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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/numatrin (B23) is a key nucleolar/nuclear matrix-associated protein required for cell growth-related functions, such as rRNA synthesis. Protein kinase CK2 (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 coordinated nuclear localization of B23 and CK2 is dynamically regulated in response to altered growth status in the cell.

Protein B23/nucleophosmin/numatrin (hereafter, B23)[1] 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 the level of 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 prostate after androgen ablation in the animal was not at the transcriptional level since B23 mRNA was present at a high level as many 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 decrease or increase in the level 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 suggesting that it plays important roles not only in the regulation of cell growth and proliferation but also in cell death by serving as a potent suppressor of apoptosis has emerged (*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 the organization of nucleolar compartments so that CK2 plays a significant role in controlling the compartmentalization of 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.

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[1]Abbreviations: B23, protein B23/nucleophosmin/numatrin; CK2, acronym for the 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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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 an experimental model. For the biochemical experimental studies of B23 in relation to CK2, we have employed the nuclear matrix fraction that 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 this work, our results demonstrate that downregulation of CK2 by various methods results in a 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 apigenin (Calbiochem). 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 figure legends. To achieve CK2 knockdown by using siRNA, both catalytic subunits α and α′ of CK2 were targeted using the following sense and antisense sequences: 5′-AUGUGGAGUUUGGGUUGUAUdTdT-3′ and 3′-dTdTUACACCUCAAACCCAACAUA-5′ (Dharmacon Research), respectively. Cyclophilin B (Dharmacon, catalog no. D-001136-01-05) was used as a control. Cells were cultured in antibiotic-free medium for at least 24 h before transfection. The siRNA and DharmaFECT-2 were diluted separately in OptiMEM medium and incubated at room temperature for 5 min. Lipid−siRNA complexes were then formed at room temperature for 20 min using different concentrations of siRNA. The complexes were diluted five times in antibiotic-free medium and added to the cells at a final concentration of 5, 10, or 50 nM siRNA (except where indicated otherwise) for different periods of transfection. The transfection of siRNA into cells was conducted 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 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 reattach over a period of 24 h. Following various cell treatments described above, medium in each well was replaced with 100 μL of fresh medium containing 100 μL/mL WST-1, and incubation was conducted 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 in individual figure legends.

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 the preparation of cell fractions; in all cases, method C was used for the preparation of cell fractions as detailed previously (*29*). Operationally, the nuclear matrix was known to contain the nucleolar component which was defined previously (*24*). Briefly, prostate tissue or cultured prostate cancer cells (pelleted ALVA-41 or PC-3 cells washed in cold PBS) were suspended [~10% (w/v)] in CSK buffer [10 mM PIPES (pH 6.8) at room temperature with 100 mM NaCl, 0.3 M sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, 4 mM vanadyl riboside, and Sigma protease inhibitor cocktail (0.02 times the final volume)]. The suspension was homogenized and centrifuged for 5 min at 600*g*. The supernatant from the 600*g* centrifugation was employed as the cytoplasmic fraction. The pellet was then suspended in the extraction buffer [10 mM Tris-HCl (pH 7.4) at room temperature with 10 mM NaCl, 3 mM $MgCl_2$, 1% Tween 40, 0.5% sodium deoxycholate, 4 mM vanadyl riboside, and Sigma protease inhibitor cocktail (0.02 times the final volume)]. The material was suspended and kept on ice for 5 min. The suspension was centrifuged again at 600*g* for 5 min, and the resulting pellet was suspended in digestion buffer [10 mM PIPES (pH 6.8) at room temperature with 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 times the final volume), 100 μg/mL RNase A, and 100 μg/mL DNase I]. The suspension was incubated at room temperature for 30 min with occasional mixing of the suspension. Subsequently, $^1/_3$ of the volume of 1 M $(NH_4)_2SO_4$ was added dropwise to the suspension to yield a

final concentration in the mixture of 0.25 M. The suspension was centrifuged for 5 min at 600*g*, and the pellet (nuclear matrix fraction) was suspended by vortexing to yield a uniform suspension in TMED with 0.2 M NaCl and Sigma protease inhibitor cocktail (0.01 times the total volume) [TMED consists of 50 mM Tris-HCl (pH 7.9) at room temperature with 5 mM $MgCl_2$, 1 mM EDTA, and 0.5 mM DTT]. Protein concentrations were estimated as described previously (*29*).

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

Immunoblot Analysis. Total lysate, cytoplasm, and NM of cultured cells or prostate tissue samples prepared as described above were employed for immunoblot analysis using mouse anti-CK2α (1:500) (BD Biosciences, San Diego, CA), mouse anti-B23 (1:200000) (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) primary antibodies. After three washes with TBS-T (Tris-buffered saline with 0.1% Tween 20), the membranes were incubated with goat anti-mouse IgG/horseradish peroxidase-conjugated secondary antibody (1:50000) (Pierce, Rockford, IL). SuperSignal West Pico chemiluminescence reagent (Pierce) was used in conjunction with Kodak Biomax ML film to detect peroxidase activity.

Immunofluorescence Staining of CK2α and B23. The cellular distribution of CK2α and B23 proteins was determined by immunofluorescence staining in ALVA-41 and PC-3 cells. The cells were grown on coverslips placed in a six-well plate. Cells on the coverslip were fixed with cold methanol for 10 min and washed three times (5 min each) in PBS. After incubation for 1 h 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, Inc.) 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-labeled (1:50) and goat anti-mouse Alexa-conjugated secondary antibodies (1:1000 dilution) (Invitrogen, Eugene, OR) were applied, and after being washed, the cells were mounted to the slides with aqueous antifading medium (Biomeda, Foster, CA). The immunofluorescence staining of cells for CK2α and B23 was visualized with an Olympus (Center Valley, PA) BX60 fluorescence microscope. The images were captured using Spot Software from Diagnostic Instruments (Sterling Heights, MI).

Detection of Apoptosis. Apoptosis in cells subjected to various doses of TBB was assessed by using the Cell Death Detection kit (TUNEL Staining, Roche) employed according to the instructions supplied by the manufacturer. After the TUNEL staining procedure had been completed, the cells were counterstained when the slides were dipped in a 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 antifading medium (Biomeda). 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 a RNAlater/PBS mixture (6:1) (Ambion, Inc., Austin, TX). Total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen, Inc., Valencia, CA), and reverse transcription for RT-PCR was conducted using RETROscript (Ambion, Inc.). 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 of 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 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s.

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 the status of B23 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 (Figure 1). As shown in Figure 1A, TUNEL analysis indicated that TBB induced apoptosis in these cells in a dose-dependent manner. Under these conditions, a dramatic reduction in the level of NM-associated B23 was observed in both the ALVA-41 and PC-3 cells, whereas no change was noticed in the B23 immunoreactive protein present in the cytoplasmic fraction (Figure 1B), 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 inhibitor TBB or apigenin for 24 h caused a dose-dependent inhibition of CK2 activity (Figure 1C). Under these experimental conditions, no change in the message level of CK2α or B23 was detected in the cells treated with CK2 inhibitors (Figure 1D,E). Analogous experiments were also undertaken by employing siRNA to downregulate the α and α′ subunits of CK2. As shown in Figure 2A, when ALVA-41 or PC-3 cells were treated with CK2 α,α′ siRNA at varying concentrations for different periods of time, there was a dose- and time-dependent effect on the 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 is in accord with previous observations of 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 the TUNEL assay (Figure 2B). The immunoreactive protein levels for B23 and CK2 catalytic subunits under the same conditions demonstrated the expected significant reduction in the magnitude of the CK2 signal in both the NM and cytoplasmic fractions, whereas B23 was not affected in the cytoplasmic fraction but its level was significantly reduced in the NM fraction (Figure 2C). These results further suggest a role for 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

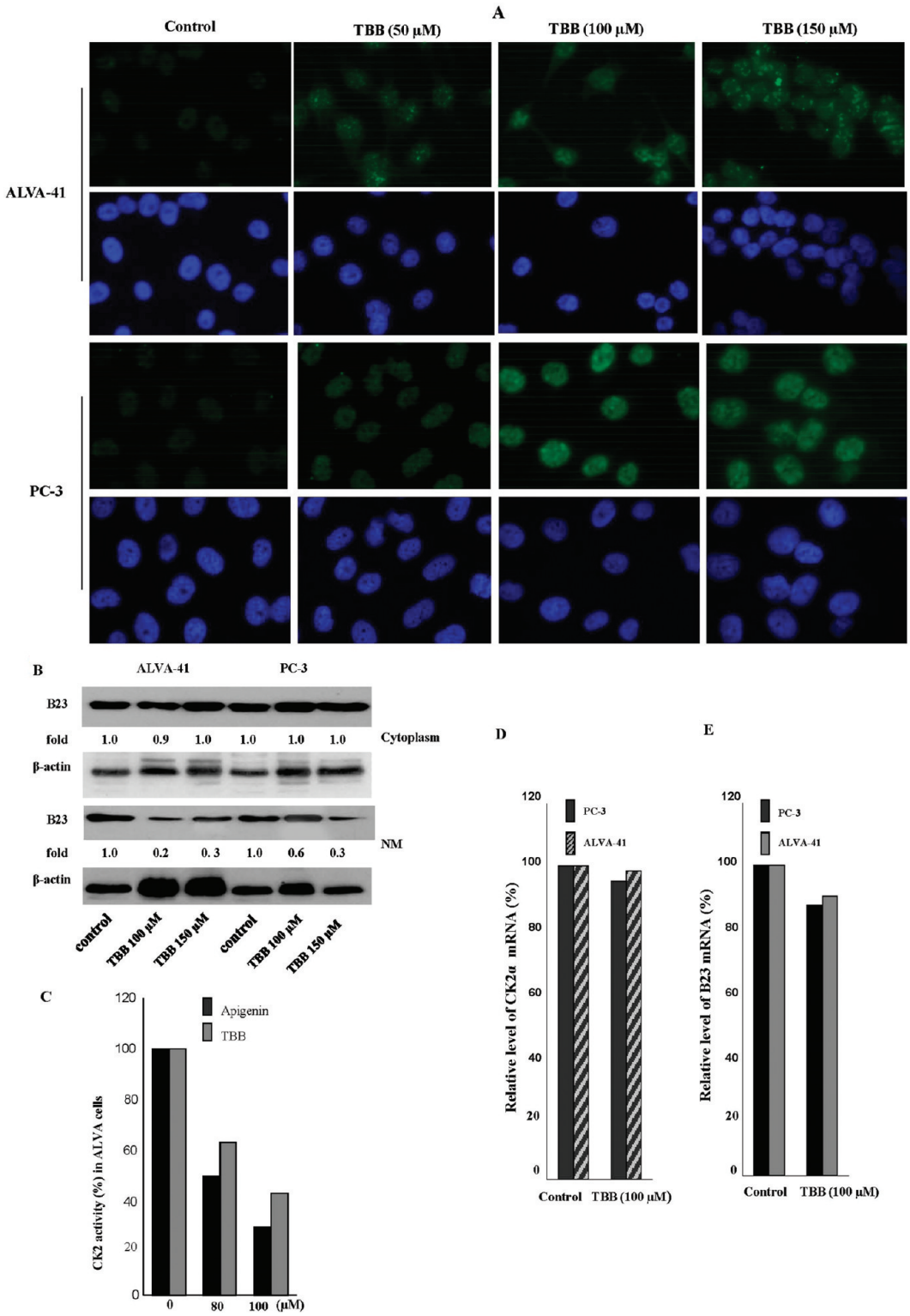

FIGURE 1: Status of B23 in the cytoplasm and NM fractions of prostate cancer cells in response to CK2 specific inhibitor TBB-mediated induction of apoptosis. (A) TUNEL staining of ALVA-41 and PC-3 cells treated with varying doses of TBB (from 50 to 150 μM). Induction of apoptosis is shown. Cells were counterstained with Hoechst 33342 to identify the nuclear component. (B) Cytoplasmic and NM-associated B23 in ALVA-41 and PC-3 cells treated with TBB at varying concentrations as described for panel 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 the left in panel B had a larger amount of protein to ensure visualization of the 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 described for panel C. (E) Measurement of B23 message in ALVA-41 and PC-3 cells under the same conditions described for panel C.

in ALVA-41 and PC-3 prostate cancer cells. The results in Figure 3A show that when small 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, which was previously demonstrated for

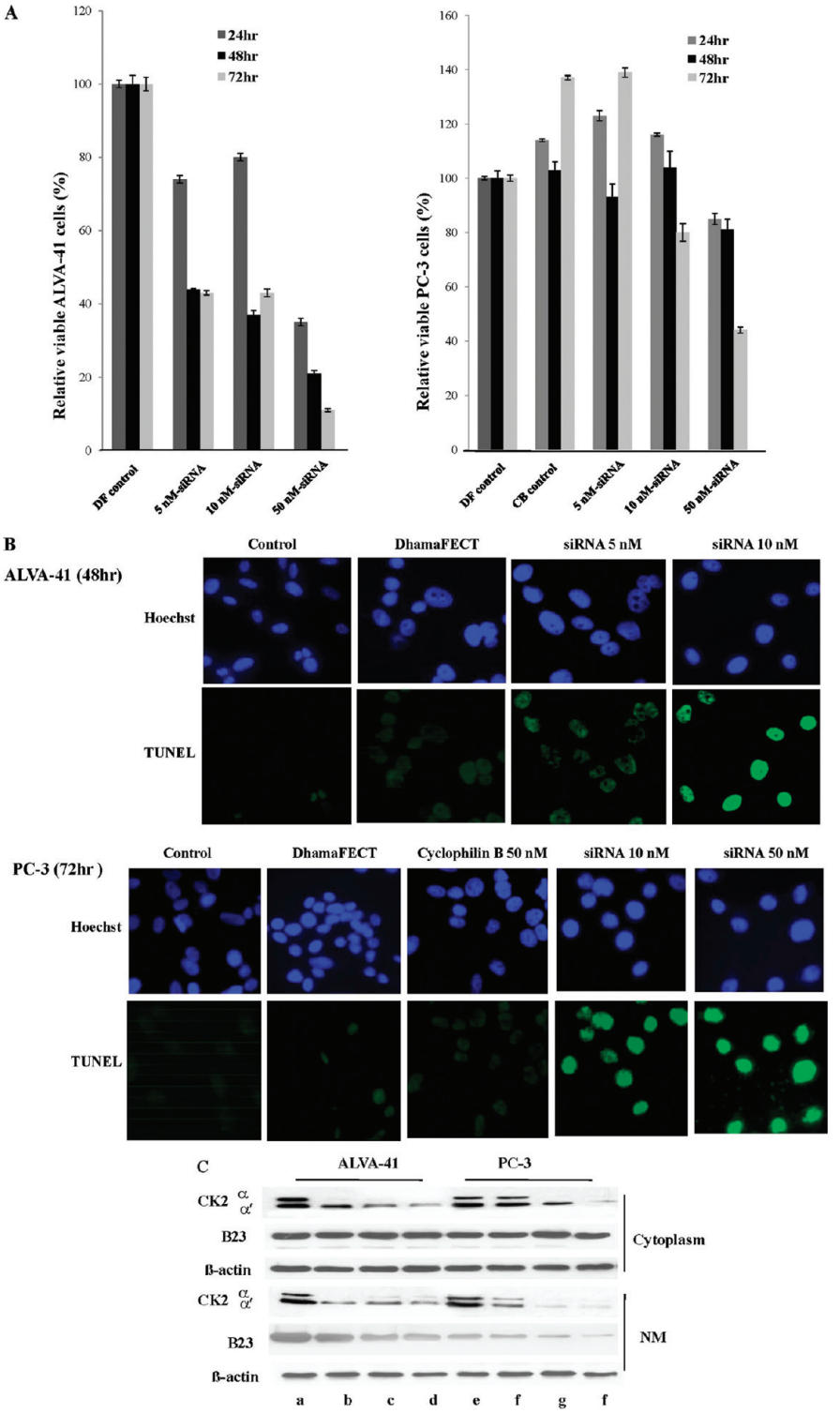

FIGURE 2: Status of B23 in the cytoplasm and NM fractions of prostate cancer cells in response to siRNA-mediated downregulation of CK2. (A) Cell viability was determined by the WST-1 assay in ALVA-41 and PC-3 cells transfected with varying concentrations of CK2 α,α′ siRNA for the periods of time shown. (B) ALVA-41 and PC-3 cells were transfected with varying concentrations of CK2 α,α′ siRNA as described for panel A. Cyclophilin B (Dharmacon, catalog no. D-001136-01-05) was employed as a control. All other details were as described in Materials and Methods. 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 α,α′ was conducted in ALVA-41 and PC-3 cells transfected with varying concentrations of CK2 α,α′ siRNA: lane a, untreated control; lane b, 5 nM CK2 α,α′ siRNA; lane c, 10 nM CK2 α,α′ siRNA; lane d, 50 nM CK2 α,α′ siRNA; lane e, untreated control; lane f, DharmaFECT control; lane g, 10 nM CK2 α,α′ siRNA; lane h, 100 nM CK2 α,α′ siRNA. The relative change in the protein bands is calculated compared to the respective control.

induction of apoptosis in prostate cancer cells by combined TRAIL and CK2 inhibitor treatment (*28*). Consistent with these conditions, etoposide, TBB, or apigenin alone at the suboptimal doses tested did not affect the NM-associated B23 in ALVA-41 cells; however, a dramatic reduction in the level of B23 was detected when these agents at suboptimal levels were combined, i.e., etoposide with TBB or etoposide with apigenin (Figure 3B), which also corresponded to increased apoptotic activity in the

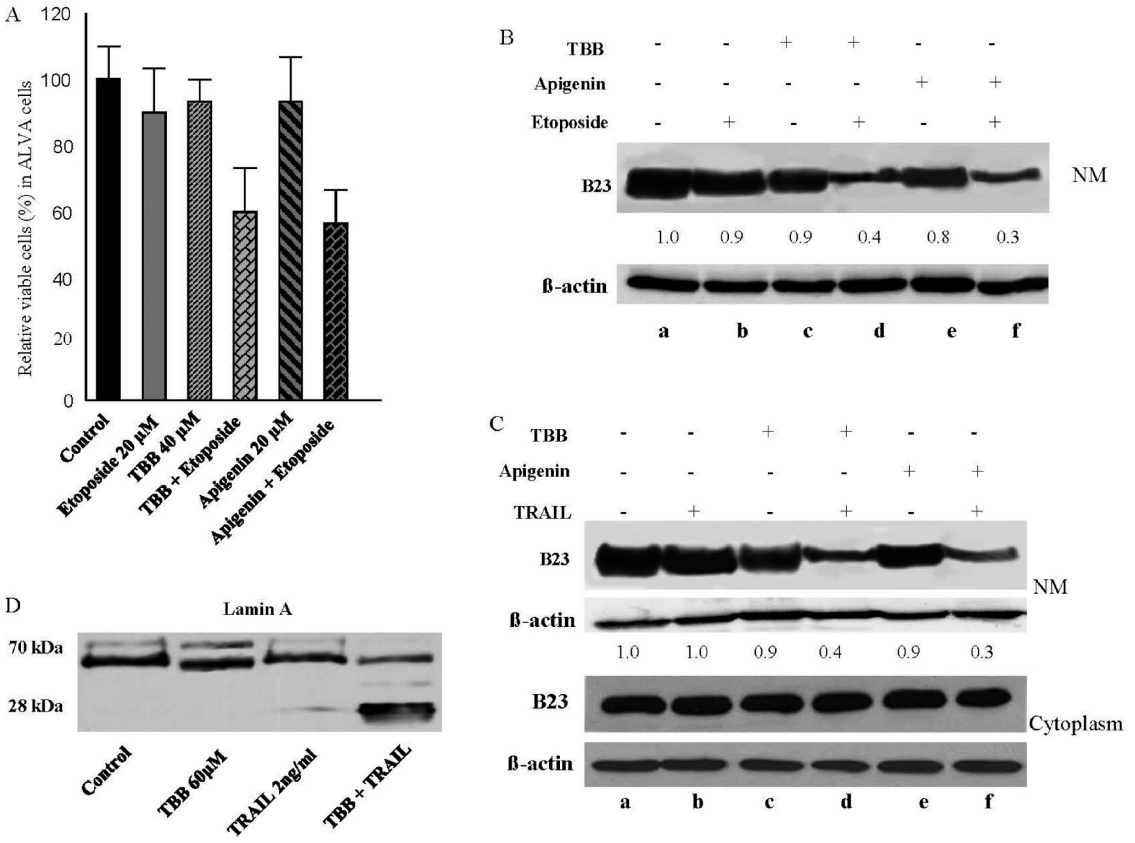

FIGURE 3: Effect of apoptosis-inducing conditions on NM-associated B23. (A) Prostate cancer cells (ALVA-41) were treated with doses of etoposide or CK2 inhibitors (TBB or apigenin) at doses that are suboptimal for induction of apoptosis. The results show a minimal effect on cell death under these conditions. When the suboptimal doses of the same inhibitors are combined, it results in induction of apoptosis, as shown in the chart. Illustrated in panel A are the data employing etoposide (an apoptosis-inducing agent) with and without CK2 inhibitor TBB or apigenin. Similar effects have previously been reported using TRAIL (an apoptosis-inducing agent) with and without TBB at suboptimal doses (*27*) (the latter conditions were employed in the experiment shown in panel 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 (suboptimal for induction of apoptosis); lane c, TBB at 40 μM (suboptimal for induction of apoptosis); lane d, etoposide with TBB (combined effect to produce apoptotic activity); lane e, apigenin at 20 μM (insufficient for induction of apoptosis); lane f, apigenin and 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, 2 ng/mL TRAIL (suboptimal dose that is insufficient for induction of apoptosis); lane c, 40 μM TBB (suboptimal dose for induction of apoptosis); lane d, TBB with TRAIL (combined effect evokes apoptotic activity); lane e, apigenin at 20 μM (insufficient dose for induction of apoptosis); lane f, TRAIL with apigenin (conditions for inducing apoptotic activity). Relative densitometric values are based on the level in the control cells. (D) Combined effect of TBB and TRAIL (both at suboptimal doses) in causing induction of apoptosis 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 TRAIL (insufficient for apoptosis induction) as indicated by a minimal cleavage of lamin A; lane d, 60 μM TBB with 2 ng/mL TRAIL resulting in potent induction of apoptosis as indicated by extensive cleavage of lamin A. These results are in accord with the corresponding effect on NM-associated B23 as shown in panel C. In all cases, an equal amount of protein was loaded in the gels, and each experiment was conducted at least three times.

cells as shown in Figure 3A. Essentially, similar results were obtained when ALVA-41 cells were treated with TRAIL and TBB or apigenin (at suboptimal doses in each case) compared with that when each individual compound was employed (Figure 3C). Confirmation of induction of apoptosis by combined treatment with TBB and TRAIL is provided in Figure 3D via demonstration of 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 CK2α (*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 CK2α. The results in Figure 4A show that treatment of ALVA-41 and PC-3 cells with 10 and 25 ng/mL TRAIL, respectively, for 24 h caused a dramatic loss of NM-associated B23 which was completely blocked by transient overexpression of CK2α. Likewise, the results in Figure 4B show that the level of 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 CK2α. A representative result is presented in Figure 4C to illustrate confirmation of transient overexpression of CK2α indicated by its enhanced message and protein in PC-3 cells. Since induction of apoptotic activity in cells is associated with the loss of CK2 in the nuclear compartment, whereas transient overexpression of CK2 promotes its additional translocation to the nuclear compartment (*31*), our results are in accord with the notion that the CK2 status in the cell and the

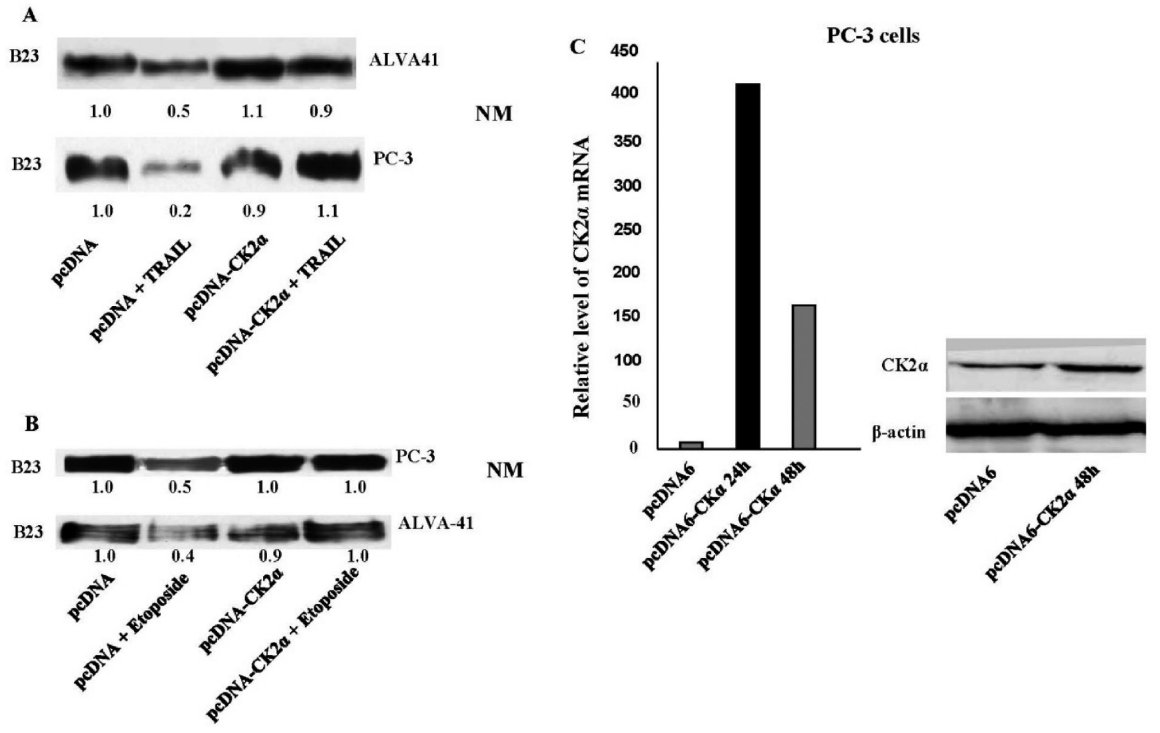

FIGURE 4: Effect of overexpression of CK2α on the status of NM-associated B23 in prostate cancer cells treated with apoptotic inducers. ALVA-41 and PC-3 cells were treated with etoposide or TRAIL at apoptosis-inducing concentrations in the absence or presence of forced overexpression of the catalytic subunit CK2α as described in Materials and Methods. (A) Treatment with TRAIL at 10 ng/mL for ALVA-41 and 20 ng/mL for PC-3 cells (for 24 h) was conducted to induce the apoptotic condition in cells. The 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 immunoreactive B23 under these conditions (not shown): lane a, control cells treated with pcDNA6; lane b, pcDNA6 with TRAIL; lane c, pcDNA6-CK2α; lane d, pcDNA6-CK2α with TRAIL. (B) Treatment of ALVA-41 and PC-3 cells with 50 μM etoposide (apoptosis-inducing level). Overexpression of CK2α was achieved as described for panel A. NM-associated immunoreactive B23 is shown: lane a, pcDNA6 control; lane b, pcDNA6 with 50 μM etoposide for 48 h; lane c, pcDNA6-CK2α; lane d, pcDNA6-CK2α with etoposide. (C) Representative experiment showing confirmation of CK2α overexpression in PC-3 cells by transient transfection with pcDNA6-CK2α illustrated by analysis of the message and protein levels.

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 data given above suggest coordinate modulation of B23 and CK2 in the nuclear compartment under various conditions, we examined the subcellular localization of B23 and CK2α and the effect of the CK2 specific inhibitor TBB on their disposition in the nuclear compartment by employing immunofluorescence studies. The results in Figure 5A show that when increasing concentrations of TBB were included in the medium, the levels of both B23 and CK2α declined 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-CK2α shows the decrease in the levels of B23 and CK2α 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 CK2α as indicated in the merged image of immunofluorescence staining for the two proteins (Figure 5B). 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, Tawfic et al. (*11*) 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 the levels of these proteins in the NM fraction isolated from these nuclei compared with the controls. 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 Figure 6A show that the B23 level in prostatic total lysate is relatively unchanged in response to androgen deprivation in the animal. Under these conditions, the significant loss of CK2 is apparent in total lysates of prostatic tissue from animals that had been castrated 6 days earlier which is reversed on androgen administration (not shown). When cytoplasmic and NM fractions isolated from the prostatic tissues were analyzed (Figure 6B), 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 was in accord with the observation with total lysate B23 under similar conditions. Also shown in Figure 6B are the results for 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

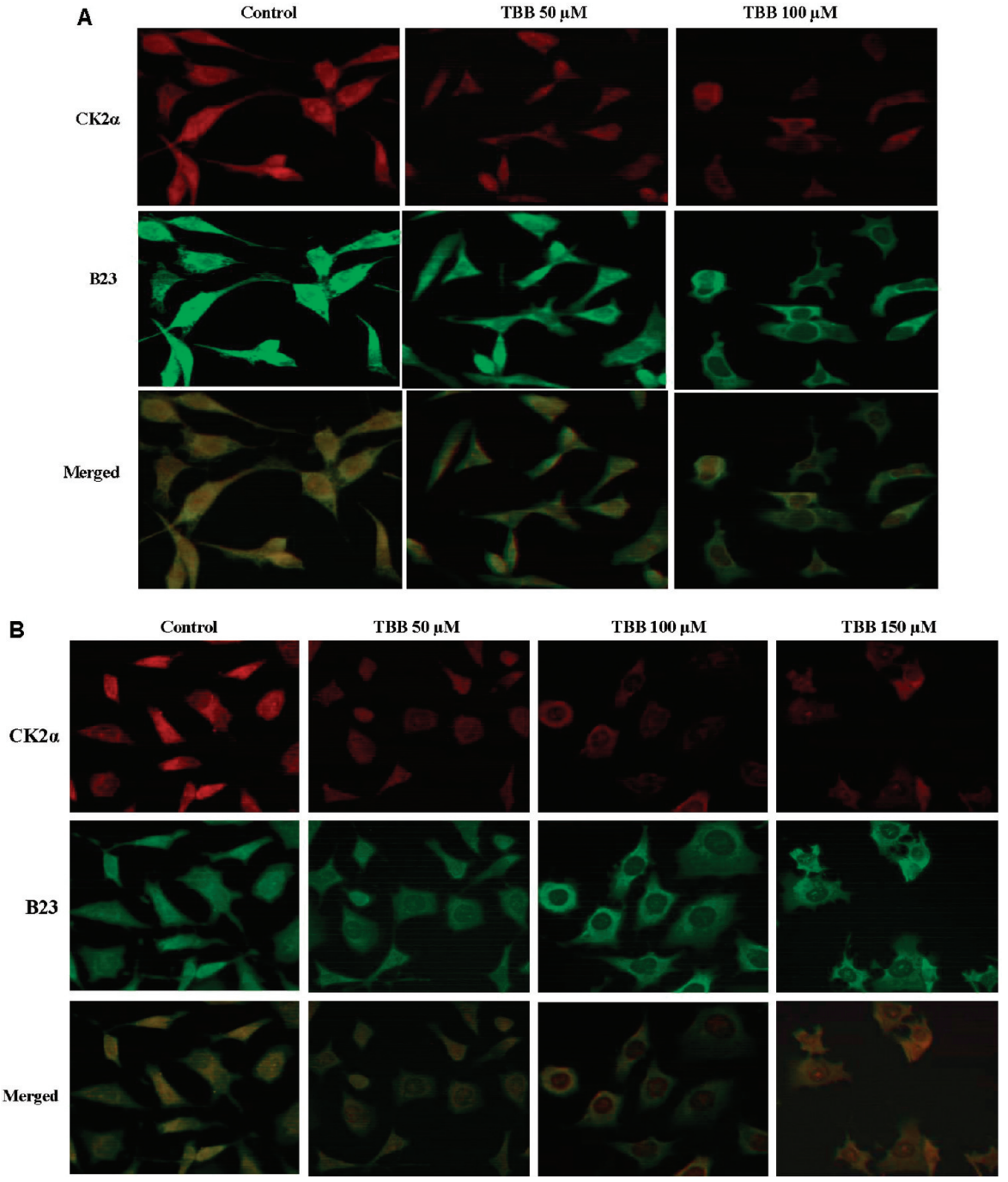

FIGURE 5: Colocalization of CK2α and B23 in the nucleus. (A) Double immunofluorescence staining for anti-CK2α and anti-B23 was conducted in ALVA-41 cells treated with varying doses of TBB, as shown. The merged image of the immunofluorescence stains for anti-CK2α and anti-B23 shows colocalization of the two proteins in the nucleus. Downregulation of CK2 by TBB treatment (producing apoptotic condition) shows coordinate loss of B23 and CK2α in the nucleus. (B) Double immunofluorescence staining for anti-CK2α and anti-B23 conducted in PC-3 cells treated with varying doses of TBB. All details are the same as for panel A. Results confirm the colocalization of CK2α and B23 in the nucleus and its reduction on induction of apoptotic conditions by TBB-mediated inhibition of CK2 as observed for ALVA-41 cells in panel A.

administration of androgen to the animal. Furthermore, changes in the NM-associated CK2 were analogous to those in B23 levels. Figure 6B 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*). These results further demonstrate the coordinate and dynamic modulation in 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 present at elevated levels 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

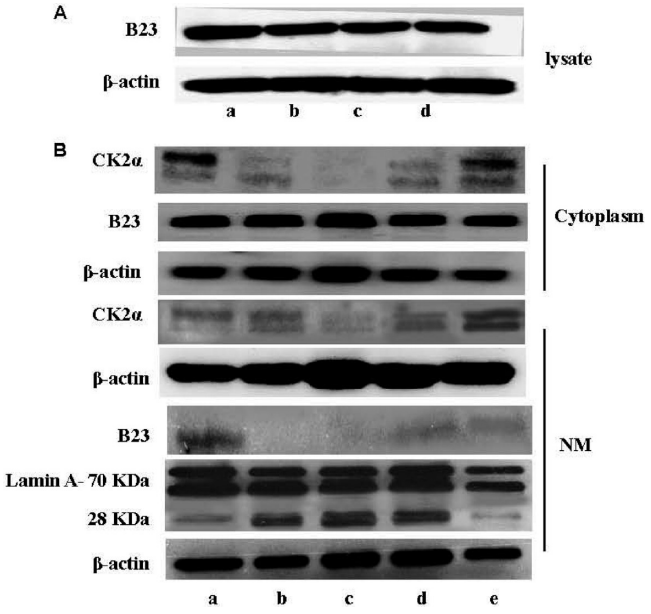

FIGURE 6: Effect of altered androgenic status on CK2α and B23 levels in rat ventral prostate tissue. Lysate, cytoplasm, and nuclear matrix fractions were isolated from prostatic tissue of rats subjected to various treatments as shown. Each fraction was analyzed by Western blotting for the presence of 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−d, rats orchiectomized for 1−3 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, rats orchiectomized 3 days earlier; lane c, rats orchiectomized 6 days earlier; lane d, rats orchiectomized 6 days earlier that were 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 a 28 kDa fragment) in the NM fraction indicates apoptotic conditions. All other details are as described in Materials and Methods.

attributed to this protein. For example, chaperone activity toward 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 ref *37*). Given the importance of B23 in cell function, a number of investigations of its response to diverse stimuli in the cell have been undertaken, 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 and 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, the increased stability of B23 was suggested to be associated with an anti-apoptotic 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., ref *37*), the dynamics of the 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 versus cytoplasmic compartment under various conditions.

The results described here are based on several experimental models for investigating 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 the 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*), this 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 has a direct impact 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., ref *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 a profound effect on cell survival. Our observations on a coordinate 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*), these results show a concordant loss of nuclear B23 under these conditions even when its level in the 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 the possibility that breakdown of B23 at least in part also plays a role in this process cannot be ruled out. On the basis of these

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 for 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 colocalize 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 downregulation of CK2 or apoptosis-inducing agents results in a loss of B23 from the nuclear matrix likely caused by a reduction in the level of 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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