# Control of the nuclear-cytoplasmic partitioning of annexin II by a nuclear export signal and by p11 binding

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## SUMMARY

**This study investigated mechanisms controlling the nuclear-cytoplasmic partitioning of annexin II (AnxII). AnxII and its ligand, p11, were localized by immunofluorescence to the cytoplasmic compartment of U1242MG cells, with minimal AnxII or p11 detected within nuclei. Similarly, GFP-AnxII and GFP-p11 chimeras localized to the endogenous proteins. Likewise, GFP-AnxII(1-22) was excluded from nuclei, whereas GFP-AnxII(23-338) and GFP alone were distributed throughout the cells. Immunoprecipitation and biochemical studies showed that GFP-AnxII did not form heteromeric complexes with endogenous p11 and AnxII. Thus, the AnxII N-tail is necessary and sufficient to cause nuclear exclusion of the GFP fusion protein but this does not involve p11 binding. A nuclear export signal consensus sequence was found in the AnxII 3-12 region. The consensus mutant GFP-AnxII(L10A/L12A) confirmed that these residues are necessary for nuclear exclusion. The nuclear exclusion of GFP-AnxII(1-22) was temperature-dependent and reversible, and the nuclear export inhibitor leptomycin B (LmB) caused GFP-AnxII or overexpressed AnxII monomer to accumulate in nuclei. Therefore, AnxII monomer can enter the nucleus and is actively exported. However, LmB had little effect on the localization of AnxII/p11 complex in U1242MG cells, indicating that the complex is sequestered in the cytoplasm. By contrast, LmB treatment of *v-src*-transformed fibroblasts caused endogenous AnxII to accumulate in nuclei. The LmB-induced nuclear accumulation of AnxII was accelerated by pervanadate and inhibited by genistein, suggesting that phosphorylation promotes nuclear entry of AnxII. Thus, nuclear exclusion of AnxII results from nuclear export of the monomer and sequestration of AnxII/p11 complex, and may be modulated by phosphorylation.**

Key words: Astrocytoma, Leptomycin B, Phosphorylation, Genistein, S100A10

## INTRODUCTION

Annexins are a family of cytosolic proteins that bind to membranes in the presence of $Ca^{2+}$ (reviewed by Gerke and Moss, 1997; Raynal and Pollard, 1994). Annexins are defined by a specific structural motif, the 'endonexin fold', contained within 4 or 8 repeat sequences that comprise the annexin 'core' and provide $Ca^{2+}$ binding sites that mediate $Ca^{2+}$-dependent binding to appropriate anionic substrates, particularly acidic phospholipids. Individual annexin proteins are distinguished primarily by their unique N-terminal extensions from the annexin core, which can contain binding regions for other proteins as well as phosphorylation sites, and thus confer functional and regulatory specificity. Annexin II (p36, calpactin I heavy chain, lipocortin II) is the best studied member of this family in this regard (reviewed by Gerke, 1989; Gerke and Moss, 1997; Raynal and Pollard, 1994; Waisman, 1995). Upon biochemical fractionation of cells and tissues using standard techniques, the majority of AnxII is tightly associated with dimers of the S100 protein p11 (S100A10, calpactin I light chain) forming an $AnxII_2/p11_2$ heterotetramer. The AnxII N-terminal residues 1-14 comprise the high-affinity binding site for p11 (Johnsson et al., 1988). The relative amounts of heterotetrameric versus monomeric AnxII are variable depending on the cell or tissue examined, from 100% heterotetrameric form in intestinal epithelium to about 50% AnxII monomer in cultured fibroblasts (Gerke, 1989; Zokas and Glenney, 1987). By contrast, endogenous p11 has never been purified from tissues or cells in the absence of AnxII, probably owing to metabolic instability of the unbound protein (Harder et al., 1993; Puisieux and Ozturk, 1996). Compared with AnxII monomer, the $AnxII_2/p11_2$ heterotetramer exhibits a markedly reduced $Ca^{2+}$ requirement for binding to phospholipid vesicles and cellular membranes. AnxII residues Y23 and S25 are physiological phosphorylation sites for src and protein kinase C, respectively and probably represent sites phosphorylated directly or indirectly after activation of certain other membrane-associated kinases, such as receptors for insulin (Biener et al., 1996), insulin-like growth factor 1 (Jiang et al., 1996), and platelet-derived growth factor (Brambilla

et al., 1991). The physiological relevance of AnxII phosphorylation is not yet understood, but in vitro phosphorylation of these sites inhibits the ability of the heterotetramer to aggregate vesicles and bundle f-actin (Waisman, 1995), and appears to decrease the affinity of $AnxII_2/p11_2$ for phospholipid binding (Powell and Glenney, 1987; Regnouf et al., 1995).

AnxII has been implicated in a variety of functional contexts, usually as a docking protein mediating the formation of membrane-based protein complexes. AnxII and the $AnxII_2/p11_2$ complex associate with plasma membranes, endosomes and exocytic vesicles, and may influence membrane-membrane or membrane-cytoskeletal interactions related to vesicular trafficking. Notably, AnxII is a prominent component of cholesterol-rich plasma membrane rafts, which also contain caveolins, src-related kinases, G-proteins and transmembrane receptors such as CD44. A recent study showed that a trans-dominant $AnxII_2/p11_2$ chimera affects CD44 clustering and the structure of the associated actin cytoskeleton, suggesting a role for AnxII in the supramolecular organization of signal transduction-related components (Oliferenko et al., 1999). In addition, p11 has been shown to associate with and affect the activities of other signaling molecules such as $cPLA_2$ (Wu et al., 1997), the Bcl2-family protein BAD (Hsu et al., 1997) and the cdc2 kinase-related PCTAIRE1 (Sladeczek et al., 1997), although whether these interactions also involve the $AnxII_2/p11_2$ complex remains to be investigated. AnxII and/or $AnxII_2/p11_2$ also appear to be present on the extracellular surface of some cell types such as endothelial cells and certain tumor cells where they can act as a receptor for plasminogen/tissue plasminogen activator (Hajjar et al., 1994; Kassam et al., 1998; Menell et al., 1999) or tenascin-C (Chung and Erickson, 1994). Finally, a function of AnxII in the nucleus that seems not to involve binding to p11 or membranes has been suggested by its purification as part of a primer recognition protein complex that enhances DNA polymerase α activity in vitro (Jindal et al., 1991). Further evidence that AnxII might play a role in promoting DNA synthesis and cell proliferation was provided by subsequent immunodepletion/reconstitution experiments in *Xenopus* oocyte nuclear extracts (Vishwanatha and Kumble, 1993) and by antisense strategies in mammalian cell lines (Chiang et al., 1999; Kumble et al., 1992). A link between AnxII and cell transformation and neoplasia was first suggested by the identification of AnxII as a major v-src phosphorylation substrate in transformed fibroblasts (Erickson and Erickson, 1980; Radke and Martin, 1979). Subsequently, AnxII expression has been found to be upregulated in several types of spontaneous neoplasms, such as pancreatic carcinoma (Vishwanatha et al., 1993), acute promyelocytic leukemia (Menell et al., 1999) and high-grade glioma (Reeves et al., 1992); in fibroblasts transformed by viral oncogenes such as v-H-ras, v-src and v-mos (Ozaki and Sakiyama, 1993); and in cells treated with mitogenic or trophic factors such EGF, FGF, NGF or TGFβ1 (Fox et al., 1991; Keutzer and Hirshhorn, 1990; Munz et al., 1997). Although AnxII and p11 are often coexpressed in cells and tissues, their relative levels vary depending on the source (Gerke, 1989; Zokas and Glenney, 1987) and detailed studies have revealed differences in AnxII and p11 expression and localization patterns within particular tissue cell types such as fibroblasts and skin keratinocytes, suggesting that AnxII monomer and $AnxII_2/p11_2$ complex may have distinct and different functions (Munz et al., 1997; Zokas and Glenney, 1987).

Overall, the available evidence suggests that AnxII and p11 are docking proteins with multiple functions, whose specific roles may depend on the cell types in which they are expressed, their localization within or outside of cells, and interactions with particular binding substrates in these locations. However, the suggestions that AnxII has functions in the nucleus or at the cell surface have been controversial since, in most cases, very little of total cellular AnxII is in these locations. Thus the detected protein could be argued to represent artefactual contaminant, and the mechanisms that might regulate its localization to these compartments are unclear. Since the functions of AnxII depend in part on its localization in cells, the present study investigated mechanisms controlling AnxII localization in neoplastic and transformed cell lines. Using molecular and pharmacologic approaches, we provide evidence that: (1) AnxII monomer readily enters the nucleus but is rapidly exported due to a functional nuclear export signal (NES) sequence that closely overlaps the p11-binding region in the AnxII N-terminus; (2) p11 binding to AnxII results in sequestration of the complex in the cytoplasmic compartment; and (3) manipulation of cellular phosphorylation can affect the nucleocytoplasmic partitioning of AnxII.

## MATERIALS AND METHODS

### Expression plasmids

5′ *Sal*I and 3′ *Bam*HI restriction sites were introduced into cDNAs encoding human AnxII and p11 (gifts of Volker Gerke, University of Munster, Germany) exactly as described previously for AnxVII (Creutz et al., 1992), and the cDNAs were ligated into these sites of pBluescript SK(+/–) (pBS; Stratagene, La Jolla, CA). 5′ *Nhe*I and 3′ *Sal*I sites were introduced into, and the termination codon removed from, hGFP(S65T) cDNA using PCR. The hGFP(S65T) and annexin cDNAs were sequentially ligated into the pCI-neo vector (Promega, Madison, WI), which had been previously modified by removing the original *Bam*HI site and introducing a new *Bam*HI site 3′ to the *Sal*I site in the multiple cloning region. The final cDNAs encoded fusion proteins with the linker: *Nhe*I-(hGFP[S65T])AAG GTC GAC ATG(AnxII/p11/AnxVII)-*Bam*HI.

A second GFP-AnxII expression vector, pEGFP-AnxII, was created by ligating the AnxII cDNA from pBS into the *Sal*I/*Xba*I sites in the multiple cloning region of pEGFP-C1 (Clontech Laboratories, Palo Alto, CA). To construct pEGFP-AnxII(1-22), pBS-AnxII was digested with *Nde*I and *Bam*HI, filled using Klenow fragment and religated, and digested with *Sal*I/*Xba*I to yield a fragment that was ligated into the *Sal*I/*Xba*I sites of pEGFP-C1. To construct pEGFP-AnxII (23-338), pBS-AnxII was digested with *Nde*I, filled using Klenow fragment, religated using a GGATCC linker to introduce a second *Bam*HI site, and digested with *Bam*HI to yield a fragment that was ligated into the *Bam*HI site of pEGFP-C1. pEGFP-AnxII(L10A/L12A) was generated by site-directed mutagenesis of pEGFP-AnxII using PCR.

### Cell lines and culture

U1242MG human astrocytoma cells (Kim-Lee et al., 1992) were provided by Alan Yates (Ohio State University, Columbus) and B31 RAT-1(v-src) transformed fibroblasts (Woodring and Garrison, 1997) by James Garrison (University of Virginia, Charlottesville). Cells were maintained in Minimal Essential Medium (MEM) supplemented with 10% fetal bovine serum and glutamine at 37°C in a 5% $CO_2$

atmosphere. Transfections were performed using Lipofectamine (Gibco BRL, Rockville, MD). Stably transfected cell populations were generated by culturing the cells in the presence of 600 μg/ml G418 for 3-4 weeks followed by fluorescence-activated cell sorting to isolate polyclonal populations of cells expressing GFP or fusion proteins. Leptomycin B (LmB) was the gift of Barbara Wolff (Novartis AG, Basel, Switzerland). Most of the experiments presented were repeated using two protocols. Either cells were rinsed with serum-free MEM, then incubated at 37°C with 100 nM LMB or 0.01% DMSO as vehicle in serum-free MEM for the indicated times; or cells were preincubated for 30 minutes with 100 nM LmB or vehicle in serum-free MEM, which was then replaced with MEM containing 10% FBS and either 200 nM LmB or vehicle, and the incubations continued for the indicated times. No significant effect of serum was seen on the localization of the examined proteins.

### Immunofluorescence

Cells cultured on poly-*d*-lysine-coated coverslips were briefly rinsed with PBS then fixed with either 4% formaldehyde in PBS or methanol for 15-30 minutes. The cells were rinsed, blocked with 10% bovine serum albumin (BSA) in PBS for 3 hours at room temperature (RT), and incubated overnight at 4°C with primary mouse monoclonal antibodies (mAbs) directed against AnxII, p11 or AnxIV (BD Transduction Laboratories, Lexington, KY) at 1:1000 dilution in PBS/3% BSA. The AnxII mAb reacted with methanol-fixed cells but not with formalin-fixed cells. The cells were then incubated with biotinylated anti-mouse (Vector Laboratories, Burlingame, CA) at 1:500 dilution in PBS/3% BSA for 2 hours at RT followed by avidin-rhodamine (Vector Laboratories) at 1:1000 dilution for methanol-fixed cells or 1:5000 dilution for formaldehyde-fixed cells. Epifluorescence microscopy was performed using a Nikon microscope equipped with an Olympus camera and Kodak ASA 400 film. Laser scanning confocal microscopy (LSCM) was performed using a Zeiss LSM 410. Cells were imaged from top to bottom in the *Z*-plane; images from the midplane of the cells were captured and stored as digital images that are shown in the figures.

### Immunoprecipitation

Stably transfected cells expressing GFP, GFP-AnxII, or GFP-p11 were scraped into ice-cold lysis buffer containing 100 mM NaCl, 16 mM Hepes pH 7.0, 0.5% Triton X-100, 2 mM EGTA, 1 mM DTT, 100 uM PMSF, 12 μg/ml leupeptin and 12 μg/ml aprotinin, and lysed with 10 strokes in a Dounce homogenizer. The lysates were centrifuged at 20,000 ***g*** for 15 minutes. The supernatants were incubated overnight with 4 μg/ml anti-GFP polyclonal antibody (Clontech), which was then precipitated using protein A-sepharose beads (Affi-Gel; Bio-Rad Laboratories, Hercules, CA). The beads were boiled in Laemmli sample buffer, which was then subjected to SDS-PAGE using 10% or 15% acrylamide gels and electrotransferred to nitrocellulose or PVDF membranes. Western blot analysis was performed using anti-AnxII or anti-p11 mAbs at 1:5000 dilution, followed by sheep anti-mouse antibody conjugated to horseradish peroxidase (Amersham Pharmacia Biotech, Piscataway, NJ) at 1:1000 dilution, with visualization by enhanced chemiluminescence.

### Triton X-100 extraction

Stably transfected cells expressing GFP-AnxII or GFP-p11 were scraped into ice-cold extraction buffer containing 150 mM NaCl, 20 mM Hepes pH 7.0, 0.2% Triton X-100, 2 mM $MgCl_2$, 1 mM DTT, 200 μM PMSF, 25 μg/ml leupeptin and 15 μg/ml aprotinin, and lysed with 10 strokes in a Dounce homogenizer. $Ca^{2+}$/EGTA buffers were added from 10× stocks in 20 mM Hepes pH 7.0 to give a final $pCa^{2+}$ of 8.17, 6.92, 5.91, 5.02, 4.065 and 3.046 as measured by a $Ca^{2+}$ electrode. The lysates were centrifuged at 20,000 ***g*** for 30 minutes. Laemmli sample buffer was added to the supernatant and pellets to give equal volumes of 1× buffer. The boiled samples were subjected to SDS-PAGE using 12% gels and electrotransferred to nitrocellulose membranes. Western blot analysis was performed using anti-AnxII mAb at 1:5000 dilution or anti-GFP polyclonal antibody (Clontech) at 1:1000 dilution, followed by anti-mouse or anti-rabbit antibodies conjugated to horseradish peroxidase, with visualization by enhanced chemiluminescence.

## RESULTS

### Localization of AnxII, p11, and GFP-AnxII or –p11 fusion proteins in U1242MG cells

The AnxII and p11 mAbs each recognized a single band of appropriate size on western blots of U1242MG cell lysates (data not shown). The localizations of AnxII and GFP fusion proteins are shown in Fig. 1. Indirect immunofluorescence demonstrated a diffuse distribution of AnxII signal throughout

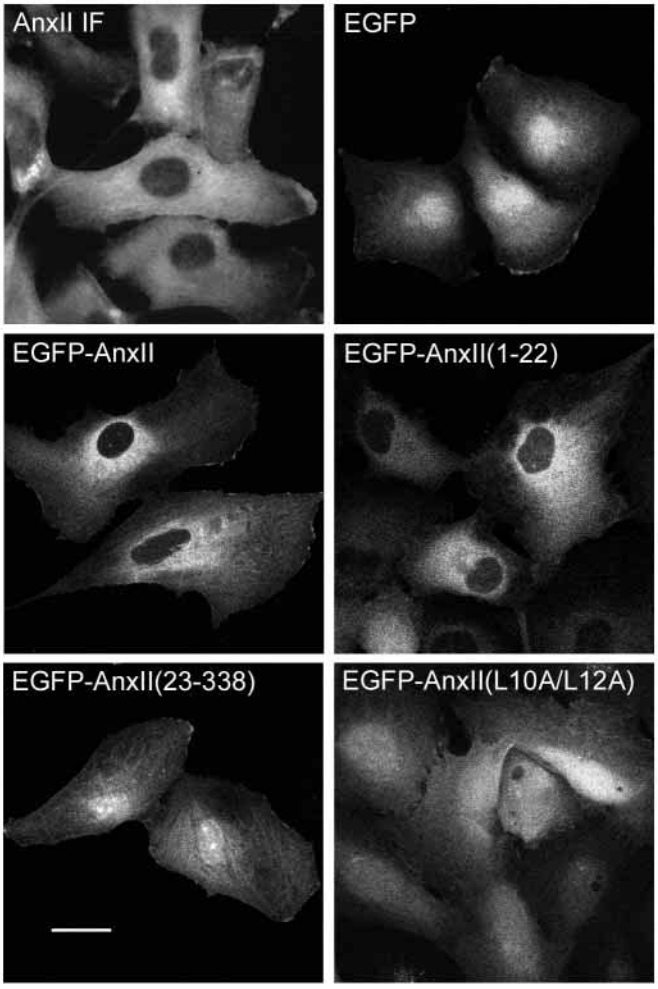


**Fig. 1.** Localization of AnxII and GFP-AnxII fusion proteins in U1242MG cells. Endogenous AnxII was visualized by indirect immunofluorescence and imaged using epifluorescence photomicrography (*AnxII IF*). Stably transfected cells expressing EGFP, EGFP-AnxII, EGFP-AnxII(1-22), EGFP-AnxII(23-338) or EGFP-AnxII(L10A/L12A) were fixed and imaged using LSCM. The association of EGFP-AnxII(23-338) with nucleoli is an artifact of fixation, apparently due to residual $Ca^{2+}$ from the wash buffer. The construct is excluded from nucleoli in living cells (not shown). Bar, 20 μm.

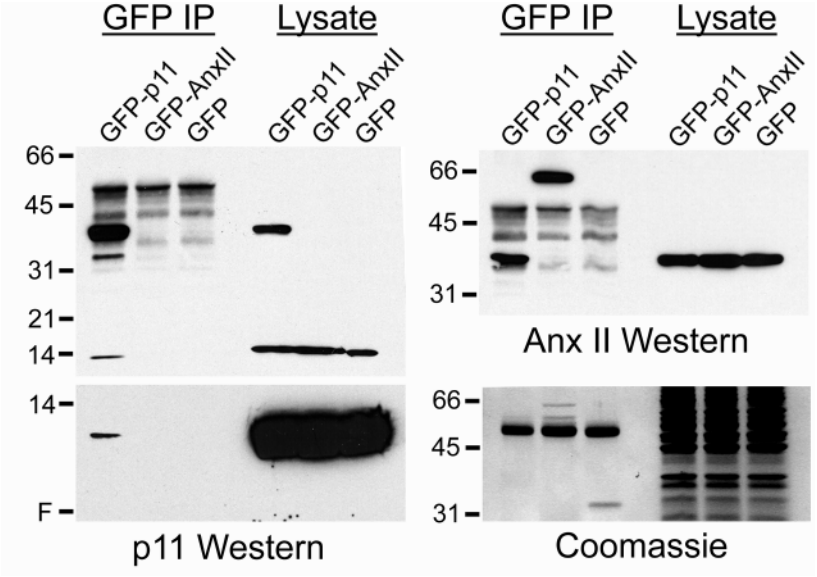


**Fig. 2.** Immunoprecipitation of GFP fusion protein complexes. Anti-GFP immunoprecipitation was performed in lysates of stably transfected U1242MG cells expressing EGFP-p11, EGFP-AnxII or EGFP. (Top-left) Western blot for p11; 10% gel transferred to nitrocellulose. The p11 mAb recognized both endogenous p11 and EGFP-p11 in the lysates. Co-immunoprecipitation of p11 was seen only with GFP-p11. (Bottom-left) The same experiment as above; 15% gel transferred to PVDF in buffer containing 30% methanol. This procedure allows separation of p11 from the dye front and maximizes transfer of p11 to the membrane. (Top-right) Western blot for AnxII; 10% gel transferred to nitrocellulose. The AnxII mAb detects EGFP-AnxII only in the immunoprecipitates, since its apparent level in the lysates is much lower than endogenous AnxII. Co-immunoprecipitation of AnxII was seen only with EGFP-p11. (Bottom-right) Coomassie Blue stained gel shows distinct bands corresponding to EGFP and EGFP-AnxII in the immunprecipitates, whereas the much fainter EGFP-p11 reproduced poorly.

the cytoplasm of U1242MG cells, with relatively little AnxII immunofluorescence detected within nuclei. AnxII immunofluorescence appeared to be increased at ruffling edges of lamellopodia in subconfluent cultures, and at cell-cell contacts in confluent cultures. The observed distribution of AnxII immunofluorescence closely resembled the patterns previously described in other cultured cells such as fibroblasts (Courtneidge et al., 1983; Nigg et al., 1983). p11 immunofluorescence in U1242MG cells showed a pattern similar to that of AnxII (see below). Likewise, GFP-AnxII and GFP-p11 in transfected cells were localized to the cytoplasmic compartment and largely excluded from nuclei. Distinct intranuclear foci of GFP-AnxII were often evident that were found to co-localize with concanavolin A binding and thus probably represent cytoplasmic invaginations (Fricker et al., 1997). By contrast, GFP alone was distributed diffusely throughout both the nuclear and cytoplasmic compartments. The annexin II N-terminal construct, GFP-AnxII(1-22), was localized to the cytoplasm and excluded from nuclei similarly to GFP-AnxII, whereas the core construct, GFP-AnxII(23-338), showed diffuse nuclear and cytoplasmic distribution such as that seen with GFP alone. Thus, the N-terminal 1-22 region of AnxII is both necessary and sufficient to direct the exclusion of the GFP fusion constructs from the nucleus. Furthermore, specific residues required for nuclear exclusion were identified by the GFP-AnxII(L10/L12A) construct, which was localized to both nuclear and cytoplasmic compartments. The nuclear versus cytoplasmic distributions of the various constructs were consistent in nearly all cells and were the same in transiently transfected cells and stably transfected cells; the occasional cells showing GFP-AnxII within nuclei also showed condensation of nuclear DAPI staining indicating M-phase or dying cells, presumably with loss of nuclear envelope integrity (data not shown).

Additional evidence that nuclear exclusion of AnxII and GFP-AnxII is a specific function of its unique N-tail was provided by the localizations of AnxIV and GFP-AnxVII (data not shown). The AnxIV mAb recognized a single 34 kDa band in western blots of U1242MG cell lysates, and by indirect IF yielded roughly similar cytoplasmic and nuclear signal intensities, with exclusion from nucleoli. GFP fused to 47 kDa AnxVII showed a diffuse distribution throughout cytoplasm and nucleus in all transfected U1242MG cells, similar to GFP alone.

### IP complex formation

Since the 1-22 region of AnxII contains the p11 binding region and substitutions at residues L10 and L12 have been shown to affect p11 binding (Becker et al., 1990), the above observations suggest that exclusion of GFP-AnxII from the nucleus involves formation of the GFP-AnxII/p11 complex. However, peptide binding data (Becker et al., 1990) and crystallographic structure of p11/AnxII N-tail complex (Rety et al., 1999) predict that fusion of GFP to the AnxII N-terminus would inhibit p11 binding. Therefore, the abilities of GFP-AnxII and GFP-p11 expressed in stably transfected U1242MG cells to form multimeric complexes with endogenous AnxII and p11 were examined by anti-GFP immunoprecipitation analyses of transfected cell lysates. As shown in Fig. 2, endogenous AnxII and p11 co-immunoprecipitated with GFP-p11 but not with GFP-AnxII or GFP alone. In the Coomassie Blue stains, GFP-AnxII and GFP are easily seen, whereas GFP-p11 could be

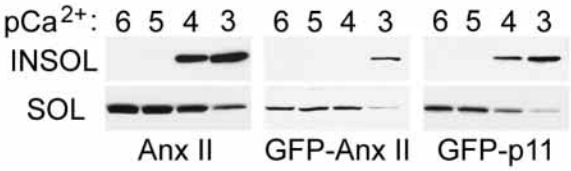


**Fig. 3.** $Ca^{2+}$-dependency of GFP-AnxII and GFP-p11 binding to Triton X-100-insoluble cell components. U1242 cells expressing GFP-AnxII or GFP-p11 were extracted with Triton X-100 in the presence of various [$Ca^{2+}$] as described in Materials and Methods. The soluble and insoluble fractions were subjected to western blot analysis using antibodies against GFP or AnxII. The $Ca^{2+}$-dependency of endogenous AnxII binding to the insoluble fraction was the same in both cell populations.

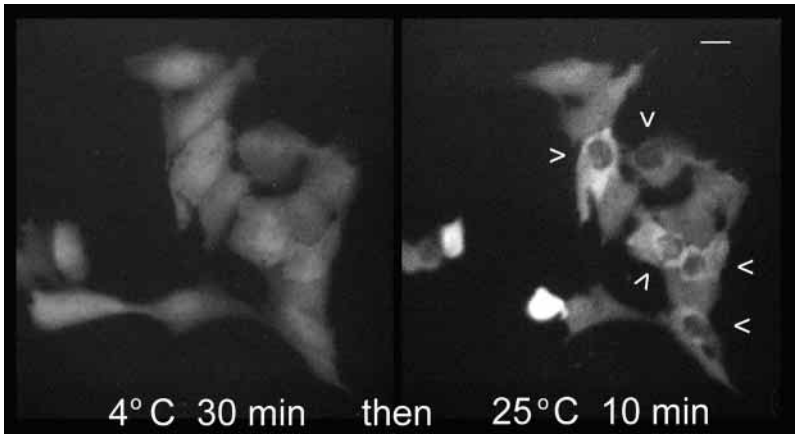


**Fig. 4.** Temperature dependency of GFP-AnxII(1-22) localization. U1242MG cells expressing GFP-AnxII(1-22) were cooled at 4°C for 30 minutes, then photographed immediately after placement on the microscope stage at RT (left) and again after 10 minutes at RT (right). Warming caused some cells to round up (bottom-left of panels). Bar, 20 μm.

identified as a faint band in the original gels but is not apparent in reproductions. Therefore, the inability to detect co-immunoprecipitation of p11 and AnxII with GFP-AnxII or GFP does not merely reflect lower levels of expression of the latter proteins versus GFP-p11 in the transfected cells. These data indicate that GFP-AnxII does not bind p11 and remains monomeric when expressed in cells, and thus p11 binding is unlikely to underlie the nuclear exclusion of GFP-AnxII. By contrast, GFP-p11 can participate in $AnxII_2/p11_2$ complex formation.

### $Ca^{2+}$-dependency of GFP-AnxII and GFP-p11 binding to Triton X-100-insoluble cell components

Formation of the $AnxII_2/p11_2$ complex decreases the $Ca^{2+}$ requirements for AnxII binding to Triton X (TX)-100-insoluble cell components, so that extraction of cells with TX-100 at 0.5 mM $Ca^{2+}$ solubilizes monomeric AnxII and leaves $AnxII_2/p11_2$ bound to membrane-associated structures (Thiel et al., 1992; Zokas and Glenney, 1987). These TX-100-insoluble structures may represent cytoskeletal proteins or cholesterol-rich rafts. p11 in the absence of AnxII does not exhibit $Ca^{2+}$-dependent binding. The $Ca^{2+}$ dependencies for GFP-AnxII and GFP-p11 to associate with the TX-100-insoluble fraction in transfected cells were examined and compared with that of endogenous AnxII (Fig. 3). Both GFP-p11 and endogenous AnxII associated with the TX-100 insoluble fraction at 100 μM $Ca^{2+}$, whereas GFP-AnxII was not detected in this fraction below 1 mM $Ca^{2+}$. Thus, in this biochemical assay, GFP-AnxII behaves as a monomer, whereas GFP-p11 and endogenous AnxII appear to be largely in complexed forms in U1242 cells, consistent with the immunoprecipitation experiments. It must be emphasized that TX-100 extraction removes the phospholipids that would bind AnxII in intact cells, so this is a nonphysiological assay that is designed to show differences in the binding characteristics of monomeric versus complexed AnxII but that does not accurately reflect the $Ca^{2+}$ concentrations required for membrane binding in intact cells. The $Ca^{2+}$ concentrations necessary to effect binding in this preparation are consistent with the concentrations needed for binding of AnxII to cytoskeletal proteins such as actin and fodrin in vitro (Gerke and Weber, 1985), which are much higher than the $Ca^{2+}$ concentrations needed for binding to phospholipid vesicles or intact cell membranes (Powell and Glenney, 1987; Raynal and Pollard, 1994).

### The AnxII N-terminus contains a consensus sequence for nuclear export

Since GFP-AnxII does not bind p11, the AnxII N-terminal sequence was examined for other motifs that could underlie its exclusion from the nucleus. The region 3VHEILCKLSLE13 conforms to consensus requirements for a leucine-rich nuclear export signal (NES; Henderson and Eleftheriou, 2000; Hope, 1997) (Table 1). The observed loss of nuclear exclusion of the GFP-AnxII(L10/L12A) mutant in the present study is consistent with the inhibition of nuclear export of other proteins when NES residues in the corresponding consensus positions were substituted with alanine.

### Nuclear exclusion of GFP-AnxII(1-22) is temperature-dependent

When cells expressing GFP-AnxII(1-22) were cooled to 4°C for 30 minutes, the chimera was no longer excluded from the

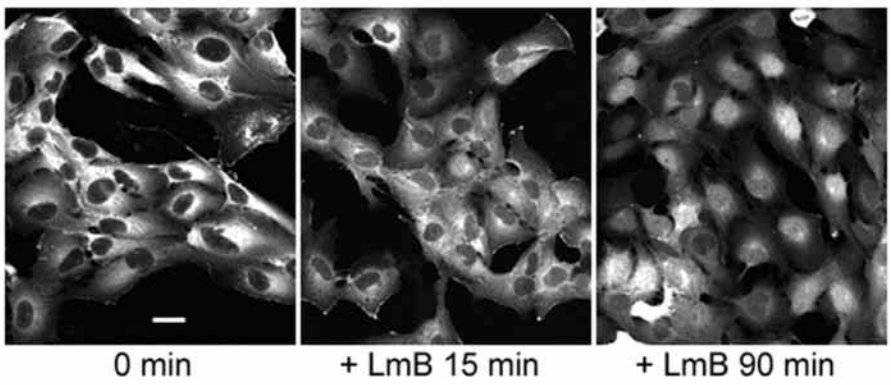


**Fig. 5.** GFP-AnxII localization over time after LmB treatment. U1242MG cells expressing GFP-AnxII were treated with LmB for various periods of time before fixation and imaging by LSCM. Nucleic acids were stained with propidium iodide to confirm nuclear localization (not shown). Bar, 20 μm.

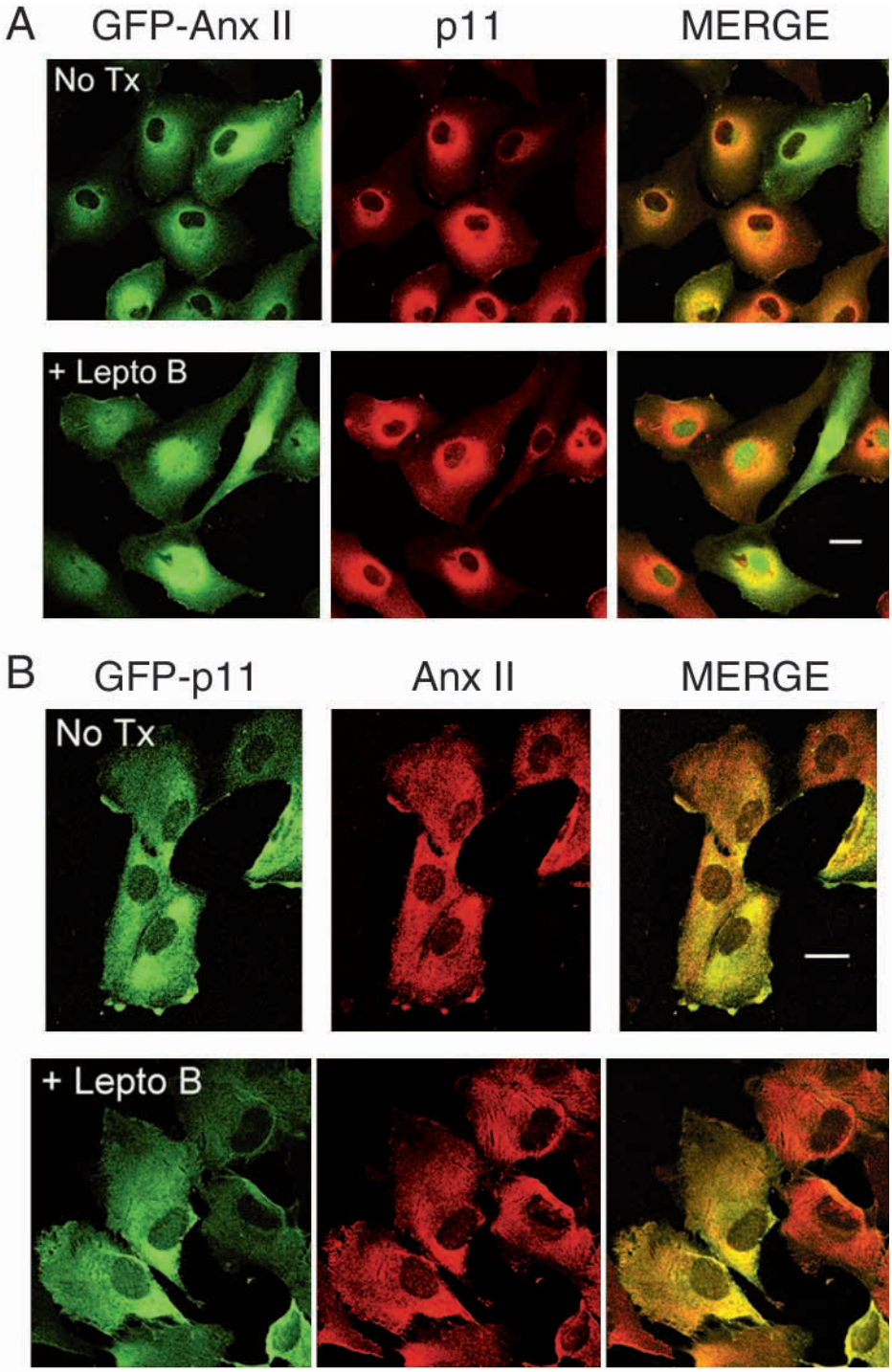


**Fig. 6.** Effect of LmB on localizations of GFP-AnxII, GFP-p11, AnxII and p11. Stably transfected U1242MG expressing GFP-AnxII (A) or GFP-p11 (B) were treated with LmB for 90 minutes, fixed, processed for indirect immunofluorescence using mAbs against p11 (A) or AnxII (B), and imaged by LSCM. Bars, 20 μm.

nuclei (Fig. 4). Within 5-10 minutes of being allowed to rewarm to room temperature, GFP-AnxII(1-22) was re-excluded from the nuclei of many cells. This reversible temperature-dependent nuclear exclusion is consistent with an active nuclear export process.

### Nuclear exclusion of GFP-AnxII is sensitive to leptomycin B

Leptomycin B (LmB) inhibits Ran/exportin-1-mediated nuclear export by blocking formation of the export complex with the cargo protein (Fornerod et al., 1997; Fukuda et al., 1997; Kudo et al., 1999; Ossareh-Nazari et al., 1997). Incubating cells expressing GFP-AnxII with LmB caused the chimera to accumulate within nuclei. This was noticeable by 15 minutes of LmB treatment, and nuclear signal was equal to that in adjacent cytoplasm in most cells by 90 minutes of treatment (Fig. 5). Thus, in untreated cells, GFP-AnxII is continuously entering the nucleus but its presence in this compartment is not obvious because it is rapidly exported, probably via a Ran/exportin-1-dependent mechanism.

### Nuclear exclusion of GFP-p11 and endogenous AnxII and p11 are not sensitive to leptomycin B in U1242MG cells

The effect of LmB on the localizations of GFP-AnxII, GFP-p11, and endogenous AnxII and p11 were examined by treating stably transfected cells for 2 hours with LmB or vehicle. GFP-AnxII transfectants were then processed for p11 indirect IF and co-localization studies, and GFP-p11 transfectants for AnxII IF and co-localization (Fig. 6). In contrast to its effect on GFP-AnxII localization, LmB treatment did not obviously promote the accumulation of GFP-p11 or of AnxII or p11 IF within nuclei. In other experiments, continuing the LmB treatment for up to 12 hours still had little effect on the localizations of GFP-p11, AnxII or p11. These observations are consistent with the biochemical evidence that GFP-p11, AnxII and p11 in the stably transfected cells are associated with each other but not with GFP-AnxII, and indicate that $AnxII_2/p11_2$ complexes are sequestered in the cytoplasmic compartment and do not enter the nucleus, whereas the GFP-AnxII monomer can traffic between the nucleus and the cytoplasm.

### Nuclear exclusion of overexpressed AnxII monomer in U1242MG cells is sensitive to leptomycin B

To determine whether nucleocytoplasmic trafficking is a bona fide property of AnxII monomer or an artifact of the GFP fusion protein, the effect of LmB on the localization of overexpressed wild-type AnxII was examined by indirect immunofluorescence in transiently transfected U1242MG cells (Fig. 7). The

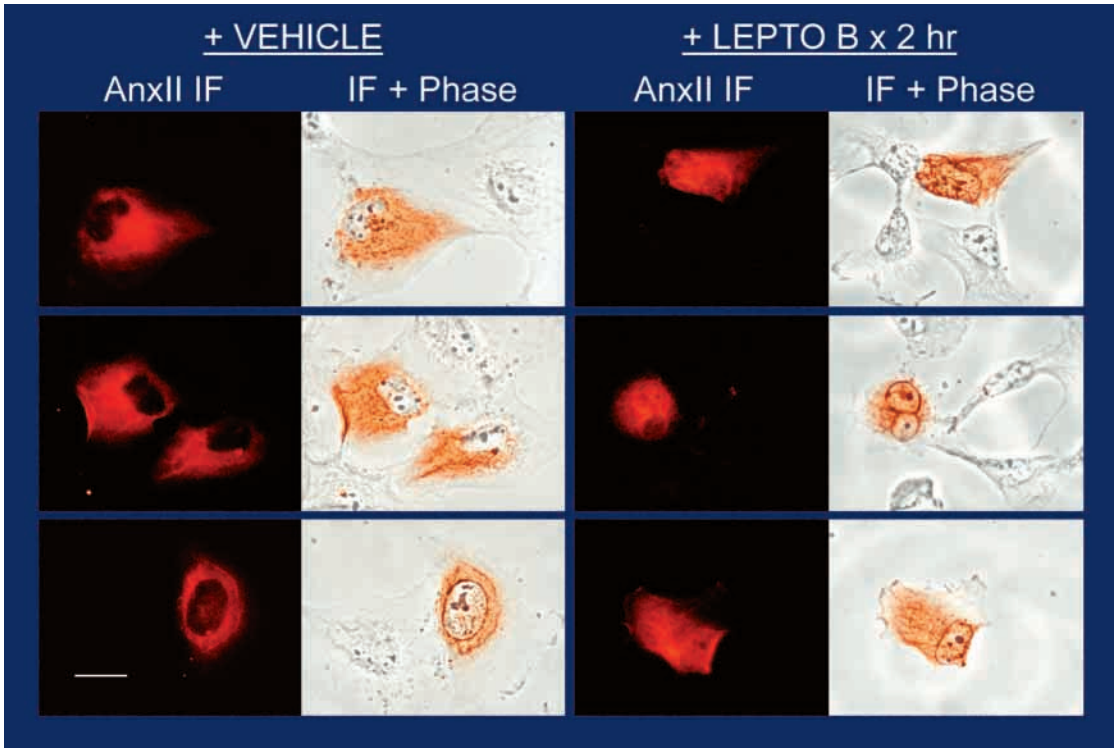


**Fig. 7.** Effect of LmB on the localization of transiently overexpressed AnxII. U1242MG were transiently cotransfected with pCI-neo-AnxII and pEGFP. Two days after transfection, the cells were incubated with 100 nM LmB or vehicle for 90 minutes, then fixed and processed for indirect immunofluorescence using mAb against AnxII. Bar, 20 μm.

transfected cells displayed a much brighter AnxII IF signal than did adjacent nontransfected cells, so that photographic and digital images of the latter could not be acquired without grossly overexposing transfected cells in the same field. The identities of the transfected cells were confirmed by cotransfection with a GFP expression vector. The overexpressed AnxII was excluded from cell nuclei in vehicle-treated cultures, but 90 minutes of treatment with LmB led to accumulation of the overexpressed protein in nuclei, while having little effect on endogenous AnxII in nontransfected cells.

### Nuclear exclusion of endogenous AnxII in v-src-transformed RAT-1 fibroblasts is sensitive to leptomycin B and can be modulated by genistein and pervanadate

AnxII has been identified as a major substrate for v-src in transformed fibroblasts and contains a single tyrosine phosphorylation site at Y23 in the N-tail. However, the consequences of tyrosine phosphorylation on the cellular biology of AnxII are poorly understood (reviewed by Gerke, 1989; Gerke and Moss, 1997; Raynal and Pollard, 1994; Waisman, 1995). To assess whether tyrosine phosphorylation affects nucleocytoplasmic trafficking of AnxII, the effects of LmB, the tyrosine kinase inhibitor genistein, and the tyrosine phosphatase inhibitor pervanadate on the localization of endogenous AnxII IF were examined in v-src-transformed RAT-1 fibroblasts (Fig. 8). The AnxII mAb recognized a single band in western blots of RAT-1(v-src) cell lysates (data not shown), and showed a cytoplasmic IF pattern and relatively little IF signal within nuclei similar to that seen in U1242MG cells and in previous studies of v-src transformed fibroblasts. Treatment of RAT-1(v-src) cells with LmB resulted in increased accumulation of AnxII IF within nuclei, with equilibration of the nuclear and cytoplasmic signal intensities

**Table 1. Nuclear export signal sequences***

| Protein | Sequence | References |
|---|---|---|
| AnxII (1-13) | S T V H E I L C K **L** S **L** E | |
| HIV-1 Rev | L Q L P P **L** E R **L** T **L** D | Malim et al., 1991 |
| MEK-1 (32-43) | A **L** Q K K **L** E E **L** E **L** D | Fukuda et al., 1996 |
| PKIα (36-47) | E **L** A L K **L** A G **L** D **I** N | Wen et al., 1995 |
| IκBα (265-275) | I Q Q Q L G Q **L** T **L** E | Arenzana-Seisdedos et al., 1997 |
| RanBP1 (178-189) | K V A E K **L** E A **L** S **V** R | Zolotukhin and Felber, 1997 |
| NES consensus | L L L x L x x L x L | |

*The NES consensus sequence in human AnxII is shown aligned with those of other proteins known to be exported from the nucleus via these sequences. The consensus sequence for a 'leucine-rich' NES is L-$X_{1-4}$-L-$X_2$-L-X-L, where L is usually Leu, Ile or Val, although other large hydrophobic residues can substitute for these (Henderson and Eleftheriou, 2000; Hope, 1997). In bold type are residues that have been directly shown to contribute to nuclear export or nuclear exclusion in the present and previous studies. The underlined residues in the AnxII sequence have been shown to be critical for p11 binding (Becker et al., 1990).

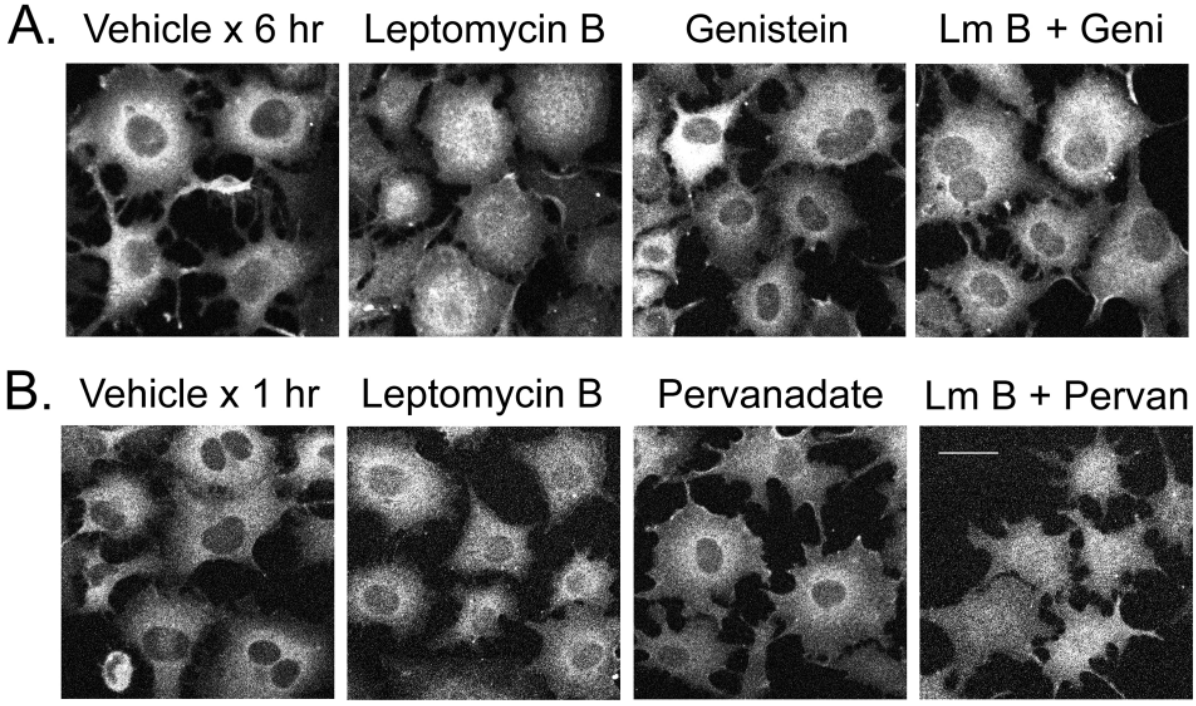


**Fig. 8.** Effect of LmB, genistein and pervanadate on AnxII localization in RAT-1(v-src) cells. In (A), cells were incubated for 6 hours in the absence or presence of 100 nM LmB, 100 μM genistein, or vehicle. In (B), cells were incubated for 1 hour in the absence or presence of 100 nM LmB, 3 μM Na pervanadate, or vehicle. Cells were then fixed, processed for indirect immunofluorescence using mAb against AnxII, and imaged by LSCM. Bar, 20 μm.

in most cells after 5-6 hours of treatment. Genistein treatment enhanced the distinction of AnxII IF intensities between the nucleus and cytoplasm in the absence of LmB, and markedly delayed the LmB-induced accumulation of AnxII IF in the nucleus. Pervanadate treatment had the opposite effect, alone causing a somewhat increased level of AnxII in nuclei and, together with LmB, allowing equilibration of nuclear and cytoplasmic signal intensities after 1 hour of treatment (Fig. 8). Since the concentration of LmB (100-200 nM) used in these experiments should maximally inhibit nuclear export (Fornerod et al., 1997; Ossareh-Nazari et al., 1997), these observations suggest that promoting tyrosine phosphorylation increases the rate of nuclear entry of AnxII.

The effect of LmB on the localizations of p11 versus AnxII were compared using stably transfected RAT-1(v-src) cells expressing GFP-p11. LmB caused a preferential accumulation of AnxII IF within nuclei (Fig. 9). The amount of GFP-p11 in nuclei sometimes also appeared to be somewhat increased, although this was variable between cells. The observations indicate that AnxII monomer enters the nucleus more readily than AnxII/(GFP)p11 heterotetramers or (GFP)p11 dimers but do not distinguish whether AnxII exists in separate monomer and heterotetramer pools, or whether AnxII/p11 complexes undergo dissociation, releasing AnxII. The p11 mAb does not recognize rat p11 and therefore the expression level and localization of endogenous p11 could not be assessed.

## DISCUSSION

Studies of the functional localization of annexins have often focused on the associations of these proteins with membrane/cytoskeletal structures in the cytoplasmic compartment but some annexins are also present within nuclei in cultured cells and tissues, including AnxI, IV, V and XI (Barwise and Walker, 1996a; Katoh et al., 1995; Mizutani et al., 1992; Raynal et al., 1992). The nuclear localization of AnxXI is mediated by its N-terminal region (Mizutani et al., 1995), which also contains a binding site for the S100 protein calcyclin, but none of the annexins contain a typical nuclear localization signal. The nuclear localization of certain annexins appears to be actively regulated. For example, serum stimulation of osteosarcoma cells and fibroblasts promotes AnxV accumulation in the nucleus (Barwise and Walker, 1996b; Mohiti et al., 1997), and the nuclear localization of AnxXI is regulated during development (Mamiya et al., 1994). The functions of these annexins in the nucleus are not understood. By contrast, AnxII shows a primarily cytoplasmic localization with low levels if any being detected in nuclei (Barwise and Walker, 1996a; Katoh et al., 1995; Raynal et al., 1992). Therefore, the suggestion that AnxII has a nuclear function has been slow in gaining acceptance. Although AnxII was reported to be detectable in purified nuclear fractions and by immunolabeling of nuclei (Arrigo et al., 1983; Vishwanatha et al., 1992), the actual physiological relevance of the minor fraction of cellular AnxII present in these preparations has remained debatable. However, AnxII immunoreactivity is increased within the nuclei of cholesteatoma keratinocytes compared with normal keratinocytes (Kim et al., 1998), providing evidence for an association of nuclear AnxII with a hyperproliferative state in vivo. In the present study, we found that AnxII contains an apparent NES sequence that overlaps

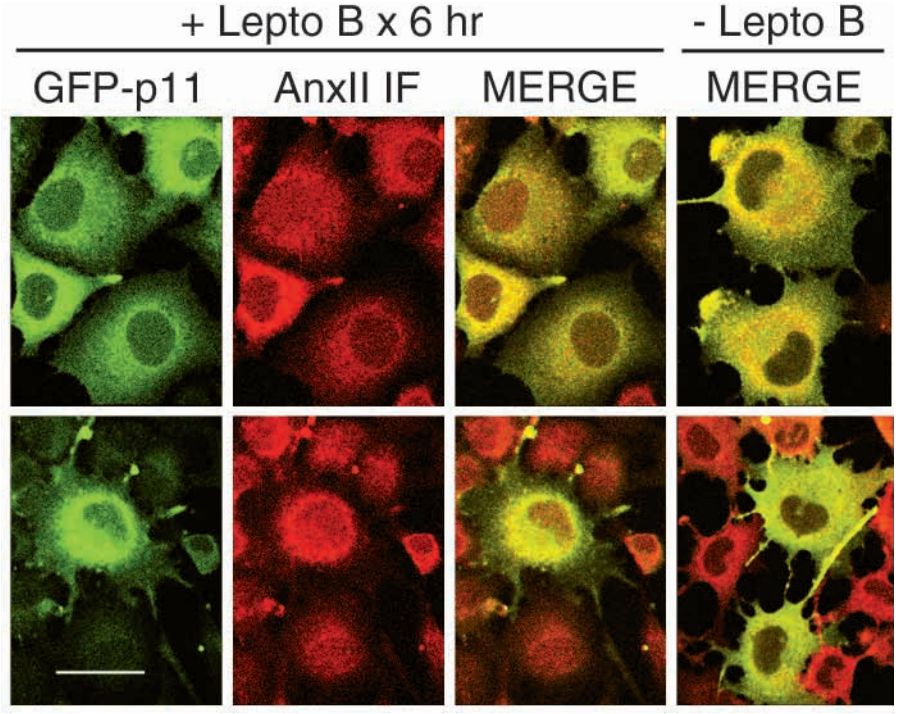


**Fig. 9.** Effect of LmB on co-localization of AnxII and GFP-p11 in RAT-1(v-src) cells. Stably transfected cells expressing GFP-p11 were treated for 5 hours with 100 nM LmB or vehicle, then fixed, processed for indirect immunofluorescence using mAb against AnxII, and imaged by LSCM. Bar, 20 μm.

the p11 binding site and, using GFP fusion constructs that disallow p11 binding to this region, showed that this sequence contributes to the temperature-dependent exclusion of the protein from the nuclei of U1242 cells. Furthermore, treatment with LmB to inhibit the Ran/exportin-mediated export pathway caused overexpressed wild-type AnxII monomer, as well as GFP-AnxII, to accumulate in the nuclei of these cells. LmB treatment of v-src-transformed fibroblasts led to increased accumulation of endogenous AnxII within their nuclei. These data indicate that AnxII monomer can readily enter the nucleus and is actively exported. Rigorous demonstration that the putative NES controlling this export is actually a crm-dependent sequence will require in vitro binding studies and/or a two-hybrid analysis. Nuclear entry of AnxII and GFP-AnxII most likely occurs via passive diffusion since AnxII does not contain a recognizable nuclear localization signal. If this is so, the ability of GFP-AnxII to bypass the nuclear diffusion barrier may be somewhat surprising given its size (about 65 kDa on denaturing SDS-PAGE). However, it may be that the ability of the GFP-AnxII fusion protein to diffuse through the nuclear pore is different from that of a typical native globular protein of similar molecular mass. GFP and AnxII each must fold into a compact structure in order to be functional, so the shape of the fusion protein might be envisioned as being dumbbell-like, with two globular proteins of 27 kDa (GFP) and 36 kDa (AnxII) tethered together by a linear linker strand. Properly oriented, such a molecule might be able to enter and pass through the nuclear pore.

The ability of LmB treatment to cause detectable increases in the nuclear accumulation of endogenous AnxII appeared to be cell line-dependent, being much more evident in v-src-transformed fibroblasts than in U1242MG cells. This suggests the existence of mechanisms that sequester AnxII in the cytoplasmic compartment, preventing its entry into the nucleus. The most likely mechanism is formation of $AnxII_2/p11_2$ complex, supported by our findings that LmB treatment had relatively little effect on the localizations of p11 and GFP-p11 in U1242MG cells. Cytoplasmic sequestration of $AnxII_2/p11_2$ could result from the size of the complex (94 kDa) preventing passage through the nuclear diffusion barrier. The complex may also be partially bound to the plasma membrane and membraneous cytoplasmic organelles at physiological $[Ca^{2+}]$, since half-maximal binding of $AnxII_2/p11_2$ to membranes occurs at a lower level of calcium than half-maximal binding of the AnxII monomer (Powell and Glenney, 1987; Raynal and Pollard, 1994). Previous observations indicate that the relative expression levels of AnxII and p11 can differ between tissue and cell types and may change under conditions related to cell proliferation and differentiation (Fox et al., 1991; Gerke, 1989; Harder et al., 1993; Munz et al., 1997; Puisieux et al., 1996; Zokas and Glenney, 1987). Therefore, differential expression of AnxII and p11 could represent a mechanism to regulate the amount of AnxII monomer that can enter the nucleus.

The bifunctionality of the AnxII N-terminal region in mediating nuclear export and p11 binding raises the possibility that p11 binds similarly to leucine-rich NES sequences in other proteins. However, p11 binding requires that the leucine-rich region be precisely located near the N-terminus, as indicated by the crystal structure of p11/AnxII peptide complex (Rety et al., 1999) and by the loss of p11 binding when GFP was fused to the AnxII N-terminus in the present study. Other proteins have been reported to bind p11, including $cPLA_2$ (Wu et al., 1997), BAD (Hsu et al., 1997), and PCTAIRE1 (Sladeczek et al., 1997), but the structural bases for these interactions have not been defined.

Our experiments with genistein and pervanadate in conjunction with LmB in v-src-transformed fibroblasts suggest that tyrosine phosphorylation represents a means of promoting the nuclear entry of AnxII, consistent with the previous detection of phosphorylated AnxII in nuclear extracts of K562 and HeLa cells (Chiang et al., 1996). The effects of genistein and pervanadate on AnxII localization became obvious only when nuclear export was inhibited with LmB, which may explain why AnxII phosphorylation by v-src appeared to have little effect on its subcellular distribution in early studies (Erickson and Erickson, 1980; Radke and Martin, 1979). We have not yet elucidated the mechanism by which this occurs, but one possibility is that phosphorylation of $AnxII_2/p11_2$ releases AnxII and allow its entry into the nucleus. Membrane binding of $AnxII_2/p11_2$ dramatically enhances the kinetics of AnxII phosphorylation by src (Bellagamba et al., 1997). In vitro phosphorylation of Y23 or S25 does not fully dissociate the complex or prevent p11 binding, although a partial

dissociation of PKC-phosphorylated complex was reported in one study (Regnouf et al., 1995). In vitro phosphorylation of AnxII on additional residues such as S11 does dissociate the complex and inhibit p11 binding (Jost and Gerke, 1996; Regnouf et al., 1995). Whether this occurs physiologically is not well-documented, although there is evidence that diphosphorylation of AnxII causes its release from the membrane in nicotine-stimulated chromaffin cells (Sagot et al., 1997). Phosphorylation of $AnxII_2/p11_2$ also somewhat decreases its affinity for membrane binding (Powell and Glenney, 1987; Regnouf et al., 1995), which might result in release of the complex from the membrane in cells. However, because of its size (94 kDa), the complex probably would still need to dissociate before AnxII could enter the nucleus. It is also possible that the effects of genistein and pervanadate seen in the present study involve a mechanism other than direct AnxII phosphorylation, since the serum-stimulated accumulation of AnxV in osteosarcoma cells is also inhibited by genistein (Mohiti et al., 1997) but there is no evidence that AnxV can be phosphorylated. The present findings indicate that there may be a number of mechanisms which serve to tightly control the concentration of AnxII within the nucleus. This might be necessary if AnxII participates in a regulated nuclear process, such as its previously suggested role as an accessory protein in DNA synthesis (Jindall et al., 1991; Vishwanatha and Kumble, 1993). The early report of AnxII association with small ribonucleoprotein particles (Arrigo et al., 1983) and the more recent demonstration of AnxII binding to cytoskeleton-associated mRNA subpopulations (Vedeler and Hollas, 2000), together with the present demonstration of AnxII export, raise the possibility of another function in RNA export and localization of ribonucleoprotein particles to particular sites. Either of these proposed functions are consistent with the reported suppression of cell proliferation by AnxII down-regulation (Chiang et al., 1999; Kumble et al., 1992). It is also possible that the physiological role(s) of AnxII is restricted to the cytoplasm, and that nuclear exclusion mechanisms serve to localize the protein to the appropriate compartment.

In conclusion, nuclear export signals have been identified in a variety of molecules and may represent a basic aspect of their functions (e.g. RNA export molecules), or may direct their localizations to cellular compartments relevant to their functions, as for signal transduction molecules and cytoskeletal actin (Henderson and Eleftheriou, 2000; Wada et al., 1998). The present study identifies nuclear export as a novel mechanism controlling the localization of a member of another class of proteins, the annexins. Since these proteins move on and off membranes in the cytoplasmic compartment in response to calcium fluxes, we can now envision a role for AnxII involving a complex cycle of membrane interactions and nuclear entry. For example, the entry of calcium into a stimulated cell could promote the binding of AnxII to the plasma membrane. In this position it may readily serve as a substrate for the cellular src kinase. After the calcium transient is completed, and the AnxII is released from the membrane, it may then travel into the nucleus as a result of its phosphorylation state. The entry of the phosphorylated annexin into the nucleus thus could provide information to the nucleus concerning the prior calcium transient, even if the calcium concentration was not elevated in the nucleus per se. The annexin may then participate in a nuclear response to the initial cell stimulation and calcium transient, perhaps by regulating DNA replication or messenger RNA processing or transport. Although such a pathway is highly speculative at present, the discovery of the nuclear trafficking of AnxII is likely to lead to new insights regarding the biological roles and regulation of this ubiquitous family of molecules.

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