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Protein B23/Nucleophosmin/Numatrin nuclear dynamics in Relation to Protein Kinase CK2 and Apoptotic Activity in Prostate Cells†

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Abstract

Protein B23/nucleophosmin/numatrin3 is a key nucleolar/nuclear matrix associated protein required for cell growth related functions, such as rRNA synthesis. Protein kinase CK2 (formerly casein kinase 2, a protein ser/thr kinase signal that is involved in cell growth and cell death) mediates phosphorylation of B23 thereby influencing its functional activity. Here we have delineated the dynamics of B23 and its link to CK2 status in response to altered growth stimuli and induction of apoptosis in cultured prostate cells and in rat prostate cells *in vivo*. Our studies employing PC-3 and ALVA-41 prostate cancer cells demonstrated colocalization of CK2 and B23 in the nucleus. Further, CK2 and B23 underwent coordinate modulation in the nucleus related to their nucleocytoplasmic shuttling in response to induction of apoptotic activity in cells caused by downregulation of CK2 or by treatment with other apoptosis-inducing agents. These alterations in nuclear association of B23 occurred in the absence of a significant change in the level of cytoplasmic B23. Similar studies in the *in vivo* model of rat prostate epithelial cells subjected to androgen deprivation (that resulted in loss of nuclear CK2 and induction of apoptosis) demonstrated dynamic modulation of nuclear matrix associated B23 without a significant change in its cytoplasmic level. These changes were reversed by androgen-mediated growth response in the prostate. Our results suggest that CK2 mediated phosphorylation of B23 is essential for its retention in the nucleus, and that coordinate nuclear localization of B23 and CK2 is dynamically regulated in response to altered growth status in the cell.

Protein B23/nucleophosmin/numatrin (hereafter, B23) is a conserved nucleolar (and nuclear matrix-associated) phosphoprotein that is localized to the granular and fibrillar regions of the nucleolus where rRNA synthesis and assembly take place (1,2). Changes in rRNA synthesis are among the earliest responses to initiation or cessation of growth signals. For example, removal of the androgenic growth signal in the rat prostate, an organ strictly dependent on the availability of androgens for growth and proliferation, results in a rapid decline in rRNA synthesis. Conversely, in the same model, stimulation of prostatic growth (by androgen administration to castrated rats) results in a rapid synthesis of rRNA for which the availability of B23 is essential (3–9). Our previous data indicated that regulation of B23 expression in the

[3]B23, protein B23/nucleophosmin/numatrin; CK2, acronym for former name casein kinase II or 2; NM, nuclear matrix; TBB, 4,5,6,7-tetrabromobenzotriazole; 5α-DHT, 5α-dihydrotestosterone; TRAIL, tumor necrosis factor related apoptosis inducing ligand.

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prostate after androgen ablation in the animal was not at the transcriptional level since B23 mRNA was present at high level as late as 6 days following androgen ablation when extensive apoptosis in prostatic cells is apparent, suggesting that under these experimental conditions the observed changes in B23 were primarily at the post-transcriptional level (10,11). Since B23 is phosphorylated by protein kinase CK2 (formerly casein kinase 2 or II), the concordant loss or increase of the two proteins in the nuclear fraction upon altered androgenic status in rat prostate further emphasized the involvement of the two proteins in the early expression of rRNA synthesis (10,11).

CK2 is a ubiquitous and highly conserved protein ser/thr kinase for which much evidence has emerged suggesting that it plays not only important roles in regulation of cell growth and proliferation but also in cell death by serving as a potent suppressor of apoptosis (12–16). CK2 is the key enzyme that mediates phosphorylation of B23 (10,11,17,18), although a phosphorylation by $p34^{cdc2}$ is also involved during mitosis (19). B23 has been implicated to have molecular chaperone activity which appears to be regulated by CK2-mediated phosphorylation (20,21). Further, phosphorylation of B23 by CK2 regulates organization of nucleolar compartments so that CK2 plays a significant role in controlling the compartmentation of the rRNA-processing (22). These observations taken together with our previous studies (10,11,23) suggest a dynamic relationship between B23 and CK2 nuclear localization in response to altered growth stimuli in the cell; however, the nature of this relationship especially in response to altered cell growth and apoptotic activity in cells has not been studied.

Here we have investigated the nature of CK2 mediated regulation of cellular B23 levels under conditions that are associated with modulation of CK2 and/or altered apoptotic activity by employing prostate cells as experimental model. For the biochemical experimental studies on B23 in relation to CK2, we have employed the nuclear matrix fraction which is known to include the nucleolar component (24). The choice of nuclear matrix (rather than whole nuclei) for the biochemical studies was based on the consideration that we have previously demonstrated the presence of B23 in the isolated nuclear matrix fraction (11), and importantly we have also demonstrated a profound rapid response of nuclear matrix associated CK2 to altered growth signals (25,26). In the present work, our results demonstrate that downregulation of CK2 by various methods results in loss of nuclear associated B23 (as evidenced by its loss in the nuclear matrix) without a significant change in the cytoplasmic levels of the protein. Institution of a growth stimulus results in the translocation of B23 along with CK2 to the nucleus where both proteins demonstrate a colocalization as demonstrated by immunofluorescence staining studies of whole cells. Since rapid translocation of CK2 to the nucleus (where it shows differential association with subnuclear compartments) in response to growth stimulus has been documented (23,25,26), the coordinate shuttling of B23 and CK2 in and out of the nucleus and their colocalization in the nuclear compartment may represent an early event in their involvement in modulating responses to growth and apoptotic stimuli in the cell.

MATERIALS AND METHODS

Treatment of Animals

Male Sprague-Dawley rats weighing 275–325 g (from Harlan-Sprague-Dawley) were used as the source of ventral prostate tissue. To achieve androgen deprivation, the animals were orchiectomized *via* the scrotal route employing isoflurane as the anesthetic agent. 5α-dihydrotestosterone (5α-DHT) was administered subcutaneously as a solution in sesame oil (1 mg/100 g of body weight); control animals received an appropriate volume of the vehicle as described previously (11). Animals were sacrificed at the end of the treatment period, and

ventral prostate tissue was removed, and subjected to preparation of lysates, or isolation of nuclear matrix and cytoplasmic fractions as described subsequently.

Cell Lines and Treatment of Cells

Prostate cancer cell lines PC-3 (androgen-insensitive) and ALVA-41 (androgen-sensitive) were maintained in RPMI-1640 (Invitrogen/GIBCO, Carlsbad, CA) supplemented with 2 mM L-glutamine and 10% FBS (for PC-3 cells) or 6% FBS (for ALVA-41 cells) in T-75 flasks (27,28). CK2 activity was altered in prostate cancer cells (ALVA-41 and PC-3) by treatment with varying concentrations (up to 150 μM) of 4,5,6,7-tetrabromobenzotriazole (TBB) (Calbiochem, San Diego, CA), or up to 100 μM of apigenin (Calbiochem, San Diego, CA). Cell viability was altered by treatment with tumor necrosis factor related apoptosis inducing ligand TRAIL (10–25 ng/ml) (R & D Systems, Minneapolis, MN), or etoposide (50 μM) (Sigma-Aldrich, St. Louis, MO), as indicated in the legends to figures. To achieve CK2 knockdown by using siRNA, both the catalytic subunits α and α′ of CK2 were targeted using the following sense/antisense sequences: 5′-AUGUGGAGUUUGGGUUGUAUdTdT-3′ and 3′-dTdTUACACCUCAAACCCAACAUA-5′ (Dharmacon Research); Cyclophilin B (Dharmacon, Catalog: D-001136-01-05) was used as a control. Cells were cultured in antibiotic-free media for at least 24 h before transfection. The siRNA and the DharmaFECT-2 were diluted separately in OptiMEM medium and incubated at room temp for 5 min. Lipid-siRNA complexes were then formed at room temp for 20 min using different concentrations of siRNA. The complexes were diluted 5 times in antibiotic-free medium and added to the cells at a final concentration of 5, 10, and 50 nM siRNA (except where indicated otherwise) for different periods of transfection. The siRNA transfection into cells was carried out using DharmaFECT-2 (0.2 μl/100 μl for ALVA-41 cells, and 0.3 μl for PC-3 cells). Transient overexpression of CK2 in PC-3 or ALVA-41 cells was achieved by transfection of cells with pcDNA6-CK2α (2.0 μg/ml using DOTAP with a 1:5 DNA/DOTAP ratio) for a period of 24 h, as described previously (27,28). In some experiments, cells transfected with pcDNA6-CK2α were subsequently treated with 10–25 ng/ml of TRAIL for 24 h, or with 50 μM etoposide for 48 h, as described previously (27,28).

Analysis of Cell Viability

The cell proliferation assay reagent WST-1 (Roche, Indianapolis, IN), was employed to determine cell viability and proliferation in PC-3 and ALVA-41 cells. An aliquot of 200 μl of a suspension of treated or untreated cells ($2–5 \times 10^3$) was placed in each well of a 96-well plate and allowed to re-attach over a period of 24 h. Following various cell treatments as above, media in each well was replaced with 100 μl of fresh media containing 100 μl/ml WST-1, and incubation was carried out at 37 °C for an additional 60 min. An automated plate reader was employed to measure OD_{450}. The results were confirmed in at least three independent experiments. Other pertinent details are mentioned under legends to individual figures.

Preparation of Whole Cell Lysates, Cytoplasm and Nuclear Matrix

Cell lysates and cell fractions (NM and cytoplasm) from rat ventral prostate tissue, and from cultured PC-3 and ALVA-41 prostate cancer cells were prepared as described previously (29). Ice-cold RIPA buffer was used for making total lysates whereas CSK buffer was employed for preparation of cell fractions, and in all cases Method C was used for preparation of cell fractions as detailed previously (29). Operationally, the nuclear matrix is known to contain the nucleolar component as was defined previously (24). Briefly, prostate tissue or cultured prostate cancer cells (pelleted ALVA-41 or PC-3 cells washed in cold PBS) are suspended (~ 10% w/v) in CSK buffer (10 mM PIPES pH6.8 at room temp + 100 mM NaCl + 0.3 M sucrose + 3 mM $MgCl_2$ + 1 mM EGTA + 0.5% Triton X-100 + 4 mM vanadyl riboside + Sigma protease inhibitor cocktail 0.02 × of the final volume). The suspension is homogenized

and centrifuged for 5 min at 600 × g. The supernatant from the 600 × g centrifugation is employed as the cytoplasmic fraction. The pellet is then suspended in the extraction buffer (10 mM Tris-HCl pH 7.4 at room temp + 10 mM NaCl + 3 mM $MgCl_2$ + 1% Tween 40 + 0.5% sodium deoxycholate + 4 mM vanadyl riboside + Sigma protease inhibitor cocktail ×0.02 of the final volume). The material is suspended and kept on ice for 5 min. The suspension is centrifuged again at 600 × g for 5 min, and the resulting pellet is suspended in the digestion buffer (10 mM PIPES pH 6.8 at room temp + 0.3 M sucrose + 50 mM NaCl + 3 mM $MgCl_2$ + 1 mM EGTA + 0.5% Triton X-100 + 4 mM vanadyl riboside + Sigma protease inhibitor cocktail ×0.02 of final volume + 100 μg/ml RNase A + 100 μg/ml DNase I). The suspension is incubated at room temp for 30 min with occasional mixing of the suspension. Subsequently, 1/3 of the volume of 1 M $(NH_4)_2SO_4$ is added dropwise to the suspension to obtain a 0.25 M final concentration in the mixture. The suspension is centrifuged for 5 min at 600 × g, and the pellet (nuclear matrix fraction) is suspended by vortexing to obtain a uniform suspension in TMED + 0.2 M NaCl + Sigma's protease inhibitor cocktail (x 0.01 of total volume) (TMED is 50 mM Tris-HCl pH 7.9 at room temp + 5 mM $MgCl_2$ + 1 mM EDTA + 0.5 mM DTT). Protein concentration was estimated as described previously (29).

Determination of CK2 Activity

CK2 activity was determined by employing Cyclex CK2 activity kit from MBL (Woburn, MA). Cells (ALVA-41 or PC-3, 0.5×10^6 in 6-well plates) were suspended in extraction buffer as suggested by the vendor, and measurement of CK2 activity in whole cell lysate was carried out according to the supplied protocol.

Immunoblot Analysis

Total lysate, cytoplasm, and NM of cultured cells or prostate tissue samples prepared as above were employed for immunoblot analysis using mouse anti-CK2α (1:500) (BD Biosciences, San Diego, CA), mouse anti-B23 (1:200,000) (Zymed, San Francisco, CA), rabbit anti-PARP (1:500) (Santa Cruz Biotechnology, Inc, Santa Cruz, CA), rabbit anti-lamin A (1:500) (Cell Signaling, Danvers, MA), and mouse anti-Actin (1:500) (Calbiochem, San Diego, CA) primary antibodies. After 3 washes with TBS-T (Tris Buffer Saline with 0.1% Tween-20) the membranes were incubated with goat anti-mouse IgG/horseradish peroxidase conjugated secondary antibody (1:50,000) (Pierce, Rockford, IL). SuperSignal West Pico chemiluminescence reagent (Pierce, Rockford, IL) was used in conjunction with Kodak Biomax ML film to detect peroxidase activity.

Immunofluorescence Staining of CK2α and B23

Cellular distribution of CK2α and B23 proteins was determined by immunofluorescence staining in ALVA-41 and PC-3 cells. The cells were grown on cover slips placed in a 6-well plate. Cells on the cover slip were fixed with cold methanol for 10 min, and washed three times (5 min/each) in PBS. After 1 h of incubation with 10% normal goat serum in PBS at room temperature cells were treated with mouse monoclonal anti-CK2α antibody (1:50 dilution) (BD, Chicago, IL) and rabbit polyclonal anti-B23 antibody (1:50 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA) in PBS (containing 2% FBS) for 2 h at room temperature. The samples were then washed three times in PBS. The goat anti-rabbit-FITC (1:50) and goat anti-mouse-Alexa conjugated secondary antibodies (1:1000 dilution) (Invitrogen, Eugene, Oregon) were applied, and after washing the cells were mounted to the slides with aqueous anti-fading medium (Biomeda, Foster, CA). The immunofluorescence staining of cells for CK2α and B23 was visualized by Olympus BX60 fluorescence microscope (Center Valley, PA). The images were captured using the Spot Software from Diagnostic Instruments (Sterling Heights, MI).

Detection of Apoptosis

Apoptosis in cells subjected to various doses of TBB was determined by using the Cell Death Detection kit (TUNEL Staining, Roche) employed according to the instructions supplied by the manufacturer. After completing the TUNEL staining procedure the cells were counterstained by dipping the slides in 1 μg/ml Hoechst 33342 solution for 1 min. They were then washed twice with PBS. Finally, the cells were mounted to the slides with aqueous anti-fading medium (Biomeda, Foster, CA). The TUNEL staining images of the cells were obtained as described above.

Real-time reverse transcription-PCR

Following the desired treatment, PC-3 and ALVA-41 cells were collected and washed twice with PBS by centrifugation at 100 × *g* for 5 min. The cells were then suspended in RNAlater/PBS (6:1) (Ambion, Inc., Austin, TX). Total RNA was isolated from cells by employing RNeasy Mini Kit (Qiagen, Inc., Valencia, CA), and reverse transcription for RT-PCR was carried out using RETROscript (Ambion, Inc., Austin, TX). Real-time PCR was performed in a total volume of 25 μl containing 5 μl of cDNA (50 ng), 1 μl of primer mix (the forward and reverse primers in water), 12.5 μl of SYBR Green Supermix (SuperArray, Frederick, MD), 0.5 μl Rox, and 6.0 μl of water. Primer sets were from SuperArray as follows: CK2α #PPH01514A; B23 #PPH19534A; and Actin #PPH00073A. The PCR conditions were as follows: an activation step at 95 °C for 10 min followed by 50 cycles of denaturation at 95 °C for 15 sec, annealing at 60 °C for 30 sec, and extension at 72 °C for 30 sec.

RESULTS

Effect of Molecular Downregulation or Chemical Inhibition of CK2 on Cytoplasmic and Nuclear associated B23 in Prostate Cancer Cells

Since downregulation of CK2 in cells results in induction of apoptosis (12,13), we determined B23 status in two types of prostate cancer cells (androgen-sensitive ALVA-41 and androgen-insensitive PC-3 cells) in response to treatment with TBB, a relatively specific inhibitor of CK2 (Fig. 1). As shown in Fig. 1*A*, TUNEL analysis indicated that TBB induced apoptosis in these cells in a dose-dependent manner. Under these conditions a dramatic reduction in the NM-associated B23 was observed in both the ALVA-41 and PC-3 cells whereas no change was noticed in B23 immunoreactive protein present in the cytoplasmic fraction (Fig. 1*B*), suggesting that nuclear B23 was translocated in response to inhibition of CK2 and the concordant induction of apoptotic activity in the cells. In separate experiments, we observed that treatment of ALVA-41 cells with varying concentrations of the CK2 inhibitors TBB or apigenin for 24 h caused a dose-dependent inhibition of CK2 activity (Fig. 1*C*). Under these experimental conditions no change in the message level of CK2• or B23 was detected in the cells treated with CK2 inhibitors (Fig. 1*D* and 1*E*). Analogous experiments were also undertaken by employing siRNA to downregulate the • and •´ subunits of CK2. As shown in Fig. 2*A*, when ALVA-41 or PC-3 cells were treated with CK2 •,•´ siRNA at varying concentrations for different periods of time, there was a dose- and time-dependent effect on WST-1 assay of cell growth. In this assay, the androgen-independent PC-3 cells were somewhat more resistant than the androgen-sensitive ALVA-41 cells, which accords with previous observations on the relative sensitivity of these cells to induction of apoptosis (27,28). The presence of apoptosis under these conditions was also confirmed by a corresponding analysis of cells using TUNEL assay (Fig. 2*B*). The immunoreactive protein levels for B23 and CK2 catalytic subunits under the same conditions demonstrated the expected significant reduction in the CK2 signal in both the NM and cytoplasmic fractions whereas the B23 was not affected in the cytoplasmic fraction but significantly reduced in the NM fraction (Fig. 2*C*). These results further suggest a role of nuclear CK2 in specifically regulating the retention of protein B23 in the nuclear compartment.

Effect of Apoptosis Inducing Agents on NM-associated B23

Since CK2 inhibition resulted in the loss of nuclear-associated B23 without any change in its level in the cytoplasm, we further examined the effects of apoptosis inducing conditions in ALVA-41 and PC-3 prostate cancer cells. The results in Fig. 3*A* show that when low doses of etoposide (20 μM), TBB (40 μM), and apigenin (20 μM) were employed there was no effect on cell viability; however, when TBB and etoposide or apigenin and etoposide were combined they produced a significant amount of apoptosis suggesting that CK2 inhibitors (TBB and apigenin) at low doses can sensitize prostate cancer cells to apoptosis-inducing agents such as etoposide, as was previously demonstrated for induction of apoptosis in prostate cancer cells by combined TRAIL and CK2 inhibitor treatment (28). Corresponding to these conditions, etoposide, TBB, or apigenin alone at the sub-optimal doses tested did not affect the NM-associated B23 in ALVA-41 cells; however, a dramatic reduction in B23 was detected when these agents at suboptimal levels were combined, i.e., etoposide plus TBB or etoposide plus apigenin (Fig. 3*B*) which also corresponded to increased apoptotic activity in the cells as shown in Fig. 3*A*. Essentially, similar results were obtained when ALVA-41 cells were treated with TRAIL plus TBB or apigenin (at suboptimal doses in each case) compared with that when each individual compound was employed (Fig. 3*C*). Confirmation of induction of apoptosis by combined treatment with TBB and TRAIL is provided in Fig. 3*D* by demonstrating cleavage of lamin A under these conditions. These results further suggest that during induction of apoptosis there is a dramatic downregulation of B23 associated with the nuclear fraction even though it does not change in the cytoplasmic fraction.

Effect of Overexpression of CK2 on NM-associated B23 in Response to Apoptosis Inducing Agents

We have previously shown that induction of apoptosis in ALVA-41 and PC-3 cells treated with 50 μM etoposide for 48 h, or TRAIL at 10 ng/ml (for ALVA-41) or 25 ng/ml (for PC-3) for 24 h, is blocked by transient overexpression of CK2• (28,30). Therefore, we examined the status of nuclear associated B23 in ALVA-41 and PC-3 cells treated with TRAIL or etoposide in the presence or absence of forced overexpression of CK2•. The results in Fig. 4*A* show that treatment of ALVA-41 and PC-3 cells with 10 or 25 ng/ml of TRAIL, respectively, for 24 h caused a dramatic loss of NM-associated B23 which was completely blocked by transient overexpression of CK2•. Likewise, the results in Fig. 4*B* show that NM-associated B23 was reduced in both ALVA-41 and PC-3 cells treated with 50 μM etoposide for 48 h (apoptosis-inducing condition) which was also blocked by transient overexpression of CK2•. A representative result is presented in Fig. 4*C* to illustrate confirmation of transient overexpression of CK2• indicated by its enhanced message and protein in PC-3 cells. Since induction of apoptotic activity in cells is associated with loss of CK2 in the nuclear compartment, whereas transient overexpression of CK2 promotes its additional translocation to the nuclear compartment (31), the present results accord with the notion that CK2 status in the cell and status of apoptotic activity can strongly influence the disposition of B23 in the nucleus.

Colocalization of B23 and CK2α in ALVA-41 and PC-3 cells

Considering that the above data suggested coordinate modulation of B23 and CK2 in the nuclear compartment under various conditions, we examined the subcellular localization of B23 and CK2• and the effect of the CK2 specific inhibitor TBB on their disposition in the nuclear compartment by employing immunofluorescence studies. The results in Fig. 5*A* show that when increasing concentrations of TBB were included in the media, both B23 and CK2• showed a decline in the immunofluorescence staining in the cells in a dose-dependent manner. The merged image of the immunofluorescence in cells stained with anti-B23 and anti-CK2• shows the decrease in B23 and CK2• in the nuclear compartment to be concordant as is

apparent, for example, in the presence of TBB at 100 μM which is an apoptosis inducing dose. Similar analysis of PC-3 cells treated with varying concentrations of TBB for 24 h also resulted in a coordinate loss of nuclear B23 and CK2• as indicated in the merged image of immunofluorescence staining for the two proteins (Fig. 5*B*). These results strongly indicate the colocalization of these two proteins in the nuclear compartment.

Dynamics of CK2 and B23 Levels in Rat Prostate Epithelial Cells in Response to Altered Androgenic Status

Rat prostate undergoes apoptosis in androgen-deprived animals which is reversed on administration of 5α-DHT. Employing this experimental model, it was previously observed that when nuclei isolated from prostates of rats subjected to androgen deprivation were examined for B23 and CK2 there was a marked reduction of these proteins in the NM fraction isolated from these nuclei compared with the controls (11). Restoration of the androgenic growth signal in the animals reversed these changes. However, the effect on cytoplasmic B23 was not examined under these conditions and the basis of the B23 reduction in the nuclear compartment was not established (11). We therefore re-examined the status of B23 in both the nuclear and cytoplasmic fractions isolated from prostate tissue from animals subjected to altered androgenic status. The results in Fig. 6*A* show that B23 level in prostatic total lysate is relatively unchanged in response to androgen deprivation in the animal. Under these conditions, significant loss of CK2 is apparent in total lysates of prostatic tissue from 6-day castrated animals which is reversed on androgen administration (not shown). When cytoplasmic and NM fractions isolated from the prostatic tissues were analyzed (Fig. 6*B*), there was essentially no change in the cytoplasmic B23 levels in prostatic epithelial cells from animals subjected to androgen deprivation for up to 6 days which accorded with the observation on total lysate B23 under similar conditions. Also shown in Fig. 6*B* are the results on CK2 levels in the cytoplasmic fraction under these conditions. However, B23 localized to the NM fraction demonstrated a dramatic loss upon androgen deprivation in the animal which was reversed on androgen administration to the animal. Furthermore, changes in the NM associated CK2 were analogous to those in B23 levels. Fig. 6*B* also demonstrates a lamin A immunoblot to indicate the existence of apoptosis in these cells in response to androgen deprivation, and its reversal on androgen administration. These results suggest that the extensive loss of nuclear B23 observed in previous studies in a similar experimental model and thought to be due to breakdown of B23 (10,11) was most likely due at least in part to a shuttling of B23 out of the nuclear fraction *in situ*. Dynamic shuttling of CK2 under similar conditions has been documented extensively (23,25,26). The present results further demonstrate the coordinate and dynamic modulation in the nuclear associated CK2 and B23 (especially in the NM compartment) in response to altered growth in the cell.

DISCUSSION

B23/nucleophosmin/numatrin is a multifunctional nucleolar (and nuclear matrix-associated) protein which is elevated in cancer and proliferating cells. An essential role for B23 in rRNA synthesis in cells is well known; however, other functions such as a role in chaperone activity and apoptosis have also been attributed to this protein. For example, chaperone activity towards proteins such as histone and NLS containing peptides including HIV-1 rev protein has been demonstrated (11,20,21,32,33). Association of B23 with ribosomal S9 has been proposed to store and protect B23 in the nucleoli thereby facilitating ribosome biogenesis (34). Other binding partners of B23 identified recently are ARF and nucleostemin; ARF acts to sequester B23 in the nucleolus thereby impeding its nucleocytoplasmic shuttling (35), and direct interaction of B23 and nucleostemin has been proposed to play a role in cell cycle regulatory mechanisms (36). Furthermore, several studies suggest that overexpression of B23 blocks apoptosis whereas its knockdown induces apoptosis although, as discussed subsequently, the

mechanism involved remains unclear (reviewed in 37). Given the importance of B23 in cell function, a number of investigations have been undertaken on its response to diverse stimuli in the cell, and in particular in response to death inducing agents in several experimental models; however, the results from these studies have been controversial with some reports showing a loss of B23 in apoptosis while others suggesting no change in its level (37–42). It was also suggested that the nature of cell death (e.g., apoptosis *vs* necrosis) may influence the response of B23 in the cell (41,42). On the other hand, increased stability of B23 was suggested to be associated with an antiapoptotic effect of *ras* during serum deprivation in transformed 3T3 cells (40). Interestingly, while several studies have implicated loss of B23 in apoptotic activity (see, e.g., 37), the dynamics of B23 protein under altered cell growth conditions have remained unclear, and the disparate results from various studies could presumably be in part due to a lack of consideration of B23 responses in the nuclear *vs* cytoplasmic compartment under various conditions.

The results described here are based on several experimental models to investigate the dynamics of B23 under conditions of cell growth and apoptosis. Together, these studies demonstrate a strong link between CK2 activity and B23 nuclear status in cells under different conditions, and also show that it is the nuclear status of B23 (especially in the NM) that primarily relates to growth status in the cell. Protein kinase CK2 plays a significant role in phosphorylation of B23, and both proteins have been shown to localize to the nuclear matrix (11,26). In this context, it is noteworthy that the chaperone activity of B23 is influenced by CK2-mediated phosphorylation (20,21). Likewise CK2 phosphorylation of B23 has been shown to play a key role in regulating the organization of nucleolar compartments (22). These studies reiterate the potential importance of CK2 mediated phosphorylation of B23, and in this regard our previous work suggested that loss of prostatic CK2 and B23 from the nuclear matrix upon removal of growth stimulus (i.e., androgen deprivation) in the rats correlated with induction of apoptosis in rat ventral prostate epithelial cells, and that these events were reversed on restoration of the androgenic growth signal by administration of 5α-DHT to castrated rats (11,26). Considering that CK2, besides its function in promoting cell growth and proliferation, has also been shown to act as a potent suppressor of apoptosis (12,13,30,43), the present work provides a basis of dynamic regulation of B23 in response to altered CK2 status under growth and apoptotic conditions in the cell suggesting a possible mode by which B23 may have a role in these processes.

Recent studies have documented that CK2 impacts directly on several components of the apoptotic machinery (13,44,45). It has also been suggested that overexpression of B23 is associated with suppression of apoptosis while its downregulation is associated with induction of apoptosis; however, a direct role for B23 in apoptosis is not apparent (e.g., 37). Since B23 plays significant roles in important cellular functions such as synthesis of rRNA which is among the earliest response to cell growth, it would stand to reason that changes in nuclear B23 would have profound effect on cell survival. Our observations on a co-ordinate link between CK2 and B23 provide a new lead to the mechanism by which nuclear B23 regulation by CK2 could play an important role in activities related to cell growth and cell death. The key features that we have identified are colocalization of CK2 and B23 in the nuclear compartment, and the apparently coordinate shuttling of both molecules in and out of the nucleus in response to altered growth status. Our results suggest that CK2 mediated phosphorylation of B23 appears to promote its nuclear retention. On the other hand, since loss of nuclear CK2 has been shown to be associated with induction of apoptosis (13,46), the present results show a concordant loss of nuclear B23 under these conditions even when its level in cytoplasmic fraction is unaltered. While several lines of evidence suggest that B23 shuttling plays a key role in the B23 nuclear status in response to altered CK2 and apoptotic signals, at present it cannot be ruled out that breakdown of B23 at least in part also plays a role in this process. Based on the present studies, we propose that a possible mechanism linking B23 with apoptotic activity in the cell could

relate to the nuclear status of CK2 which by regulating the level of B23 phosphorylation may relate it to apoptotic activity in the cell. Further support to this notion comes from our data showing the specific loss of nuclear associated B23 when cells are sensitized to induction of apoptosis by moderate downregulation of CK2.

In summary, we have presented data to suggest for the first time that both CK2 and B23 co-localize in the nuclear matrix fraction in the cell, and phosphorylation of B23 by CK2 appears to be essential for its nuclear retention. Induction of apoptotic conditions such as by downregulation of CK2 or by apoptosis-inducing agents results in loss of B23 from the nuclear matrix likely caused by reduction in CK2-mediated phosphorylation. Thus, nuclear retention of B23 depending on the nuclear status of CK2 may reflect the coordinate nature of their critical role in cell growth and cell survival.

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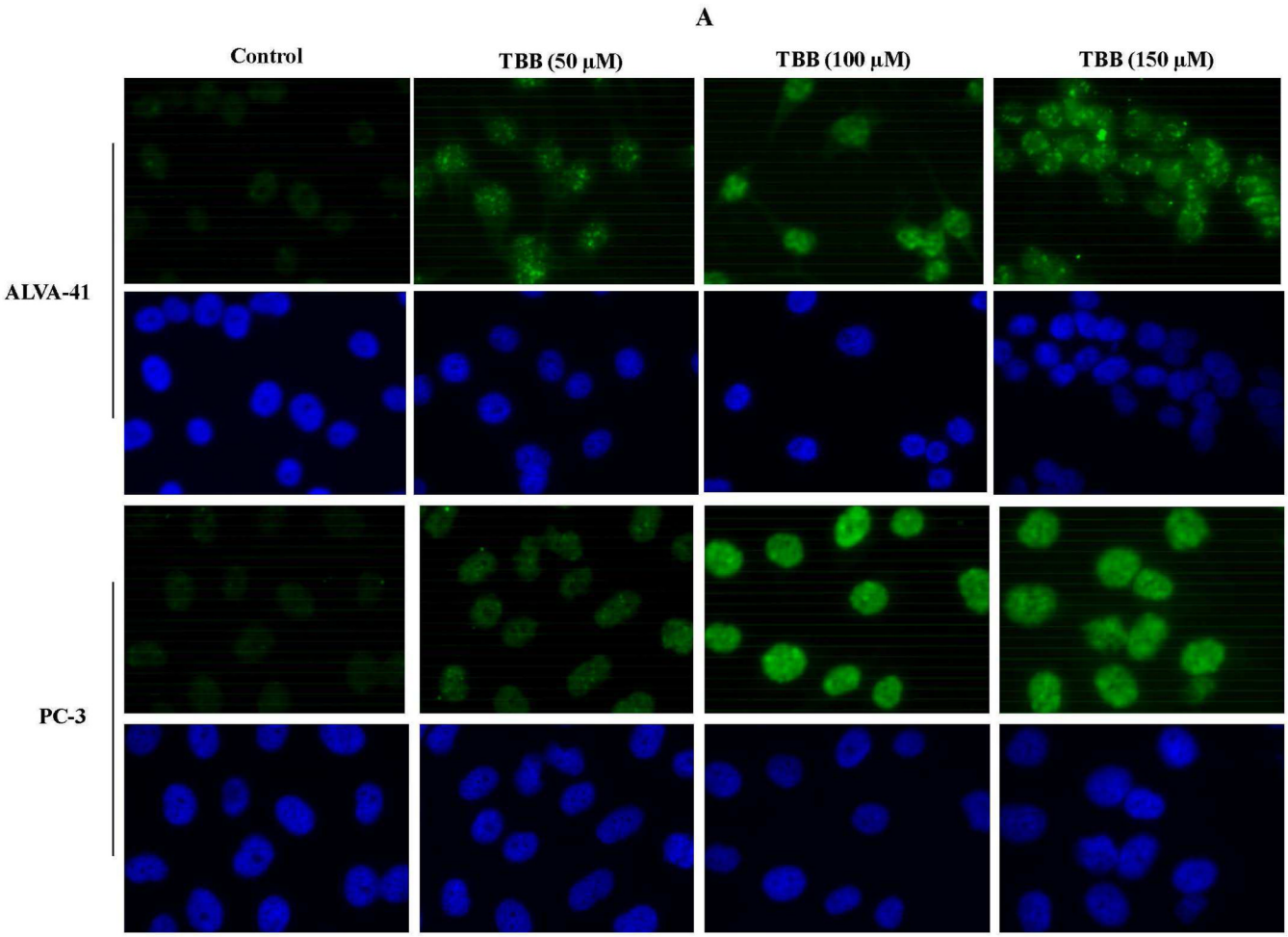


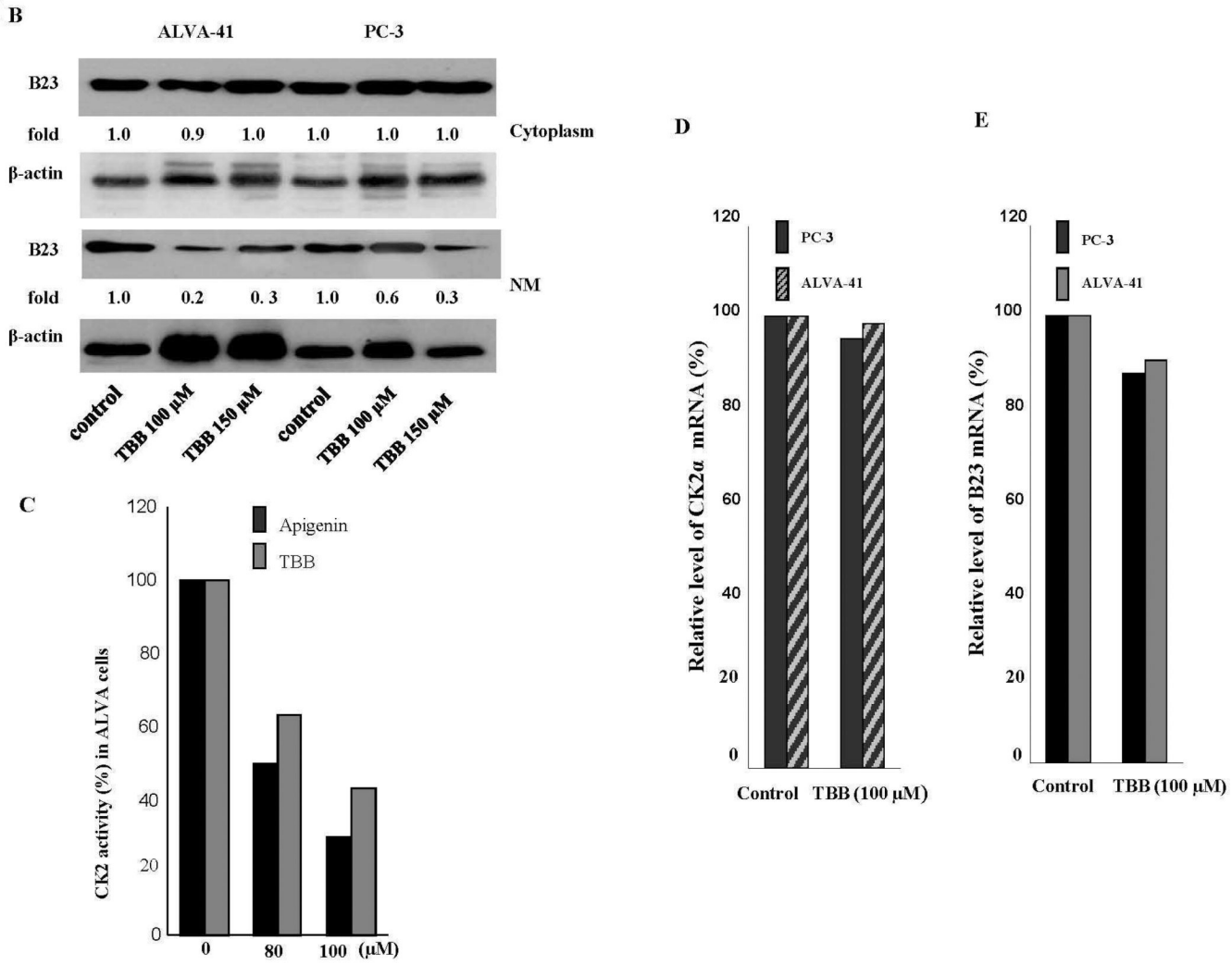
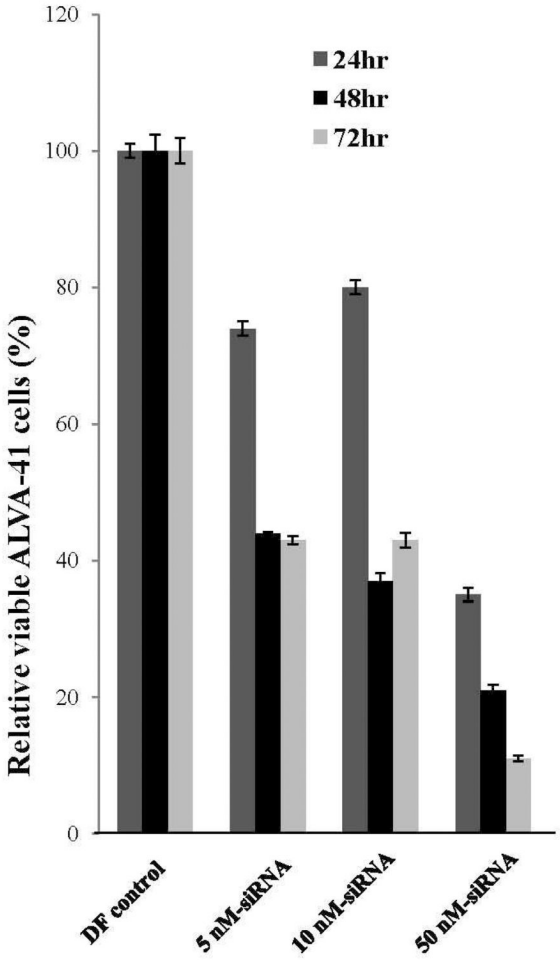
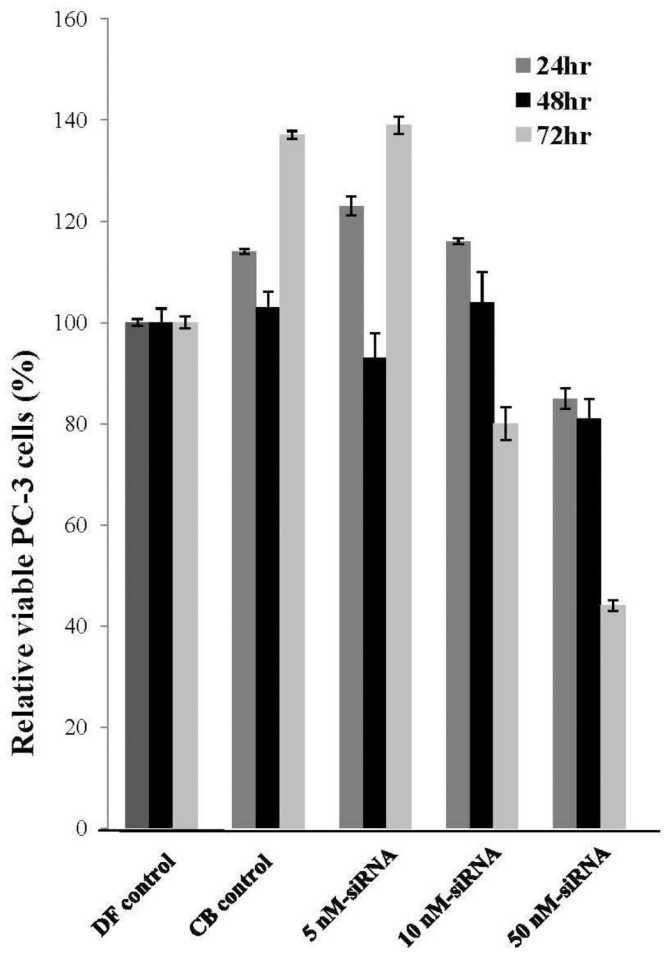


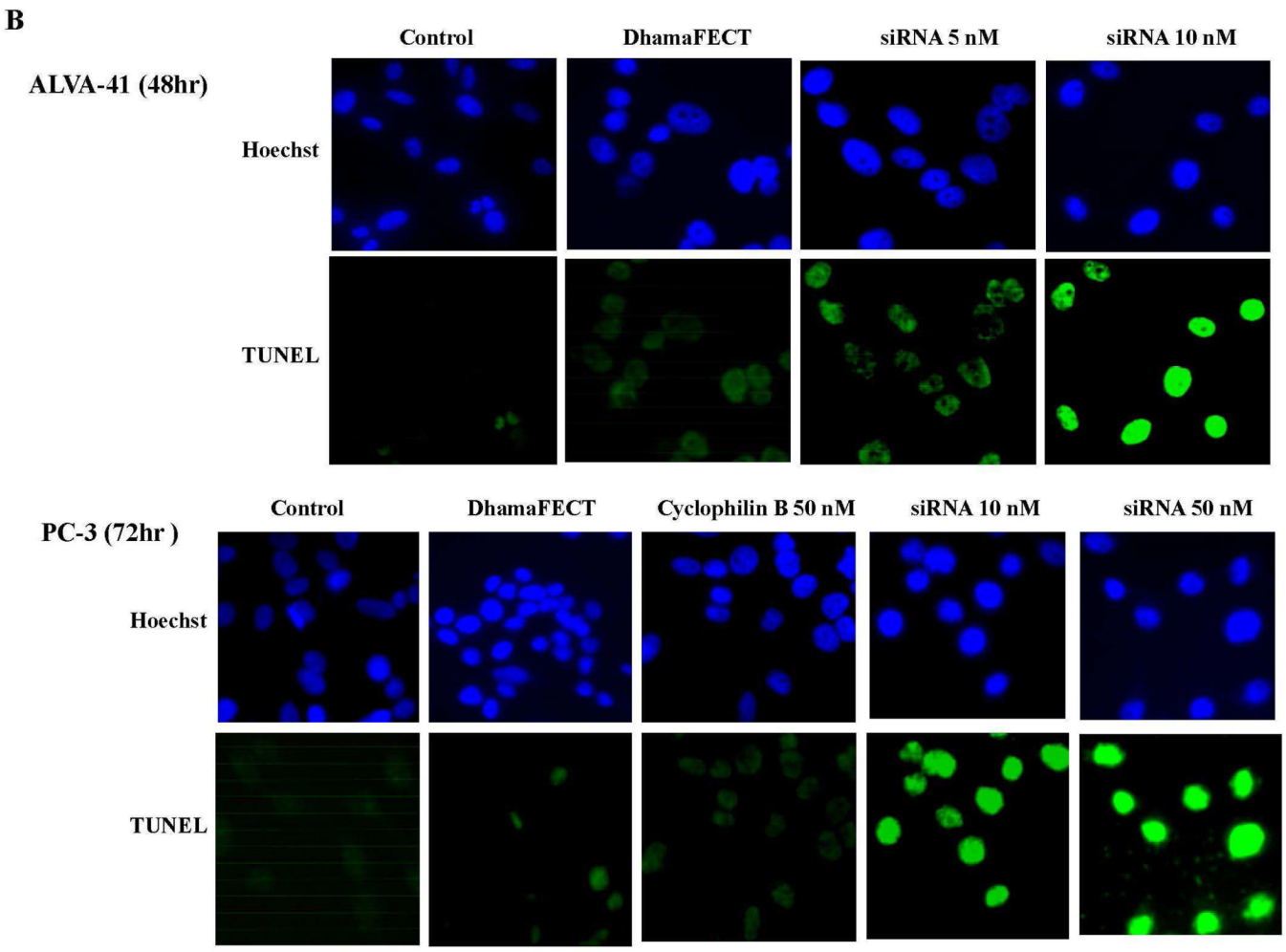
FIGURE 1.

Status of B23 in the cytoplasm and NM fractions of prostate cancer cells in response to CK2 specific inhibitor TBB mediated induction of apoptosis. *A*, TUNEL staining of ALVA-41 and PC-3 cells treated with varying doses of TBB (from 50 to 150 μM). Induction of apoptosis is shown. Cells were counterstained with Hoechst 33342 to identify nuclear component. *B*, cytoplasmic and NM associated B23 in ALVA-41 and PC-3 cells treated with TBB at varying concentrations as under *A*. The relative change in the protein bands is calculated compared to the respective control and normalized to the β-actin in the sample (e.g., lanes 2 and 3 from left in Fig. 1B had a higher amount of protein to ensure visualization of B23 signal in the nuclear matrix). *C*, effect of TBB and apigenin on CK2 activity at the concentrations of the inhibitors shown. *D*, measurement of the message expression for CK2α in ALVA-41 and PC-3 cells under the same conditions as for *C*. *E*, measurement of B23 message in ALVA-41 and PC-3 cells under the same conditions as for *C*.

A







C

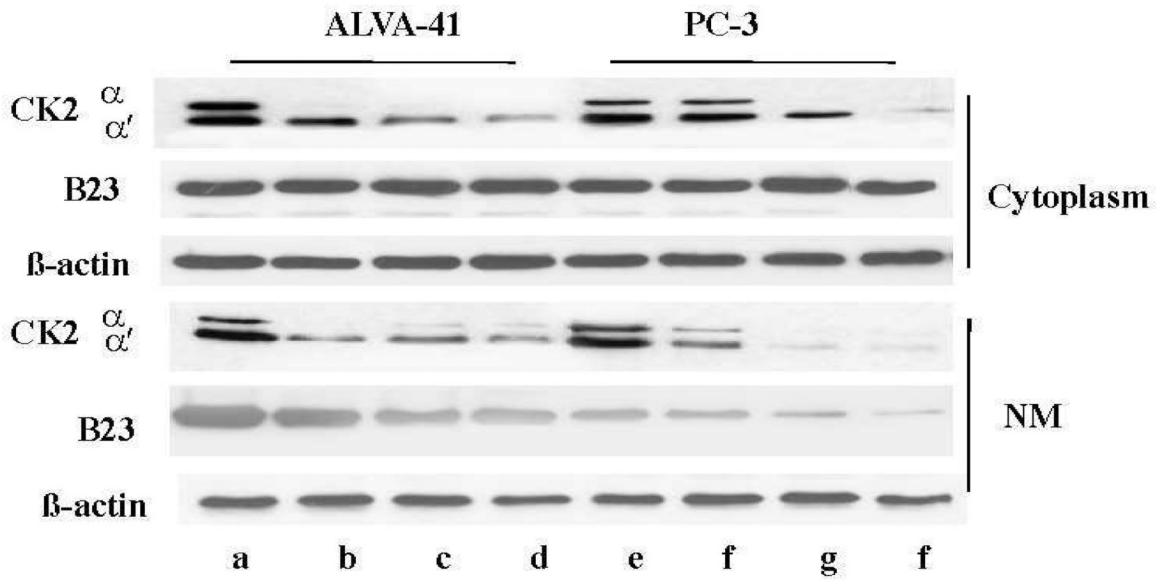


FIGURE 2.

Status of B23 in the cytoplasm and NM fractions of prostate cancer cells in response to siRNA-mediated downregulation of CK2. *A*, cell viability was determined by WST-1 assay in ALVA-41 and PC-3 cells transfected with varying concentrations of CK2••′ siRNA for the periods of time shown. *B*, ALVA-41 and PC-3 cells were transfected with varying concentrations of CK2αα′ siRNA as under *A*. Cyclophilin B (Dharmacon, Catalog: D-001136-01-05) was employed as a control. All other details were as described under Experimental Procedures. TUNEL staining shows the induction of apoptosis in cells treated with CK2 siRNA. Cells were counterstained with Hoechst 33342 to identify nuclei. *C*, immunoblot analysis of cytoplasmic and NM associated B23 and CK2••′ was carried out in ALVA-41 and PC-3 cells transfected with varying concentrations of CK2••′ siRNA. Lane *a*, untreated control; lane *b*, 5 nM CK2••′ siRNA; lane *c*, 10 nM CK2••′ siRNA; lane *d*, 50 nM CK2αα′ siRNA; lane *e*, untreated control; lane *f*, DharmaFECT control, lane *g*, 10 nM CK2αα′ siRNA; lane *h*, 100 nM CK2αα′ siRNA. The relative change in the protein bands is calculated compared to the respective control.

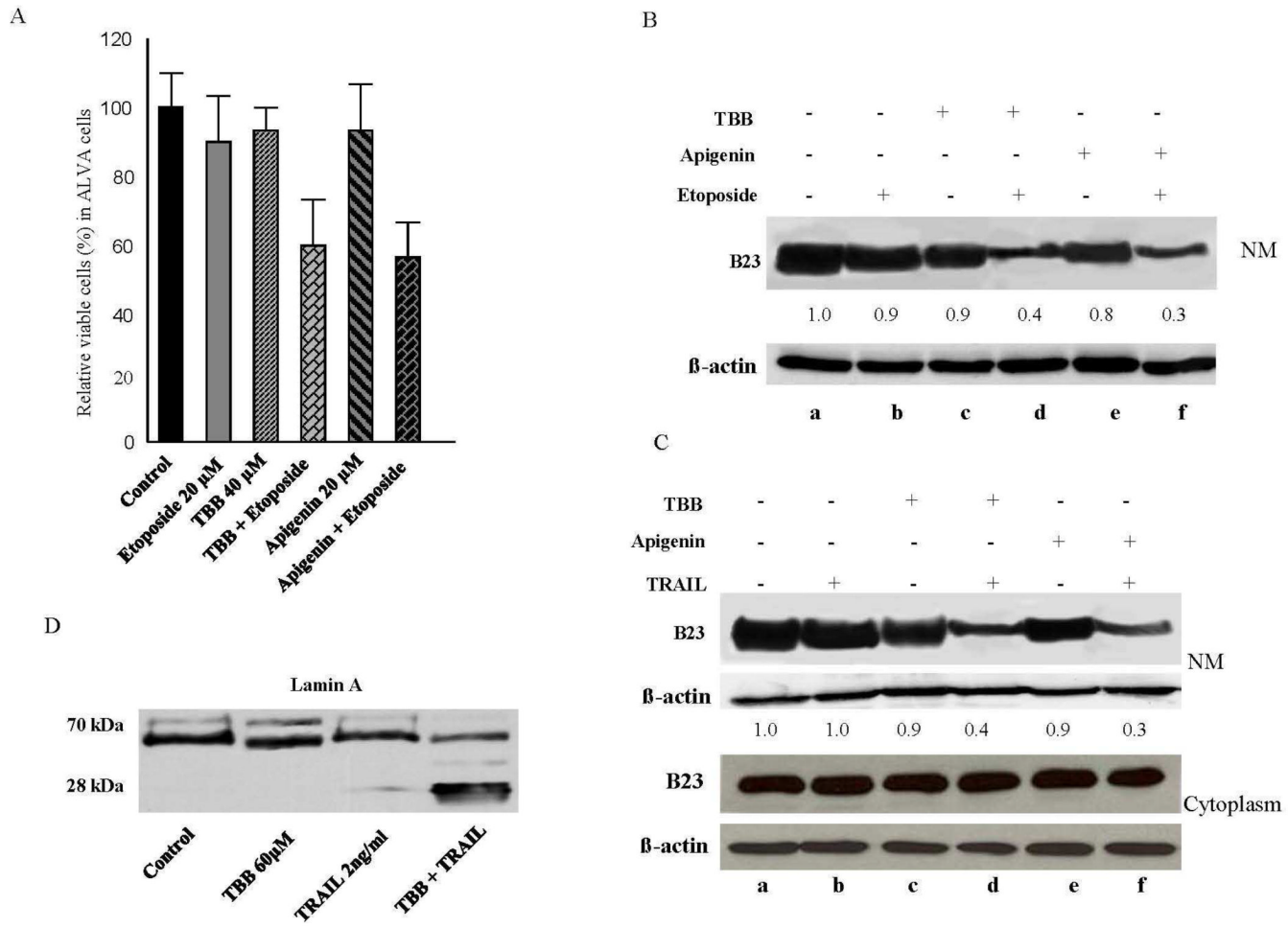


FIGURE 3.

Effect of apoptosis inducing conditions on NM-associated B23. *A*, prostate cancer cells (ALVA-41) were treated with doses of etoposide, or CK2 inhibitors (TBB or Apigenin) at doses that are sub-optimal for induction of apoptosis. The results show a minimal effect on cell death under these conditions. When the sub-optimal doses of the same inhibitors are combined it results in induction of apoptosis, as shown in the chart. Illustrated in *A* are the data employing etoposide (an apoptosis-inducing agent) with and without CK2 inhibitors TBB or apigenin. Similar effects have previously been reported using TRAIL (an apoptosis-inducing agent) with and without TBB at sub-optimal doses (27) (the latter conditions were employed in the experiment shown under *C*. *B*, NM-associated B23 immunoreactive protein in cells treated with TBB, apigenin and etoposide, as shown. Lane *a*, control; lane *b*, 20 μM etoposide (sub-optimal for induction of apoptosis); lane *c*, TBB at 40 μM (sub-optimal for induction of apoptosis); lane *d*, etoposide plus TBB (combined effect to produce apoptotic activity); lane *e*, apigenin at 20 μM (insufficient for induction of apoptosis); lane *f*, apigenin plus etoposide (combined effect to produce apoptotic activity). Densitometric values are calculated relative to the level in control cells. *C*, NM-associated B23 immunoreactive protein in cells treated with TBB, apigenin and TRAIL as shown. Lane *a*, control; lane *b*, TRAIL 2 ng/ml (sub-optimal dose which is insufficient for induction of apoptosis); lane *c*, TBB at 40 μM (sub-optimal dose for induction of apoptosis); lane *d*, TBB plus TRAIL (combined effect evokes apoptotic activity); lane *e*, apigenin at 20 μM (insufficient dose for induction of apoptosis); lane *f*, TRAIL plus apigenin (conditions to induce apoptotic activity). Relative densitometric values are based

on level in the control cells. *D*, combined effect of TBB and TRAIL (both at sub-optimal doses) in causing induction of apoptosis is illustrated by cleavage of lamin A in treated cells, as shown. Lane a, control; lane *b*, 60 μM TBB (insufficient for induction of apoptosis) showing no cleavage of Lamin A; lane *c*, 2 ng/ml of TRAIL (insufficient for apoptosis induction) as indicated by a minimal cleavage of Lamin A; lane *d*, 60 μM TBB plus 2 ng/ml of TRAIL resulting in potent induction of apoptosis as indicated by extensive cleavage of Lamin A. These results accord with the corresponding effect on NM-associated B23 as shown under *C*. In all cases, equal amount of protein was loaded in the gels, and each experiment was confirmed at least three times.

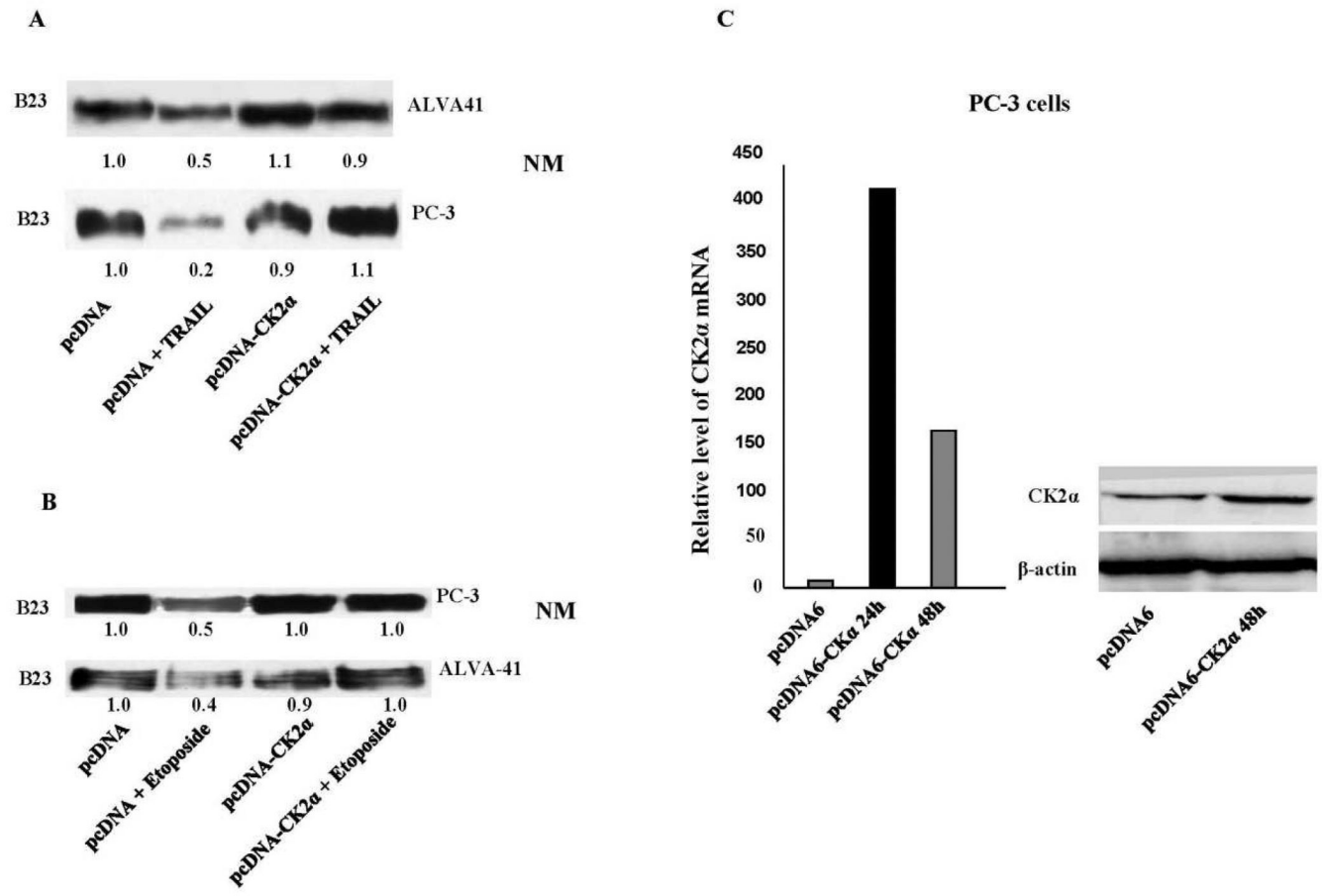
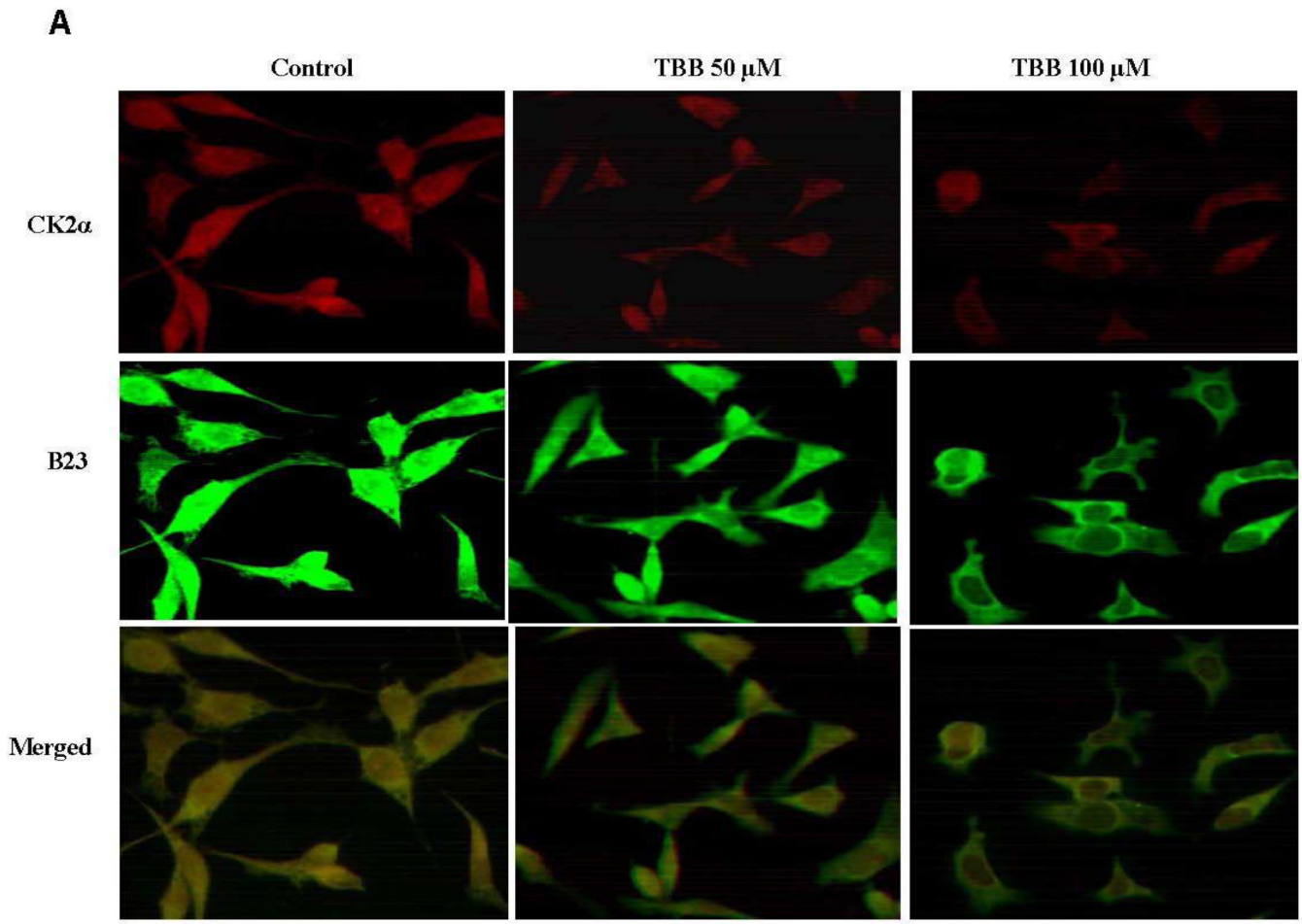


FIGURE 4.

Effect of overexpression of CK2α on the status of NM-associated B23 in prostate cancer cells treated with apoptotic inducers. ALVA-41 and PC-3 cells were treated with etoposide or TRAIL at apoptosis inducing concentrations in the absence or presence of forced overexpression of the catalytic subunit CK2α as described under Experimental Procedures. *A*, treatment with TRAIL at 10 ng/ml for ALVA-41 and 20 ng/ml for PC-3 cells (for 24 h) was carried out to induce apoptotic condition in cells. pcDNA6-CK2α expression vector (2.0 μg/ml) was employed to achieve overexpression of CK2α in ALVA-41 and PC-3 cells, as indicated. NM-associated B23 is shown for each experimental condition; there was no change in cytoplasmic B23 immunoreactive B23 under these conditions (not shown). *Lane a*, control cells treated with pcDNA6; *lane b*, pcDNA6 plus TRAIL; *lane c*, pcDNA6-CK2α; and *lane d*, pcDNA6-CK2α plus TRAIL. *B*, treatment of ALVA-41 and PC-3 cells with 50 μM etoposide (apoptosis-inducing level). Overexpression of CK2α was achieved as described under *A*. NM-associated immunoreactive B23 is shown. *Lane a*, pcDNA6 control; *lane b*, pcDNA6 plus 50 μM etoposide for 48 h; *lane c*, pcDNA6-CK2α; and *lane d*, pcDNA6-CK2α plus etoposide. *C*, a representative experiment showing confirmation of CK2α overexpression in PC-3 cells by transient transfection with pcDNA6-CK2α is illustrated by analysis of the message and protein levels.



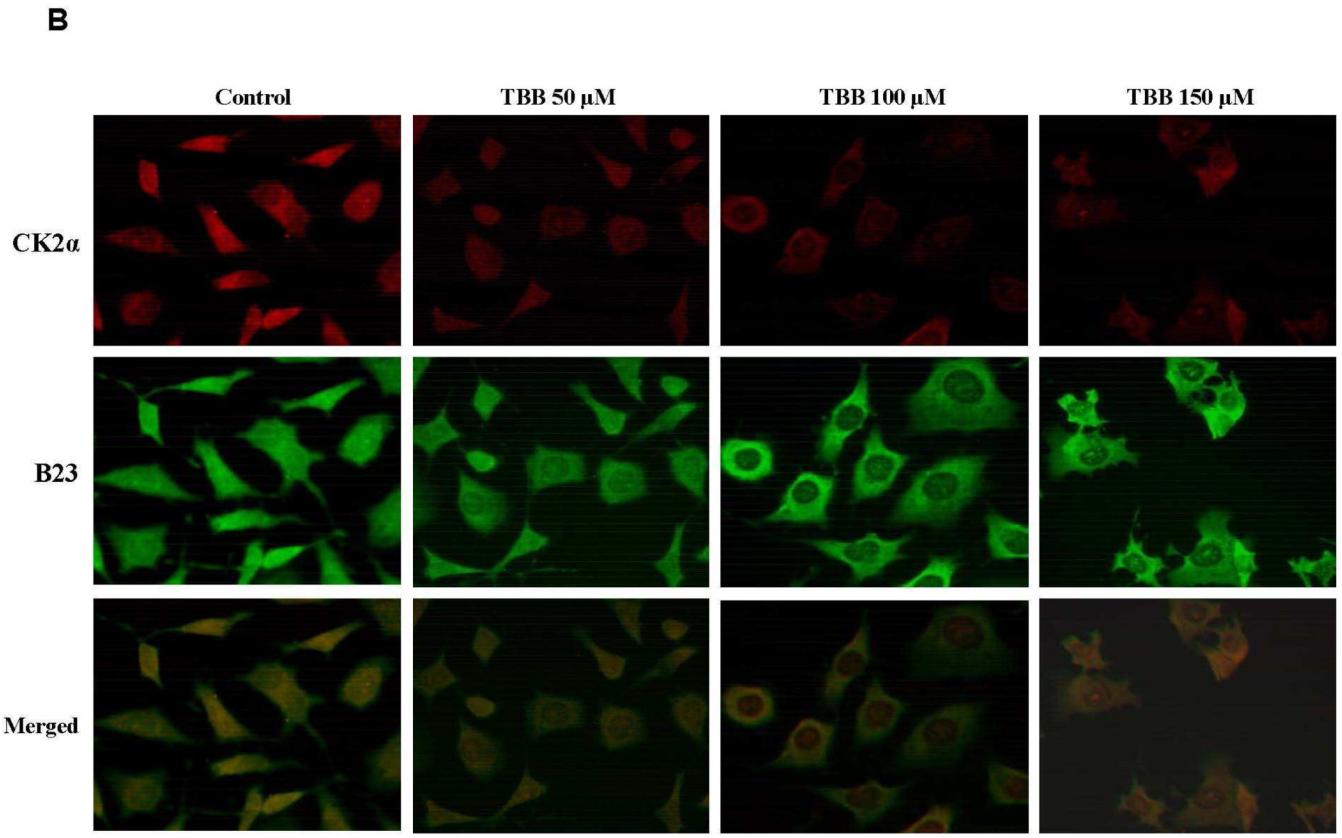


FIGURE 5.

Colocalization of CK2α and B23 in the nucleus. *A*, double immunofluorescence staining for anti-CK2α and anti-B23 was carried out in ALVA-41 cells treated with varying doses of TBB, as shown. Merged image of the immunofluorescence stains for anti-CK2α and anti-B23 shows co-localization of the two proteins in the nucleus. Downregulation of CK2 by TBB treatment (producing apoptotic condition) shows co-ordinate loss of B23 and CK2α in the nucleus. *B*, double immunofluorescence staining for anti-CK2α and anti-B23 was carried out in PC-3 cells treated with varying doses of TBB. All details are the same as for *A*. Results confirm the co-localization of CK2α and B23 in the nucleus and its reduction on induction of apoptotic condition induced by TBB mediated inhibition of CK2 as observed for ALVA-41 cells under *A*.

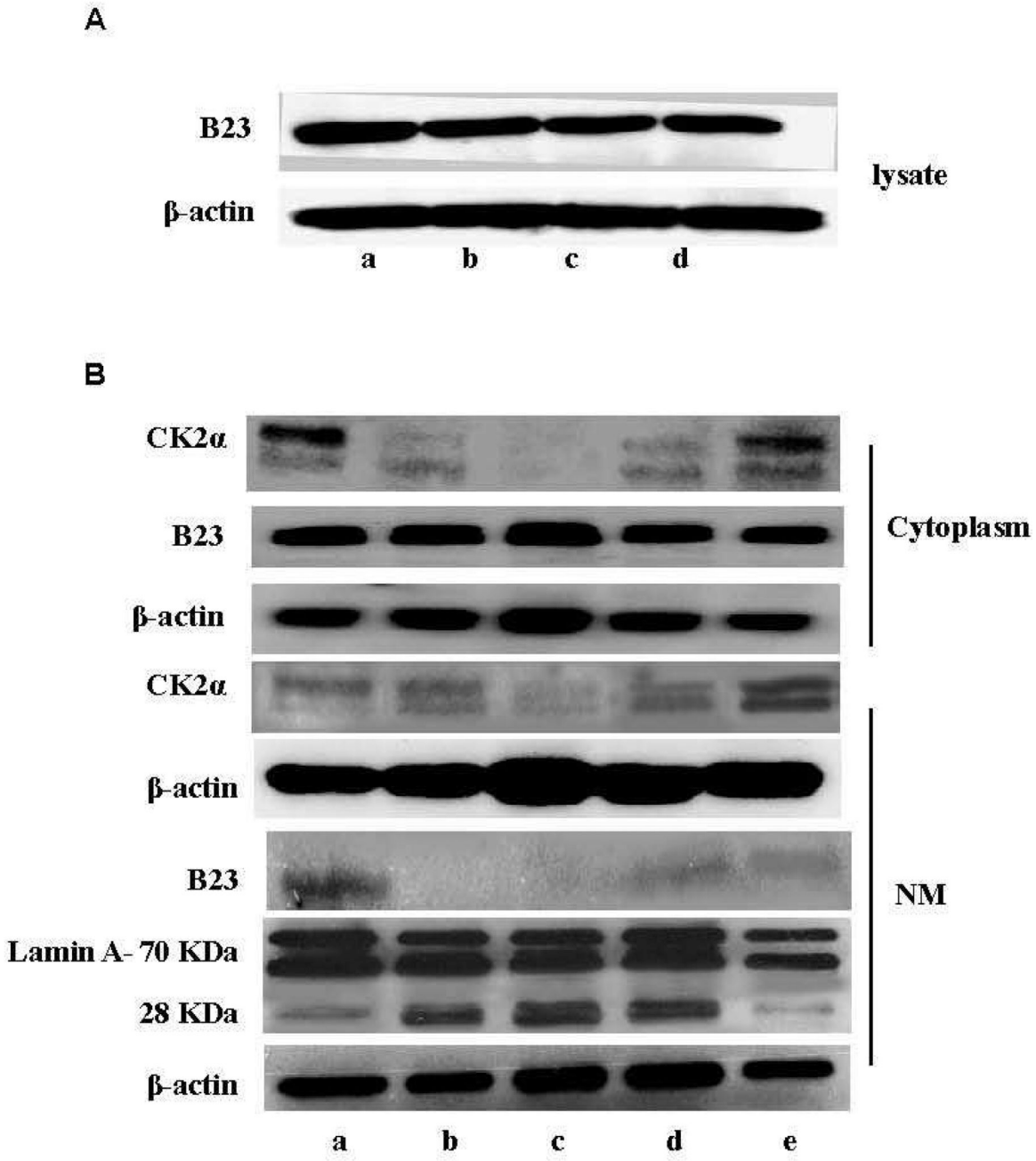


FIGURE 6.

Effect of altered androgenic status on CK2α and B23 levels in rat ventral prostate tissue. Lysate, cytoplasm and nuclear matrix fractions were isolated from prostatic tissue of rats subjected to various treatments as shown. Each fraction was analyzed by Western blotting for the presence of immunoreactive CK2α, B23 and lamin A. *A*, level of B23 in total lysates from prostatic tissues isolated from rats with altered androgenic status. *Lane a*, control rats; *lanes b* to *d*, rats orchiectomized for 1, 2, and 3 days, respectively. *B*, detection of protein CK2α, B23 and lamin A in cytoplasm and NM fractions isolated from prostate tissue of normal rats, or rats subjected to altered androgenic status. *Lane a*, control rats; *lane b*, 3-day orchiectomized rats; *lane c*, 6-day orchiectomized rats; *lane d*, 6-day orchiectomized rats given 5α-DHT for 4 days; *lane e*,

same as *lane d* except that 5α-DHT was administered for 7 days. Breakdown of lamin A (to 28 kDa fragment) in the NM fraction indicates apoptotic condition. All other details are as described under Experimental Procedures.