

## Annexin II expression is reduced or lost in prostate cancer cells and its re-expression inhibits prostate cancer cell migration

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**While studying Bim, a BH3-only proapoptotic protein, we identified an ~36 kDa protein, which was abundantly expressed in all five strains of primary normal human prostate (NHP) epithelial cells but significantly reduced or lost in seven prostate cancer cell lines. The ~36 kDa protein was subsequently identified as annexin II by proteomic approach and confirmed by Western blotting using an annexin II-specific antibody. Conventional and 2D SDS-PAGE, together with Western blotting, also revealed reduced or lost expression of annexin I in prostate cancer cells. Subcellular localization studies revealed that in NHP cells, annexin II was distributed both in the cytosol and underneath the plasma membrane, but not on the cell surface. Prostate cancer cells showed reduced levels as well as altered expression patterns of annexin II. Since annexins play important roles in maintaining Ca<sup>2+</sup> homeostasis and regulating the cytoskeleton and cell motility, we hypothesized that the reduced or lost expression of annexin I/II might promote certain aggressive phenotypes of prostate cancer cells. In subsequent experiments, we indeed observed that restoration of annexin II expression inhibited the migration of the transfected prostate cancer cells without affecting cell proliferation or apoptosis. Hence, our results suggest that annexin II, and, likely, annexin I, may be endogenous suppressors of prostate cancer cell migration and their reduced or lost expression may contribute to prostate cancer development and progression.**

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### Introduction

We are studying the role of Bcl-2 family proteins in regulating prostate cancer cell apoptosis. While studying Bim, a BH3 domain-only proapoptotic protein, we

found that an unknown ~36 kDa protein was reduced or lost in most of the prostate cancer cell lines studied (Liu *et al.*, 2002). Through the use of mass spectrometry proteomic analysis, here we report that this 36 kDa protein is annexin II. Annexin I is similarly decreased or lost in prostate cancer cells.

Annexins are a family of Ca<sup>2+</sup>-dependent, phospholipid-binding proteins. More than 20 different annexin isoforms have been identified (Raynal and Pollard, 1994; Morgan and Fernandez, 1997). Annexin II, also known as p36, lipocortin II, and calpactin I heavy chain, was initially identified as one of the major phosphorylation targets of pp60<sup>v-src</sup> (Elkso and Erikson, 1980). Annexin II exists as a monomer, heterodimer, or heterotetramer (AII<sub>t</sub>). AII<sub>t</sub> is composed of two copies each of annexin II and p11 or II<sub>2</sub>-p11<sub>2</sub> (Raynal and Pollard, 1994; Morgan and Fernandez, 1997). p11 is a member of the S100 protein family and is also called S100A10, calpactin I light chain, or annexin II light chain (Morgan and Fernandez, 1997). While the precise function of annexin II is unclear, it has been shown to be involved in Ca<sup>2+</sup>-dependent exocytosis, endocytosis, cell–cell adhesion (Mai *et al.*, 2000), proliferation (Chiang *et al.*, 1999), cell surface fibrinolysis (Hajjar and Acharya, 2000), and osteoclast formation and bone resorption (Takahashi *et al.*, 1994). Recent studies suggest that annexin II might be linked to tumorigenesis (Mai *et al.*, 2000). Annexin I (also known as lipocortin I) is a mediator of anti-inflammatory glucocorticoids, possibly because of its ability to inhibit phospholipase A2 and leukocyte diapedesis (Raynal and Pollard, 1994; Morgan and Fernandez, 1997). Annexin I has also been reported to regulate apoptosis in myelo-monocytic cells (Solito *et al.*, 2001).

Recently, the expression of both annexins I and II has been reported to be reduced or lost in prostate cancer cells *in vivo* (Paweletz *et al.*, 2000; Chetcuti *et al.*, 2001). However, the relevancy of this finding to prostate cancer development has not been studied. Here, we show that re-expression of annexin II in prostate cancer cells inhibits their migratory capacity without affecting cell survival or proliferation. These results suggest that annexin II (and, likely, annexin I) may normally suppress prostate cancer progression by inhibiting cell migration.

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## Materials and methods

### Cells and reagents

Five primary strains of normal human prostate (NHP) epithelial cells, NHP1 – NHP5, and seven prostate cancer cell lines, PPC-1, MDA PCa 2b (MDA 2b), LNCaP, C4-2, C5, PC3, and Du145, were cultured as described previously (Liu *et al.*, 2002; Tang *et al.*, 2002).

Mouse monoclonal anti-annexin I and -annexin II antibodies were purchased from BD PharMingen (San Diego, CA, USA). Rabbit polyclonal and mouse monoclonal (clone 14A8) anti-Bim antibodies were bought from Calbiochem (San Diego, CA, USA) and Alexis Biochemicals (San Diego, CA, USA), respectively. Mouse monoclonal anti-actin antibody was purchased from ICN (Indianapolis, IN, USA). Monoclonal anti-BrdU (5-bromo-2'-deoxyuridine) antibody (hybridoma culture supernatant) was kindly provided by Dr Martin Raff. Goat polyclonal anti-LDH (lactate dehydrogenase) antibody was obtained from Chemicon International (Temecula, CA, USA). The secondary antibodies, goat anti-rabbit or -mouse IgG or donkey anti-goat IgG conjugated to horseradish peroxidase, or goat anti-mouse IgG conjugated to FITC or Texas Red, were bought from Amersham Biosciences (Piscataway, NJ, USA) or Santa Cruz. Liposome FuGENE 6 was purchased from Roche (Indianapolis, IN, USA). Lipofectamine 2000 was obtained from Gibco (Carlsbad, CA, USA). All other chemicals were purchased from Sigma (St Louis, MO, USA).

### Two-dimensional gel electrophoresis (2D PAGE) analysis

NHP2 or LNCaP cells ( $5 \times 10^6$ ) were resuspended in 100  $\mu$ l of a modified RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.5% Triton X-100, 10 mM EDTA). The cells were subjected to freezing and thawing three times, followed by sonication ( $3 \times 15$  s). The cell lysate was centrifuged at 14000  $g$  for 15 min at 4°C. An amount of 50  $\mu$ g of proteins was treated with DNase and RNase followed by overnight acetone precipitation at -20°C. The protein pellet was resuspended in 185  $\mu$ l of rehydration buffer (9.8 M urea, 2% CHAPS, 100 mM DTT, 0.1% (w/v) BioLytes, and trace amount of bromophenol blue), and incubated at room temperature for 1 h for complete protein solubilization. The protein mixture was then centrifuged at 15000  $g$  for 30 min at ambient temperature. The supernatant (50  $\mu$ g/sample) was loaded onto an 11-cm focusing tray and overlaid with a precast IPG dry strip, pH 3–10, with the gel side down and then covered with mineral oil. Isoelectric focusing was performed by following a manufacturer-recommended protocol using Bio-Rad PROTEAN IEF Cell (Hercules, CA, USA). After rehydrating for 12 h at 50 V, the focusing was carried out automatically with the following program, 250 V for 15 min, from 250 to 5000 V for 2.5 h, and a final focusing step at 8000 V for 55000 Vh. Focused IPG strips were equilibrated in 5 ml of equilibration solution (150 mM Tris-HCl, pH 8.8, 6 M Urea, 30% v/v glycerol, 2% SDS with 2.5% DTT added for the first 10 min and 2% iodoacetamide added for the last 10 min). SDS-PAGE was carried out using a precast Criterion 8–16% gradient gel in Criterion cell, at 20 V for 10 min and then at 200 V for 45–55 min. Gels were then fixed in 10% methanol–7% acetic acid for 40 min, stained with SYPRO Ruby protein stains (Bio-Rad) overnight, and then destained in 10% methanol–7% acetic acid for 1 h. The images of the stained 2D gels were taken on a Vistra FluorImager SI (Amersham Bioscience) and analysed using PDQUEST software package (Bio-Rad).

### Western blotting

For Western blotting of 1D gels, the whole cell lysates were prepared in RIPA buffer containing a protease-inhibitor cocktail (Sigma). Protein concentrations were determined by MicroBCA kit (Pierce, Rockford, IL, USA). A measure of 30  $\mu$ g whole cell lysate from each sample was loaded on a 10% precast SDS-polyacrylamide gel (Bio-Rad). After gel electrophoresis, proteins were transferred to a nitrocellulose membrane. The membrane was sequentially probed, stripped, and reprobed (Liu *et al.*, 2002) with polyclonal or monoclonal anti-Bim, or anti-annexin I, -annexin II, -actin, or -LDH antibodies. The intensity of the annexin II and LDH bands was determined by scanning on Image Station 440 CF (Kodak) and analysed using the Kodak 1D 3.5 software. For 2D gels, following protein transfer, the blot was probed with the polyclonal anti-Bim antibody. Secondary antibodies used were goat anti-mouse or -rabbit IgG or donkey anti-goat IgG conjugated to HRP and protein bands were revealed using enhanced chemiluminescence (ECL).

### MALDI-TOF MS analysis

Duplicate 2D-PAGE gels were simultaneously run for each sample. One gel was used in Western blotting (see above) and the other was used for protein spot picking. The protein spot that matched the protein identified by the anti-Bim antibody in NHP2 cells, together with another three protein spots that were prominently expressed in NHP2 cells but lost in LNCaP cells (see Figure 1 for details), were manually cut out of the gel. They were subjected to in-gel tryptic digestion based on the Rosenfeld procedure (Rosenfeld *et al.*, 1992). The tryptic digests were analysed on a Voyager DePRO™ MALDI-TOF MS system (Applied Biosystems, Foster City, CA, USA). The samples were mixed with matrix at 1 : 1 ratio on a 100-well steel MALDI target for analysis in a total volume of 1  $\mu$ l. The  $\alpha$ -cyano-4-hydroxy cinnamic acid matrix was prepared at 2 mg/ml in 50% ACN/0.3% trifluoroacetic acid solvent. Samples were analysed in the positive-ion mode with delayed extraction. The cocrystallized target spot was ionized with a UV nitrogen laser (337 nm) at 20 Hz, accelerated at 20 kV, and analysed in the reflector mode. Each sample mass spectrum is the average of 200 laser shots. The instrumental parameters used are as follows: bin size – 0.5 ns, delay time – 100 ns, mass range – 800–3200 Da, low mass gate 750 Da, grid – 78.4% of accelerating voltage, guide wire – 0.005% of accelerating voltage.

An automated database search was performed on the Proteomic Solution 1 data station (Applied Biosystems). Peptide mass lists were filtered to remove trypsin autolysis peaks. The mass list for each sample was entered in the search program, MS-Fit 3.3.1, in the Protein Prospector suite. The Swiss-PROT database was searched using a 15 ppm peptide mass tolerance for tryptic digest and a maximum of three missed cleavages and carbamidomethylation of the cysteines. The search was performed for all species with unrestricted pI and molecular weight. The protein represented by the highest scoring match is reported and peptide sequences assigned according to the best match.

Following the analysis of each sample by peptide mass mapping, one ion from each MS spectrum was subjected to postsource decay (PSD) analysis in order to further confirm the protein identity by peptide sequencing. The PSD spectra of each ion were acquired in automated mode and are a composite of several segments of spectrum each acquired at a different PSD mirror ratio (each mirror ratio focusing a certain  $m/z$  region). The mirror ratios ranged from 1.000 to

0.03 with the actual number of mirror ratios depending on the mass of the ion fragmented. Each segment of the spectrum was the average of 250 laser shots.

For MALDI-PSD spectra, the raw data was smoothed and the mass list exported to the MS-Tag peptide fragmentation database search engine in the Protein Prospector suite. Similar parameters were used *vide infra*, except that the parent ion mass tolerance was 20 ppm and the fragment ion tolerance was 800 ppm. The protein identified in each case as the highest scoring match was identical to that identified in the peptide mass mapping analysis. Since the two methods use different data sets and independent database searches, the resulting identification has a very high confidence level.

#### Immunofluorescence studies

Various cells were grown on 18 mm<sup>2</sup> circle glass coverslips (6 × 10<sup>3</sup>/coverslip). For cell surface labeling, cells were fixed in 4% paraformaldehyde (PFA) at 4°C for 15 min. For cytoplasmic labeling, cells were permeabilized with 1% Triton X-100 in PBS after fixation. Coverslips were blocked in 30% goat whole serum, then incubated in primary antibodies (1 : 1000) and secondary antibody (goat anti-rabbit IgG-FITC; 1 : 2000). Finally, cells were incubated with 1 μg/ml DAPI (4', 6-diamidino-2-phenylindole) to label all nuclei. Coverslips were mounted on slides using Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA) and observed under an Olympus BX40 epifluorescence microscope. Images were captured with MagnaFire software and processed in Photoshop.

#### Construction of annexin II expression vector

Full-length human annexin II cDNA was amplified by RT-PCR using forward primer 5'-GGCCCAGCTAGCTT-CAAAATGTCTACTG-3' and reverse primer 5'-AC-CATTTGTCGACGCTCAGGCCGTGT-3' based on the cDNA sequence of annexin II (accession number NM\_004039). RT-PCR was performed as described previously (Liu *et al.*, 2002). The PCR product (1070 bp), which contained the full coding region of annexin II as confirmed by sequencing, was cloned into pCRII-TOPO (Invitrogen, Carlsbad, CA, USA).

To construct the annexin II expression vector, the annexin II coding sequence was released from pCRII-TOPO by *Bam*HI and *Xho*I digestion and subcloned into the mammalian expression vector pIRES-hrGFP-1a (Stratagene, La Jolla, CA, USA). The orientation of the gene in the vector was determined by restriction enzyme digestion and sequencing. The resultant annexin II expression vector was designated phrGFP-annexin II, in which the annexin II cDNA was driven by a CMV promoter and hrGFP (humanized *Renilla* GFP) was translated from the internal ribosomal entry site (IRES). To characterize the annexin II expression vector, phrGFP-annexin II, or empty vector pIRES-hrGFP, was transfected into 293 cells. At 48 h after transfection, cells were observed under a fluorescence microscope and then harvested for Western blotting.

#### GFP-BrdU double fluorescence microscopy

LNCaP or DU145 cells grown on glass coverslips were either untransfected or transiently transfected with empty vector pIRES-hrGFP or phrGFP-annexin II with either FuGENE 6 (for Du145 cells) or Lipofectamine 2000 (for LNCaP cells). At 48 h after transfection, cells were pulsed with 10 μM BrdU for 4 h, followed by fixation in 4% PFA at 4°C for 20 min to

preserve GFP and permeabilization in 1% Triton X-100 for 20 min at room temperature. Then cells were treated with DNase (100 μg/ml) for 70 min to denature DNA and stained for BrdU (Tang *et al.*, 2002). Briefly, cells were incubated with the anti-BrdU antibody followed by goat anti-mouse IgG conjugated to Texas Red. Cells were counterstained with DAPI. The results were expressed as percentage of BrdU<sup>+</sup> cells (mean ± s.e.).

#### Migration assays

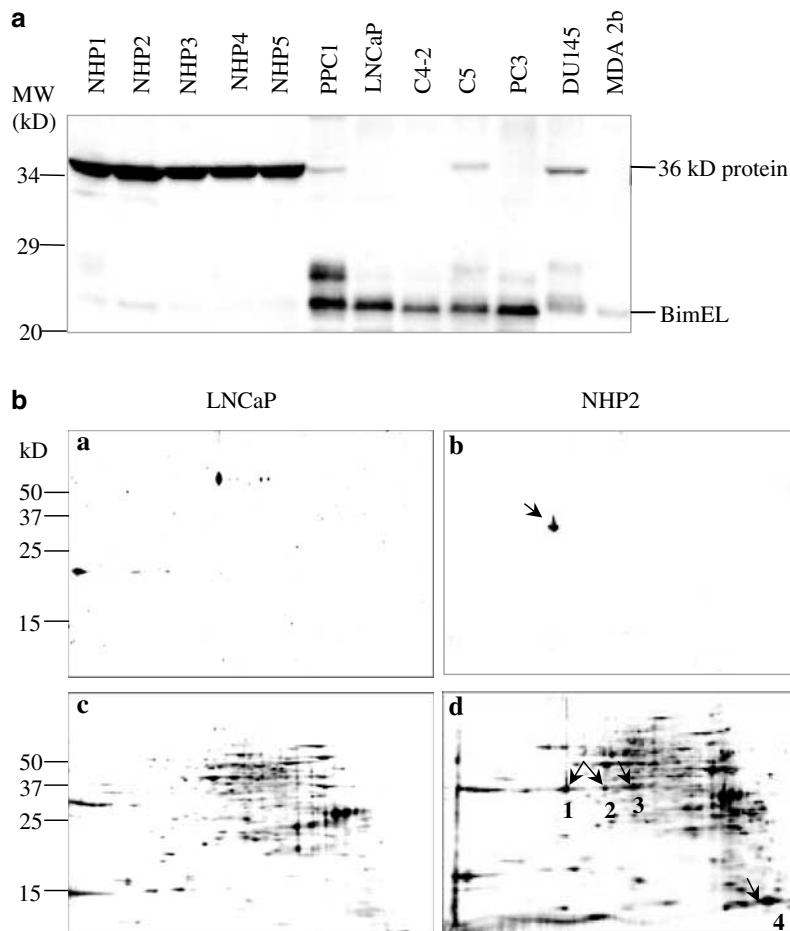
NHP2, LNCaP, or DU145 cells were plated on the Boyden chamber transwell filters (8 μm pore) at 2.5 × 10<sup>4</sup>/well in serum-free medium (RPMI-1640). Medium containing 5% FBS was added to the bottom chambers, and cells were cultured at 37°C and 5% CO<sub>2</sub>. After 12 h, LNCaP and Du145 cells were transfected with either pIRES-hrGFP or phrGFP-annexin II, using either FuGENE 6 (for Du145 cells) or Lipofectamine 2000 (for LNCaP cells). Cells were observed under a regular phase-contrast microscope or an Olympus IX-ILL30 inverted fluorescence microscope, 48 h after transfection. The GFP<sup>+</sup> cells on both top (i.e. unmigrated cells) and bottom (i.e. migrated cells) sides of the filter were counted. For NHP2 cell migration, 48–96 h after plating, cells migrated through the filter were either directly counted on an inverted microscope by focusing on the bottom of the plane or counted after removing the cells on the top of the filter (i.e. unmigrated cells) followed by staining with 1% Giemsa. Triplicate wells were performed for each condition and the results were expressed as the rate of migration, that is, percentage (mean ± s.e.) migrated GFP<sup>+</sup> cells out of the total GFP<sup>+</sup> cells (i.e. migrated + unmigrated). The experiments were repeated three times.

## Results and discussion

#### Reduced or lost expression of annexins I and II in prostate cancer cells

We are studying the regulation of prostate cancer cell apoptosis by Bcl-2 family proteins. When we performed a Western blotting on a panel of NHP and prostate cancer cells using a polyclonal anti-Bim (a BH3-only proapoptotic Bcl-2 protein) antibody, we detected an unexpected ~36 kDa protein, which was strongly expressed in all five primary NHP strains but reduced or lost in most of the prostate cancer cells (Figure 1a). The contrasting expression patterns suggest that this 36 kDa protein might play a role in prostate cancer development or progression. To identify this unknown protein, we employed proteomic approach. Whole cell lysates from NHP2 (that expressed abundant 36 kDa protein) and LNCaP (which did not express the 36 kDa protein) cells were separated by 2D gel electrophoresis. After transferring the proteins to nitrocellulose membrane, the blot was probed with the polyclonal anti-Bim antibody, which detected a specific ~36 kDa protein spot in NHP2 (Figure 1b, panel b) but not in LNCaP cells (Figure 1b, panel a).

The duplicate 2D gels (Figure 1b, panels c and d) run in parallel for each sample were stained with SYPRO Ruby protein stain. The protein spot in NHP2 cells (Figure 1b, panel d, arrow 1) that matched the spot



**Figure 1** A ~36 kDa protein is significantly reduced or lost in prostate cancer cells. (a) A total of 30  $\mu$ g of whole cell lysates from each cell type was loaded on a 15% SDS–polyacrylamide gel. The membrane was probed with a polyclonal anti-Bim antibody. In addition to the expected BimEL (an isoform of Bim) band, a prominent ~36 kDa protein was also detected by the antibody, which was abundantly expressed in all five strains of NHP cells but reduced or lost in prostate cancer cell lines. (b) Whole cell lysates (50  $\mu$ g) was treated with DNase and RNase for each sample (NHP2 and LNCaP) and then loaded onto a precast IPG dry strip, pH 3–10, for focusing. Conventional SDS–PAGE was carried out using a precast Criterion 8–16% gradient gel. Duplicate gels were run in parallel for each sample. With each cell type, one gel was used in Western blotting with the anti-Bim antibody (a and b) and the other gel was stained with SYPRO Ruby protein stains (c and d). The arrow in b indicates the 36 kDa protein spot 1 (panel d) identified by the anti-Bim antibody. The arrows in d indicate the four protein spots excised for MS analysis

detected by the anti-Bim antibody (Figure 1b, panel b, arrow 1) was manually excised. Another two protein spots of similar MW but different pIs (Figure 1b, panel d, arrows 2 and 3), which were not detected by the anti-Bim antibody but were reduced in LNCaP cells, were also excised for comparative purposes. The fourth protein spot, which was very abundantly expressed in NHP2 (Figure 1b, panel d, arrow 4) but nearly undetectable in LNCaP cells (Figure 1b, panel c), was also picked up. These four proteins were subjected to in-gel tryptic digestions. The MALDI-TOF MS analysis and peptide mass mapping of the tryptic digests subsequently identified the protein spots 1 and 2 as annexin II (Figure 2a, right panel), spot 3 as annexin I (Figure 2a, left panel), and spot 4 as S100A2, a calcium-binding protein (Lee *et al.*, 1992). To confirm the peptide mass mapping results, we picked one of the

peptide peaks for each protein obtained from MALDI-TOF MS (e.g. annexin II shown in Figure 2b) and used PSD fragmentation (e.g. annexin II in Figure 2c) to provide sequence information for an independent database search. The results confirmed their identities (Figure 2a, underlined, bold, and italicized sequences).

To verify the MALDI-TOF results, the same Western blot as shown in Figure 1a was reprobed for annexins I and II. As shown in Figure 3, both annexins I and II were indeed reduced or lost in most prostate cancer cells. For example, when normalized to LDH, annexin II was undetectable in LNCaP, C4-2, and PC3 cells, reduced by ~50–70% in PPC-1, C5, and Du145 cells, and reduced by ~15-fold in MDA 2b cells, as revealed by densitometric scanning (Figure 3).

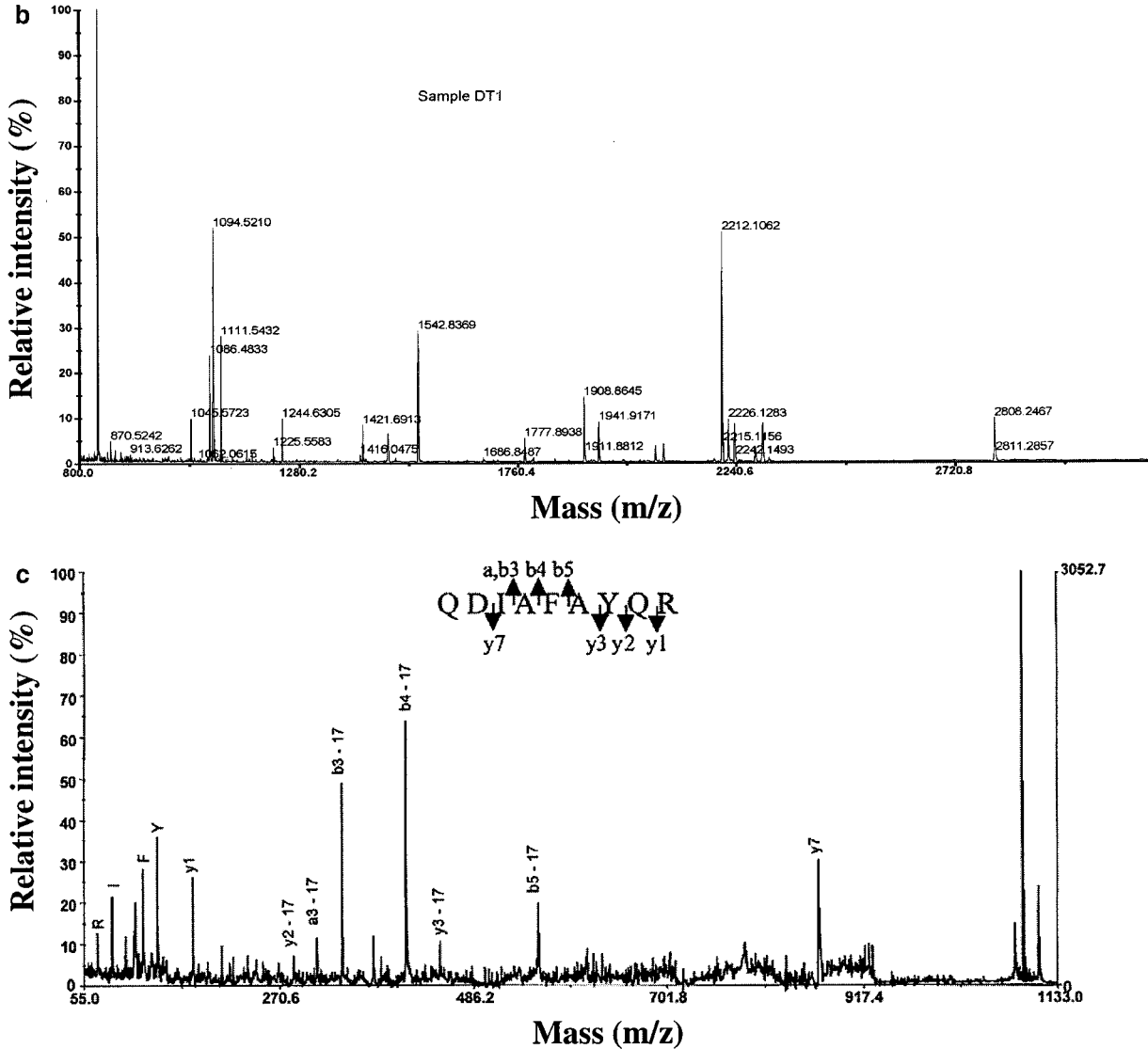
Annexin II in protein spot 1 (Figure 1b (b and d)) was detected on Western blotting by the polyclonal but not

**a Annexin I (protein spot 3)**

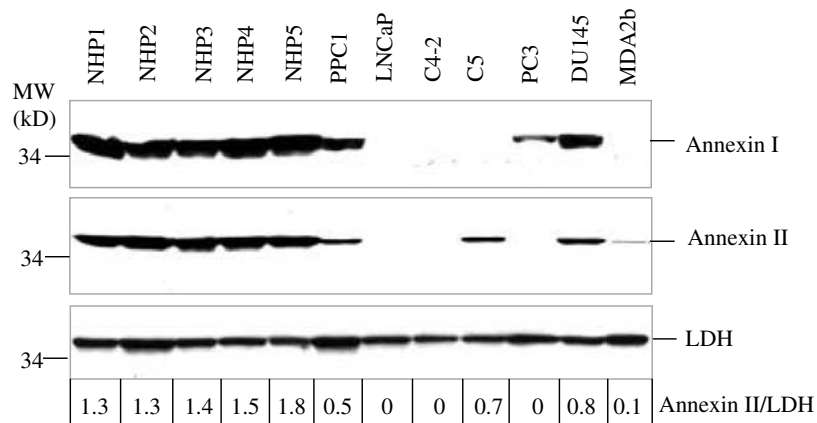
MAMVSEFLKQAWFIENEEQEYVQTVKSSKG 30  
 GPGS AVSPYPTFNPS SDVAALHKAI MVKGV 60  
 DEATII DILTKRNNARQIQI KAAYLOETGK 90  
PLDETLK KAL TGHL EEVVLALLKTPAQFDA 120  
DELRAAMKGLGTDEDTLIEILASRTNKEIR 150  
 DINRVYREELKRDLAKDI TS DTS GDFRNAL 180  
 LSLAKGDRSEDFGVNEDLADSDARAL YEAG 210  
 ERRKGT DVNVFNTI L TTRSYPQLRRVFQKY 240  
 TKYS KHD MNKVL DL ELKGD I EKCLTAI VKC 270  
 ATSKPAFFAEK L HQAMKGVGTRHKALIRIM 300  
 VSRSEI DMNDI KAF YQKMYG I SL CQAILDE 330  
 TKGDYEKILVALCGGN 346

**Annexin II (protein spots 1 and 2)**

MSTVHEILCKLSLEGDHSTPPSAYGSVKAY 30  
TNFDAERDALNIETAIKTKGVDEVTVNII 60  
 TNRSNAQRQDI AFAYQRRTKKELASALKSA 90  
 LSGHLETVILGLLKTPAQYDASELKASMKG 120  
 LGTDEDSLIEIICSRINQELQEINRVYKEM 150  
 YKTDLEKDII SDTSGDFRKL MVALAKGRRRA 180  
EDGSVI DYELI DQARDL YDAGVKRKGTDV 210  
 PKWISI MTERSVPHLQKVFDRYKSYSPYDM 240  
 LESIRKEVKGDLEN AFLNLVQCI QNKPLYF 270  
 ADRLYDS MKGKGTRDKVLI RIMVSRSEVDM 300  
 LKIRSEFKRKYGKSLYYIQQDTKGDYQKA 330  
 LLYLCGGDD 339



**Figure 2** (a) The three protein spots (Figure 1b, d, arrows 1–3) were analysed by MALDI-TOF and PSD and identified as annexin I (protein spot 3, left panel) and annexin II (protein spots 1 and 2, right panel), respectively. The *underlined* aa show the peptide sequences matching the spectrum peaks obtained from MALDI-TOF. The *underlined, bold, and italic* aa show the sequences further analysed by PSD. The AER sequence in annexin II that also exists in the epitope of the peptide polyclonal anti-Bim antibody is indicated. (b) shows the MALDI-TOF spectrum of annexin II from gel spot DT1 (protein spot 1 in Figure 1b, panel d). The annexin II peptides with matched masses are 1086, 1094, 1111, 1225, 1244, 1421, 1542, 1908, and 2064. The trypsin autolysis peaks are 842, 1045, 2212, and 2808. (c) shows the MALDI-PSD spectra acquired for the peptide at  $m/z=1111.5432$ , which was identified as QDIAFAYQR from annexin II in a search of the Swiss-Prot database with MS-Tag. The a, b, y and ammonium ions are labeled with loss of  $\text{NH}_3$  shown as -17



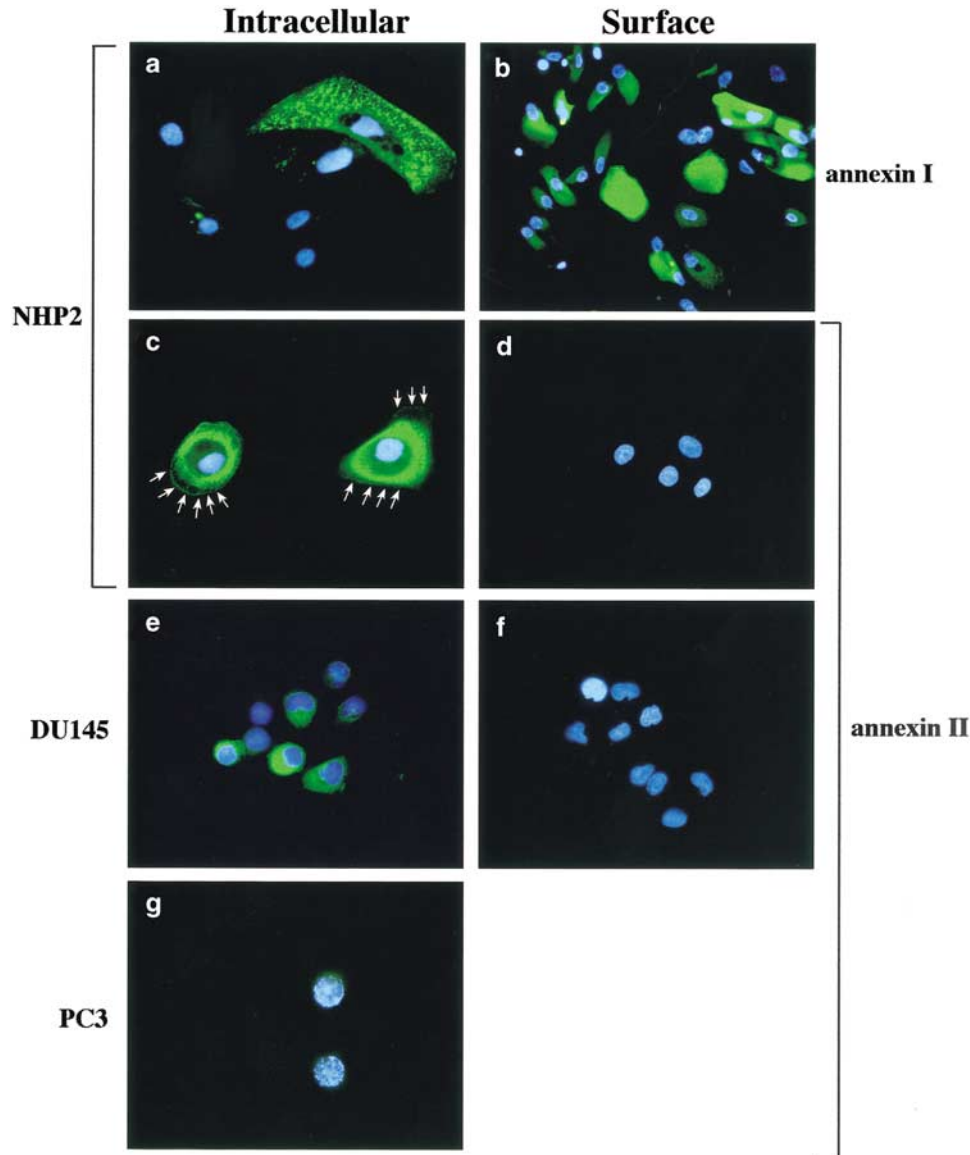
**Figure 3** Annexins I and II are reduced or lost in prostate cancer cell lines. The same blot as used in Figure 1a was stripped and reprobed for annexin I (~36 kDa), annexin II (~36 kDa), and LDH (~35 kDa). Note that annexin I was undetectable in LNCaP, C4-2, C5, MDA 2b, and Tsu-Pr cells and reduced in PC3 cells (upper panel). Similarly, annexin II was undetectable in LNCaP, C4-2, and PC3 cells and reduced in all other prostate cancer cells (middle panel). The ratio of annexin II/LDH after densitometric scanning for each cell type is presented at the bottom of the figure

by the monoclonal anti-Bim antibody (not shown). Since the polyclonal anti-Bim antibody was raised against a peptide sequence, and the monoclonal anti-Bim antibody was raised against the mouse BimS full-length sequence (which contains the peptide sequence), these results suggest that annexin II is detected by the polyclonal anti-Bim antibody most likely because of fortuitous crossreaction. Indeed, when the epitope of the polyclonal anti-Bim antibody (AERPPQLPR-GAPTSLQTEPQDRSP) was aligned with the annexin II amino-acid sequence, only the first tripeptide AER was shared by both sequences (Figure 2a, right panel, boxed), which might be too short to account for the crossreaction, consistent with the fact that the monoclonal anti-Bim antibody did not crossreact with the annexin II band (not shown). The annexin II in protein spot 2 (Figure 1b, d) was not detected by the polyclonal anti-Bim antibody on Western blot (Figure 1b, b), either because the protein level was too low, or more likely because this protein spot represented a post-translationally modified annexin II (thus a different pI), which was not recognized by the antibody.

Recently, two groups (Pawelczak *et al.*, 2000; Chetcuti *et al.*, 2001) reported reduced/lost expressions of annexins I and II in human prostate cancer tissues. Interestingly, annexin I has been reported to be upregulated in human mammary adenocarcinoma cells (Ahn *et al.*, 1997; Pencil and Toth, 1998). Similarly, annexin II has been shown to be upregulated in a variety of tumor cell lines including pancreatic (Vishwanatha *et al.*, 1993), lymphoma (Chiang *et al.*, 1996), and small cell lung (Cole *et al.*, 1992) cancer cell lines. Also, annexin II expression is elevated in glioblastoma (Reeves *et al.*, 1992) and hepatocellular carcinomas (Frohlich *et al.*, 1990). These observations suggest that annexins I and II may play a unique role in maintaining the prostate homeostasis and their reduced expression may thus contribute to prostate cancer development and/or progression.

#### *Reduced as well as altered expression patterns of annexin II in prostate cancer cells*

We focused our subsequent studies on annexin II, because it was initially identified by the polyclonal anti-Bim antibody (Figure 1) and also because relatively more is known about its physiological functions. To understand how reduced annexin II expression may contribute to prostate cancer development, we first studied its normal distribution in NHP cells and then compared with its expressions in prostate cancer cells. As a comparison, we also studied the subcellular distribution of annexin I. Immunofluorescent staining using an affinity-purified monoclonal anti-annexin I antibody revealed that, in NHP2 cells, annexin I was mostly expressed on the cell surface (Figure 4b) with only a small proportion of the cells showing distinct intracellular granular staining (Figure 4a). In sharp contrast, immunolabeling with an affinity-purified monoclonal anti-annexin II antibody revealed that annexin II in NHP2 cells was distributed in the cytosol as well as underneath the plasma membrane (Figure 4c; arrows), but not on the cell surface (Figure 4d). A quantitative analysis detected the subplasmalemmal localization of annexin II in ~60% of the NHP2 cells, whereas 100% of the NHP2 cells showed cytoplasmic staining of annexin II (Table 1). The submembranous distribution of annexin II, previously also observed in other cells (Huang *et al.*, 1986; Ma and Ozers, 1996; Zobiak *et al.*, 2001), suggests potential annexin II-membrane interactions. Interestingly, annexin II may also be exported outside of cells and the extracellular annexin II has been identified as both soluble and membrane-bound (Hajjar *et al.*, 1994). For example, membrane-bound annexin II has been found in several cell types including endothelium (Hajjar *et al.*, 1994), keratinocytes (Ma *et al.*, 1994), and glioma and smooth muscle cells (Chung and Erickson, 1994). Nevertheless, in our multiple experiments with NHP2 cells, we did not



**Figure 4** Reduced levels and altered expression patterns of annexin II in prostate cancer cells. NHP2, PC3, and Du145 cells were used in either surface (b,d,f) or intracellular (a,c,e,g) staining of annexin I (a,b) or annexin II (c-g) using the affinity-purified antibodies, as detailed in Materials and Methods. The secondary antibody used was goat anti-mouse IgG coupled to FITC. Cell nuclei were counterstained with DAPI. The arrows in c indicate submembranous staining pattern of annexin II in NHP2 cells. Original magnifications:  $\times 200$  in panel b and  $\times 400$  in all other panels

observe cell surface-bound annexin II or soluble annexin II deposited in extracellular matrix (Figure 4d and data not shown), although we could easily detect the cell surface localization of annexin I (Figure 4b).

Next, we studied the distribution of annexin II in PC3 and Du145 prostate cancer cells. Consistent with the Western blotting data (Figure 3), annexin II expression was reduced in most DU145 cells (Figure 4e) and undetectable in PC3 cells (Figure 4g; Table 1). Similar to NHP2 cells, no cell-surface labeling of annexin II was observed in Du145 cells (Figure 4f) or PC3 cells (not shown). More interestingly, annexin II in Du145 cells was distributed exclusively in the cytosol with no submembranous association (Figure 4e; Table 1), sug-

gesting that prostate cancer cells not only show reduced but also altered patterns of annexin II expression.

Similar immunolocalization experiments revealed reduced expression of annexin I in PC3 and Du145 cells (not shown), consistent with the Western blotting data.

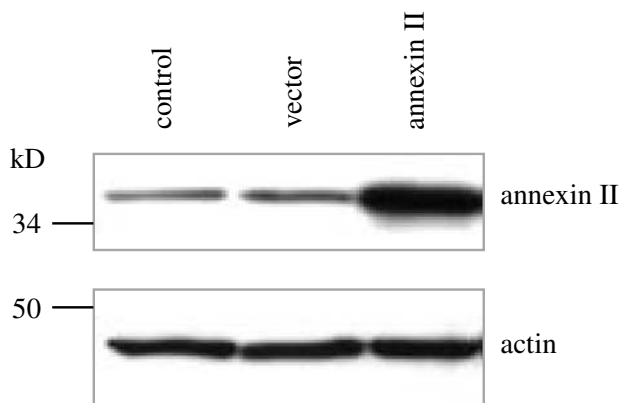
*Annexin II does not affect prostate cancer cell-cycle progression or apoptosis*

Although our observations so far (Figures 1–4; Table 1) and others' (Paweletz *et al.*, 2000; Chetcuti *et al.*, 2001) suggest that reduced or lost expression of annexin II (or annexin I) may contribute to prostate cancer development/progression, this possibility has not been directly

**Table 1** Expression of annexin II in NHP2, PC3, and Du145 cells<sup>a</sup>

Cells	Annexin II expression (%)	
	Submembranous	Cytosolic
NHP2	60	100
Du145	0	100
PC3	0	0

<sup>a</sup>Annexin II expression and localization were detected by immunofluorescence microscopy as detailed in Materials and Methods. An average of 500–1000 cells were counted for each cell type and the results were expressed as % of the total



**Figure 5** Characterization of the annexin II expression vector. Log-phase 293 cells were either untransfected (control), or transfected with empty vector (pIRES-hrGFP) or with annexin II expression vector phrGFP-annexin II (annexin II). At 48 h post-transfection, the cells were harvested and the whole cell lysates were used in Western blotting with antibodies against annexin II or actin

tested. Since annexin II is reduced in prostate cancer cells, the molecule may normally suppress prostate cancer development by restricting cell proliferation, promoting programmed cell death (apoptosis), or by limiting cell migration and invasion.

We first examined the effect of annexin II re-expression on the proliferation and apoptosis of prostate cancer cells. The published data about the effect of annexin II on cell proliferation is controversial and seems to be cell type-dependent. For instance, Takahashi *et al.* (1994) reported that annexin II increased osteoclast formation and bone resorption, possibly by stimulating the osteoclast precursor cell proliferation in bone marrow (Menna *et al.*, 1999). However, annexin II suppresses lymphocyte proliferation (Aarli *et al.*, 1997). To determine whether annexin II affects the proliferation of prostate cancer cells, we attempted to restore the annexin II expression in LNCaP cells that had lost its expression (Figure 3) as well as to overexpress annexin II in Du145 cells that showed reduced expression (Figure 3), using a GFP-tagged annexin II expression vector (phrGFP-annexin II). When transiently transfected into 293 cells, increased annexin II protein was detected (Figure 5),

suggesting that the expression plasmid encodes the expected product.

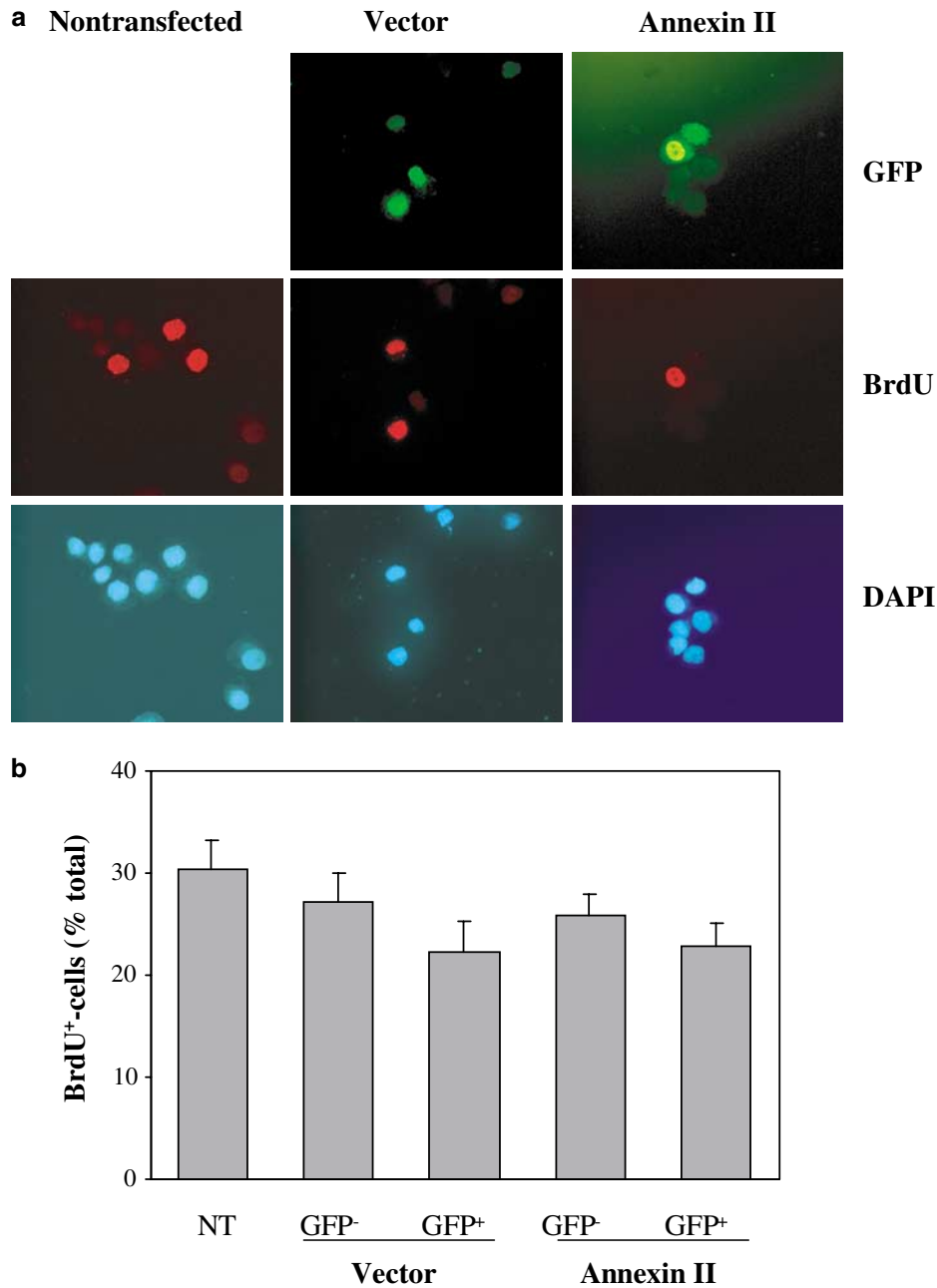
We then studied, using BrdU incorporation assays, the cell-cycle progression in DU145 and LNCaP cells transiently transfected with the vector alone or annexin II-encoding vector. We employed a recently developed BrdU labeling protocol (Tang *et al.*, 2002) that allows the detection of BrdU<sup>+</sup> cells without jeopardizing the GFP signal so that we could compare the percentage of the S-phase cells in isogenic GFP<sup>+</sup> and GFP<sup>-</sup> cells. As shown in Figure 6a, there was no significant difference in BrdU incorporation (labeled as red) between vector-transfected and annexin II-transfected GFP<sup>+</sup> (green) DU145 cells. Quantification revealed similar and slightly decreased, but statistically insignificant, % BrdU<sup>+</sup> cells in both vector- and annexin II-transfected cells (Figure 6b). The low level of GFP cytotoxicity has been observed previously in other cell systems (Tang *et al.*, 2002). Similar transfection experiments in LNCaP cells also did not reveal any of the effect of annexin II expression on cell proliferation (not shown).

Finally, in both Du145 and LNCaP cells, enforced expression of annexin II neither induced nor inhibited apoptosis in these cells (not shown), consistent with the fact that NHP cells normally express abundant annexin II (Figures 3 and 4). Together, the results suggest that it is unlikely that annexin II inhibits prostate cancer development by suppressing proliferation or promoting apoptosis.

#### *Annexin II inhibits migration of prostate cancer cells*

Several lines of evidence suggest that annexin II may negatively regulate prostate cancer cell motility and migration. First, annexin II has been shown to be associated with the cytoskeleton (Pol *et al.*, 1997). Second, annexin II in most (i.e. ~60%) NHP2 cells is submembranously distributed (Figure 4a). Annexin II underneath the plasma membrane has been proposed to form a complex with p11, a member of the S100 family of EF-handed calcium-binding protein (Zokas and Glenn, 1987). This complex, comprising a heterotetramer of two molecules each of annexin II and p11 (i.e. II<sub>2</sub>-p11<sub>2</sub>), is thought to participate in the regulation of the supramolecular organization of F-actin and intermediate filament (Ikebuchi and Waisman, 1990; Bianchi *et al.*, 1995). Third, although Du145 did not completely lose annexin II expression (Figures 3 and 4e), the decreased annexin II proteins in Du145 cells were exclusively localized to the cytosol (Figure 4e; Table 1). Finally, LNCaP cells and their derivatives, C4-2 and C-5, as well as MDA 2b cells express low levels of F-actin (Navone *et al.*, 1997; Tang *et al.*, 1998, 2002), and these cells also express lowest levels of annexin II (Figure 3), again suggesting a potential connection between annexin II and cytoskeletal elements such as actin microfilament.

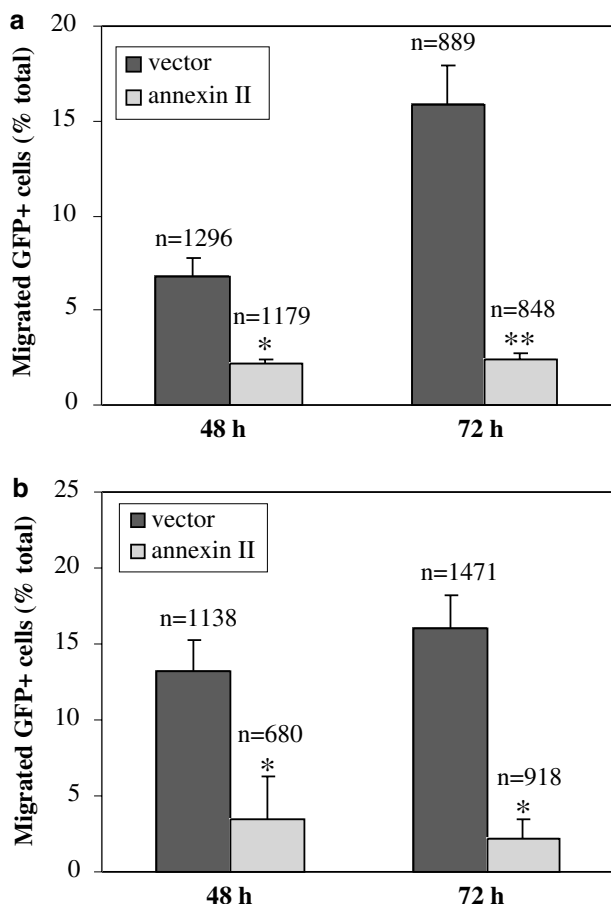
In order to directly assess the effect of annexin II on cell motility and migration, we carried out a trans-well migration assay in modified Boyden chambers using NHP2 cells that expressed abundant endogenous annexin II, Du145 cells that expressed reduced levels



**Figure 6** Overexpression of annexin II does not affect cell-cycle progression as revealed by BrdU incorporation assays. DU145 cells grown on coverslips were either untransfected or transfected with the empty vector pIRES-hrGFP (vector) or with phrGFP-annexin II (annexin II). Cells were pulsed with BrdU (10  $\mu$ M), 48 h after transfection, and then processed for BrdU immunostaining as detailed in Materials and Methods. The cell nuclei were counterstained with DAPI. (a) Representative micrographs showing that some GFP<sup>+</sup> cells (top panels) in vector-only or annexin II-transfected cells both incorporate BrdU (red; middle panels). Cells were counterstained with DAPI to identify all cells (bottom panels). (b) The percentage of BrdU<sup>+</sup> cells was determined in both GFP<sup>-</sup> and GFP<sup>+</sup> isogenic cell populations. On average, 500–1000 cells were counted for each condition. The results are expressed as the mean  $\pm$  s.e. obtained from three independent experiments. NT, nontransfected. Vector, pIRES-hrGFP. Annexin II, phrGFP-annexin II. No statistically significant difference was observed among various conditions (Student's *t*-test). Note that both transfection and GFP slightly inhibited BrdU incorporation, as previously observed in other cell systems (Tang *et al.*, 2002)

of annexin II, or LNCaP cells that did not express annexin II (Figure 3). NHP2 cells did not migrate across the Boyden chamber membrane within 96 h in our multiple repeat experiments (not shown). By contrast,

~15% of GFP<sup>+</sup>-Du145 or LNCaP cells transfected with the vector alone migrated across the membrane within 72 h (Figure 7a, b). Overexpression of annexin II in Du145 (Figure 7a) or restored expression of annexin



**Figure 7** Annexin II inhibits migration of DU145 (a) or LNCaP (b) cells. Cells on trans-well filters were transfected with either pIRES-hrGFP (vector) or with phrGFP-annexin II (annexin II). The GFP<sup>+</sup> cells on both sides of the filter were counted, 48 or 72 h after transfection. The results are expressed as the percentage of the total GFP<sup>+</sup> cells migrated across the membrane. The bars represent the mean  $\pm$  s.e. from three independent experiments. The total numbers of cells counted are indicated (*n*). \* $P < 0.01$ , \*\* $P < 0.001$  (Student's *t*-test). Note that NHP2 cells did not migrate across the Boyden chamber membrane (see text)

II in LNCaP (Figure 7a) cells significantly inhibited their migration 48 h after transfection. The inhibitory effect became even more prominent 72 h after transfection: the migration rate in annexin II-transfected cells remained essentially unchanged whereas the rate was more than doubled in the empty vector-transfected cells (Figure 7b).

In conclusion, the use of proteomic analysis reveals reduced or lost expressions of annexins I and II in prostate cancer cells. Our observations concur with others' data showing reduced annexins I and II expression in prostate cancer cells *in vivo* (Pawletz *et al.*, 2000; Chetcuti *et al.*, 2001). Chetcuti *et al.* recently reported that 100% prostate cancer specimens (31 cases in total) examined lacked the 36 kDa annexin II immunostaining and these cancer tissues also lacked annexin II mRNA expression. Southern analysis of cancer DNA, however, did not reveal any noticeable

deletions/mutations in the annexin II gene (Chetcuti *et al.*, 2001), suggesting that the loss of annexin II protein expression results from transcriptional or post-transcriptional suppression. Indeed, treatment of LNCaP cells, which have completely lost annexin II expression (Figure 3, this study), with the DNA methyltransferase inhibitor 5-aza-deoxycytidine reactivated the annexin II expression in these cells (Chetcuti *et al.*, 2001), suggesting that promoter hypermethylation might be responsible for the gene silencing. Interestingly, another annexin molecule, annexin 7, is also lost in prostate cancer cells and has been proposed as a prostate tumor suppressor (Srivastava *et al.*, 2001). Different from annexin II, the loss of annexin 7 expression appears to be because of loss of heterozygosity, thus indicative of gene mutations (Srivastava *et al.*, 2001). It is of interest to note that S100A2, which, like p11 or S100A10, belongs to the S100 family of proteins, is also reduced in LNCaP cells (protein spot 4 in Figure 1b, panel d), as observed in breast cancer cells (Lee *et al.*, 1992) and recently reported by others in prostate cancer cells (Gupta *et al.*, 2002). Another S100 protein, S100A4, is also reduced in prostate cancers (Gupta *et al.*, 2002). Since both annexin I and annexin II form complexes with various S100 proteins, these observations together suggest that annexins with their S100 binding partners may represent a family of proteins that generally inhibit prostate tumorigenesis.

How may reduced annexin I/II expression contribute to prostate cancer development? Subsequent mechanistic studies focused on annexin II suggest that it probably inhibits prostate cancer development by inhibiting cell migration without affecting proliferation or apoptosis. Exactly how the re-expressed annexin II inhibits the migratory ability of prostate cancer cells remains to be determined. It is possible that these annexin II molecules may form complexes with p11 underneath plasma membrane and bind to cytoskeletal molecules, and the II<sub>2</sub>-p11<sub>2</sub> complexes then stabilize cytoskeleton and inhibit cell migration (Thiel *et al.*, 1992; Bianchi *et al.*, 1995; Zobiak *et al.*, 2001). The II<sub>2</sub>-p11<sub>2</sub> complexes may also regulate or stabilize, through unknown mechanisms, the intermediate filament, a major cytoskeletal component that helps to maintain the mechanical stability of cells and serves as attachment sites for cell organelles and cytoplasmic molecules, which in turn limits cell migration (Ikebuchi and Waisman, 1990; Bianchi *et al.*, 1995). Regardless of the molecular mechanisms, prostate cancers lose or suppress annexin II expression, annexin II re-expression inhibits prostate cancer cell migration (that is important for prostate cancer progression and metastasis), and soluble annexin II inhibits the migration of lung carcinoma cells (Balch and Dedman, 1997). These observations suggest that restoration of annexin II expression may represent a novel anti-prostate cancer therapeutic approach. We are currently addressing the physiological functions of annexin II (and I) in NHP cells by attempting to downregulate their expression using the RNAi technique.

### Abbreviations:

2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; aa, amino acids; BrdU, 5-bromo-2'-deoxyuridine; DAPI, 4', 6-diamidino-2-phenylindole; hrGFP, humanized *Renilla* green fluorescence protein; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; MS, mass spectrometry; NHP, normal human prostate epithelial cells; PSD, postsource decay.

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