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Cell cycle and post-transcriptional regulation of annexin expression in IMR-90 human fibroblasts

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Based on the finding that the expression of some annexins varies dramatically as a function of cellular proliferation state [Schlaepfer and Haigler (1990) J. Cell Biol. **111**, 229–238], it has been proposed that the cellular level of the annexins might be critical for the regulation of cell growth. To further test this hypothesis, we have studied the expression of various annexins in normal human IMR-90 fibroblasts synchronized by serum deprivation. Using immunoblotting, the cellular content of annexins (Anxs) II, V and VI was found to vary by less than 10% during the cell cycle. However, Anx IV expression increased by 50% during S-phase and the levels of Anxs I and VII were reduced by 40% in early G2/M. However, using RNase protection assays, the mRNAs of Anxs I and VII were found to be uniformly expressed throughout the cell cycle, suggesting that down-regulation of both proteins in G2/M occurred through a post-transcriptional process. In addition, cells transfected with Anx VII cDNA were shown to contain an amount of Anx VII similar to wild-type cells, despite the elevation of Anx VII mRNA content in transfected cells by approx. 2 orders of magnitude. Vector misconstruction or possible secretion of the overexpressed protein were ruled out using appropriate controls. Therefore, as with cell-cycle regulation, Anx VII expression in transfected cells is also controlled by post-transcriptional mechanisms. Furthermore, using pulse–chase analysis, we have determined that annexin VII, and other Anxs, have a slow turnover rate, consistent with the limited changes of expression throughout the cell cycle. Taken together, these results question the hypothesis that cellular expression of Anxs plays a general role in cell growth and support the concept that post-transcriptional mechanisms may control levels of Anxs I and VII.

INTRODUCTION

The annexins (Anxs) constitute a family of Ca^{2+}-dependent phospholipid-binding proteins including 13 members with a similar structure, characterized by the presence of four or eight repeats of a 70-amino acid segment and a variable N-terminal extremity (see [1–3] for reviews). The main property they share is their ability to bind to negatively charged phospholipids in the presence of Ca^{2+}, which appears to be responsible for their anti-phospholipase, anti-coagulant, anti-inflammatory, and anti-protein kinase C activities, as well as their capacity to drive Ca^{2+}-dependent aggregation of secretory granules. In addition, various Anxs possess a transmembrane Ca^{2+} channel activity. The Anxs have also been implicated in the regulation of endosomal vesicle trafficking and in cell–matrix interactions. Nevertheless, these many properties have complicated any interpretation as to the specific physiological role of these proteins.

An additional interesting feature of the annexins is their potential role in the control of cell proliferation. This hypothesis emerged initially with the description of Anxs I and II as substrates of the protein tyrosine kinase activities *in vivo*, associated either with the epidermal growth factor receptor or that encoded by the Rous sarcoma virus respectively [4,5]. Anx II has been shown to activate DNA polymerase α *in vitro* [6], but whether this process is involved in the control of cell proliferation remains to be established. Nevertheless, major evidence for a role of the annexins in the regulation of cell growth *in vivo* was produced by Schlaepfer and Haigler who reported that the expression of Anxs I and V was dramatically altered in human normal fibroblasts depending on the status of cell proliferation [7]. Proliferating cells were shown to contain three- to four-fold higher levels of Anx I and three- to four-fold lower levels of Anx V than quiescent cells. Other reports have also shown differential expression of annexins during cell differentiation [7–9] and embryo development [10–12]. However, since cell proliferation is governed at the cell cycle level, a crucial question remained unanswered of whether Anx expression was actually regulated during the cell cycle. In this paper, we have synchronized normal cultured human fibroblasts and analysed the expression of various Anxs during the different phases of the cell cycle.

MATERIALS AND METHODS

Synchronization and lysis of the cells

IMR-90 (CCL-186, ATCC, Rockville, MD, U.S.A.), NIH 3T3 and HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and an antibiotic cocktail (Biofluids, Rockville, MD, U.S.A.). IMR-90 cells used in cell-cycle studies were subcultured 1:3 and used between passage 10s and 15. For synchronization, newly confluent cells were rinsed once and incubated in serum-free medium. After 72 h, the cell cycle was reactivated by passaging the cells 1:3 in medium with 10% serum. Progression through the cell cycle was monitored using [^{3}H]thymidine incorporation (5 μCi/ml, 4 h) and anti-cdc2 immunoblotting. For immunoblotting analysis, cells were scraped from the flask in the following buffer: 20 mM Tris (pH 7.5)/0.5% SDS/0.5% Triton X-100/1 mM EGTA/1 mM EDTA/5 μg/ml leupeptin/

Abbreviations used: Anx, annexin; DMEM, Dulbecco's modified Eagle's medium; RT-PCR, reverse-transcriptase PCR.

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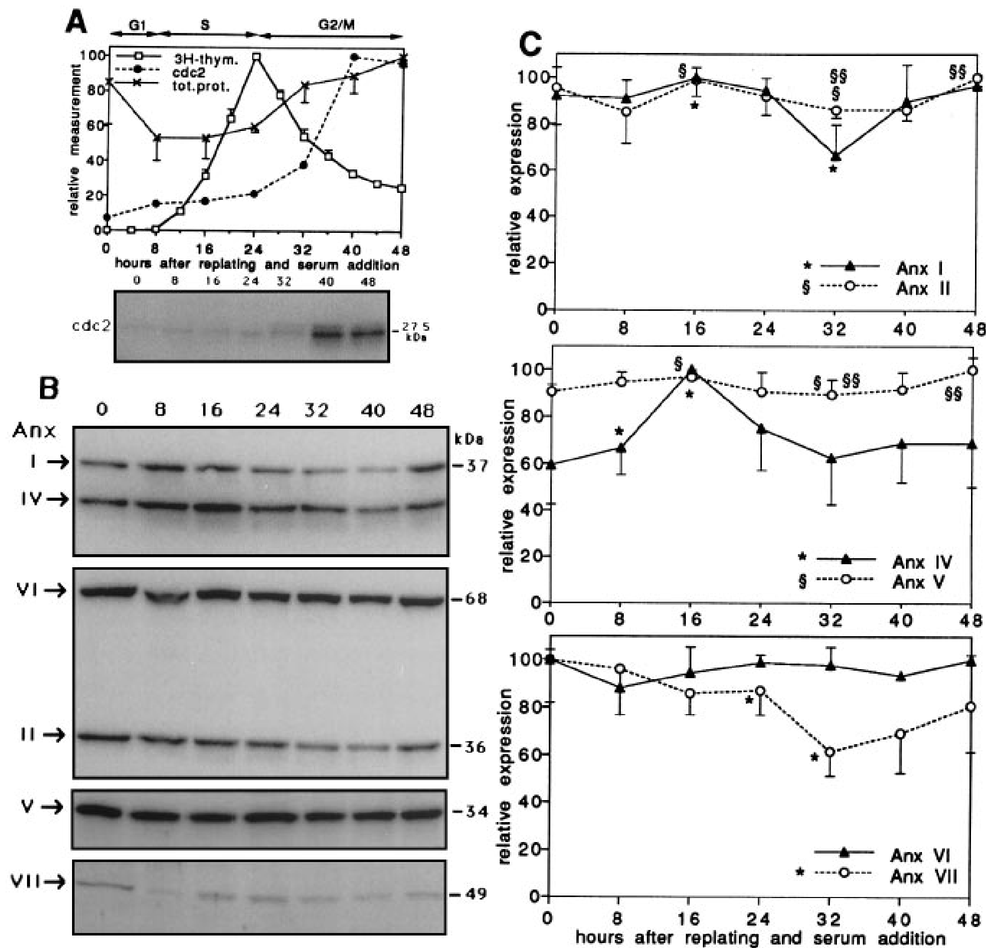

Figure 1 Expression of the Anxs throughout the cell cycle

Confluent human fibroblasts were incubated for 72 h in serum-free medium. At $t = 0$, the cells were replated 1:3 in 10% serum medium. Cells were harvested at the indicated time after cell cycle reactivation. (**A**) Synchronization of the cells. DNA synthesis was measured by [^{3}H]thymidine incorporation (□). Expression of cdc2 was analysed using immunoblotting of crude cell lysate (see gel, 50 μg of total cell protein per lane). Measurements were obtained by phosphorimaging (●). The amount of total cell proteins at the indicated time is also plotted (×). (**B**) Immunoblot analysis of the Anxs in crude lysates from one representative experiment. A panel of specific anti-annexin antibodies was used as primary antibodies. Each blot was probed with two or three different antibodies that recognized proteins of distinct M_r. ^{125}I-labelled anti-IgG antibodies and phosphorimaging were used as the detection method. The apparent M_r of the immunodetected proteins was determined with M_r standards. Total proteins per lane 20 μg, except Anx VII, 60 μg per lane. (**C**) Quantitative analysis of Anx immunoblots by image density integration of phosphorimager signals. Values are means ± S.E.M. of four independent experiments similar to (**B**). The symbols * and § indicate, for one given Anx, two sets of data significantly different from each other (paired t test, $P < 0.05$).

5 μg/ml aprotinin/1 mM PMSF. Homogenization was completed with a 10 s sonication. Protein assays were performed using the μBCA kit (Pierce, Rockford, IL, U.S.A.) with bovine serum albumin as a standard. The samples were then lyophilized and resuspended in Laemmli's sample buffer [13]. To perform mRNA analysis, total RNAs were prepared using the RNAzol procedure (Cinna/Biotecx, Friendswood, TX, U.S.A.).

Immunoblotting analysis

Total cell proteins or extracts of metabolically labelled Anxs were subjected to SDS/PAGE (Novex, San Diego, CA, U.S.A.). The gel content was electroblotted onto nitrocellulose using the Mini Trans-Blot device (BioRad, Hercules, CA, U.S.A.). Immunodetection was performed with a panel of antibodies which specifically detect various Anxs. Anxs I, II, IV and VI were detected using a monoclonal antibody kit (Zymed, San Francisco, CA, U.S.A.). Anx V was revealed using a polyclonal rabbit antibody raised against human Anx V [14]. Anx VII was detected with a rabbit antibody against a synthetic peptide RDLEKDIRSDTSG. A monoclonal antibody (Zymed) was used to detect the cell-cycle-dependent kinase cdc2. Following incubation with primary antibodies, the filters were exposed to either goat ^{125}I-labelled anti-rabbit IgG (polyclonal antibodies) or goat ^{125}I-labelled anti-mouse IgG (monoclonal antibodies) (ICN, Costa Mesa, CA, U.S.A.). The immunolabelled bands were visualized with a Molecular Dynamics PhosphorImager and quantified using the image analysis software NIHimage. Using purified recombinant human Anx VII, we have determined that the signal was linear with respect to the amount of standard in the range 1 ng–1 μg.

mRNA analysis using RNase protection assay

Templates for the RNase protection assay were constructed using PCR amplification (Perkin-Elmer Cetus, Norwalk, CT, U.S.A.) of either Anx I, Anx V or Anx VII cDNA to obtain fragments of different lengths, which would give respectively 150,

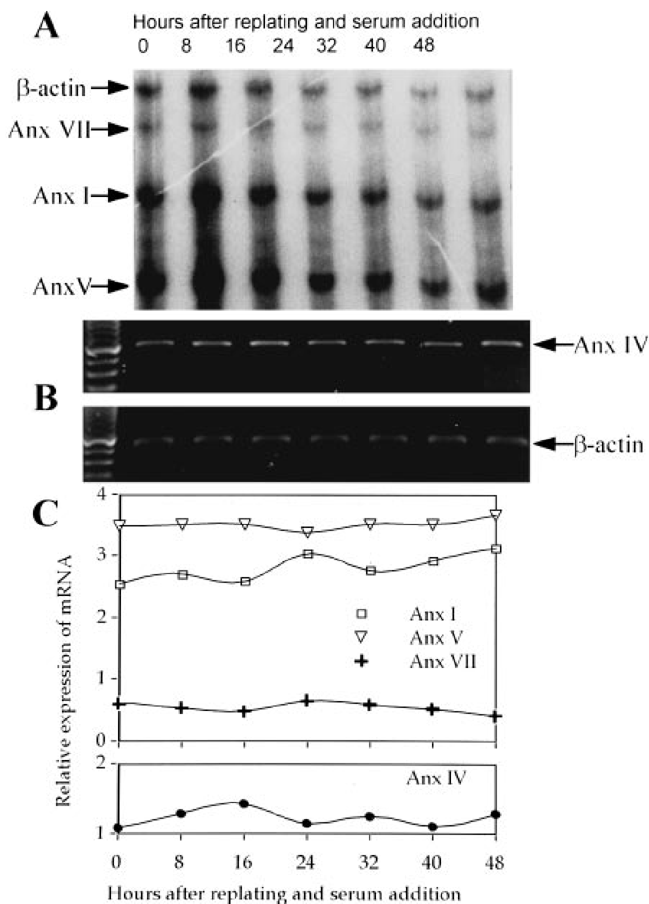

Figure 2 Expression of the mRNAs of Anxs I, IV, V and VII during the cell cycle of human fibroblasts

IMR-90 cells were synchronized as in Figure 1 and total RNAs were extracted at the indicated time after cell cycle reactivation. (**A**) For each sample, the levels of mRNAs of Anxs I, V, VII and β-actin were determined using RNase protection assay. The RNase-protected products were separated by denaturing polyacrylamide gel electrophoresis, revealed by autoradiography and identified according to their size. (**B**) RT PCR was used to analyse the level of mRNAs of Anx IV and β-actin. RT PCR products were run on agarose gel and stained with ethidium bromide. (**C**) The expression of mRNAs was quantified by densitometric analysis of the above images, relative to the expression of the β-actin mRNA. These data are representative of two independent experiments.

100 and 210 bp RNase protected products. The PCR products were digested with *Eco*RI and cloned into the pSP72 vector (Promega, Madison, WI, U.S.A.). The primer sets used for each cDNA are:

Anx I: sense TGCAGAATTCCTCAAGCAGGCCTGG

antisense GAGTCGAATTCTTATGGCCTTATGCA-AGGCAGCGA

Anx V: sense TGCAGAATTCATGGCACAGGTTCTCAG-AGGC

antisense GAGTCGAATTCTGTGCCCAAGCCTT-TCATA

Anx VII: sense TGCAGAATTCATGTCATACCCAGGC-TAT

antisense GAGTCGAATTCTCCAGGGGCTGG-ATAACCTCCA

To synthesize RNA probes, the plasmids containing either Anx I, Anx V or Anx VII cDNA fragments were linearized, and [α-^{32}P]UTP-labelled antisense RNA was obtained using an *in vitro* transcription kit (Ambion, Austin, TX, U.S.A.) with either SP6 or T7 RNA polymerase, depending on the orientation of the fragments in pSP72. Following transcription *in vitro*, the samples were treated with DNase I, phenol/chloroform extracted and purified using a Sephadex G-25 push column (Stratagene, La Jolla, CA, U.S.A.). The samples were then run on an 8 M urea/6 % polyacrylamide sequencing gel. After autoradiography, the gel piece corresponding to the full-length transcript was excised and eluted with 350 μl of 0.5 M ammonium acetate/ 1 mM EDTA/0.1 % SDS by incubating overnight at 37 °C.

RNase protection assays were performed using a kit from Ambion. For each reaction, 50 μg of total RNA from synchronized cells was resuspended in 20 μl of hybridization buffer containing approx. 200000 c.p.m. of antisense RNA from Anx I, Anx V, Anx VII and β-actin, and hybridized at 45 °C overnight. Afterwards, samples were digested by addition of a mixture containing RNase A and T1 (Ambion) at 37 °C for 30 min. RNases were denatured and RNA precipitated by using 300 μl of inactivation–precipitation buffer (Ambion). Thereafter samples were electrophoresed onto a sequencing polyacrylamide gel. A sequencing reaction using M13 as template and a universal primer was also run for gel calibration. Finally, the gel was processed for autoradiography.

mRNA analysis using reverse transcription-PCR

Anx IV mRNA level was determined using the Access reverse transcriptase (RT) PCR system (Promega, Madison, WI, U.S.A.) with two primers yielding a 519 bp RT PCR product (sense GGCCATGGCAACCAAAGGAGG; antisense CTCACGAG-AGCATCGTCCAG). As a control, the β-actin mRNA level was also analysed using specific primers yielding a 420 bp RT PCR product. All reactions were performed according to Promega's protocol using 0.2 μg of total RNA and 30 cycles of amplification, conditions found to be within the linear range of amplification of both Anx IV and β-actin. RT PCR products were analysed using agarose gel electrophoresis and ethidium bromide staining followed by computer densitometry analysis.

Construction of expression vectors and transfection

The cDNA of Anx VII was inserted into the mammalian expression vector pZIPneoSV(X) [15,16] after intermediate cloning in pSP72-NotI (see Figure 3A; both plasmids were kindly provided by S. Hermouet and J. S. Gutkind, N.I.H.). Briefly, the entire coding region of Anx VII cDNA previously cloned in pTrc [17] was amplified by PCR using the sense oligonucleotide TGCGCTAGCGGATCCGCTGCTGCTGGGGTCAGAAT-GTCATACCC including a restriction site *Nhe*I. The antisense primer GAGCTCGAGGAATTCCTACTGGCCCACAATA-GCCAGAAGAA carries a site *Xho*I. The PCR product was digested with *Nhe*I and *Xho*I, then cloned into pSP72-NotI using the compatible sites *Sal*I and *Xba*I. The construct was then digested with *Not*I and the 1.4 kb Anx VII cDNA fragment was inserted into the unique cloning site *Not*I in pZIPneoSV(X). Thereafter, the construct was sequenced using the Sequenase protocol (USB, Cleveland, OH, U.S.A.). Protein expression of the cDNA cloned in pSP72-NotI was verified using an *in vitro* transcription and translation kit (Promega, Madison, WI, U.S.A.). NIH 3T3 and HeLa cells were transfected with pZIP-AnxVII using a calcium phosphate precipitation kit (BRL, Gaithersburg, MD, U.S.A.). Stable transfectants were selected

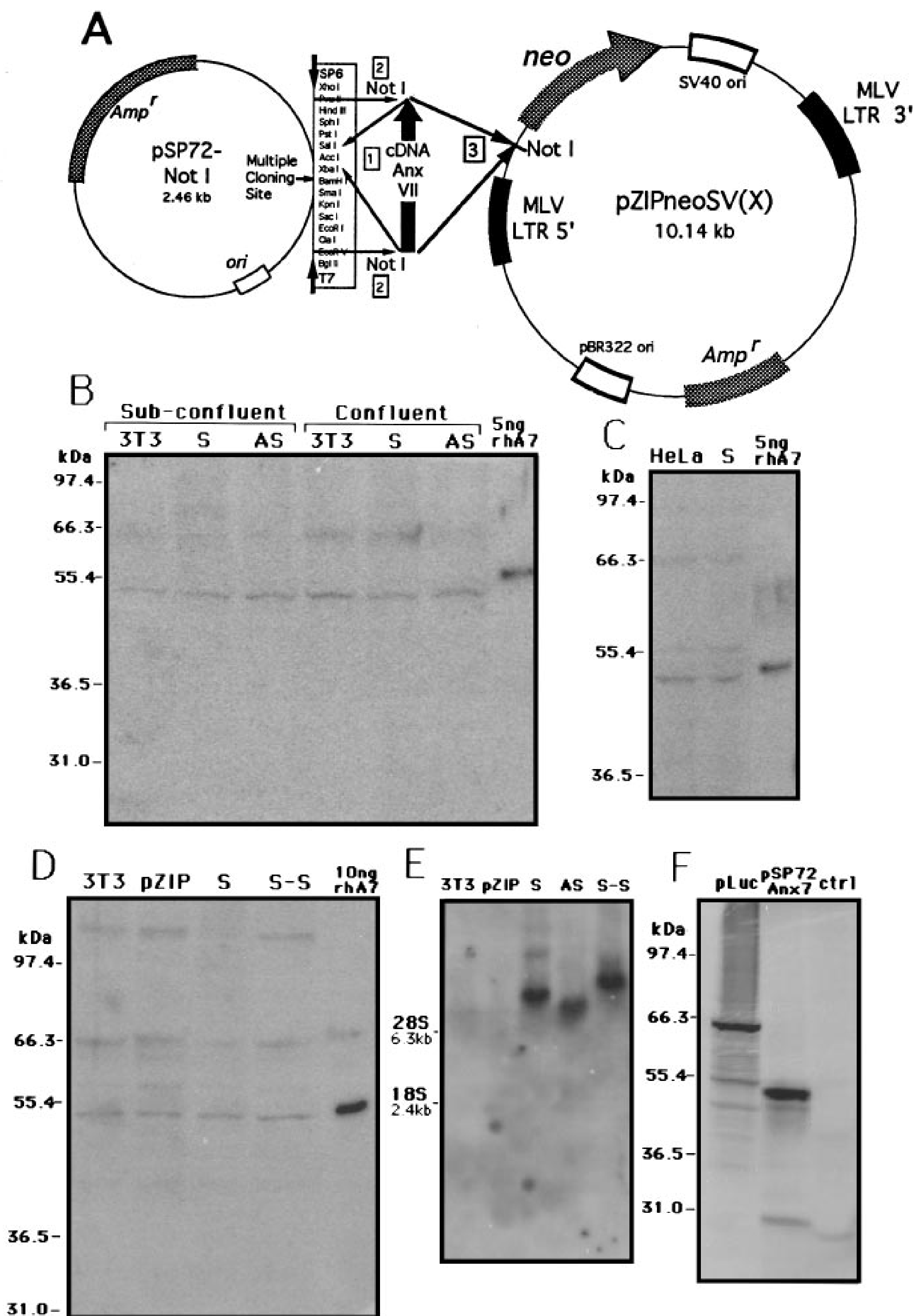

Figure 3 Anx VII expression is down-regulated by a post-transcriptional mechanism

(**A**) The cDNA of human Anx VII was inserted into the mammalian expression vector pZIPneoSV(X) after intermediate cloning in pSP72-NotI (see the Materials and methods section for details). The plasmid pZIPneoSV(X)–Anx VII was used to permanently transfect NIH 3T3 or HeLa cells, using calcium phosphate precipitation and G418 selection. (**B**) Anti-annexin VII immunoblot of 100 μg of total proteins from NIH 3T3 wild-type (3T3) or transfected with pZIP carrying one copy of Anx VII cDNA in sense (S) or antisense (AS) orientation: 5 ng of rhA7/5 ng of recombinant human Anx VII. (**C**) Same as (**B**) with HeLa cells. (**D**) Immunoblotting analysis of Anx VII in culture medium from NIH 3T3 wild-type (3T3) or transfected with the vector only (pZIP) or vector with Anx VII cDNA in sense orientation; one copy (S) or two tandem copies (S–S). Before collecting the culture medium, 20 mM EGTA was added for 5 min to release extracellular Anxs that might associate Ca^{2+}-dependently with cell membranes. (**E**) Northern blot, using human Anx VII cDNA as probe, of 40 μg of total RNA from NIH 3T3 wild-type (3T3) or transfected with the vector only (pZIP) or with the vector carrying one copy of Anx VII cDNA, in sense (S) or antisense (AS) orientation, or two tandem copies in sense orientation (S–S). (**F**) Transcription and translation of pSP72-Anx VII *in vitro*. The cDNA of Anx VII cloned in pSP72-NotI was expressed *in vitro* using the T7 promoter of pSP72 and T7 RNA polymerase for transcription, and a rabbit reticulocyte lysate for translation. The assay was performed in the presence of [^{35}S]methionine. Labelled proteins were analysed by SDS/PAGE and phosphorimaging. pLuc is a plasmid provided with the expression kit for T7-expression of 65 kDa luciferase. Control: assay performed with pSP72-NotI only as template.

using the antibiotic G418 (500 μg of active compound/ml, BRL). Cellular expression of the transfected cDNA was determined by Northern and immunoblotting, and quantified by phosphorimaging.

Metabolic labelling and Anx extraction

Nearly confluent cells were labelled with the following medium: 89 % methionine-free DMEM/1 % DMEM/10 % dialysed fetal bovine serum/20 μCi/ml [^{35}S]methionine. After 48 h, cells were washed with PBS, then subcultured 1:3 in regular culture medium for 1 to 7 days. Thereafter, the cells were washed and frozen. The different samples were then thawed all at once and processed simultaneously at 0–4 °C. To extract the Anxs, a brief version of the calcium/EGTA procedure was performed [18]. Cells were scraped into buffer H: 50 mM Tris, pH 7.4/50 mM NaCl/1 mM EDTA and various protease inhibitors [18]. After a 5 s sonication, 2 mM $CaCl_2$ and 20 mM $MgCl_2$ were added. The homogenate was mixed for 5 min on ice, then spun at 14000 ***g*** for 15 min. Thereafter, the pellet was sonicated and recentrifuged twice in homogenization buffer H with $CaCl_2$ and $MgCl_2$. Finally, the pellet was sonicated in buffer H containing 20 mM $MgCl_2$ and 5 mM EGTA. After shaking for 10 min on ice, the preparation was centrifuged for 15 min at 14000 ***g***. Supernatant proteins were resolved by SDS/PAGE and stained with Coomassie Blue. Radiolabelled proteins were revealed by phosphorimaging. Immunoblotting was also performed to identify the various Anxs present in the extract. Quantitative measurements of ^{35}S signals were determined by integration of the phosphorimager data using the NIHimage software. A densitometric analysis of Coomassie-stained gels was also performed. ^{35}S measurements were ratioed with densitometric data to take into account possible variations of the amount of various Anxs in the different extracts. Calculation of Anx half-life ($t_{\frac{1}{2}}$) was based on the plot of log(labelling) versus time. The first-order rate constant k was determined from the slope $= -k/2.3$ and $t_{\frac{1}{2}}$ was calculated from $t_{\frac{1}{2}} = 0.693/k$ [19].

RESULTS

To study the expression of the Anxs during the cell cycle in normal human cells, we have used IMR-90 cells which are non-transformed, non-immortalized cultured, human, diploid lung fibroblasts that can be arrested in G0 phase by serum deprivation [20]. As shown in Figure 1(A), [^{3}H]thymidine incorporation and cdc2 expression indicate that the cells synchronously re-enter the first cycle of division after replating in the presence of serum, with the cycle lasting approx. 48 h. The expression of the Anxs at different stages of the cell cycle was determined in total protein extracts from cells harvested at different times after replating, using immunoblotting with a panel of specific anti-annexin antibodies. A typical experiment is shown in Figure 1(B) and quantitative data from four independent experiments have been analysed and plotted in Figure 1(C). The results indicate that the levels of Anxs II, V and VI vary by less than 10 % during the cell cycle. In contrast, the expression of Anxs I and VII is reduced by 40 % in early G2/M, and Anx IV increases by 50 % during S-phase. To determine whether changes in Anx levels were caused by variations in mRNA expression, the levels of various Anx mRNAs during the cell cycle were measured by RNase protection assays or RT PCR and compared with the expression of the β-actin mRNA. Figure 2 shows that expression of Anx V mRNA is very stable, as expected from protein results. The mRNAs of Anxs I and VII are also steadily expressed, suggesting that down-regulation of both proteins in G2/M occurs through a post-transcriptional process. In addition, a moderate increase of Anx IV mRNA level was observed during the S-phase that could account for the rise of protein expression during this phase.

To determine the significance of Anx homeostasis in cell growth control, we have permanently transfected mammalian cells with human Anx VII cDNA cloned in the expression vector pZIPneoSV(X) (Figure 3A). Northern blot analysis of total RNA from transfected cells (Figure 3E) shows that cells transfected with one (S) or two (S–S) copies of Anx VII cDNA in the sense orientation have a very high level of Anx VII mRNA compared with control cells (3T3 and pZIP), whereas the endogenous 2.4 kb mRNA is hardly visible. Phosphorimager data indicated a difference of approx. 2 orders of magnitude (results not shown). The larger size of mRNAs detected in transfected cells is due to the polycistronic mRNA produced by pZIPneoSV(X), containing both the Anx VII and *neo* genes. However, the immunoblotting analysis displayed in Figures 3(B) and 3(C) shows that cells transfected with the sense constructs (lanes S) contain levels of Anx VII similar to wild-type cells (lanes 3T3 and HeLa). Analysis of culture medium (Figure 3D) indicates that transfected cells (S and S–S) do not release more Anx VII than control cells (3T3), thereby ruling out a possible secretion of the putative overexpressed protein. As an additional control, Anx VII was efficiently expressed *in vitro* from the cDNA inserted in pZIP, using a transcription and translation assay (Figure 3F). Hence, it appears that expression of Anx VII is maintained at a constant level, despite a tremendous increase in mRNA. In addition, cells were transfected with pZIP expressing Anx VII mRNA in antisense orientation. Strikingly, NIH 3T3 (Figure 3B, lane AS) or HeLa (not shown) does not have a reduced level of endogenous Anx VII, despite a high cell content in antisense RNA detected by Northern blotting (Figure 3E, lane AS). Similarly, attempts to down-regulate the expression of Anx VII, and also Anxs I and V, in various cell types using phosphorothioate antisense oligonucleotides have not been successful (P. Raynal, H. B. Pollard and M. Srivastava, unpublished results, [21]).

To determine whether the stability of the Anxs was an important factor in maintaining their expression, we measured their turnover rate in IMR-90 human fibroblasts using pulse–chase analysis. The Anxs were partially purified using a conventional procedure of co-precipitation with cell membrane and Ca^{2+}, followed by EGTA elution [18]. The proteins thereby extracted were identified by immunoblotting. Out of eight major bands visible on Coomassie-stained SDS/PAGE of the extracts, five were identified using specific anti-annexin antibodies: Anxs I, II, VI, VII and Anx IV, which co-migrated with Anx V (Figure 4A). Pulse–chase analysis of IMR-90 was achieved by incubating nearly confluent cells with [^{35}S]methionine for 48 h, followed by subculture at a 1:3 ratio with non-radioactive methionine. After 1 to 7 days, Anxs were extracted and separated by SDS/PAGE, and ^{35}S-labelling was detected and quantified using a phosphorimager (Figures 4B and 4C). Half-life calculations vary from 38.5 h for Anx VII to 94.7 h for Anxs IV–V, indicating that the Anxs have a slow turnover rate.

DISCUSSION

We have tested whether the expression of the Anxs is modulated during the cell cycle of non-transformed and non-immortalized cells. Our results indicate that the cell content of Anxs II, V and VI remain constant during the cell cycle of synchronized normal human fibroblasts. The fact that Anx V is steadily expressed in

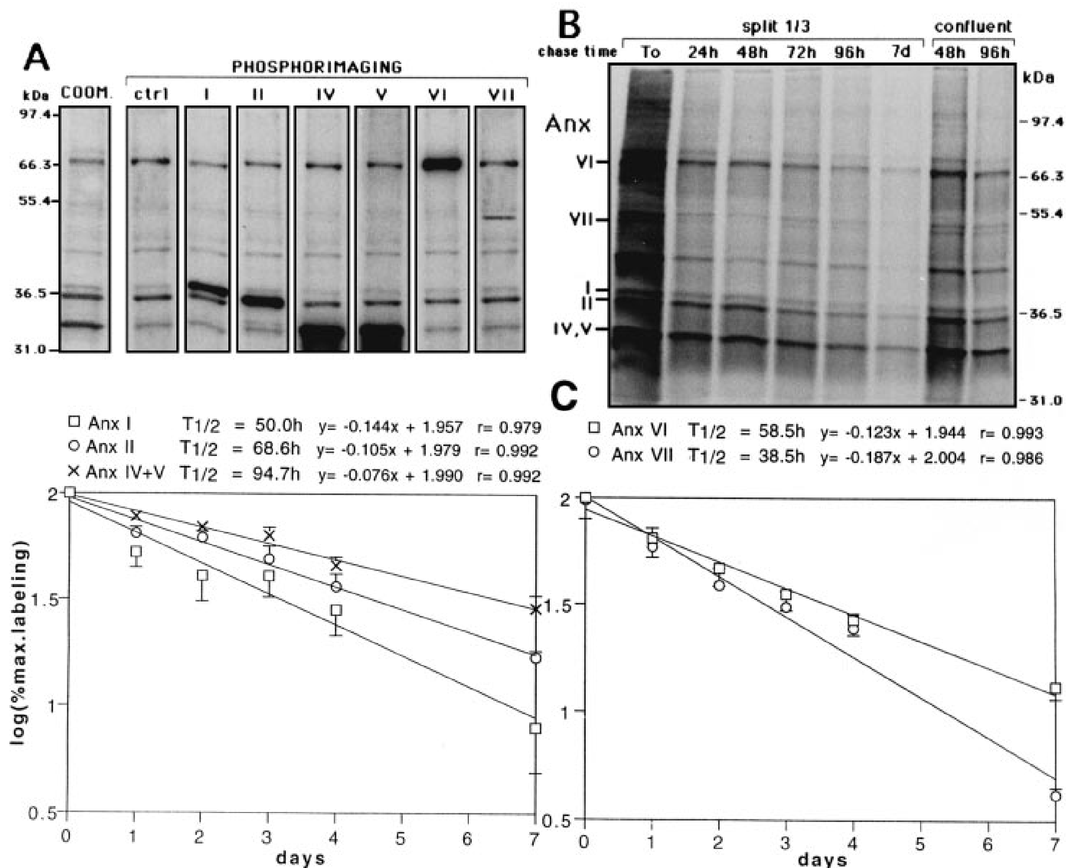

Figure 4 Determination of the turnover rate of the Anxs

(**A**) Identification of Anxs in Ca^{2+}/EGTA extracts from [^{35}S]methionine-labelled IMR-90 cells. Partial purification of Anxs was performed by co-precipitation of Anxs with cell membranes and Ca^{2+}, followed by EGTA elution. An extract of labelled cells was split into eight identical fractions resolved by SDS/PAGE. One lane was stained with Coomassie Blue (Coom) and the rest were immunoblotted with specific anti-annexin antibodies (I, II, IV, V VI and VII) and appropriate ^{125}I-labelled anti-IgG antibodies. Therefore phosphorimaging detection reveals the signals from both ^{35}S metabolic labelling and ^{125}I immunoblotting, except for the control lane (ctrl) that has not been immunoblotted. Thus the signal appearing after immunoblotting differentially from the ctrl lane identifies the Anx recognized by the respective antibody. (**B**) Anx extracts from pulse–chased IMR-90. Nearly confluent cells were incubated with [^{35}S]methionine during 48 h, then either subcultured 1 : 3 or kept confluent for the indicated time with non-radioactive methionine. Afterwards, Anxs were Ca^{2+}/EGTA extracted, separated by SDS/PAGE and ^{35}S labelling was revealed using phosphorimaging. The identification of the various proteins is based on (**A**). (**C**) Determination of the turnover rate of the Anxs. Cells were labelled as in (**B**) and then split 1 : 3 at the beginning of the chase. For each chase time, quantitative measurements of the ^{35}S labelling of each Anx were obtained from the phosphorimager, and plotted versus time. Values are means ± S.E.M. of three independent experiments.

IMR-90 cells was unexpected. Using cultured human foreskin fibroblasts, a cell type apparently very close to IMR-90, Schlaepfer and Haigler have shown that Anx V expression in quiescent cells is increased by 3- to 4-fold compared with proliferating cells [7]. These data had been interpreted to suggest that intracellular levels of Anx V were crucial for cell growth control. However, our study clearly indicates that cell division can proceed independently of the Anx V intracellular level. Further studies are certainly required to determine the generality and significance of the variations of Anx V expression, if any, between resting and proliferating cells. As for Anx V, we found that intracellular levels of Anx II were independent of the cell cycle. This contrasts with studies showing that Anx II varies moderately during the cycle of transformed mammalian cells [22]. In addition, the steady expression of Anx VI during the cell cycle is somewhat surprising, since the growth of carcinoma cells A431 has been shown to depend on the Anx VI intracellular level [23]. These results could be based on the many differences existing between transformed and normal mammalian cells and point to the possibility of Anxs being of importance in the transition to the transformed state.

By contrast, the expression of Anxs I, IV and VII in IMR-90 cells is modestly regulated in a cell-cycle-dependent manner. However, the mechanisms regulating expression of Anxs I and VII are not related to transcriptional control since RNase protection assays showed that Anxs I and VII mRNAs were uniformly expressed during the cell cycle. This suggests that cell-cycle regulation of Anxs I and VII expression in these cells occurs only at a post-transcriptional level. Supporting the existence of such a mechanism, our study with transfected cells implied that a post-transcriptional process down-regulated the expression of transfected Anx VII mRNA. Anx VII was chosen because its expression is modulated during the cycle of IMR-90 cells and its endogenous expression is very low, representing only approx. 0.01 % of total cell proteins in NIH 3T3 and HeLa cells (P. Raynal, H. B. Pollard and M. Srivastava, unpublished results). However, transfected cells keep their Anx VII to a very low level, despite a dramatic increase of mRNA content. The mechanism of this post-transcriptional regulation of Anx VII is unknown, but it may well be related to the differences observed between mRNA and protein levels during the cycle of IMR-90 fibroblasts. In addition, antisense RNA did not alter the cell content of Anx

VII, even at concentrations of antisense RNA approx. 100 times higher than endogenous mRNA. However, considering the small turnover rate of the Anxs, it does not seem unexpected that an antisense strategy failed to inhibit Anx VII expression. Indeed, we showed, using pulse–chase analysis, that the Anxs in human fibroblasts IMR-90 have long half-lives, in the range 1.5–4 days. A similar value has been reported for bovine Anx VII in non-dividing chromaffin cells [21]. As a matter of comparison, the half-lives of cdc2 (Figure 1A) or the transcription factor c-*jun* [24] are in the region of 1 h.

In conclusion, taken together, these results raise reasonable doubts about the concept that cellular expression of Anxs plays a general role in cell growth. Alternatively, a putative function of the Anxs in cell growth control could be regulated by different mechanisms, possibly phosphorylation or modification of intracellular localization. Studies addressing these issues are certainly required to determine the actual role of the Anxs in cell growth and differentiation.

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