Annexin II up-regulates cellular levels of p11 protein by a post-translational mechanism

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Annexin II (p36) and p11, which belong to two different families of calcium-binding proteins, are able to form a heterotetrameric protein complex $(p36)_2(p11)_2$ called calpactin I. As these proteins were detectable only in the presence of each other in a variety of cell lines, we studied the mechanisms of regulation of cellular levels of annexin II and p11. In cells expressing p11 messenger RNA, p11 protein is undetectable unless annexin II is also expressed. As an example, the hepatoblastoma HepG2 cell line displays no detectable annexin II nor p11 protein, although it expresses p11 mRNA. The overexpression of annexin II by gene transfer into HepG2 cells leads to the up-regulation of the cellular levels of p11 by a post-translational mechanism. In the presence of annexin II, there is no major change in the p11 transcript levels, but the half-life of the p11 protein is increased more than 6-fold. Thus, the degree of expression of annexin II, which varies according to different states of cellular differentiation and transformation, is an essential factor in the regulation of cellular levels of p11.

INTRODUCTION

Annexin II (calpactin I heavy chain, p36) was initially identified as a substrate of the transforming protein ($pp60^{src}$) of Rous sarcoma virus (see [1,2] for reviews). Subsequently, it was shown to be one of the most actively phosphorylated substrates for many different receptor- and non-receptor-protein kinases [3–6]. Although it was implicated in many different cell processes, the exact cellular functions of annexin II remain unclear. It is involved in signal transmission pathways such as the transmission of the external signal to the cytoskeleton [7], the transduction of the calcium-related mitogenic signals [2], or the regulation of the activity of phospholipase A_2 and phospholipase Cγ, both of which are involved in the intracellular signal transduction [8,9]. The same molecule was also shown to play an essential role in membrane fusion events during exocytosis and endocytosis [10–12]. At last, in addition to these membrane-related events, annexin II was recently shown to be implicated in nuclear processes. Indeed, it stimulates DNA polymerase α in lagging-strand DNA synthesis [13], its immunodepletion inhibits DNA replication in *Xenopus* egg extracts [14] and its cellular levels are subject to mammalian cell cycle regulation [15].

Annexin II is present in cells in monomeric as well as tetrameric forms. In its tetrameric form, two molecules of annexin II bind to two molecules of a smaller protein, p11, to form the calpactin I complex $(p36)_2$–$(p11)_2$ [16,17]. The p11 protein belongs to a family of small dimeric proteins which share sequence homologies with S100α and S100β [18–20]. In the tetrameric complex, p11 modulates some biological properties of annexin II. For example, the binding of p11 to annexin II results in an increased affinity for calcium and lipid, and an inhibition of the annexin II phosphorylation [21,22]. Based on these data, p11 is considered to be a regulator of the annexin II functions. p11 is present exclusively in the cytoskeleton, whereas the cellular distribution of annexin II is more diffuse [23]. As a tetrameric complex, annexin II is mainly localized to the cytoskeleton. As a monomer it appears to be cytosolic and nuclear [13]. The physiological significance of such a distribution may indicate that the two pools of annexin II participate in different cellular processes. In this case, the formation of the $(p36)_2$–$(p11)_2$ complex may be the main mechanism of regulation of the dual p36 functions. Using a large panel of cell lines and expression vectors, we studied how the cellular levels of p11 and annexin II are regulated. We show here that annexin II is a key regulator of cellular levels of the p11 protein.

MATERIALS AND METHODS

Cell lines

Cell lines employed in these studies were obtained from the American Type Culture Collection, Rockville, MD, U.S.A. Epithelial cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal-calf serum, 1 % (w/v) non-essential amino acids and glutamine (2 mM) (Gibco Laboratories, NY, U.S.A.). Lymphoid cell lines were grown in RPMI 1640 medium containing 10 % (v/v) fetal-calf serum and glutamine (2 mM) (Gibco Laboratories, NY, U.S.A.).

Metabolic labelling and immunoprecipitation

Subconfluent cells were grown for 1 h in methionine-free medium containing 10 % dialysed fetal-calf serum. Cells were labelled with 0.2 $mCi \cdot ml^{-1}$ [^{35}S]methionine (specific radioactivity > 1000 $Ci \cdot mmol^{-1}$; Amersham, Bucks., U.K.) for 1 h in 10-cm-diam. culture dishes. Then, after 0, 1, 2, 6 and 12 h of chase, cells were washed in PBS (10 mM phosphate/140 mM NaCl, pH 7.2) and proteins extracted in 1 ml of lysis buffer [50 mM Tris/HCl, pH 7.4, 0.25 M NaCl, 0.1 % (v/v) Triton X-100, 5 mM EDTA, 50 mM NaF]. All solutions contained a cocktail of protease inhibitors (50 $mg \cdot l^{-1}$ PMSF, 10 $mg \cdot l^{-1}$ soybean trypsin inhibitor, 1 $mg \cdot l^{-1}$ aprotinin). Immunoprecipitation experiments were performed as described previously [24]. The monoclonal antibody p11 subunit clone no. Z015 (Zymed, San Francisco, CA, U.S.A.) was used for the study of p11 protein. Briefly, samples were precleared with 40 μl of 50 % (v/v) Protein-A–Sepharose sus-

Abbreviations used: CMV, cytomegalovirus; HSV TK, herpes simplex virus thymidine kinase.

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pension (Pharmacia Biotech, Sweden) for 2 h at 4 °C. Two microlitres of p11 monoclonal antibody were added. After an overnight incubation on a rocker at 4 °C, and a 5 min centrifugation at 13000 ***g***, supernatants were added to Eppendorf tubes containing 40 μl of Protein-A–Sepharose beads and analysed as described [24].

Western immunoblotting

Cells were washed twice in PBS and proteins solubilized for 30 min at 4 °C in the lysis buffer [50 mM Tris/HCl (pH 7,4), 0.25 M NaCl, 1 mM EDTA, 0.1 % Triton X-100, 50 mM NaF] containing a cocktail of protease inhibitors (0.2 mg·l^{-1} leupeptin, 0.2 mg·l^{-1} aprotinin, 2 mg·l^{-1} tosylphenylalanine chloromethane, 2 mg·l^{-1} soybean trypsin inhibitor, 10 mg·l^{-1} PMSF). Lysates were centrifuged for 15 min at 13000 ***g*** at 4 °C. The supernatants were harvested, adjusted to a protein concentration of 1 mg·ml^{-1}, and stored frozen at −70 °C until use. Cell lysates adjusted to equal amount of proteins (100 μg) were separated by SDS/PAGE and electrotransferred to Immobilon P membrane (Millipore Continental Water Systems, Bedford, U.S.A.). Immunodetection of p36 was performed using the AF5 monoclonal antibody [25] and p11 with the monoclonal antibody p11 subunit clone no. Z015 (Zymed, San Francisco, CA, U.S.A.). p36 and p11 were then detected with a murine peroxidase-conjugated antibody and Amersham enhanced chemiluminescence detection reagent.

Construction of expression vectors

A full-length human annexin II cDNA was cloned from FOCUS cDNA library made in lambda GT 11 phage, using a partial human annexin II cDNA [25] as a ^{32}P-labelled probe. A 1.4 kbp fragment was then PCR-amplified using the following primers carrying artificially created *Bam*HI restriction sites (forward: 5′-ATAGCGGCCGCGGATCCTGGAGCCCGTCAGTATC-3′; reverse: 5′-TAAGCTTATCGATGGATCCGGCGCTCAGCTGGAA-3′).

PCR product was cut with *Bam*HI, purified and ligated into the pCMV vector constructed by Baker et al. [26] and kindly provided by S. Friend (MGH Cancer Center, Boston, MA, U.S.A.). Three clones were selected by restriction enzyme analysis and nucleic acid sequencing. The vector pCMV-p36wt contains a wild-type annexin II cDNA placed under the promoter/enhancer sequences of the cytomegalovirus (CMV). The vector pCMV-p36m contains a PCR-mediated mutation at the first nucleotide of codon 10 (A to G) of annexin II cDNA which yields a lysine to glutamic acid change located in the p11-binding site of annexin II [27,28]. Finally, the vector pCMV-p36wo has the wild-type p36 cDNA in wrong orientation with respect to the CMV promoter/enhancer sequences. All vectors share the same neomycin resistance gene placed under the control of the herpes simplex virus thymidine kinase (HSV TK) gene promoter [26]. p11 expression vector was constructed using a similar approach, except that a p11 cDNA was cloned by reverse PCR-amplification using RNA obtained from normal human lymphocytes. The following primers derived from the published human p11 cDNA sequence were used: p11-F, 5′-ATAGGATCCAAGGCTTCAACGGACC-3′; p11-R, 5′-ATGGATCCTTAAGCGACCCTTTGGAC-3′.

Both primers had artificially created *Bam*HI restriction sites in their 5′ termini. PCR products were first cloned into pCRII (InVitroGen, The Netherlands). Then, after sequence analysis, the *Bam*HI–*Bam*HI fragment was cloned into the pCMV vector.

Cellular transfections

Exponentially growing cells were transfected by the calcium phosphate method [29], using 10 μg of plasmid DNA. Transfected cells were incubated in a medium containing geneticin (0.5 mg/ml) (Gibco Laboratories, NY, U.S.A.) for 3–4 weeks, to obtain antibiotic-resistant colonies.

Northern blot analysis

Total cellular RNA was extracted by the guanidinium thiocyanate extraction procedure and analysed as previously described [25].

RESULTS

The intracellular levels of p11 and annexin II proteins were studied in a large collection of cell lines. It is noticeable that, in spite of a wide variation of their levels among the tested cell lines, the amount of p11 appeared tightly coupled to the amount of annexin II, suggesting a co-regulation (Figure 1).

We have shown previously that annexin II was overexpressed in most of the hepatocarcinoma cell lines and primary hepatocarcinomas [25], whereas annexin II and p11 were undetectable

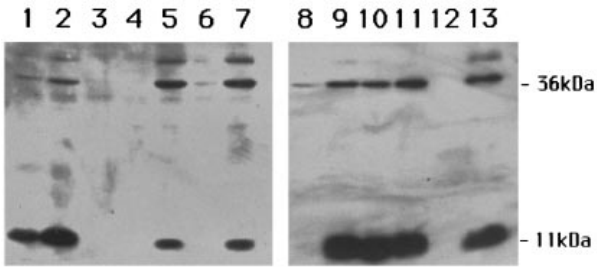

Figure 1 Western blot analysis of annexin II (p36) and p11 levels in human tumour cell lines and CV1 cells

Lane 1, CV1 (monkey kidney fibroblast-like); lane 2, GCT (fibrous histiocytoma); lane 3, Colo320DM (colon adenocarcinoma); lane 4, HL60 (promyelocytic leukaemia); lane 5, HT29 (colon adenocarcinoma); lane 6, SKNSH (neuroblastoma); lane 7, RS4.11 (leukaemia); lane 8, Jurkat (T-cell leukaemia); lane 9, A704 (renal adenocarcinoma); lane 10, ACHN (renal adenocarcinoma); lane 11, Caki 1 (clear cell carcinoma); lane 12, IMR32 (neuroblastoma); lane 13, LS180 (colon adenocarcinoma).

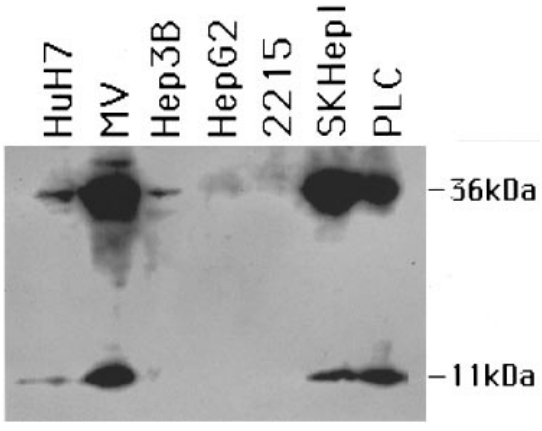

Figure 2 Western blot analysis of annexin II (p36) and p11 levels in human hepatoma cell lines

Abbreviations: MV, Mahlavu; 2215, 2.2.15 clone of HepG2; PLC, PLC/PRF/5.

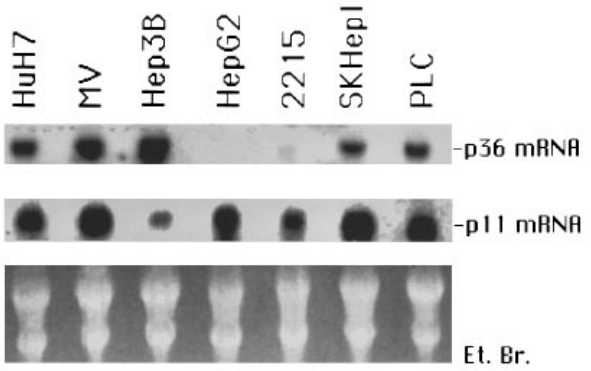

Figure 3 Northern blot analysis of annexin II (p36) and p11 mRNAs from human hepatoma cell lines

Abbreviations: MV, Mahlavu; 2215, 2.2.15 clone of HepG2; PLC, PLC/PRF/5; Et.Br., ethidium bromide.

in normal hepatocytes [25,30,31]. Consequently, annexin II and p11 levels were studied in greater detail in seven hepatoma-derived cell lines. Both proteins were easily detectable in four hepatocarcinoma cell lines (HuH7, Mahlavu, SKHepI, PLC/PRF/5). One cell line (Hep3B) had low amounts of annexin II and p11 proteins. Both annexin II and p11 were undetectable in a hepatoblastoma cell line (namely HepG2) as well as in the HepG2/2.2.15-derived cell line (Figure 2).

We next studied p11 and annexin II messenger RNA levels in the same set of hepatocellular carcinoma (HCC) cell lines by Northern blotting (Figure 3). As expected from protein studies, both p11 and annexin II transcripts were easily detectable in Huh7, Mahlavu, SKHep1 and PLC/PRF/5 cells. However, in Hep3B cells, p36 transcripts were abundant, whereas p36 protein levels were low (see Figure 2). Furthermore, HepG2 and HepG2/2.2.15 cells that displayed no detectable annexin II nor p11 proteins expressed p11 transcripts. This discrepancy between

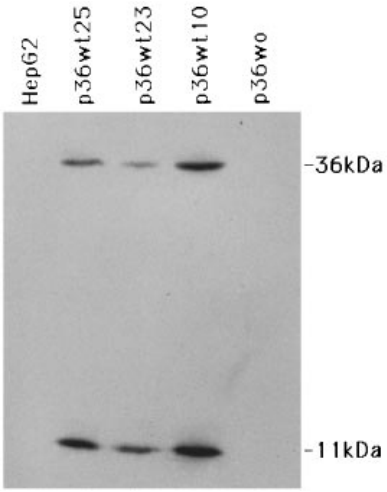

Figure 4 Western blot analysis of annexin II and p11 in HepG2-derived clones

p36wt25, p36wt23, p36wt10 are clones selected after transfection with wild-type annexin II cDNA. p36wo is a clone selected after transfection with an expression vector containing the annexin II cDNA in the wrong orientation with respect to CMV promoter/enhancer sequences.

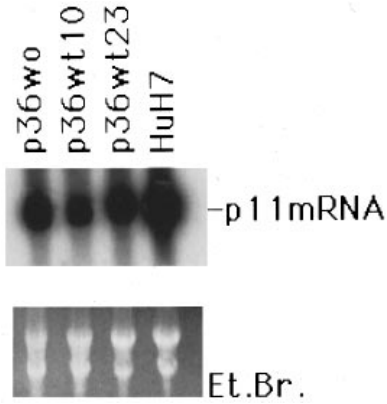

Figure 5 Northern blot analysis of p11 mRNAs from HepG2-derived clones and HuH7 hepatoma cell line

p36wo is a clone selected after transfection of HepG2 with an expression vector containing the annexin II cDNA in the wrong orientation with respect to CMV promoter/enhancer sequences. p36wt10 and p36wt23 are clones selected after transfection of HepG2 with wild-type annexin II cDNA. Abbreviation: Et.Br., ethidium bromide.

transcript and protein levels suggest that messenger RNA levels are not the only determinant in the regulation of cellular levels of both p11 and annexin II.

To investigate the effect of annexin II on cellular levels of p11, we transfected an annexin II expression vector in the hepatoblastoma-derived cell line HepG2. As shown in Figure 1, annexin II and p11 proteins are undetectable in this cell line. HepG2 is a well-differentiated cell line which is commonly used to study liver-specific functions. It is a minimally deviant cell line, displaying most of the phenotypic and biochemical characteristics of differentiated hepatocytes [32]. After cellular transfection, several clones expressing high levels of annexin II were selected and studied for the levels of p11 protein. All clones expressing high levels of annexin II also displayed high levels of p11 protein (Figure 4). As control, a cellular transfection was carried out with an expression vector containing the annexin II cDNA in wrong orientation with respect to CMV promoter/enhancer sequences (HepG2-p36wo). After geneticin selection, resistant colonies had no detectable annexin II protein nor p11 protein.

To study the mechanisms of p11 accumulation in annexin II-expressing clones, the expression of endogenous p11 gene was studied by RNA Northern blotting. Equal amounts of p11 transcripts were detected in annexin II-positive and -negative cell lines (Figure 5). This observation indicates that the accumulation of p11 protein in annexin II-expressing clones is not due to an overexpression of p11 gene, but to a post-transcriptional regulation. As annexin II and p11 form a tetrameric complex, we next tested whether annexin II expression was able to increase the metabolic stability of p11 protein. An annexin II-negative clone (HepG2-p36wo) and an annexin II-positive clone expressing high levels of annexin II (HepG2-p36wt10) were subjected to pulse–chase experiments after metabolic labelling with [^{35}S]methionine. As shown in Figure 6, the rate of p11 synthesis was similar in HepG2-p36wo and HepG2-p36wt10 cells. However, the half-life of p11 in the annexin II-positive clone (> 12 h) was longer, as compared with the annexin II-negative clone (< 2 h). Thus, the expression of annexin II caused a significant increase in the half-life of p11.

To test the hypothesis that annexin II induces a stabilization of p11 by formation of the heterotetramer complex $(p36)_2$–$(p11)_2$,

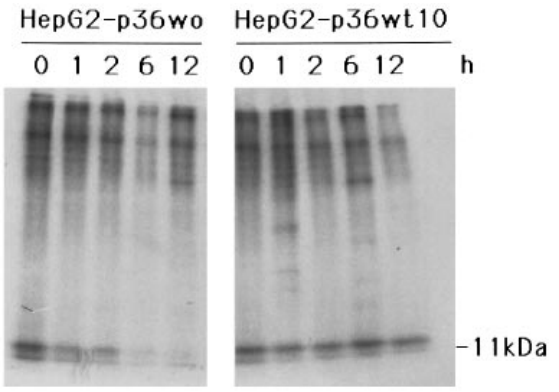

Figure 6 Autoradiogram of an immunoprecipitation using the anti-p11 monoclonal antibody after [^{35}S]methionine pulse–chase labelling

Cells were labelled with [^{35}S]methionine, followed by a chase with unlabelled medium for 1 to 12 h. HepG2-p36wo: clone selected after transfection with an expression vector containing the annexin II cDNA in wrong orientation with respect to CMV promoter/enhancer sequences. HepG2-p36wt10: clone selected after transfection with wild-type annexin II cDNA.

we selected a p36 cDNA containing a point mutation at the first nucleotide of codon 10 (A to G). This mutation provokes an amino acid substitution of the basic lysine residue (amino acid 9 of the mature protein) by an acidic glutamic acid residue. To form the calpactin I complex, p11 binds to the N-terminal 12 amino acid residues of annexin II that act as an amphiphatic helix [28]. The exact same substitution at codon 10 was previously shown to result in a 35-fold decrease in the ability of binding of annexin II to the p11 protein [28]. After transfection and selection

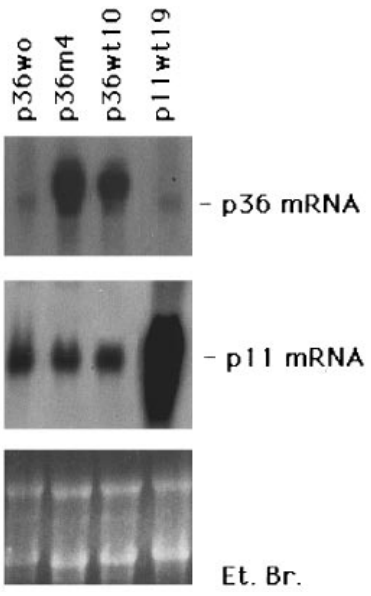

Figure 7 Northern blot analysis of annexin II (p36) and p11 mRNAs in HepG2-derived clones

p36wo: clone selected after transfection with an expression vector containing the annexin II cDNA in wrong orientation with respect to CMV promoter/enhancer sequences. p36m4: clone selected after transfection with a mutant p36 cDNA. p36wt10: clone selected after transfection with wild-type annexin II cDNA. p11wt19: clone selected after transfection with wild-type p11 cDNA. Abbreviation: Et.Br., ethidium bromide.

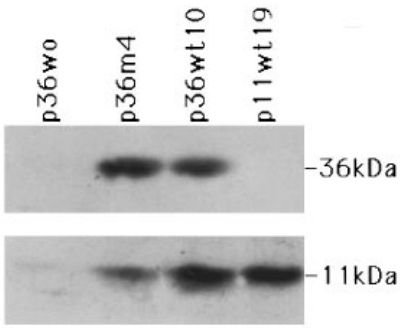

Figure 8 Western blot analysis of annexin II (upper lane) and p11 (lower lane) in HepG2-derived clones

p36wo: clone selected after transfection with an expression vector containing the annexin II cDNA in wrong orientation with respect to CMV promoter/enhancer sequences. p36m4: clone selected after transfection with a mutant p36 cDNA. p36wt10: clone selected after transfection with wild-type annexin II cDNA. p11wt19: clone selected after transfection with wild-type p11 cDNA.

of clones overexpressing this mutant annexin II (HepG2-p36m) (Figure 7), levels of p11 were studied by Western immunoblotting in comparison with HepG2-derived cell lines overexpressing the wild-type annexin II (HepG2-p36wt) (Figure 8). Amounts of p11 in mutant p36 cell lines were higher than in the HepG2 parental cell line but much lower than in wild-type p36-expressing cell lines. This observation strongly suggests that the stabilization of p11 is due to the direct interaction with annexin II via the heterotetramer complex formation.

Taken together, our experiments (based on the overexpression of annexin II in HepG2 cells) showed that annexin II is able to up-regulate cellular levels of p11 protein through a direct interaction. It has been shown previously in human fibroblasts that the turnover rate of the soluble form of p36 (considered as monomeric) was shorter than that of the insoluble form (considered as calpactin I complex) [23]. Thus, we asked whether the overexpression of p11 in HepG2 cells was able to increase annexin II protein levels. Using a similar approach, we constructed a p11 expression vector and obtained several clones from the HepG2 cell line after stable tranfection with the p11 expression vector. Owing to the high levels of stably transfected p11 cDNA expression (Figure 7) these clones displayed detectable levels of p11 protein (Figure 8). However, none of them displayed detectable levels of annexin II protein. These results suggest that p11 protein is unable to up-regulate annexin II levels in HepG2 cells. This is most likely due to the fact that the expression of the annexin II gene is weak or absent in HepG2 cells, and that p11 protein is unable to increase the levels of annexin II transcripts in these cells (Figure 7).

DISCUSSION

Owing mostly to its multiple intracellular localizations, annexin II was implicated in three general cellular processes: signal transduction, membrane fusion events during exocytosis and endocytosis, and DNA replication. The first two phenomena appear to involve the heterotetrameric form, $(p36)_2$–$(p11)_2$, which is associated with the cytoplasmic face of the plasma membrane and the submembranous cytoskeleton. This complex, called calpactin I, has an increased affinity for Ca^{2+} and phospholipid when compared with the monomeric annexin II [21,22]. On the other hand, the DNA replication process seems to involve a weak nuclear subfraction of the monomer. Indeed, a recent study

shows that about 5% of total annexin II, identified as the primer recognition protein PRP1, associates with the glycolytic enzyme 3-phosphoglycerate kinase (PRP2) to form a complex that interacts with DNA polymerase α [13]. Consequently, this dual activity requires from the cell a tight regulation of annexin II levels, since weak alterations in the proportion of the nuclear population of this protein could significantly affect DNA synthesis.

Our results confirm previous studies showing that there is a close correlation between protein levels of p11 and annexin II in different cell types [23]. In most of the cell lines, transcriptions of annexin II and p11 genes appear to be correlated. However, some others display different transcript amounts, suggesting a post-translational regulation of the protein levels. Using a cell line that displays no detectable annexin II and p11 proteins, we demonstrated that overexpression of annexin II provoked an increase of p11 levels due to the stabilization of the protein by complex formation. Therefore, the presence of a stable p11 protein requires the presence of annexin II. This could explain why p11 protein always appears to be present in the tetrameric form. Our observations also suggest that the main role of p11 is to regulate the annexin II functions.

Taken together, the results suggest that two different processes (transcriptional and post-translational) are required to regulate levels of annexin II and p11. A transcriptional regulation was described in different cell types such as virus-transformed rat culture cell lines [33], human leukaemias [34], and PC12 pheochromocytoma cells where both annexin II and p11 transcription are induced after nerve growth factor (NGF)-induced differentiation [35]. This co-regulation could occur through similar sequence elements, such as the βDRE (β-globin direct-repeat element) motif found in both annexin II and p11 promoter regions [36–38]. The increase of p11 expression would favour the formation of the calpactin I complex. This form of annexin II, which has higher affinity to Ca^{2+} but is not susceptible to phosphorylation by $pp60^{v\text{-}src}$ and serine threonine kinases, can associate with the membrane skeleton and may be directly involved in cellular differentiation and/or membrane-related processes. Moreover, a post-translational regulation may be involved to avoid the release of free monomeric p36 in different intracellular compartments. Stabilization of the constituent polypeptides of multisubunit protein complexes was described in different models including spectrin, immunoglobulins or acetylcholine receptor [39–42]. Interestingly, in these cases, transcription of the different constituents does not appear to be co-regulated. Thus, the stoichiometry of the complex is only determined through the binding of proteins to each other and not by the relative molar ratios of the components synthesized. Consequently, one component is limiting and other polypeptides are in excess. Such a mechanism, in the absence of a post-transcriptional regulation, can only be used in the case of inactive or very unstable monomers. For the calpactin I complex the existence of an excess of one component could dysregulate DNA replication processes either by increasing or decreasing monomeric annexin II. It is noteworthy that the monomeric subunits of spectrin turnover with a half-life of 50 min, as compared with 15 h for annexin II [23]. It is then conceivable that the cell regulates, initially, the equilibrium between annexin II and p11 by a transcriptional monitoring. Secondly, to avoid release of monomeric p36 from the calpactin I complex, a reciprocal stabilization is required. The regulation of annexin II and p11 levels by both transcriptional and post-translational mechanisms is a good example of the tight regulation of functions of a multisubunit protein complex without affecting processes involving monomers.

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