.

In other experiments, GL15 cells were plated on glass coverslips in Costar tissue culture plates at a density of 5×10^4 cells/well (1.6 cm in diameter) and cultured as above for one day. Cells were washed free of the culture medium and fixed as above. Fixed cells were permeabilized with 0.1% Triton X-100 in TBS for 10 min at room temperature, and exposed to 3% H_2O_2 in methanol for 15 min to quench any endogenous peroxidase activity. Cells were then incubated in buffer A for 3 h at room temperature followed by incubation with the anti-

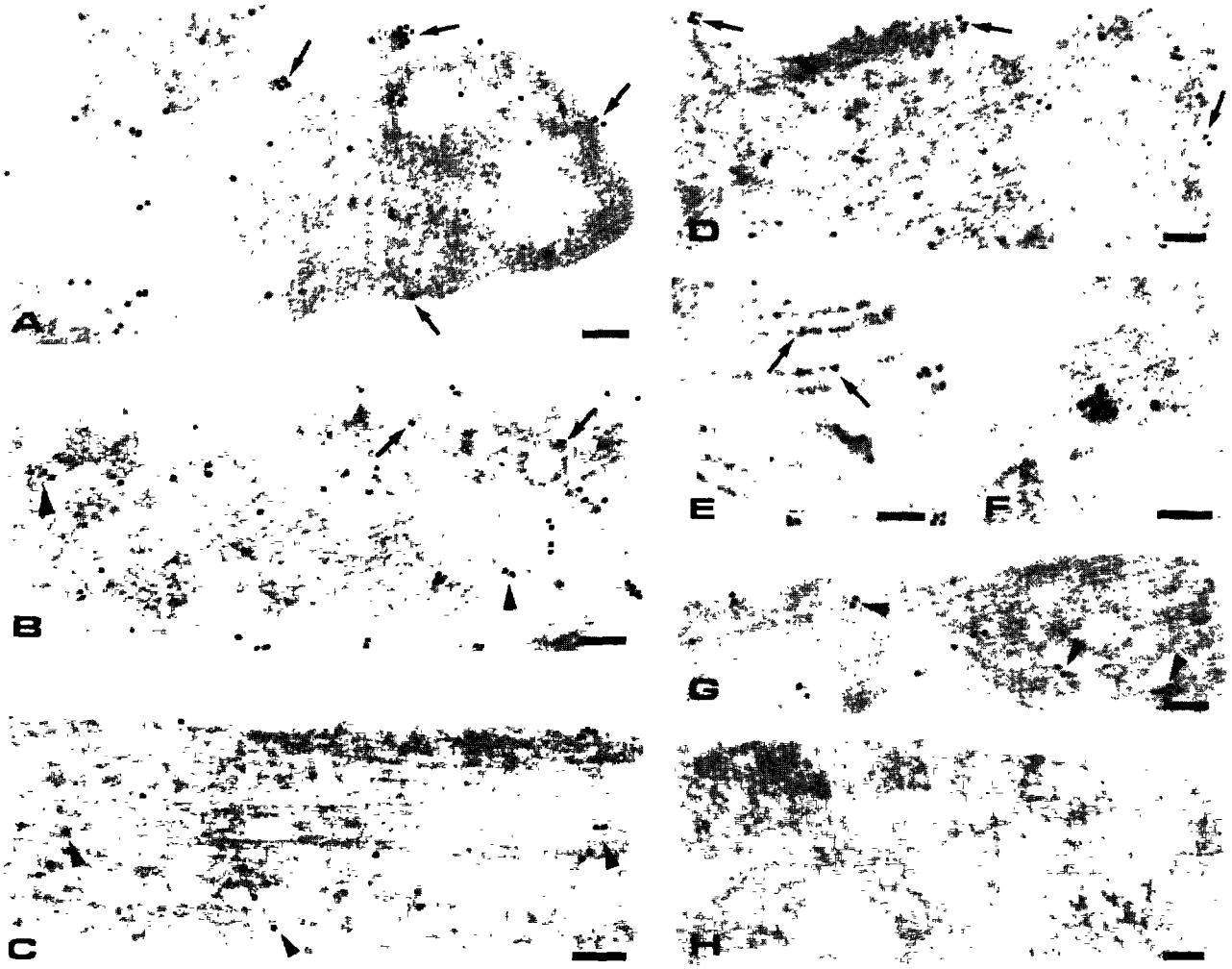

Fig. 1. Immunocytochemical localization of annexin V in human GL15 glioma cells. Electron microscope analysis by post-embedding immunocytochemistry. Immune reaction product is observed on plasma membranes (arrows in A and D), in association with intermediate filaments in the cell body (arrowheads in B, also see D and F) and in cell processes (arrowheads in C and G), on membranes limiting cytoplasmic vacuoles (arrows in B), and on membranes of the endoplasmic reticulum (arrows in E). Annexin V is also found diffusely in the cytoplasm (A,B,D). Control sections show no immune reaction product (H). Bars: A–H = 0.1 μm.

annexin V antiserum (1:100 in 1% BSA in TBS) overnight at 4°C. This step was followed by sequential incubations of cells with a sheep-anti-rabbit IgG antiserum (1:100 in 1% BSA in TBS) and with a horseradish peroxidase-anti-peroxidase complex (1: 200 in 1% BSA in TBS), each for 1 h at room temperature. Washings between antisera were done with 0.2% Triton X-100 in TBS. The immune reaction product was developed using 0.3 mg of diaminobenzidine (DAB)/ml of 50 ml mM Tris-HCl, pH 7.5, containing 0.02% H_2O_2, in the dark. Cells were washed in TBS and mounted in a permanent aqueous medium on glass slides for light microscopy. Control experiments were done as described above.

2.3. *Immunochemical analyses*

To quantitate annexin V in GL15 cells, cells were plated on 35-mm plastic Petri dishes and cultivated for 1, 2, 4, 7, 11, or 18 days. On the day of experiments, cells were washed free of the medium with TBS and extracted with 0.2 ml of a solution of 3% SDS in 50 mM Tris-HCl, pH 7.5 for 15 min at room temperature, after which 2-mercaptoethanol was added to a final concentration of 5%. Cell extracts were boiled for 5 min and subjected to SDS-PAGE (7.5% acrylamide) [24] in parallel with known amounts of annexin V which served as a reference. The polypeptides so separated were electroblotted onto nitrocellulose paper and subjected to immunostaining [25] using the anti-annexin V antiserum (1:1000). The immune reaction product was developed using DAB as described above. Immunoreactive bands on nitrocellulose sheets were subjected to densitometry for quantification of annexin V. Cells grown in parallel dishes were used to measure the total protein content [26]. To measure the amount of annexin V/cell, cells were plated in 96-multiwell plates at a density of 7×10^3 cells/well and cultivated as above for 1, 2, 4, 7, 11, or 18 days. On the day of experiments, cells were processed as described for light microscopy (see section 2.2.), except that the immune reaction product was developed with a solution of 4 mM *o*-phenylenediamine in 50 mM citric acid, 100 mM Na_2HPO_4, pH 5.0, containing 0.01% H_2O_2 (50 μl/well for 10 min in the dark), followed by 50 μl of 4 N H_2SO_4 to terminate the reaction. The immune reaction product was measured spectrophotometrically at 492 nm using a microplate reader photometer. In control experiments, the primary antiserum was omitted. Cells seeded in parallel 96-multiwell plates were used to calculate the number of cells/well at the different post-plating days by a tetrazolium-based (MTT) colorimetric assay [27].

3. RESULTS AND DISCUSSION

The anti-annexin V antiserum recognized both bovine isoforms of annexin V, but only one polypeptide in extracts from GL15 glioma cells by immunoblotting (not shown). This polypeptide comigrated with annexin V (CaPB33) in SDS gels, indicating that GL15 cells express only the lower M_r isoform of annexin V.

By immunocytochemistry using the immunogold (post-embedding) method, annexin V was found diffusely in the cytoplasm as well as associated with plasma membranes, intermediate filaments, and membranes of the endoplasmic reticulum (Fig. 1).

Fig. 2 documents changes occurring in the expression of annexin V in Gl15 cells as a function of cellular growth state, as investigated by immunocytochemistry at the light microscope level. Cells were cultivated for 1–18 days in the presence of serum with renewal of the medium every other day. The maximum expression of the protein/cell was observed between day 1 and day 2 after plating, as judged by light microscopy (Fig. 2A,B), with a progressive decrease in the expression of annexin V during the subsequent post-plating days. From the eleventh day only a faint immunoreactivity could be seen in these cells (Fig. 2E,F). Under the present exper-

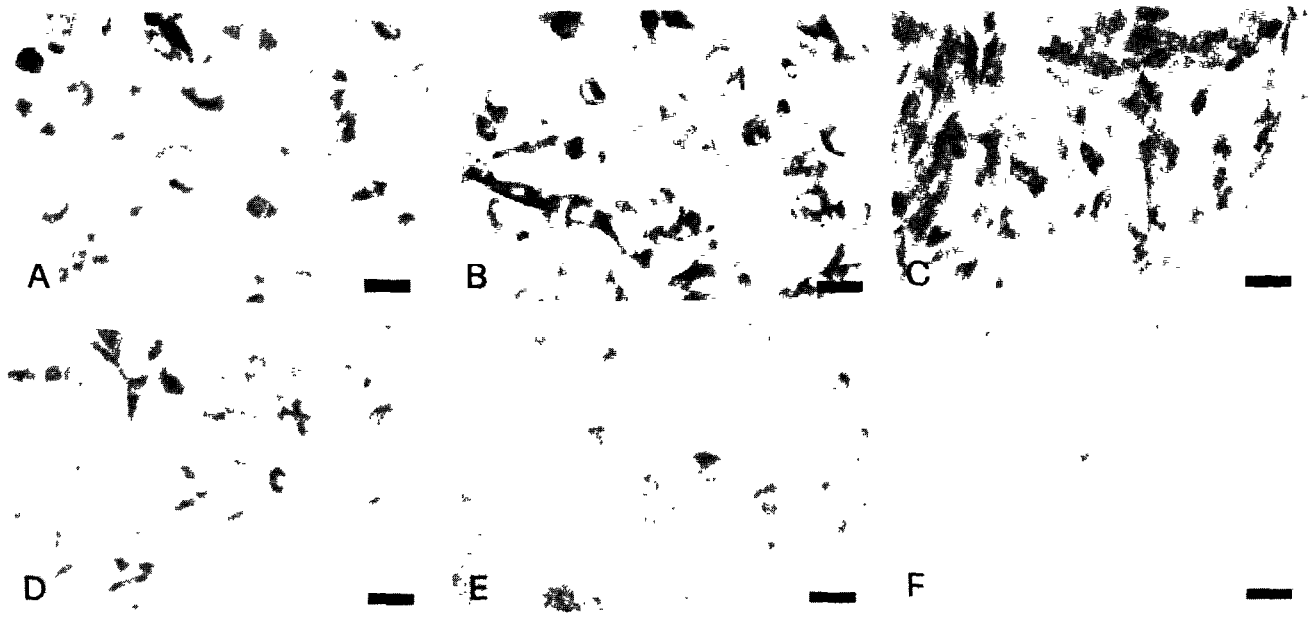

Fig. 2. Immunocytochemical localization of annexin V in human GL15 glioma cells as a function of cellular growth state. Cells were plated on glass coverslips in tissue culture cluster plates and cultivated for 1 (A), 2 (B), 4 (C), 7 (D), 11 (E), or 18 (F) days in the presence of serum, before immunocytochemistry. The culture medium was renewed every other day. The maximum expression of the protein/cell is apparently observed between the first (A) and second (B) post-plating day. Then, the intensity of the immune reaction product progressively declines, although some residual immunoreactivity is still observable at the eighteenth post-plating day (F). Bars: A–F = 50 μm.

imental conditions, confluency was apparently attained around the fourth post-plating day (Fig. 2C).

A more thorough analysis of the proliferation pattern of GL15 cells by cell count and protein determination (Fig. 3) revealed that the maximum expression of annexin V/cell occurred at day 1, and that the annexin V content declined to very low levels as cells proliferated, such that at day 18 the annexin V content/cell was about 4% of that at day 1 (Fig. 3A). Also, in the time period investigated these cells proliferated in an almost linear way when cultivated in the presence of serum (Fig. 3B). Immunoblots of SDS-extracts of GL15 cells cultivated for 1–18 days showed that at day 1 the annexin V concentration was 8.33 μg/mg of protein, becoming 0.41 μg/mg of protein at day 18 (Table I). As the data in Fig. 3 and Table I suggested that the expression of annexin V in GL15 cells negatively correlated with cell proliferation, several culture conditions were tested to see whether or not the expression of annexin V could be correlated with cell differentiation.

(1) When cells were cultivated in the presence of serum without renewing the medium, cell proliferation was linear up until day 7, and decreased significant thereafter (Fig. 4, ■). Under these conditions, the annexin V content/cell decreased to nearly the same levels as in the reference experiment (serum renewed every other day) from day 1 to day 11, and significantly increased from day 11 to day 18 as compared to the reference experiment (Fig. 4, ●).

(2) When cells were grown in the presence of serum

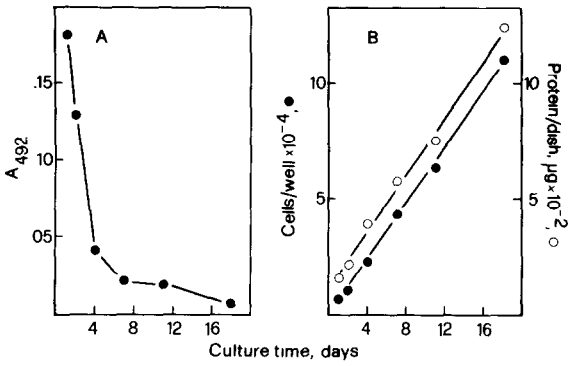

Fig. 3. Immunochemistry of annexin V in human GL15 glioma cells as a function of culture time. (A) The annexin V content/cell was measured on cells cultivated in 96-multiwell plates for the time indicated in the presence of DMEM supplemented with fetal calf serum. The medium was renewed every other day. Annexin V immunochemistry was done as described in section 2.3. Results are expressed as A_{492} vs. the culture time. (B) Cells were plated in 96-multiwell plates (●) or in 35-mm plastic Petri dishes (○) and cultivated as described above. Cells plated on 96-multiwell plates were used for cell counting by the MTT method (●), whereas cells plated on Petri dishes were used for protein determination (○). Under the present experimental conditions, the immunocytochemical counterpart of which was illustrated in Fig. 2, the annexin V content/cell fell down to very low levels by the end of the first post-plating week (A), whereas cells proliferated in an almost linear way during the time period investigated (B).

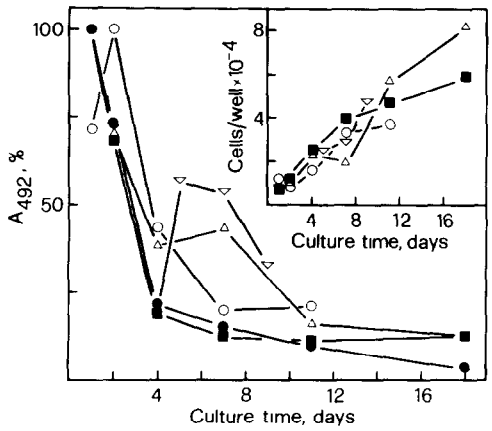

Fig. 4. Immunochemistry of annexin V in human GL15 glioma cells as a function of culture time under different culture conditions. The percent amount of annexin V/cell was plotted vs. the culture time. (●) Data in Fig. 3A were included for comparison. (■) Cells were cultivated as in (●), except that the culture medium was not renewed (△) Cells were cultivated as in (●), except that at day 2 the medium was substituted for by DMEM alone. (▽) Cells were cultivated as in (●), except that at day 4 the medium was substituted for by DMEM alone. (○) Cells were plated in DMEM alone and cultivated for 11 days without renewing the medium. Cell counts under the different culture conditions are reported in the inset. Note that changing the culture medium at day 4 to DMEM alone (▽) resulted in a 3-fold increase in the annexin V content/cell during the next three days along with an arrest in cell proliferation. Also, when cells were plated and cultivated in DMEM alone (○), the maximum content of annexin V/cell was observed at day 2 and its level remained significantly higher than in the reference experiment (●) between day 4 and day 7.

for two days and in DMEM without serum thereafter (Fig. 4, △), the decrease in the annexin V content/cell was less steep from day 2 to day 4, as compared to the reference experiment, increased to some extent from day 4 to 7, and decreased thereafter to values signifi-

Table I

Content of annexin V in human GL15 glioma cells as a function of cellular growth state

Post-plating day	Annexin V (μg/mg of protein)
1	8.33 ± 0.64
2	6.07 ± 0.58
4	2.11 ± 0.18
7	1.13 ± 0.08
11	0.94 ± 0 03
18	0.41 ± 0.04

GL15 glioma cells were plated at a density of 2×10^5 cells/dish in parallel 35-mm plastic Petri dishes and cultivated for 1–18 days in DMEM supplemented with 20% fetal calf serum. The medium was renewed every other day. At intervals as indicated, cells were washed free of the medium with TBS and solubilized with either SDS plus 2-mercaptoethanol for SDS-PAGE and immunostaining with the anti-annexin V antiserum after transfer of separated polypeptides onto nitrocellulose paper, or 0.1 M NaOH plus 0.19 M Na_2CO_3 for protein determination. Known amounts of annexin V were subjected in parallel to immunoblotting and served as reference to quantitate annexin V in immunoblots of SDS-extracts. Figures represent the mean of three determinations ± S.E

Fig. 5. Immunocytochemistry of annexin V in human GL15 glioma cells cultivated in DMEM alone for 11 days. Conditions were as described in Fig. 4, except that cells were cultivated in the absence of serum for 1 (A), 2 (B), 4 (C), 7 (D), or 11 (E) days without renewing the medium. Note that under these conditions cells acquired a stellate shape and expressed significant levels of annexin V even at day 11. Bars: A–E = 15 μm.

cantly higher than those registered in the reference experiment. This pattern was congruent with cell counts and protein determination, in that the increase in annexin V content/cell observed between day 4 and day 7 coincided with an arrest of cell proliferation.

(3) When cells were cultivated for four days in the presence of serum with renewal of the medium every other day, followed by serum deprivation, the proliferation rate sensibly decreased during the next three days (Fig. 4, ▽). During this time period, the annexin V content/cell increased by more than thrice, as compared to the reference experiment.

(4) Since the results of experiments (2) and (3) suggested that the annexin V content/cell negatively correlated with proliferation, one additional experiment was done, in which cells were cultivated for 11 days without renewing the medium in the absence of serum since the beginning. In this case, in addition to cell count, protein determination, and measurement of annexin V immunoreactivity in cells seeded in 96-multiwell plates, immunocytochemistry at the light microscope level was carried out. Under these conditions (Fig. 4, ○), cells stopped proliferating from day 1 to day 2, restarted proliferating from day 2 to day 7, albeit at a significantly slower rate than in the reference experiment, and almost arrested between day 7 and day 11. The maximum expression of annexin V content/cell was registered at day 2. At day 4, cells contained twice as much annexin V as the corresponding cells of the reference experiment, and, in spite of some proliferation between day 7 and day 11, the annexin V content remained constant in this time period. All these data were consis-

tent with the possibility that the expression of annexin V in GL15 glioma cells is related to differentiation rather than to proliferation. Immunocytochemistry at the light microscope level (Fig. 5) showed that annexin V was expressed in cells cultivated for as long as eleven days in the absence of serum without renewal of the medium, and that serum deprivation induced these cells to acquire a stellate shape (compare Fig. 5 with Fig. 2).

No annexin V immunoreactivity was found in neurons [20–22] or in rat chromaffin cells [20]. On the other hand, by immunochemical dosage, annexin V was found to be expressed in extremely low amounts in NGF-untreated rat pheochromocytoma (PC12) cells and to be expressed in high amounts in NGF-treated PC12 cells as long as these cells were exposed to NGF [28]. Also, the amount of annexin V in cultured fibroblasts, as measured immunochemically, was maximal at confluency and remained constant during the post-confluency time, at which time no further cell proliferation occurred [28]. These data were interpreted as indicating that annexin V accumulated in the above cell types during cell differentiation, rather than during cell proliferation. In the case of GL15 glioma cells, by both immunochemistry and immunocytochemistry the annexin V level was found to be high during the first two post-plating days when cells were cultivated in the presence of serum, and to progressively decline to almost zero while cells were still actively proliferating. However, whenever cell proliferation decreased or arrested, reaccumulation of annexin V was observed, in agreement with the conclusion that annexin V is mostly expressed during cell differentiation. Qualitatively identical results were obtained with antisera specific to S-100 protein, the glial fibrillary acidic protein, and annexin VI (unpublished data), suggesting that these latter proteins, too, are expressed and exert their activities in differentiated cells.

In conclusion, we have offered evidence for association of annexin V with membranes and cytoskeletal structures in vivo. This subcellular distribution was seen whenever annexin V was allowed to accumulate in glioma cells, i.e. soon after plating and/or after serum deprivation. This latter treatment resulted in remarkable changes in cell morphology, suggestive of differentiation. The above sites, i.e. membranes and intermediate filaments, thus appear to be physiological targets of annexin V, although the functional link between these two subcellular localizations of the protein is still missing. Recently, evidence for Ca^{2+}-dependent self-association of several annexins in vitro has been presented [4]. An attractive possibility might therefore be that annexin V, the dimerization of which has been described [16,29,30], serves the function of connecting cytoskeletal structures to the lipid bilayer of cell membranes. Accordingly, annexin V accumulates in glioma cells during differentiation.

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REFERENCES

[1] Crompton, M.R., Moss, S E and Crumpton, M J. (1988) Cell 55, 1–3.
[2] Klee, C.B. (1988) Biochemistry 27, 6645–6653.
[3] Burgoyne, R.D and Geisow, M J. (1989) Cell Calcium 10, 1–10.
[4] Zacks, W.J. and Creutz, C E. (1990) J. Bioenerg. Biomembr. 22, 97 120.
[5] Römisch, J. and Pâques, E.-P. (1991) Med. Microbiol. Immunol. 180, 109–126.
[6] Crumptom, M.J. and Dedman, J.R. (1990) Nature 345, 212
[7] Donato, R , Giambanco, I , Aisa, M.C., Ceccarelli, P. and Di Geronimo, G. (1988) Cell Biol. Int. Rep. 12, 565–566.
[8] Donato, R., Giambanco, I., Pula, G and Bianchi, R. (1990) FEBS Lett. 262, 72–76.
[9] Learmomth, M.P., Howell, S A , Harris, A.C.M., Amess, B., Patel, Y., Giambanco, I., Bianchi, R , Pula, G., Ceccarelli, P., Donato, R , Green, B.N and Aitken, A. (1992) Biochim. Biophys. Acta 1160, 76–83.
[10] Pula, G., Bianchi, R., Ceccarelli, P., Giambanco, I. and Donato, R (1990) FEBS Lett. 277, 53–58.
[11] Bianchi, R., Giambanco, I., Ceccarelli, P., Pula, G. and Donato, R. (1992) FEBS Lett. 296, 158–162
[12] Huber, R., Schneider, M., Mayr, I., Römisch, J. and Pâques, E.-P. (1990) FEBS Lett. 275, 15–21.
[13] Rojas, E., Pollard, H B., Haigler, H.T., Porra, C. and Burns, A.L. (1990) J. Biol. Chem. 265, 21207–21215
[14] Shidara, Y. (1984) Acta Obstet. Gynaecol. Jpn. 36, 2583–2592.
[15] Reutelingspreger, C.P.M., Hourstra, G. and Heneker, H.C. (1988) Eur. J. Biochem. 98, 1572–1578
[16] Funakoshi, T., Haimark, R.L., Hendrickson, L.E., McMullen, B.A. and Fijikawa, K (1987) Biochemistry 26, 5572–5578
[17] Schlaepfer, D.D., Jones, J. and Haigler, H.T. (1992) Biochemistry 31, 1886–1891.
[18] Pepinski, R.B., Tizard, R., Mattaliano, R.J., Sinclair, L K., Miller, G.T., Browing, J.L., Chow, E.P., Burne, C., Huang, K.-S., Pratt, D., Watcher, L., Hession, C., Frey, A.Z. and Wallner, B.P. (1988) J. Biol. Chem. 263, 10799–10811.
[19] Kaetzel, M.A., Hazarika, P and Dedman, J.R. (1988) J. Biol. Chem 264, 14463–14470.
[20] Giambanco, I , Pula, G., Ceccarelli, P., Bianchi, R. and Donato, R. (1991) J Histochem. Cytochem. 39, 1189–1198.
[21] Spreca, A., Rambotti, M.G., Giambanco, I., Pula, G., Bianchi, R., Ceccarelli, P. and Donato, R. (1992) J. Cell. Physiol. 152, 587–598
[22] Woolgar, J.A., Boustead, C.M and Walker, J.H. (1990) J. Neurochem. 54, 62–71.
[23] Bocchini, V., Casalone, R., Collini, P., Rebel, G. and Lo Curto, F (1991) Cell Tissue Res. 265, 73–82.
[24] Laemmli, U.K. (1970) Nature 227, 680–685.
[25] Towbin, H., Staehelin, T and Gordon, J. (1979) Proc. Natl Acad Sci. USA 76, 4350–4354.
[26] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol Chem. 193, 265–275.
[27] Pagé, M , Bejaoui, N., Cinq-Mars, B. and Lemieux, P. (1988) Int. J. Immunopharmacol 10, 785–793.
[28] Schlaepfer, D D. and Haigler, H.T. (1990) J. Cell Biol 111, 229–238.
[29] Ahn, N.G., Teller, D.C., Bienkowski, M.J., McMullen, B.A , Lipkin, E.W. and de Haen, C. (1988) J. Biol. Chem 263, 18657–18663.
[30] Giambanco, I., Pula, G., Bianchi, R. and Donato, R. (1990) FEBS Lett. 267, 171–175.