Expression of Annexins as a Function of Cellular Growth State

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Abstract. Annexins are a structurally related family of Ca^{2+} binding proteins of undetermined biological function. Annexin I (also called lipocortin 1) is a substrate for the EGF-stimulated tyrosine kinase and is postulated to be involved in mitogenic signal transduction. To investigate further the involvement of lipocortin 1 in cell proliferation, we measured lipocortin 1 levels in normal diploid human foreskin fibroblasts (HFF) to determine whether its expression changed as a function of growth status. For comparison, the expression of annexin V (also called endonexin II) was measured in HFF cells. Endonexin II is a protein with similar Ca^{2+} and phospholipid binding properties as lipocortin 1, but it is not a substrate for tyrosine kinases. Quiescent HFF cell cultures were induced to proliferate by either subculture to lower cell density, EGF stimulation, or serum stimulation. In all three protocols, proliferating HFF cells contained three- to fourfold higher levels of lipocortin 1 and three- to fourfold lower levels of endonexin II than quiescent HFF cells. In contrast, the expression of annexin II (also called calpactin I) and annexin IV (also called endonexin I) remained relatively unchanged in growing and quiescent HFF cells. Lipocortin 1 synthesis rate was eightfold higher and its turnover rate was 1.5-fold slower in proliferating compared to quiescent HFF cells. Endonexin II synthesis rate remained constant but its turnover rate was 2.2-fold faster in proliferating compared to quiescent HFF cells. In a separate set of experiments, annexin expression levels were measured in cultures of rat PC-12 cells, a pheochromocytoma that ceases proliferation and undergoes reversible differentiation into nondividing neuronlike cells in response to nerve growth factor (NGF). After NGF treatment, PC-12 cells expressed fivefold higher levels of endonexin II and 32-fold higher levels of calpactin 1. Lipocortin 1 and endonexin I were not expressed in PC-12 cells. In summary, lipocortin 1 expression exhibited a positive correlation with cell proliferation in HFF cells. The increased expression of endonexin II in quiescent HFF cells and differentiating PC-12 cells implies that this protein may play a more prominent role in nondividing cells.

Annexins are a family of structurally related proteins that bind to certain phospholipids in a Ca^{2+}-dependent manner (for reviews, see references 7 and 12). The phospholipids to which they bind are preferentially located on the cytosolic face of the plasma membrane. Since they were independently discovered by several different laboratories that were interested in different biological problems, these proteins have been given several unrelated, and often overlapping names including lipocortins, calpactins, synexin, chromobindins, endonexins, calelectrins, calcimedins, and placental anticoagulant proteins. The relationships of these proteins are illustrated in Table I of reference 21. Each annexin has an amino terminal domain that has only limited sequence similarity with the others, while all annexins have a core domain consisting of either a four- or eightfold repeat of a conserved amino acid sequence that is ~72 amino acids in length. Despite detailed structural information, the biological functions are not clearly defined for any of the annexins. Some of the more attractive proposals for annexin function include involvement in regulation of membrane traffic and exocytosis (3, 9, 31), mediation of cytoskeletal-membrane interactions (12, 47), and mitogenic signal transduction (10, 20, 33).

Since the different annexins have unique amino-terminal domains, it is compelling to speculate that this domain confers a unique biological function to each annexin. Many of the proteins are phosphorylated by protein kinase C in the amino terminal domain (17, 41, 45), thereby raising the possibility that phosphorylation may modulate their function. Two of the proteins, annexin I (also called lipocortin 1) and annexin II (also called calpactin 1), are phosphorylated on a conserved tyrosine residue in the amino terminal domain by the EGF receptor/kinase (8, 20, 33) and by $pp60^{src}$ (13, 15), respectively. The phosphorylation of lipocortin 1 by the EGF receptor/kinase exhibits many characteristics expected of a physiological substrate for a protein tyrosine kinase: (*a*) the EGF receptor/kinase affinity for lipocortin 1 in vitro is very high, apparent K_m = 50 nM (20); (*b*) the phosphorylation occurs in intact cells in a growth and EGF-dependent manner (14, 39) and; (*c*) the stoichiometry of phosphorylation in cultured diploid fibroblasts changes from <1% in quiescent cells to ~25% in EGF-stimulated cells (D. D.

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Schlaepfer and H. T. Haigler, unpublished results). Thus, lipocortin 1, and possibly other annexins, may be involved in intracellular transduction of mitogenic signals. Phosphorylation of lipocortin 1 at tyrosine 21 reduced by fivefold the amount of Ca^{2+} required for half-maximal association of the protein with phospholipid vesicles (40) and increased by 10-fold the sensitivity to tryptic cleavage at lysine-26 (20). However, the physiological significances of these observations are not yet known.

Another indication that the individual annexins have specialized roles comes from the observation that different annexins have unique tissue and cell distributions (7, 16, 35). A comprehensive expression survey is not available, because the members of this family have only recently been defined. It is known that the expression of annexins can be modulated by the stage of development (5, 28, 30), differentiation (22, 24, 36, 46), and thyroid status (37). Initially, it was proposed that the synthesis of lipocortins was induced by glucocorticoids (34, 38, 44); however, recent studies have been inconsistent with this proposal (1, 2, 23, 24, 32).

Since lipocortin 1 appears to be a physiological substrate for the EGF receptor kinase and thus has been implicated in the regulation of cell growth, we have investigated its expression as a function of growth in human diploid foreskin fibroblasts (HFF).[1] For comparison, we also measured the expression of annexin V (also called endonexin II), a protein with Ca^{2+} and phospholipid binding properties similar to those of lipocortin 1 (11, 19, 25, 26, 29, 42). Endonexin II does not have a tyrosine residue in its amino terminal domain and is not a protein kinase substrate (41). The study reported herein shows that mitogenic stimulation of normal diploid fibroblasts caused an increase in lipocortin 1 and a decrease in endonexin II expression. We also observed increased endonexin II and calpactin 1 expression in PC-12 cells that had stopped dividing and had differentiated in response to nerve growth factor (NGF).

Materials and Methods

Cell Culture

Experimental cultures of diploid human foreskin fibroblasts (HFF) (passages 10–16) were plated in 35-mm cell culture dishes containing Dulbecco's modified Eagle's medium (DME), supplemented with 10% calf serum, penicillin (50 U/ml) and streptomycin (50 μg/ml). Stock cultures were maintained in T-75 flasks with complete media changes every 5 d. HFF cells were subcultured by limited trypsin treatment. Briefly, confluent cells were exposed to trypsin 0.25% without Ca^{2+} and Mg^{2+} (No. 610-5050; Gibco Laboratories, Grand Island, NY) for 10 min at 37°C, harvested by centrifugation (300 g), washed with PBS, and resuspended in complete media. Stock HFF cells were subcultured (1:6) once every 3 wk.

Experimental cultures of rat adrenal pheochromocytoma cells (PC-12) (No. CRL 1721; American Type Culture Collection, Rockville, MD) were plated in collagen-coated 35-mm cell culture dishes containing RPMI 1640 (Mediatech, Herndon, VA), supplemented with 1% heat-inactivated horse serum, penicillin (50 U/ml) and streptomycin (50 μg/ml). NGF (50 ng/ml) (2.5S NGF; Sigma Chemical Co., St. Louis, MO) was added daily in a sterile PBS solution containing 0.1% BSA. 50% of the experimental culture medium was replaced every 2 d. Stock cultures were maintained in collagen-coated T-75 flasks in a growth media containing 85% RPMI 1640, 10% heat-inactivated horse serum, and 5% FBS. 75% of the media was replaced every 2 d. When the stock PC-12 cells became confluent, they were removed from the collagen substrate by forced pipetting, harvested by centrifugation, passed through a 22-gauge needle to obtain a single cell suspension and subcultured at a 1:6 ratio.

1. *Abbreviations used in this paper*: HFF, human foreskin fibroblast; NGF, nerve growth factor.

Cellular Protein Extraction

All experimental 35-mm cell culture dishes were rinsed with PBS, and the protein extracted at room temperature by the addition of 125 μl of extraction buffer (62 mM Tris, pH 6.8, 2.5 mM $MgCl_2$, 1 mM EGTA, 1% SDS [wt/vol], 1% Triton X-100 [vol/vol], 5 μg/ml leupeptin, and 5 μg/ml aprotinin) per 35-mm dish. The cells were dislodged with a rubber policeman and 10 μl of a DNase I and RNase solution (1 mg/ml each in 20 mM Hepes, pH 7.4, containing 5 mM $MgCl_2$) was added to reduce viscosity. Extracts from several 35-mm culture dishes were pooled for each experimental time point and duplicate aliquots were assayed for protein content by the BCA micro protocol method (Pierce Chemical Co., Rockford, IL). The remaining experimental extracts immediately were frozen in liquid N_2, lyophilized, and then resuspended in the same volume of Laemmli SDS sample buffer (27).

Cellular growth rates were determined by measuring changes in total cellular protein. Cell numbers were determined with the use of a hemocytometer. By measuring both total protein content and cell numbers, we found that there was an average of 3×10^3 cells/microgram of total protein and that this value did not significantly change as a function of cell density.

SDS-Gel Electrophoresis and Western Immunoblot Analysis

20 μg of cell protein from aliquots of the experimental extracts were subjected to SDS-PAGE (27) and electrophoretically transferred to Immobilon PVDF membranes (Millipore Continental Water Systems, Bedford, MA) by the method by Towbin et al. (43). Molecular mass standards used for calibration were purchased (Bio-Rad Laboratories, Richmond, CA). After staining with Coomassie blue, the Immobilon membranes were incubated for 2 h at 37°C in 2% powdered milk (wt/vol) in a TBS solution (50 mM Tris-HCl, pH 7.7, 150 mM NaCl, 0.2% NaN_3) to saturate the Immobilon membrane binding capacity. The membranes were washed in TBS (5 min), then exposed to specific rabbit polyclonal antiserum (1:400 dilution) in a 0.25% gelatin-TBS solution for 2 h at 23°C. Polyclonal rabbit antiserum was raised to placental lipocortin 1, endonexin I and endonexin II as described (20, 26). The membranes were sequentially washed with TBS, TBS containing 0.05% NP-40 (2×), and then TBS again. After washing, the membranes were exposed to ^{125}I-labeled protein A (37.8 μCi/μg) (ICN Radiochemicals, Irvine, CA) at 5×10^5 cpm/ml in 0.25% gelatin-TBS solution for 1 h at 23°C. The membranes were washed with TBS and TBS containing NP-40 as before, dried, and autoradiography was performed with Kodak XAR-5 film with intensifying screens at −70°C. The immunoreactive protein bands that were visualized by autoradiography were excised and the amount of associated radioactivity was determined with a gamma-counter.

By comparing radioactivity associated with an immunoreactive band in the experimental sample with standard curves constructed from Western blots of purified human placental annexin proteins (20), the nanogram amounts of specific annexin protein in the experimental samples were calculated. In each experiment, the immunoblots of the experimental cell extracts and the purified protein standards were coincubated in the same primary antibody and ^{125}I-labeled protein A solutions at the same time. The ^{125}I-labeled protein A signal was linear with respect to the annexin protein standards in the range tested (10–500 ng). There was no detectable protein/antibody cross-reactivity when antilipocortin 1, antiendonexin I, or antiendonexin II was used to probe 500 ng of a different purified annexin protein (lipocortin 1, calpactin 1, endonexin I, and endonexin II). Polyclonal antiserum raised to chicken calpactin I was a generous gift from Dr. Tony Hunter (6). This antiserum failed to recognize 500 ng of endonexin I or endonexin II, but it did cross-react with lipocortin 1 at ~15% the intensity with which it reacted with calpactin 1. Thus, the anticalpactin 1 antibody could not be used to obtain precise measurements of calpactin 1 expression in HFF cells because of cross-reactivity with lipocortin 1 that had a similar migration by SDS-PAGE. The anticalpactin 1 antibody was used to quantitate PC-12 calpactin 1 expression because these cells did not express detectable amounts of lipocortin 1.

Metabolic Labeling and Immunoprecipitation

The rates of synthesis of annexin proteins were determined by pulse-labeling and immunoprecipitation. HFF cells were subcultured (1:10) into

35-mm cell culture dishes at a density of 1 × 10^5 cells/dish in 3 ml of DME supplemented with 10% calf serum, penicillin (50 U/ml) and streptomycin (50 μg/ml). At the indicated time, cultures were washed two times with PBS and then pulse-labeled in 1 ml methionine-free DME (No. 320-1970 AJ; Gibco Laboratories) supplemented with 10% dialyzed calf serum and 250 μCi of [^{35}S]methionine (>1,000 Ci/mmol) (Amersham Corp., Arlington Heights, IL) for 3 h. The labeling media was removed, the cells were washed three times in PBS, and lysed in 125 μl immunoprecipitation extraction buffer (10 mM NaH_2PO_4 pH 7.2, 0.5% SDS [wt/vol], 2% β-mercaptoethanol [vol/vol], 2 mM EDTA, 2 mM PMSF, and 0.1 mM leupeptin). The lysate was boiled for 2 min, clarified by centrifugation (16,000 *g*, 10 min) and stored frozen at −70°C. Since the total amount of cell protein in each dish increased throughout the experiment, duplicate culture dishes were processed to determine total cell number and total cell protein for each experimental time point. Samples were thawed, diluted fourfold with PBS containing 1% Triton X-100 (vol/vol), and divided into two equal parts for immunoprecipitation with either rabbit polyclonal antilipocortin 1 or antiendonexin II. An aliquot of each sample was removed and precipitated with TCA to determine the amount of [^{35}S]methionine incorporated into total cell protein. Immunoprecipitations were then performed on 50 μg total protein was described (14). The antigen was eluted from the protein A-Sepharose (Sigma Chemical Co.) with Laemmli SDS sample buffer (27) and analyzed by SDS-PAGE. The gels were treated with EN^{3}HANCE (New England Nuclear, Boston, MA), dried, and exposed to Kodak XAR-5 film with intensifying screens at −70°C. The immunoprecipitated protein bands that were visualized by autoradiography were excised, the gel pieces were dissolved in 90% PROTOSOL (New England Nuclear), and the associated radioactivity was determined by scintillation counting.

The half-lives of annexin proteins were determined by pulse-chase labeling followed by immunoprecipitation. HFF cells were subcultured (1:10) at a density of 1 × 10^5 cells/dish as described above. At either day 1 or day 5 after the initial subculture, the growth media was removed and the 35-mm cell dishes were washed three times with PBS. The cells were pulse-labeled in 1 ml of methionine free DME (No. 320-1970 AJ; Gibco Laboratories) supplemented with 10% dialyzed calf serum and 250 μCi [^{35}S]methionine (>1,000 Ci/mmol) (Amersham Corp.) for 2.5 h. The labeling media was removed, the cultures were rinsed three times with PBS, and then incubated in the original growth media supplemented with an additional 1 mM unlabeled methionine. The cells were harvested at the indicated chase times and the radioactivity associated with immunoprecipitated lipocortin 1 and endonexin II was determined as described above.

Results

Regulated Expression of Lipocortin 1 and Endonexin II as a Function of Cell Growth

The following experiments were performed to determine whether the expression of annexins varied as a function of cell growth in cultured diploid HFF. HFF cells from a confluent stock T75 flask were subcultured into 80 35-mm dishes (1 × 10^5 cells/dish), an ∼10-fold increase in available growth surface area. The cells were allowed to grow without any media changes. Cellular extracts taken at several time points were analyzed for lipocortin 1, endonexin II, and for total cellular protein content (Figs. 1 and 2). The initially sparse cultures grew rapidly for the first 4 d as indicated by an increase in both cell density and total cellular protein content per dish (Fig. 2). As cells became quiescent at days 6–8 (∼6 × 10^5 cells/dish), the growth rate decreased and the total protein content reached a plateau value (Fig. 2).

Immunoblot analysis of cellular extracts showed the same qualitative pattern at all time points: a single protein band that reacted with polyclonal antiserum specific to the annexins (Fig. 1). But quantitative immunoblot analysis of the cellular extracts showed that there were significant changes in the expression of certain annexins as a function of time after subculture (Fig. 2). As seen visually in the immunoblot autoradiograms in Fig. 1, and quantitatively in Fig. 2, lipocortin 1 expression increased from 0.5% of total cellular protein at the time of plating to a peak of 2.0% at day 3 and then declined with time back to the initial level. In striking contrast, endonexin II expression was highest (2.0% of total cellular protein) initially, decreased to ∼0.5% at day 3 and then rose with time to the initial level (Figs. 1 and 2).

Since several different annexins have been identified in vertebrates, the growth-dependent expression of other members of this gene family also were measured in HFF in experiments like those described in Figs. 1 and 2. Immunoreactive endonexin I expression remained relatively constant

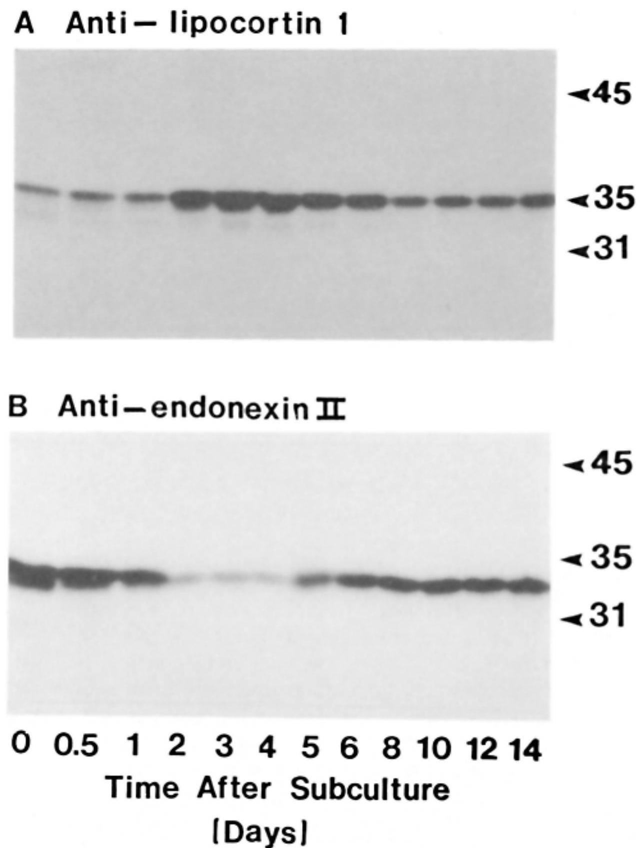

Figure 1. Immunoblot analysis of lipocortin 1 and endonexin II expression in diploid human fibroblasts subcultured at a 1:10 ratio. Stock confluent HFF cells were subcultured (1 × 10^5 cells/35-mm dish) in 3 ml of DME containing 10% calf serum and allowed to proliferate without a media change throughout the experiment. Cell lysates were prepared at each time point indicated and 20 μg total protein from each extract was resolved by SDS-PAGE and transferred to Immobilon membranes (see Materials and Methods). The Immobilon membranes were incubated with antiserum specific for either lipocortin 1 (*A*) or endonexin II (*B*). Immunoreactive protein was visualized by incubating with ^{125}I-labeled protein A followed by autoradiography. The day 0 gel lane represents a 20-μg protein extract sample from the confluent stock fibroblasts used to generate the 1:10 subculture experimental 35-mm dishes. The day 0.5 gel lane represents a 20-μg protein extract sample from the experimental 35-mm dishes 12 h after subculture. Autoradiograph exposure time (at −70°C with an intensifying screen) was 3 h. Coomassie blue staining of the Immobilon membranes before immunoblotting showed that the gross visual pattern of protein bands did not change significantly as a function of time after plating.

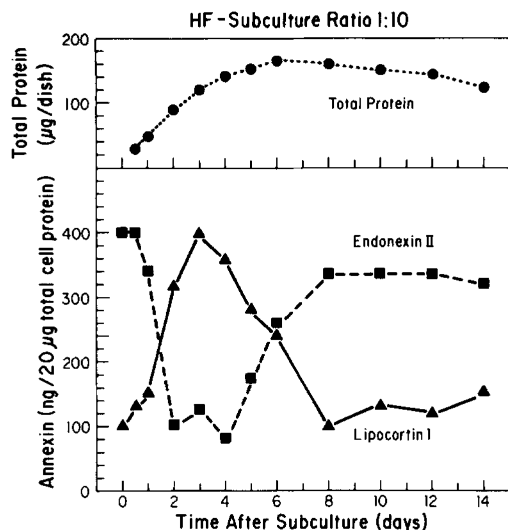

Figure 2. Quantitation of lipocortin 1 and endonexin II expression in diploid human fibroblasts subcultured at a 1:10 ratio. The immunoreactive protein bands from identical experiments as described in Fig. 1 were excised from the Immobilon membranes and the radioactivity was determined with a gamma-counter. The measured radioactivity was compared to standard curves constructed using purified lipocortin 1 and endonexin II to calculate the amount of immunoreactive lipocortin 1 (*triangles*) and endonexin II (*squares*) in the cell extracts for each experimental time point (see Materials and Methods). The total protein (*circles*) in the 35-mm subculture dishes at each time point was determined as described in Materials and Methods. On average, there were 3×10^3 cells/microgram of total cell protein. These results are an average of three separate experiments.

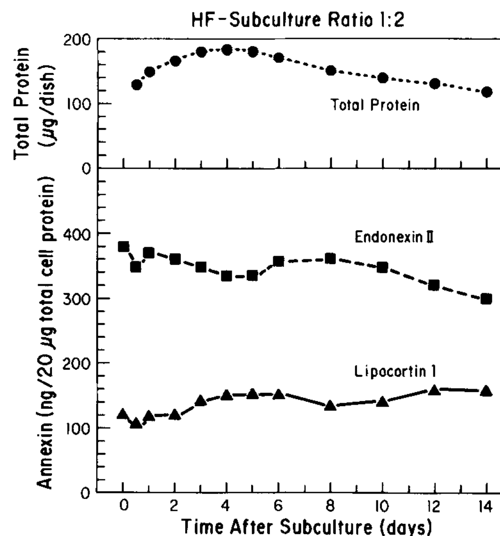

Figure 3. Quantitation of lipocortin 1 and endonexin II expression in diploid human fibroblasts subcultured at a 1:2 ratio. Stock confluent HFF cells were subcultured (5×10^5 cells/35-mm dish) in 3 ml of DME containing 10% calf serum and allowed to proliferate without a media change throughout the experiment (14 d). Immunoblot analysis was performed with 20 μg total protein from each extract, exactly as described in the legend of Fig. 1, and subjected to quantitative analysis as described in the legend of Fig. 2. *Triangles*, lipocortin 1; *squares*, endonexin II; and *circles*, the total protein/35-mm dish. These results are an average of two separate experiments.

throughout the experimental time period within a range of 0.08–0.13% of the total cell protein (data not shown). Using similar methods, attempts to quantitatively measure calpactin 1 expression were only partially successful due to cross-reactivity between anticalpactin 1 antiserum and lipocortin 1 (see Materials and Methods). However, by calculating the contribution of lipocortin 1 cross-reactivity to the total immunoreactive signal, it was determined that calpactin 1 was expressed at relatively high levels (~1% of total cellular protein), and the expression did not significantly change (less than twofold) as a function of growth state (data not shown). Thus, of the annexins investigated, only lipocortin 1 and endonexin II underwent large changes in expression as a function of growth state. Although the expression of lipocortin 1 and endonexin II changed approximately fourfold, the inverse complementary nature of the changes resulted in <20% change in the sum of the two at any given time point. It may be significant that the increase in lipocortin 1 and decrease in endonexin II coincided with the period of rapid cell growth and the return to their initial states coincided with the time at which the cells became quiescent and underwent density inhibition to cell growth.

To determine whether the lipocortin 1 and endonexin II expression changes were correlated with the cellular growth state or were the result of the manipulations involved in the subculture procedure such as the trypsin treatment used to remove the cells from the stock culture flasks, the following experiment was performed. HFF cells were subcultured as described in the legend of Fig. 1, except experimental cultures were plated at a high density (1:2 subculture ratio, 5×10^5 cells/dish) so that only minimal cellular growth was possible (Fig. 3). Under these conditions, there were only minor changes in the expression of lipocortin 1 and endonexin II (Fig. 3). Thus, subculture alone did not result in changes in expression of these two proteins. Subculture at a 1:4 ratio (2.5×10^5 cells/dish) resulted in approximately a fourfold increase in lipocortin 1 expression 2 d after plating. This increase in lipocortin 1 expression was followed by a return to the initial values that occurred more rapidly compared to cultures subcultured at a 1:10 ratio (data not shown).

Cellular Expression of Lipocortin 1 and Endonexin II in Quiescent HFF Cells after Media Replacement

To investigate the hypothesis that lipocortin 1 and endonexin II expression correlated with the cellular growth state, the growth rate was manipulated by serum and medium starvation and subsequent refeeding. Stock confluent HFF cells were subcultured at a 1:10 ratio (1×10^5 cells/dish) and allowed to grow without a media change for 16 d. The total cell protein content reached a plateau value (~180 μg/dish), and the total cell number remained constant (5×10^5 cells/dish) from day 6 to day 16, indicative of a density inhibited, quiescent cell state. On experimental day 16, the cell media was replaced completely with fresh DME supplemented with 10% calf serum (Fig. 4). This caused an approximate doubling of cell density (1.1×10^6 cells/dish by day 19; Fig. 4) and total cell protein within 3 d (Fig. 4; day 16–19). No further growth was stimulated by an additional

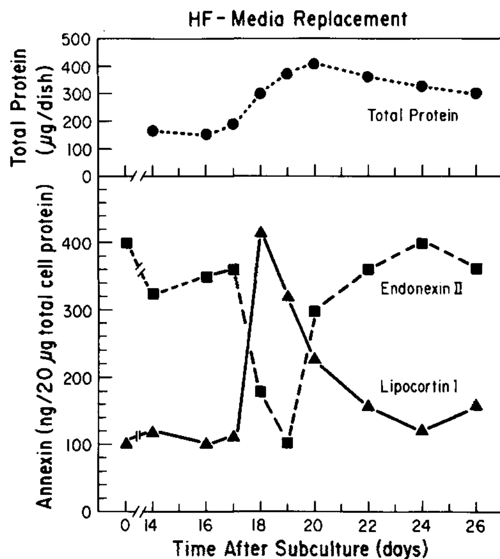

Figure 4. Lipocortin 1 and endonexin II expression in quiescent diploid human fibroblasts after mitogenic stimulation with fresh media. Stock confluent HFF cells were subcultured at a 1:10 ratio (1×10^5 cells/35-mm dish) in 3 ml of DME containing 10% calf serum and allowed to proliferate without a media change until subculture day 16 at which time the cells were quiescent. The media was completely replaced with fresh DME containing 10% calf serum on subculture days 16 and 21. Immunoblot analysis was performed with 20 μg total protein from each extract, exactly as described in the legend of Fig. 1, and subjected to quantitative analysis as described in the legend of Fig. 2. The day 0 data point represents a 20-μg protein extract sample from the confluent stock fibroblasts that were used to generate the original 1:10 subculture. The day 16 extract was obtained immediately before the culture media was replaced. *Triangles*, lipocortin 1; *squares*, endonexin II; and *circles*, the total protein/35-mm dish. These results are an average of two separate experiments.

media change on day 21 (Fig. 4). Immunoblot analysis of extracts showed that on day 18, 2 d after the media was replaced, there was a fourfold increase in lipocortin 1 protein expression followed by a return to the initial low value over the next 3 d (Fig. 4). In an opposite fashion, there was a 3.5-fold decrease in endonexin II expression during the second and third days after the media was replaced (Fig. 4). The media change on day 21 did not stimulate growth and did not significantly affect lipocortin 1 or endonexin II expression (Fig. 4).

Regulated Expression of Lipocortin 1 and Endonexin II in Confluent HFF Cells after Epidermal Growth Factor (EGF) Addition

The following experiment was performed to determine whether lipocortin 1 and endonexin II expression in confluent HFF cells would be affected by EGF-stimulated cell growth and division. Stock confluent HFF cells were subcultured at a 1:10 ratio and allowed to proliferate (9×10^5 cells/dish by day 16) with regular media changes on days 5, 10, 15, and 20. The addition of EGF (50 ng/ml) to the culture media of the confluent cells on day 16 stimulated a further round of cell division. There was an approximately twofold

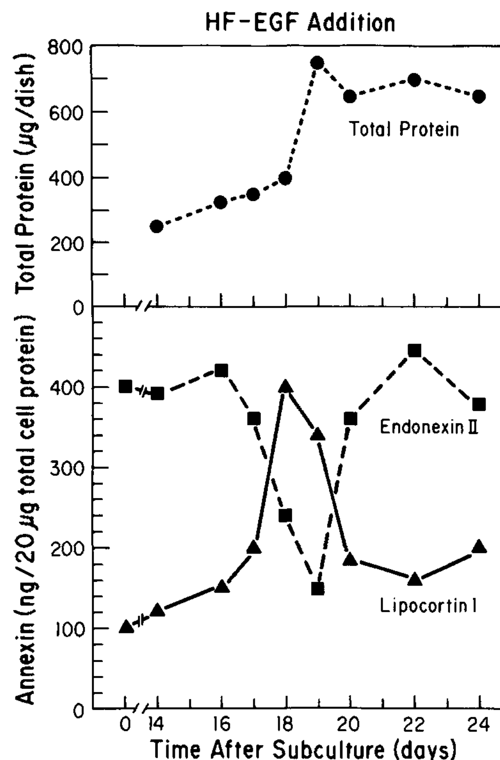

Figure 5. Lipocortin 1 and endonexin II expression after EGF addition to confluent diploid human fibroblasts on subculture day 16. Stock confluent HFF cells were subcultured at a 1:10 ratio (1×10^5 cells/35-mm dish) and allowed to proliferate with media changes on subculture days 5, 10, 15, and 20. EGF (50 ng/ml) was added to the culture media on subculture day 16. Immunoblot analysis was performed with 20 μg total protein from each extract, exactly as described in the legend of Fig. 1, and subjected to quantitative analysis as described in the legend of Fig. 2. The day 0 data point represents a 20-μg protein extract sample from the confluent stock fibroblasts that were used to generate the original 1:10 subculture. The day 16 data point represents an extract obtained immediately before EGF addition. *Triangles*, lipocortin 1; *squares*, endonexin II; and *circles*, the total protein/35-mm dish. These results are an average of two separate experiments.

increase in the monolayer cell density (2.2×10^6 cells/dish by day 19; Fig. 5) and in total protein (days 16–19, Fig. 5) within 3 d after EGF addition. During the period of EGF-stimulated cell growth, there was an approximately threefold increase in lipocortin 1 expression and an approximately threefold decrease in endonexin II expression (Fig. 5). After the period of EGF-stimulated cell growth, the lipocortin 1 and endonexin II expression returned to the levels detected before stimulation.

A control set of experimental cells were subjected to the same protocol of media replacement described in Fig. 5, but the cells did not receive EGF addition on day 16. Within the experimental window of days 16–24, there were no significant changes in lipocortin 1 and endonexin II expression in this control set of healthy and confluent HFF cells (data not shown). Thus, the cellular changes in lipocortin 1 and endonexin II expression observed within the confluent HFF cells appears to be correlated with some aspect of fibroblast growth or division that was stimulated by the EGF addition.

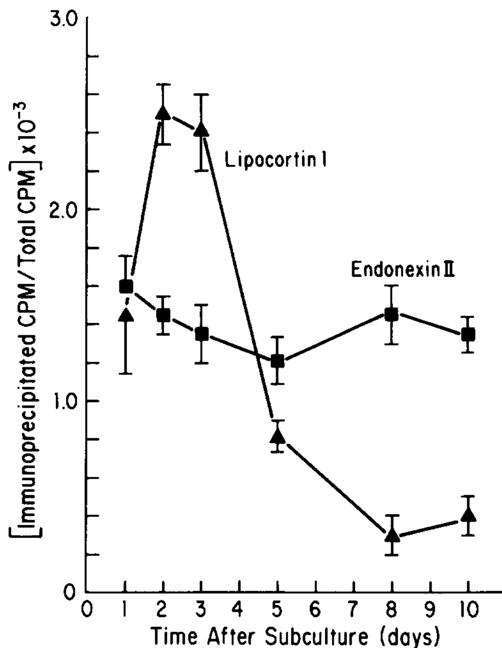

Figure 6. Lipocortin 1 and endonexin II rate of synthesis in diploid human fibroblasts subcultured at a 1:10 ratio. Stock confluent HFF cells were subcultured (1 × 10^5 cells/35-mm dish) as described in the legend of Fig. 1. At the indicated time after the initial subculture, the media was removed, and the HFF cells were pulse-labeled in 1 ml with 250 μCi [^{35}S]methionine for 3 h as described in Materials and Methods. Cell lysates were prepared, the total acid precipitable [^{35}S] in aliquots of each extract was determined, and immunoprecipitations were performed on 50 μg total protein from each extract with antiserum to either lipocortin 1 or endonexin II. The immunoprecipitated proteins were resolved by SDS-PAGE, visualized by autoradiography, and quantitated by scintillation counting as described in Materials and Methods. The experiment was repeated two times and the results were averaged (± standard deviation). *Triangles*, lipocortin 1, and *squares*, endonexin II.

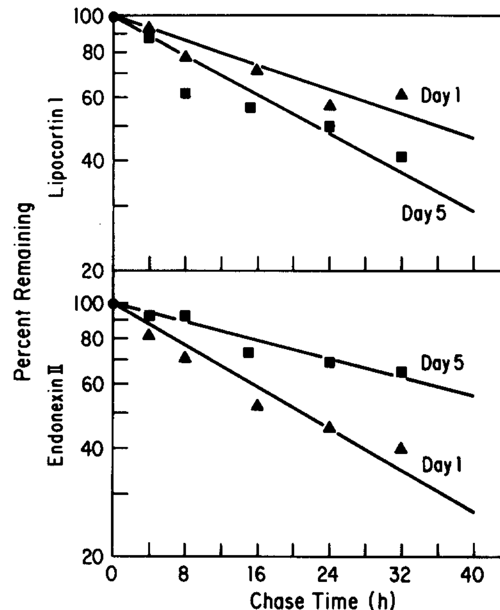

Figure 7. Pulse-chase analysis of lipocortin 1 and endonexin II in diploid human fibroblasts subcultured at a 1:10 ratio. Stock confluent HFF cells were subcultured (1 × 10^5 cells/35-mm dish) as described in the legend of Fig. 1. The HFF cells were allowed to proliferate without a media change for either 1 or 5 d. At days 1 or 5, the HFF cells were pulse-labeled with 250 μCi/ml [^{35}S]methionine for 2.5 h, and then rinsed with PBS and incubated in the original growth media containing an excess of unlabeled methionine for the indicated times. The amount of radioactivity associated with either lipocortin 1 or endonexin II was determined by immunoprecipitation, followed by autoradiography and scintillation counting as described in Materials and Methods. Lines were drawn through the data points through the use of exponential regression analysis that was constrained to 100% at $t = 0$. Labeling that initiated on day 1 (*triangles*): lipocortin 1 ($R^2 = 0.95$, $t_{1/2} = 35$ h) and endonexin II ($R^2 = 0.98$, $t_{1/2} = 21$ h). Labeling that initiated on day 5 (*squares*): lipocortin 1 ($R^2 = 0.95$, $t_{1/2} = 23$ h) and endonexin II ($R^2 = 0.96$, $t_{1/2} = 46$ h). The results are an average of two separate experiments.

Synthesis and Turnover of Lipocortin 1 and Endonexin II as a Function of Cell Growth in HFF Cells

The rates of synthesis and turnover of lipocortin 1 and endonexin II were measured to gain insights into the level at which the growth-dependent regulation of annexin expression occurs. Stock confluent HFF cells were subcultured at a 1:10 ratio (1 × 10^5 cells/dish) as described in the legend of Fig. 1. At the indicated time points, the HFF cells were pulsed-labeled with [^{35}S]methionine and the annexins were immunoprecipitated from the cell lysates (see Materials and Methods). Lipocortin 1 synthesis was maximal during the period in which the HFF cells were highly proliferative (Fig. 6, days 2 and 3) and decreased eightfold as the cells became quiescent (Fig. 6; days 8 and 10). In contrast to these results, the rate of endonexin II synthesis remained relatively constant throughout the experiment at a value intermediate to that of the lipocortin 1 extremes (Fig. 6). Since the rate of endonexin II synthesis was relatively constant while its cellular expression levels were changing (Fig. 2), we considered the possibility that the expression changes were due to the regulation of the endonexin II degradation rate.

The turnover rates of endonexin II and lipocortin 1 were measured on either day 1 or 5 after the initial subculture: the days in which the rate of change in annexin expression was maximal (Fig. 2). HFF cell cultures were pulse-labeled with [^{35}S]methionine for 2.5 h, chased with the original growth media supplemented with 1 mM unlabeled methionine, and, at the indicated times, the annexins were immunoprecipitated from the cell lysates (see Materials and Methods). The time course of endonexin II radioactive decay could be approximated by a single exponential curve. From these data, it was estimated that the $t_{1/2}$ of endonexin II turnover increased from 21 h in proliferating cells labeled on day 1 to 46 h in quiescent cells labeled on day 5 (Fig. 7). However, it should be noted that other physiological changes may have occurred during the relatively long chase period. In fact, the decay curve for endonexin II in cultures labeled on day 1 is not exactly linear in the semilog plot (Fig. 7) and may indicate that degradation occurred at a more rapid rate at the earlier time points of the chase.

The turnover rate of lipocortin 1 also changed as a function of HFF cell growth. However, the differences in lipocortin

1 turnover varied less, and in an opposite manner compared to the differences in endonexin II turnover. The $t_{1/2}$ of lipocortin 1 turnover decreased from 35 h in proliferating cells labeled on day 1 to 23 h in quiescent cells labeled on day 5 (Fig. 7).

Thus, the observed growth-dependent lipocortin 1 expression change (Fig. 2) is due to changes in both its rate of synthesis (Fig. 6) and degradation (Fig. 7) while alterations in endonexin II expression appear to be achieved primarily by changes in its rate of degradation (Fig. 7). The measured turnover rates of endonexin II do not quite quantitatively account for the observed changes in its expression (Fig. 2). However, the half-life during the period of maximal endonexin II decrease may be shorter than the one measured in the experimental window.

Expression of Annexins in Rat Adrenal PC-12 Cells as a Function of NGF Addition and Cellular Differentiation

Since the expression of certain annexins in HFF cells changes as a function of cell growth and division, experiments were designed to study the relative expression of annexins in a rapidly proliferating cell line that was stimulated to differentiate into nondividing cells. The cells chosen were PC-12 cells (18), a cell line that ceases mitotic division and initiate neurite outgrowth in response to NGF. PC-12 cells were subcultured (2×10^5 cells/dish) into collagen-coated 35-mm experimental cell dishes in minimal media with or without the addition of NGF (50 ng/ml) on a daily basis. Control cells were allowed to proliferate without NGF addition for 10 d. At this point, high density clusters of rounded cells were observed (1×10^6 cells/dish). In the NGF-treated experimental group, growth was slower and the cells had begun to produce a neurite outgrowth by day 3 and a more defined network of neurites by day 5 (data not shown). However, all cells did not differentiate in response to NGF and some clusters of cells continued to proliferate (by visual inspection, ~50% of the cells had assumed a stellate appearance by day 5). The effects of NGF were in large part reversible because the majority of the neurites retracted within 2 d of NGF removal from the cultures.

Neither control nor NGF-treated cells contained detectable amounts of lipocortin 1 or endonexin I by immunoblot analysis. The control PC-12 cells expressed a constant low level endonexin II (at 0.04% of total cell protein) and calpactin 1 (at 0.02% of total cell protein). These levels did not vary significantly as a function of time after plating (data not shown). However, NGF addition to the PC-12 cell cultures caused a fivefold increase in endonexin II expression (to 0.2% of total protein) and a 32-fold increase in the expression of calpactin 1 (to 0.65% of total protein) by day 5 of NGF treatment (Fig. 8). The level of endonexin II expression returned to the initial control value over a period of 3 d after NGF removal and a shift to complete growth medium (Fig. 8). The level of calpactin 1 expression was slower to return to the control baseline value after NGF removal and a shift to a growth promoting culture media (Fig. 8). The observed changes in calpactin 1 and endonexin II expression in response to NGF might be even greater in cultures in which a higher percentage of the cells differentiate in response to the growth factor.

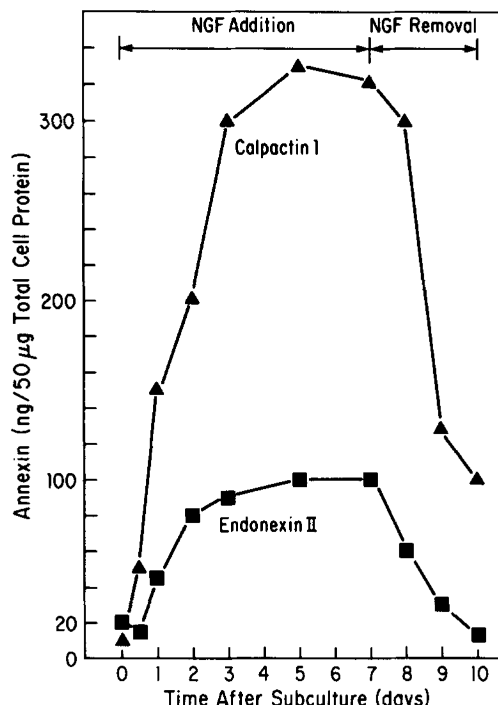

Figure 8. Endonexin II and calpactin 1 expression in PC-12 cells as a function of NGF-stimulated differentiation. Stock PC-12 cells were subcultured into collagen-coated 35-mm dishes (2×10^5 cells/dish) in a minimal growth media (RPMI 1640 containing 1% heat-inactivated horse serum) in the presence of NGF (50 ng/ml). The cultures were treated with NGF for 7 d then shifted to NGF-free growth media (RPMI 1640 supplemented with 10% heat-inactivated horse serum and 5% FBS) for either 1, 2, or 3 d. Immunoblot analysis was performed with 50 μg total protein from each extract, exactly as described in the legend of Fig. 1. The immunoblot was subjected to quantitative analysis as described in the legend of Fig. 2. Endonexin II expression is indicated by squares and calpactin 1 expression is indicated by triangles. the day 0 point represents a sample from the stock PC-12 cells that were used to set up the experimental subculture and did not receive NGF treatment. These results are an average of two separate experiments. Control PC-12 cultures that were maintained throughout the 10-d experimental period in minimal growth media without NGF showed constant endonexin II and calpactin 1 expression at ~0.04% and 0.02% of total protein, respectively (data not shown).

Discussion

The Ca^{2+}-binding proteins in the annexin family are attracting intensive investigation because of their potential involvement in Ca^{2+}-mediated stimulus-response coupling. One of the annexins, lipocortin 1, has properties that make it a candidate for involvement in three different intracellular signal transduction pathways. (*a*) It is phosphorylated by the EGF receptor/kinase (10). (*b*) It is phosphorylated in vitro by protein kinase C (41); and (*c*) although there is no direct evidence in intact cells, it potentially can change its cellular location in response to Ca^{2+} fluxes by virtue of its ability to undergo reversible Ca^{2+}-dependent binding to plasma membranes (40). Despite their potential importance, the biological function is not clearly defined for any of the eight or more proteins in the annexin family. In the absence of direct assays, functional insights can be sought by determining the

cell types that express these proteins and parameters that affect their expression. Previous studies have shown that different annexins have unique tissue and cell distributions (7, 16, 35). But beyond the basic observation that differential expression indicates specialized function, these surveys of protein and gene expression in different cell types have not provided significant insights into the functional role played by these proteins.

We have studied lipocortin 1 in cultured cells to determine if its expression changed as a function of growth status. We also concentrated our study on endonexin II because it has Ca^{2+} and phospholipid binding properties similar to lipocortin 1, but it is not phosphorylated by the EGF receptor/kinase or other known protein kinases (41, 42). We focused our study on normal diploid HFF because they express relatively high levels of both lipocortin 1 and endonexin II, because they have a well-defined growth response to EGF (4), and because lipocortin 1 phosphorylation in intact HFF cells has been characterized (14). We found that the level of lipocortin 1 expression increased three to fourfold when quiescent HFF cells were stimulated to proliferate. The increase in expression was seen when cell division was induced by either providing quiescent cells more surface area for growth (Fig. 2), providing fresh medium and serum to nutrient and serum-starved cultures (Fig. 4), or EGF-stimulation of cultures grown to confluence in fresh medium and serum (Fig. 5). The increased expression of lipocortin 1 may be associated with the fact that maximal phosphorylation of lipocortin 1 also occurs in proliferating HFF cells (D. D. Schlaepfer and H. T. Haigler, unpublished results), but the functional correlation of this association remains to be determined. Additionally, we conclude that the regulation of lipocortin 1 expression was due to changes in both its rate of synthesis (Fig. 6) and its rate of degradation (Fig. 7) in HFF cells.

Under the above conditions, there was a concomitant three- to fourfold decrease in endonexin II expression (Fig. 2, 4, and 5) in proliferating HFF cells or in quiescent HFF cells that were stimulated to undergo cell division. Since the rate of endonexin II synthesis was found to remain constant (Fig. 6), we conclude that the regulation of its expression was primarily due to changes in its rate of degradation (Fig. 7). For comparison, the expression of endonexin I and calpactin 1 were measured and found to remain relatively unchanged in growing and quiescent HFF cells. When the fibroblasts were experimentally induced to proliferate, the magnitude of the lipocortin 1 increase and the endonexin II decrease in expression was similar. This results in the sum expression of these four annexins remaining approximately constant at 3.5% of the total cell protein throughout all stages of HFF cell growth.

It is important to determine whether the reciprocal expression of lipocortin 1 and endonexin II in fibroblasts with respect to growth indicates a causal relationship. If lipocortin 1 and endonexin II are indeed involved in cell replication, it will be interesting to determine whether these proteins interact with the same cellular machinery. It may be possible that lipocortin 1 plays a role in a stimulatory growth-regulatory pathway and endonexin II is part of an inhibitory growth-regulatory pathway. Examination of the growth response time course and annexin expression does not provide a clear answer to this question because the changes in expression occur with a time course that slightly precedes or is simultaneously with cell replication. The increase in lipocortin 1 expression and the decrease in endonexin II expression began ~1 d after the mitogenic stimulus and their levels reached a maximum or minimum, respectively, 2 to 3 d after stimulation (Figs. 2, 4, and 5). When quiescent fibroblasts were stimulated to undergo a single round of replication with fresh medium and serum (Fig. 4) or with EGF (Fig. 5), the expression of lipocortin 1 and endonexin II returned from the altered levels to the original basal levels over a period of ~2 d. This relatively slow change in expression levels may be correlated with the fact that these proteins have measured half-lives on the order of over 20 h (Fig. 7).

Contrary to the growth-regulated expression results in the normal diploid HFF cells, experiments conducted with the A431 transformed cell line showed that not all cultured cells regulate the expression of lipocortin 1 and endonexin II in a growth or density-dependent manner. The A431 cells, which lack normal density-dependent growth regulation, expressed constant levels of lipocortin 1 and endonexin II. These levels were similar to those found in rapidly growing fibroblasts; i.e., relatively high levels of lipocortin 1 and low levels of endonexin II (data not shown). Although these observations are consistent with the proposal that there is a positive correlation between rapid cell proliferation and lipocortin 1 expression and a negative correlation between rapid cell replication and endonexin II expression, a number of other properties of the epithelial A431 cell line could have contributed to these results.

Expression of annexins also was investigated in cultured pheochromocytoma PC-12 cells. These transformed cells can be induced to reversibly differentiate into nondividing neuronlike cells with NGF (18). Our results confirm and extend previous qualitative studies which showed that these cells do not contain lipocortin 1 (34) and have increased expression of calpactin 1 after NGF treatment (16). In the control PC-12 cells, endonexin II, and calpactin 1 were expressed at a constant low level. However, when the PC-12 cells were induced to reversibly differentiate into neuronlike cells, the cellular expression of endonexin II increased fivefold and the expression of calpactin 1 increased 32-fold (Fig. 8). The elevated level of endonexin II returned to the initial baseline value over a period of 3 d after NGF removal and a shift a growth promoting media. These results support the proposal that the increased endonexin II expression in quiescent HFF cells and in differentiated PC-12 cells may be correlated with a prominent yet undefined functional role in nondividing cells.

A primary reason for investigating the potential role that annexins play in cell growth is the observation that lipocortin 1 and calpactin 1 are cellular substrates for protein tyrosine kinases. Also, a previous study showed a correlation between increased cell replication and increased expression of calpactin 1 (also called p36). The study showed that plating primary chicken myoblasts from dissociated chick limb buds onto culture dishes resulted in the increased expression of calpactin 1 (5). These observations of increased calpactin 1 expression in cultured myoblasts may be analogous to the observed results of increased lipocortin 1 expression in HFF cells that were stimulated to grow by low density (1:10) subculture (Fig. 2). However, we did not observe an increased expression of calpactin 1 in growing HFF cells. Other studies also fail to demonstrate a positive correlation be-

tween the expression of these two annexin tyrosine kinase substrates and rapid cell proliferation. For instance, immunohistochemical studies of the developing central nervous system of the rat embryo showed that lipocortin 1 expression was localized specifically to a small group of cells in the neural tube (30). Lipocortin 1 first appeared to these cells at about the time when they stopped dividing and underwent terminal differentiation. Similarly, the most dramatic increase in calpactin 1 expression was observed in NGF-treated PC-12 cells that underwent differentiation into nondividing cells (Fig. 8). It is possible that in these nondividing cells, these annexin proteins may play a increased role in some differentiated cell function such as the crosslinking of secretory vesicles (31). Overall, the results of these studies of annexins in different cell types show that their expression patterns are regulated in an independent and complex manner.

In summary, the demonstration that annexin expression is growth-regulated in certain cell types adds more indirect evidence to the proposal that these proteins may be involved in some aspect of cell replication or differentiation. Direct evidence is needed to evaluate this proposal. Since all vertebrate cells investigated to date express multiple forms of annexins, each potentially having a different cellular role, an effective investigation of annexin function may be facilitated by studying a lower organism that expresses fewer annexin gene products.

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