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Cell Cycle Dependent Regulation of Protein Kinase CK2 Signaling to the Nuclear Matrix

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Abstract Protein kinase CK2 is a ubiquitous protein serine/threonine kinase that is involved in cell growth and proliferation as well as suppression of apoptosis. Several studies have suggested that the kinase plays a role in cell cycle progression; however, changes in enzyme activity during phases of cell cycle have not been detected. Nuclear matrix is a key locus for CK2 signaling in the nucleus. We therefore examined CK2 signaling to the nuclear matrix in distinct phases of cell cycle by employing synchronized ALVA-41 prostate cancer cells. Removal of serum from the culture medium resulted in G0/G1 arrest, and a reduction in the nuclear matrix-associated CK2 activity which was rapidly reversed on addition of serum. Arresting the cells in G_0/G_1 phase with hydroxyurea and subsequent release to S phase by serum gave similar results. Cells arrested in the G_2/M phase by treatment with nocodazole demonstrated an extensive reduction in the nuclear matrix-associated CK2 which was reversed rapidly on addition of serum. Changes in the immunoreactive CK2 protein were concordant with the activity data reflecting a dynamic trafficking of the kinase in distinct phases of cell cycle. Under the same conditions, CK2 activity in total cellular lysate remained essentially unaltered. These results provide the first direct evidence of discrete modulations of CK2 in the nuclear matrix during the cell cycle progression. Inducible overexpression of CK2 in CHO cells yielded only a modest increase in CK2 activity even though a significant increase in expression was apparent at the level of CK2α-specific message. Stably transfected ALVA-41 cells, however, did not show a significant change in CK2 levels despite increased expression at the message level. Not surprisingly, both types of the stably transfected cells failed to show any alteration in cell cycle progression. Distribution of the CK2 activity in the cytosolic versus nuclear matrix fractions in normal cells appears to be different from that in the cancer cells such that the ratio of nuclear matrix to cytosolic activity is much higher in the latter. Considering that nuclear matrix is central to several nuclear functions, this pattern of intracellular distribution of CK2 may have implications for its role in the oncogenic process. J. Cell. Biochem. 88: 812–822, 2003. Published 2003 Wiley-Liss, Inc.†

Key words: protein kinase CK2; cell cycle; nuclear matrix; prostate; cancer cells; stable transfection

Protein kinase CK2 (formerly casein kinase 2 or II) is a ubiquitous and highly conserved serine/threonine kinase that has long been considered to be involved in many cellular functions including cell growth and proliferation [for reviews see, Litchfield and Lüscher, 1993; Ahmed, 1994; Allende and Allende, 1995; Pinna and Meggio, 1997; Ahmed, 1999; Guerra and Issinger, 1999; Ahmed et al., 2000; Tawfic et al., 2001]. The kinase activity has been consistently observed to be elevated in the various neoplasias (human as well as animal models) that have been examined [Rayan et al., 1985; Seldin and Leder, 1995; Kelliher et al., 1996; Landesman-Bollag et al., 1998, 2001; Guerra and Issinger, 1999; Tawfic et al., 2001]. It has also been shown that CK2 plays a role in suppression of apoptosis [Ahmed et al., 2002; Guo et al., 2001] which may be particularly relevant to tumor biology since cancer cells are known to demonstrate a deregulation of apoptosis.

CK2 is a tetrameric protein consisting of the regulatory β_2 subunits linked to the catalytic α_2,

Huamin Wang and Shihui Yu contributed equally to this work.

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or α'_2, or $\alpha\alpha'$ subunits. The enzyme is localized in the cytoplasmic and nuclear compartments, and its dynamic shuttling to the nuclear compartment in response to a variety of physiological as well as stress signals has been demonstrated [Ahmed, 1999; Guo et al., 1999; Gerber et al., 2000; Martel et al., 2001; Yu et al., 2001; Davis et al., 2002]. The nuclear matrix appears to be a significant target for CK2 nuclear signaling [Tawfic and Ahmed, 1994a,b; Tawfic et al., 1996; Ahmed, 1999; Guo et al., 1999; Yu et al., 1999]. Nuclear matrix is a subnuclear proteinaceous structure that has been implicated in the organization of chromatin thereby playing a key role in a variety of growth related nuclear functions including cell proliferation [Berezney, 1991; Getzenberg et al., 1991; Nickerson and Penman, 1992; Stein et al., 2000; Nickerson, 2001]. Concordant with the view that CK2 is essential for cell viability and proliferation, a number of studies have suggested that it plays a role in cell cycle progression. For example, in temperature sensitive mutants of yeast cells the inactivation of CK2 produced cell cycle arrest at either the G_1/S or G_2/M transitions [Hanna et al., 1995]. Likewise, it was shown that antibodies to CK2 blocked cell cycle progression through various phases [Pepperkok et al., 1994]. On the other hand, studies based on measurement of CK2 activity in phases of cell cycle have tended to be disparate. Original observations on CK2 activity in human primary fibroblasts and other established cells found no significant activity changes throughout the cell cycle [Schmidt-Spaniol et al., 1993]. However, in other work it was reported that HeLa cells fractionated by centrifugal elutriation yielded populations of cells in different phases with varied CK2 activity as measured in total cell extracts [Marshak and Russo, 1994]. In accord with the earlier observations [Schmidt-Spaniol et al., 1993], a recent study employing both centrifugal elutriation and chemical synchronization of human fibroblasts also failed to find major fluctuations in CK2 activity during any stages of cell cycle except for the stimulation of the activity in quiescent cells following serum stimulation [Bosc et al., 1999]. The basis of these disparate observations remains unclear.

Immunofluorescence analysis of the intracellular localization of CK2 in human primary fibroblasts, 3T3 cells, and fibrosarcoma (HT1080) cells suggested that it was largely localized in the nucleus irrespective of the stage of the cell cycle [Schmidt-Spaniol et al., 1993]. Other studies have suggested greater nuclear localization of the kinase in cells stimulated to proliferate [Ahmed et al., 1993b; Filhol-Cochet et al., 1994; for reviews see Ahmed, 1994, 1999; Ahmed et al., 2000]. Immunohistochemical studies have also suggested that a more intense staining for CK2 is observed in the nuclei compared with the cytoplasm of cancer cells whereas the signal is diffused in different compartments in the normal cells [Yenice et al., 1994; Faust et al., 1999].

Much recent evidence supports the notion that within the nucleus, distinct domains are present which may serve as targets for specific signals [for a review see Stein et al., 1998, 2000]. Considering the nuclear matrix as a key locus for CK2 nuclear signaling [for reviews see Tawfic et al., 1996, 2001; Ahmed, 1999, 2000], we reasoned that even though changes in CK2 in various phases of cell cycle were not apparent when measured as total activity, they might be reflected as discrete dynamic modulations at the level of the nuclear matrix. Here, by utilizing synchronized ALVA-41 cells, we have demonstrated that significant changes in the nuclear matrix associated CK2 activity and protein occur in cells arrested in specific phases of cell cycle. Release of cells from a specific block by introduction of serum is accompanied by rapid changes in the nuclear matrix-associated CK2 activity and protein. Further, because CK2 has been consistently found to be upregulated in cancers, it has been of interest to examine the biological consequences of elevated CK2 in cells by attempting forced overexpression of the subunits of the enzyme. To that end, cells stably transfected with CK2-α were found to demonstrate a high level of expression of the message but not of the CK2 protein, or an effect on cell cycle progression. This was the case for attempted overexpression of CK2 in normal (e.g., CHO) cells as well as cancer (ALVA-41) cells, suggesting that the cells resist stable overexpression of CK2 protein. Of note, the relative distribution of the kinase between the cytoplasmic and nuclear matrix fractions was found to be distinctly different in normal versus cancer cells.

MATERIALS AND METHODS

Materials

Cell lines. Prostate cancer cell line ALVA-41 was received from Dr. Richard C. Ostenson,

U.S. Department of Veterans Affairs, American Lake VA Medical Center, Tacoma, WA. Cell line CHO AA8 Tet-Off which is a stably transformed cell line generated by transfecting CHO (Chinese hamster ovary) cells with the pTet-Off plasmid was purchased from Clontech Laboratories (Palo Alto, CA). These cells were maintained in Ham's F-12 medium containing 6% FBS, 2 mM glutamine, and 100 μg/ml G418. PC-3 cells were from the American Type Culture Collection (Manassas, VA). They were maintained in RPMI 1640 supplemented with 2 mM L-glutamine, 25 mM HEPES, 10% FBS in an atmosphere containing 5% CO_2.

Chemicals. Defined fetal bovine serum (FBS) was purchased from HyClone Laboratories (Logan, UT). The RPMI-1640 culture medium was obtained from Life Technologies, Inc. (Grand Island, NY). The specific peptide substrate for CK2 was synthesized by Peptide Technologies Corp. (Gaithersburg, MD). Antibodies against the α and β subunits of CK2 were purchased from Calbiochem-Novabiochem International (San Diego, CA) and Santa Cruz Biotechnology (Santa Cruz, CA). The [γ-^{32}P]ATP was purchased from ICN Biomedicals (Costa Mesa, CA). Nocodazole and hydroxyurea were purchased from Sigma (St. Louis, MO). All reagents were of the highest purity available.

Methods

Cell culture and various treatments. Cells (ALVA-41) were initially grown under normal culture conditions at 37°C in 75 cm^2 culture flasks in RPMI-1640 plus 6% FBS and 2 mM glutamine. The cells were subjected to different treatments to achieve arrest in various phases of cell cycle following which they were labeled with propidium iodide for FACS analysis. The various treatments were: (a) control normal cells grown in 6% FBS; (b) cells grown in serum-free medium for 36 h; (c) cells grown in the presence of 100 ng/ml of nocodazole for 16 h; and (d) cells grown in 10 mM hydroxyurea for 16 h. These conditions respectively yield a cell distribution of 58% in G_0/G_1 phase, 65% in G_0/G_1 phase, 2.6% in G_0/G_1 phase, and 63% in G_0/G_1 phase. The culture conditions for these cells were altered to promote cell cycle progression to the next phase, as described in the text and legends to tables and figures.

Stable transfection of cells with CK2α. Insertion of cDNA for CK2-α was made into the cloning site of the tetracycline response plasmid pBI, a vector that contains two cloning sites for bicistronic constructs. This construct was used to transfect the CHO AA8 Tet-Off cells using DOTAP (Roche Molecular Biochemicals, Indianapolis, IN) as the transfection agent. These cells were grown in Ham's F-12 medium with 6% FBS, 2 mM glutamine, 400 μg/ml G418, and 200 μg/ml hygromycin for selection of the stable pBI-CK2-α transformants. After the stable clones had been selected, 100 μg/ml of G418 and 100 μg/ml of hygromycin were used for maintenance, and 4 μg/ml of tetracycline was included to suppress expression of the CK2-α subunit.

For the preparation of ALVA-41 cells with stable CK2-α overexpression, the cDNA for CK2-α [Yu et al., 1999] was inserted into the multiple cloning site of the expression vector pcDNA6 (Invitrogen, Carlsbad, CA). This construct was transfected into ALVA-41 cells using DOTAP as the transfection agent. The cells were grown in RPMI-1640 with 6% FBS, 2 mM glutamine, and 8 μg/ml blasticidine S for selection of stable transformants. Once the stable transfectants were selected the blasticidine S was reduced to 2 μg/ml for maintenance.

Cell cycle analysis. Following various treatments, cells were harvested by scraping in 15 ml original medium, and centrifuged at 600 *g* for 5 min at room temperature. The pellet was rinsed in 10 ml of normal saline containing 0.5 mM of PMSF and 2.0 μg/ml of leupeptin. The rinsed cells (collected by centrifugation) were gently suspended in 1.0 ml of hypotonic fluorochrome solution (50 μg/ml propidium iodide in 0.1% sodium citrate, 0.1% Triton X-100, and 50 μg/ml RNase). The cells were allowed to stain overnight at 4°C in the dark. These cells were analyzed on a FACSort (Becton Dickinson) employing the Cell Quest software program. Each experiment was performed in triplicate, and at least 10,000 forward scatter gated events were collected for each sample.

Preparation of nuclear matrix and cell lysate. The cells treated as above were utilized for isolation of the nuclear matrix. The nuclear matrix was prepared immediately from the washed cells using a previously described procedure referred to as Method C [Tawfic et al., 1997]. The recovery of protein in the nuclear matrix fraction was determined by a modified Coomassie staining procedure [Ahmed et al., 1996].

For preparation of cell lysates, cells from the above described treatments were collected and homogenized in the CSK buffer referred to in Method C described previously [Tawfic et al., 1997]. The homogenized material was subjected to centrifugation at $600\,g$ for 5 min, and the supernatant material was designated as the total lysate.

Assay of CK2 activity. The CK2 enzyme activity associated with the nuclear matrix fractions was measured using a specific peptide substrate, R-R-R-A-D-D-S-D-D-D-D-D [Marin et al., 1994]. Nuclear matrix protein (~ 2.5 μg) was incubated for 20 min at 37°C with 30 mM Tris-HCl, pH 7.45, 150 mM NaCl, 40 mM β-glycerophosphate, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.5 mM PMSF, 10 μg/ml leupeptin, 50 μM ATP (γ-^{32}P-ATP specific activity of 3×10^6 cpm/nmol ATP) with or without 200 μM peptide substrate in a 100 μl final volume. [Guo et al., 2001]. The same procedure was employed for assaying the CK2 activity in the total cell lysate fraction.

Western blot analysis for CK2. For immunodetection of CK2, a 50–70 μg sample of the nuclear matrix protein was separated on a 10% acrylamide gel containing 4 M urea and 0.1% SDS for Western blotting, as described previously [Guo et al., 1999; Yu et al., 1999]. The primary antibodies, anti-CK2α and anti-CK2β were used at 2 μg/ml and were incubated with the blot for 2 h at room temperature. The secondary antibody, alkaline phosphatase conjugated anti-mouse IgG, was used at 0.5 μg/ml and was incubated with the blots for 2 h at room temperature. Immobilized alkaline phosphatase was visualized using 5-bromo-4-chloroindolyl phosphate and Nitro Blue Tetrazolium as described previously [Guo et al., 1999; Yu et al., 1999].

Immunofluorescence analysis for CK2 in cells. ALVA-41 cells were grown on chamber slides where they were subjected to various treatments, and then were fixed with cold (−20°C) methanol and blocked with 5% BSA in Tris buffered saline (BSA Blocker) for 1 h at 4°C. The primary antibody was a goat IgG anti-CK2-α which was diluted × 50 in the BSA Blocker solution. Fixed cells were incubated overnight at 4°C with this antibody. The FITC-conjugated rabbit anti-goat IgG (× 50 dilution in the BSA Blocker) was incubated with the cells for 2 h at room temperature in the dark. All washes after fixing and antibody incubations were done with TBS at 4°C. Slides were mounted with Gel/Mount from Biomeda (Foster City, CA), and fluorescence images were obtained using a Nikon Axiovert microscope equipped with a digital camera. The wavelength for FITC filter was $E_x = 420-490$.

Slot blot analysis. Total RNA was purified from cultured cells using Trizol reagent (Life Technologies, Inc., Grand Island, NY). It was evaluated by agarose gel electrophoresis and absorbance at 260 and 280 nm. The RNA was diluted in 10 × SSC (1.5 M NaCl, 0.15 M Na-citrate) with 6.15 M formaldehyde, and was then heated for 15 min at 67°C. A slot blot on Nytran membrane filter (Schleicher and Schuell, Keane, NH) was prepared by applying 30 μg RNA per slot. The RNA was bound to the Nytran membrane by employing a UV-cross-linker. The cDNA probe for CK2-α [Ahmed et al., 1993a] and a synthetic oligonucleotide probe for β-actin were labeled using α-^{32}P-deoxyATP and a random primed labeling kit (Roche Molecular Biochemicals, Indianapolis, IN). Hybridizations were carried out overnight at 42°C with the probe diluted in HYB-9 solution (Gentra Systems, Inc., Minneapolis, MN). The blots were subjected to several high stringency washes at 50°C with 0.1 × SSC containing 0.1% SDS prior to autoradiography.

RESULTS

Chemical Synchronization of ALVA-41 Cells in Various Phases of Cell Cycle

In order to delineate the dynamics of CK2 signaling in the nuclear matrix of cells in different phases of cell cycle, we synchronized the ALVA-41 cells by utilizing the standard chemical reagents that are known to arrest cells in specific phases. Figure 1 shows a representative FACS analysis of cells subjected to various treatments. Also, the data in Table I provide the corresponding information on the percent of cells in various phases of cell cycle. As shown in Table I, under normal conditions (treatment "a"), the relative percent distribution of ALVA-41 cells in G_0/G_1, S, and G_2/M phases was of the order of 58, 36, and 18, indicating the steady state of cell cycle progression in these cells. Removal of serum (treatment "b") resulted in a larger accumulation of the cells in G_0/G_1 phase, which was reversed within 4 h on addition of serum as evidenced by an apparent increase

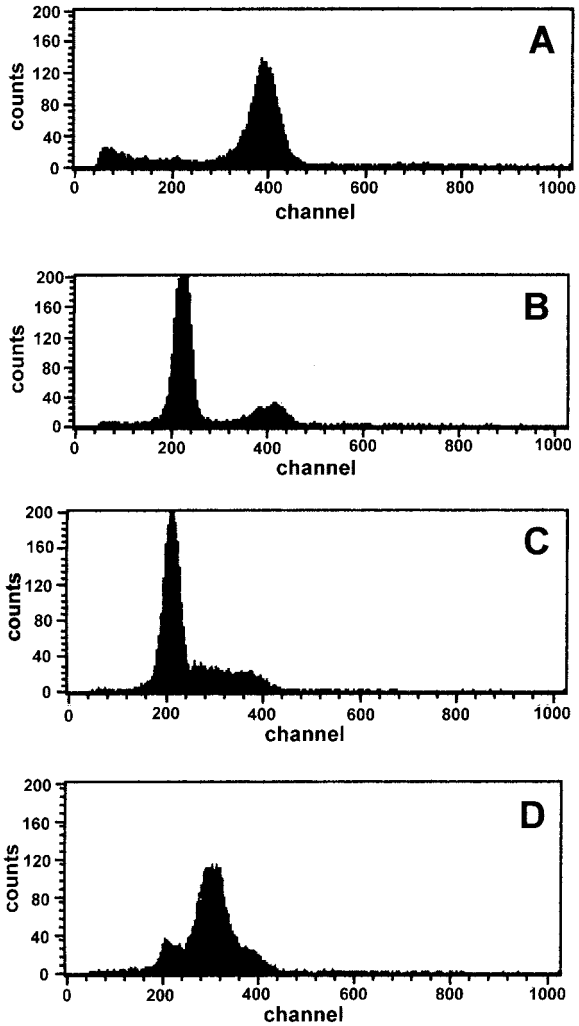

Fig. 1. Representative FACS analysis of ALVA-41 cells treated with various drugs to achieve cell cycle arrest in various phases. Cells were grown under culture conditions as described under Table I, and subjected to FACS analysis as described under Methods. **A**: cells treated with 100 ng/ml nocodazole for 16 h (treatment ''e'' under Table I); (**B**) cells treated with 100 ng/ml of nocodazole as under (A), followed by 10% FBS for 4 h (treatment ''f'' under Table I); (**C**) cells treated with 10 mM hydroxyurea for 16 h (treatment ''g'' under Table I); (**D**) cells treated with 10 mM hydroxyurea as under (C), followed by 10% FBS for 4 h (treatment ''h'' under Table I).

TABLE I. Distribution of Synchronized ALVA-41 Cells in Various Phases of Cell Cycle

Cell culture treatment*	Percentage of cells in cell cycle phase		
	G_0/G_1	S	G_2/M
a	57.9 ± 4.7	35.5 ± 4.6	18.4 ± 3.6
b	64.9 ± 3.5	17.2 ± 3.6	13.7 ± 3.8
c	59.5 ± 6.7	18.2 ± 4.3	20.5 ± 6.7
d	56.9 ± 3.2	25.2 ± 4.8	17.4 ± 6.0
e	2.6 ± 2.3	11.8 ± 4.7	79.5 ± 11.9
f	66.7 ± 6.8	11.5 ± 3.3	16.0 ± 4.0
g	62.6 ± 4.3	25.9 ± 2.6	3.9 ± 1.8
h	18.2 ± 8.3	82.0 ± 4.8	7.3 ± 5.2

*Treatment of the ALVA-41 cells was as follows: Treatment "a" (normal control) represents cells grown in a medium consisting of RPMI-1640 containing 25 mM HEPES, 2 mM glutamine, and 6% FBS in a 5% CO_2 atmosphere at 37°C. For treatment "b", cells were grown to 60–80% confluency under treatment "a" conditions and then the medium was changed to RPMI-1640 containing 25 mM HEPES, 2 mM glutamine, without any added serum, and allowed to grow for 36 h in a 5% CO_2 atmosphere at 37°C. Treatment "c" represented cells grown as under treatment "b" followed by replacement of the culture medium with RPMI-1640 containing 25 mM HEPES, 2 mM glutamine, and 10% FBS, and incubated for 4 h in a 5% CO_2 atmosphere at 37°C. Treatment "d" involved growing of cells as under "a" until 60–80% confluency was achieved followed by changing of the medium to RPMI-1640 containing 25 mM HEPES, 2 mM glutamine without added serum for 4 h at 37°C in a 5% CO_2 atmosphere. Treatment "e" involved cells grown as under "a" to 60–80% confluency followed by a change of the medium to RPMI-1,640 containing 25 mM HEPES, 2 mM glutamine, 6% FBS, and 100 ng/ml nocodazole, and allowing the cells to grow for 16 h at 37°C in a 5% CO_2 atmosphere. Treatment "f" was similar to the treatment "e" except that it was followed by replacement of the culture medium with RPMI-1640 containing 25 mM HEPES, 2 mM glutamine, and 10% FBS and the incubation was carried out for another 4 h at 37°C in a 5% CO_2 atmosphere. Treatment "g" was similar to treatment "e" except that 10 mM hydroxyurea was included in place of nocodazole. Treatment "h" was similar to "f" except that hydroxyurea was included in place of nocodazole. The data are tabulated from FACS analysis of cells grown under various culture conditions, and are presented as percent distribution (mean ± SEM) based on three separate determinations. The percentage of cells in different phases as shown in the Table do not add up to exactly 100 in each case because of the standard errors obtained from multiple experiments in the FACS analysis. The 100% total, however, is within the range of the standard error as shown in the tabulated data.

in the population of the G2/M phase cells (treatment "c"). The effect of removal of serum begins to appear within 4 h (treatment "d" compared with treatment "b"). Treatment of cells with nocodazole arrested them in G2/M phase of cell cycle so that 80% of the cells were present in this phase (treatment "e" in Table I and Fig. 1A). Addition of FBS under these conditions resulted in a rapid shift of the cells to G_0/G_1 phase so that 67% of the cells had shifted to this phase within 4 h of treatment with serum (Table I, treatment "f", and Fig. 1B). The cells were also arrested in the G_0/G_1 phase on treatment with hydroxyurea which resulted in accumulation of 63% of the cells in this phase of cell cycle (Table I, treatment "g", and Fig. 1C). Release of cells arrested in the G_0/G_1 phase by addition of serum to hydroxyurea treated cells evoked a rapid cell cycle progression so that within 4 h after serum treatment 82% of the cells were in the S phase.

Analysis of CK2 Activity and Protein in the Nuclear Matrix of Chemically Synchronized ALVA-41 Cells

Previous studies have shown that there is minimal change in the total CK2 protein and activity in cells examined at different stages of cell cycle [Schmidt-Spaniol et al., 1993; Bosc et al., 1999]. Since nuclear matrix appears to be a major locus of CK2 nuclear signaling, it seemed reasonable to focus on this fraction for a study of the CK2 dynamics in various phases of cell cycle. The results in Figure 2 show CK2 activity in the cell lysate (panel A), and the nuclear matrix (panel B) fractions isolated from chemically synchronized ALVA-41 cells in different phases of the cell cycle, as described under Table I and Figure 1. Measurement of CK2 activity in the total cell lysate indicates that it remains relatively constant during different cell cycle phases (Fig. 2A). This accords with the previous observations on the regulation of cellular CK2 activity during the cell cycle [Schmidt-Spaniol et al., 1993; Bosc et al., 1999]. However, significant changes became apparent on determining the CK2 activity and the immunoreactive protein corresponding to CK2 α and β subunits intrinsic to the nuclear matrix preparations (Fig. 2B and Fig. 3). The removal of serum from the medium resulted in a moderate but significant reduction in the CK2 activity in the nuclear matrix fraction which was reversed rapidly (i.e., within 4 h) to control levels (Fig. 2B, lanes a–c). Analogous corresponding changes were apparent in the immunoreactive protein for subunits α and β of CK2 (Fig. 3B,C, lanes a–c). An extensive reduction (~80%) was observed in the nuclear matrix-associated CK2 activity in ALVA-41 cells arrested in the G_2/M phase by treatment with nocodazole (Fig. 2B, lane d). It is noteworthy that under these conditions there was also a dramatic loss of immunoreactive protein corresponding to the α subunit of CK2 reaching a minimal level (Fig. 3A, lane d). The reduction in the protein corresponding to the β subunit was less severe (Fig. 3B, lane d). Remarkably, the release of ALVA-41 cells from G_2/M phase by addition of serum resulted within 4 h in a significant (100%) increase in CK2 activity as well as the immunoreactive protein levels corresponding to the α and β subunits of CK2 (lane e in Fig. 2B and Fig. 3A,B) compared with the activity and levels in the arrested cells. Arresting the cells in the G_0/G_1 phase by treatment with hydroxyurea resulted in a reduction of CK2 activity and protein (lane f in Fig. 2B and Fig. 3A,B) which was rapidly (i.e., within 4 h) reversed on serum treatment. Thus, it appears that nuclear matrix association of CK2 is reduced in the G_0/G_1 and G_2/M phases, with the latter showing a much greater decrease.

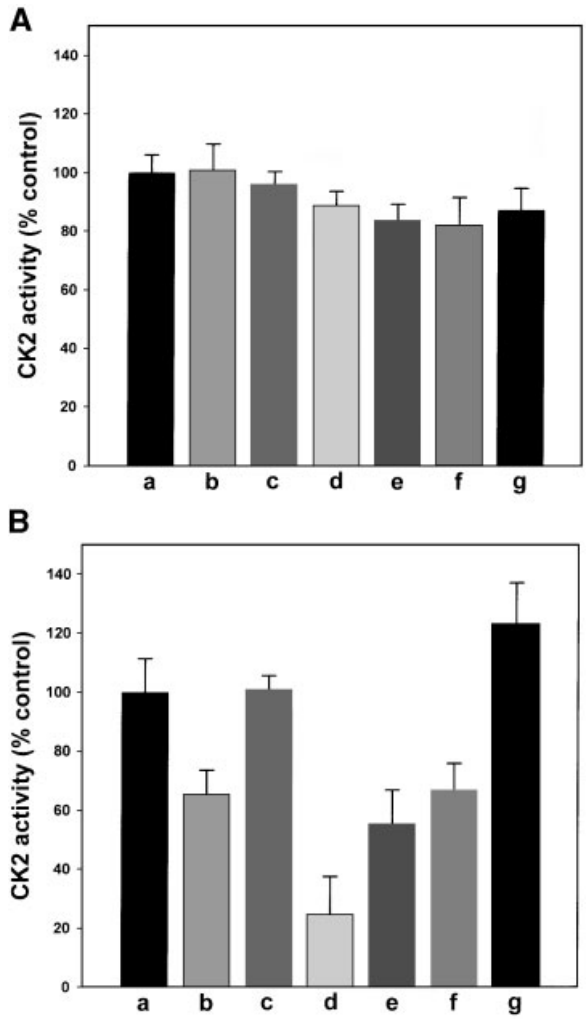

Fig. 2. Cell cycle dependent CK2 activity in lysates and in the nuclear matrix fractions isolated from ALVA-41 cells synchronized across cell cycle, and upon release from the specific block. ALVA-41 cells were subjected to various treatments as described under Table I. Cell lysate and nuclear matrix preparations were isolated from these cells and examined for CK2 activity as described under Methods. **Panel A** demonstrates the CK2 activity in the cell lysate; **panel B** shows the CK2 activity in the corresponding nuclear matrix preparations. Panels A and B: **lanes a–c**, cell treatment was respectively as under ''a''–''c'' in Table I; **lanes d–g**, cell treatment was respectively as under ''e''–''h'' in Table I. The data are based on seven different assays each in triplicate.

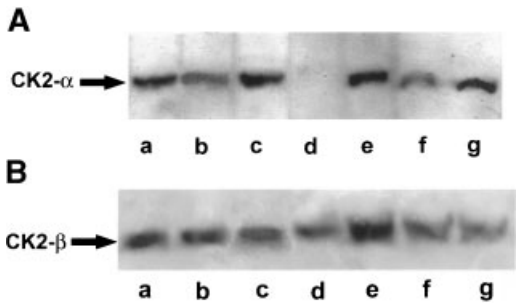

Fig. 3. Immunoreactive protein corresponding to the α and β subunits of CK2 in the nuclear matrix isolated from ALVA-41 cells synchronized across the cell cycle, and upon release from the specific block. The immunoblots for the α and β subunits of CK2 in the nuclear matrix preparations from ALVA-41 are shown in panels (**A**) and (**B**), respectively. The **lanes** (**a–g**) in both panels correspond to those described in Figure 2.

Further, rapid shifts in CK2 association with the nuclear matrix fraction occur under conditions that promote progression of cell cycle from G_0/G_1 to S phase, and from G_2/M phase to G_0/G_1 phase.

Immunofluorescence Analysis of CK2 in the ALVA-41 Cells Arrested in and Released From G_0/G_1 Phase of Cell Cycle

Figure 4 shows the dynamics of nuclear association of CK2 in ALVA-41 cells under different growth conditions. When cells are grown in serum-depleted medium, immunofluorescence for the α subunit of CK2 is observed in a diffuse pattern in the nuclear and cytoplasmic compartments (Fig. 4A). For comparison, Figure 4E shows the cells grown under the normal serum conditions without any treatment; in this case, the CK2 immunofluorescence is also diffuse and present in both the nuclear and cytoplasmic

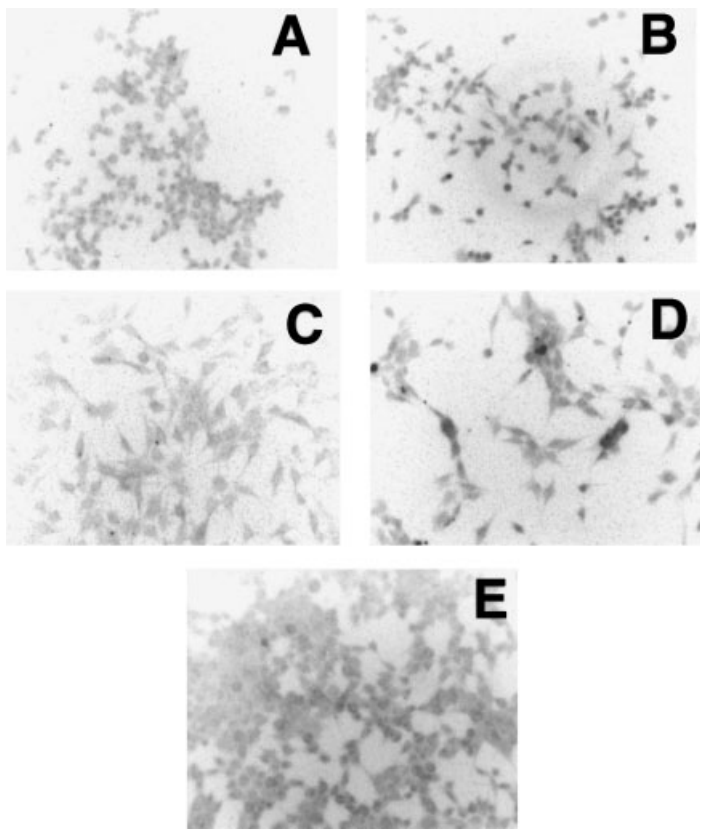

Fig. 4. Dynamics of CK2-α in ALVA-41 cells demonstrated by immunofluorescence staining following cell cycle block with hydroxyurea and subsequent serum-mediated release. Cells were grown on chamber slides and treated as follows. **Panel A**: ALVA-41 cells grown in serum-free RPMI-1640 for 36 h (as for treatment ''b'' under Table I); (**panel B**) cells grown as under Panel A followed by 4 h in RPMI-1640 with 10% FBS; (**panel C**) cells treated with 10 mM hydroxyurea for 20 h; (**panel D**) cells treated with 10 mM hydroxyurea for 20 h as under panel C followed by 4 h in RPMI-1640 with 10% FBS; (**panel E**) ALVA-41 cells grown under normal serum conditions for 36 h. All images are shown at a magnification of ×200.

compartments. Upon subsequent treatment with 10% serum, a marked shift in the immunofluorescence to the nuclear compartment becomes apparent within the 4 h period examined, as judged by the much stronger intensity of immunofluorescence in the nuclear compartment than in the cytoplasm (Fig. 4B). This result is commensurate with the shuttling of the CK2 to the nuclear compartment in response to the serum stimulus. Exposure of cells to hydroxyurea for 16 h to block the cells in the G_0/G_1 phase also resulted in a diffuse pattern of CK2 immunofluorescence in the cells (Fig. 4C). However, a remarkable shift in the immunofluorescence was noted in these cells on removal of the hydroxyurea block by addition of serum so that within 4 h an intense CK2 immunofluorescence was apparent in the nuclear compartment (Fig. 4D). Together, these observations accord with the data on CK2 activity and immunoreactive protein in the nuclear matrix under similar conditions, described in Figures 2 and 3.

Stable Overexpression of α Subunit of CK2 in Cells

We planned to examine the effects of overexpression of CK2 in cells with a view to determine its effects on cell cycle progression. Two types of cells were generated. The first was the CHO Tet-off cell line transfected with pBI-CK2-α in which CK2 overexpression is suppressed by tetracycline. The mRNA expression data for CK2-α in these cells upon removal of the tetracycline from the medium showed relative signal intensities of CK2-α mRNA in a slot blot as 1.0, 29.7, 1.6, and 25.1, respectively (the corresponding values for β-actin probe were 1.0, 1.5, 0.8, and 1.0, respectively). These results indicated the incorporation of the transgene and its expression in the CHO cells, and also suggested that the expression tended to decline over time. The second cell line was ALVA-41 that was stably transformed with pcDNA6-CK2-α. The selected clones were examined for expression of the CK2-α mRNA. The results showed that the CK2-α mRNA was overexpressed by about 10-fold compared with the control cells (data not shown). The CK2 activity was also examined in the cytosol and the nuclear matrix isolated from the stably transfected CHO and ALVA-41 cells. Table II shows that when tetracycline was removed from the medium the induction of CK2 expression in the CHO cells resulted in a modest increase of 13% in the cytosolic fraction, whereas it was increased by about 40% in the nuclear matrix fraction suggesting a preferential increase in the latter, as was observed previously in transient transfection experiments [Yu et al., 1999]. The stably transfected ALVA-41 cells, however, demonstrated little change in the CK2 activity despite the increase in the expression of the message for CK2-α mentioned above. Of note, in the stably transfected CHO cells a preponderant amount of the CK2 activity was localized in the cytosolic fraction while only 23% of the activity was present in the nuclear matrix fraction. On the other hand, the ALVA-41 and PC-3 cells showed an almost equal amount of CK2 activity in the cytosol and nuclear matrix (Table II). These results accord with our previous observations based on activity measurements or immunohistochemical localization of CK2 in normal versus tumor specimens [Gapany et al., 1995; Faust

TABLE II. CK2 Activity in the Lysate and Nuclear Matrix Fraction Isolated From CHO and ALVA-41 Cells Stably Transfected With Expression Plasmids pBI-CK2-α and pcDNA6-CK2-α, Respectively

		CK2 activity in	
Cells	Conditions or treatments	Cell lysate	Nuclear matrix
CHO	+tetracycline, 48 h	76.1 ± 2.6	17.6 ± 1.6
	−tetracycline, 48 h	86.3 ± 0.9	24.6 ± 1.5
ALVA-41	pcDNA6 control	65.5 ± 3.9	66.4 ± 5.3
	pcDNA6-CK2-α	64.5 ± 1.0	60.4 ± 1.1
PC-3	None	64.9 ± 2.8	86.3 ± 0.4

CHO cells stably transfected with the Tet-off inducible expression plasmid pBI-CK2-α were grown without (induced system) or with (non-induced system) 4 μg/ml tetracycline for 48 h. ALVA-41 cells were stably transfected with pcDNA6 (control) or pcDNA6-CK2-α. As described in the text, both CHO and ALVA-41 cells showed a substantial increase in the CK2-α mRNA. Prostatic PC-3 cells were included as a control cancer cell line. Lysate and nuclear matrix fractions were isolated from the cells and CK2 activity associated with these fractions was determined as described under Methods. CK2 activity is expressed as nmol ^{32}P/mg protein/h (mean ± SEM).

et al., 1996, 1999], and in other tumor cells in culture [Guo et al., 1999]. Thus, it appears that the distribution of CK2 in the tumor cells compared with normal cells is distinctly different such that the tumor cells demonstrate a much higher level of CK2 in the nuclear compartment than in the cytosol.

An analysis of cell cycle in the CHO Tet-off cells was also carried out. However, based on FACS analysis, no significant change in cell cycle progression was apparent in stably transfected CHO cells maintained in the presence or absence of tetracycline over 4 days (data not shown). This apparent lack of effect on the cell cycle may relate to the modest change in the induced levels of CK2 protein. Similar results were observed with the stably transfected ALVA-41 cells (data not shown).

DISCUSSION

In the present work, we have provided evidence that discrete spatio-temporal regulation of CK2 can be detected in the nuclear matrix fraction even when no distinct changes in the total cellular activity of the kinase are apparent in different phases of cell cycle. In this regard, the latter observations on a lack of change in the CK2 activity in cell extracts accord with the previous work on cell lines different from those employed by us [Schmidt-Spaniol et al., 1993; Bosc et al., 1999]. The earlier work had also examined the immunofluorescence of CK2 in the cell nucleus during the cell cycle progression [Schmidt-Spaniol et al., 1993]. The immunofluorescence analysis of ALVA-41 cells arrested by hydroxyurea treatment followed by their release on the addition of serum indicated that the diffuse pattern of immunostaining in the cells changed to a more intense nuclear staining following the addition of serum. Presumably, confocal microscopy would also distinguish the discrete changes in CK2 in the subnuclear fractions since we could clearly discern them in the nuclear matrix fraction isolated from the cells subjected to different manipulations, as shown in Figure 2. Various reports from this laboratory have indicated that CK2 is localized in the nucleus in distinct subnuclear loci such as chromatin and nuclear matrix where it may undergo specific dynamic changes in response to different stimuli.

The nuclear matrix appears to be a particularly responsive target for CK2 signaling in the nucleus, not only for growth related activities but also in response to stress [for reviews see Ahmed, 1999; Ahmed et al., 2000, 2002]. For example, it was demonstrated that translocation of CK2 to chromatin [Gerber et al., 2000] and the nuclear matrix compartments [Davis et al., 2002] occurred in cells subjected to heat shock. Interestingly, even the treatment of isolated nuclei to heat shock resulted in a trafficking of CK2 to the nuclear matrix within the nucleus [Davis et al., 2002]. In the present work, our results clearly indicate that CK2 is reduced in the nuclear matrix in G_0/G_1 and G_2/M phases of the cell cycle, and that release from these phases evokes a rapid increase in the nuclear matrix-associated CK2. Thus, even though CK2 appears to be generally unregulated in terms of its total activity, this and several other studies support the notion that its spatio-temporal status in specific loci may be a key means of its functional regulation [Ahmed, 1999; Ahmed et al., 2000; Faust and Montenarh, 2000; Martel et al., 2001; Yu et al., 2001]. Of note, similar modes of regulation of signaling molecules in other systems have been recognized [Stein et al., 1998; Haj et al., 2002; Zaccolo and Pozzan, 2002].

In the present work, we also attempted to determine the effects of overexpression of CK2 on cell cycle progression. We achieved stable Tet-off inducible overexpression of CK2-α in CHO cells, and stable transfection of CK2-α in prostate cancer cells (ALVA-41). However, in both cases, we did not detect a significant overexpression of CK2 protein even though a marked elevation of the message for CK2-α was noted in both cell types. Thus, the lack of effect on the cell cycle progression in either of the two cells may not be surprising. Indeed, similar results were obtained in studies undertaken with different cell types [Li et al., 1999; Vilk et al., 1999]. It has been suggested that an autoregulatory mechanism at the level of transcription may control the level of CK2 in the cell [Pyerin and Ackermann, 2001]. However, the resistance to forced stable overexpression of CK2 protein suggests that post-transcriptional mechanisms may also be involved, especially when one considers that in cancer cells the upregulation of CK2 is primarily at the level of the protein [Tawfic et al., 2001]. On the other hand, it is noteworthy that transient overexpression of CK2 protein in cells results in a differential enhancement in the CK2 associated with the nuclear matrix [Yu et al., 1999]. Transient overexpression of CK2-α subunit has also been

shown to produce certain biological effects, including protection of cells against chemical-mediated apoptosis in several cell lines [Guo et al., 2001], and a change in the morphology of 3T3 cells [Li et al., 1999]. Thus, it remains an important challenge to determine the mechanism(s) underlying the deregulation of CK2 in cancer cells. It appears that a collaboration with other molecule(s) or gene(s) in the cell (such as myc or tal) may be critical to evoke such a change in cellular CK2 associated with the process of oncogenesis [Landesman-Bollag et al., 2001]. Further, it is noteworthy that in double transgenic models of oncogenesis employing overexpression of CK2-α, the oncogenic potential was imparted even though the overexpression was detected only at the mRNA level [Landesman-Bollag et al., 2001].

We have previously reported that normal cells compared with tumor cells demonstrate a diffuse pattern of CK2 immunostaining while the cancer cells show a significantly more intense staining in the nucleus [Faust et al., 1999]. An expansion of these observations in the present work has shown that distribution of CK2 in the cytoplasmic and nuclear matrix compartments in CHO (a non-cancer cell line) versus the ALVA-41 cancer cells was distinctly different, and accorded with the aforementioned immunohistochemical data. Thus, it appears that the relative distribution of CK2 activity in the nuclear matrix and cytoplasmic fractions has a distinct pattern in the normal versus the cancer cells. The significance of such a change in the distribution of CK2 in the context of the oncogenic process in the cell is not clear at present.

In summary, we have demonstrated, for the first time, that despite a lack of apparent change in the total cellular activity of CK2 in different phases of cell cycle, significant discrete alterations in the nuclear matrix-associated CK2 are apparent under the same conditions. This work further emphasizes the role of CK2 trafficking to the nuclear matrix as a means of its functional regulation and signal transduction. We have also shown that cells in culture when stably transfected with CK2-α resist overexpression of CK2 protein or activity even though they show a significant elevation in the level of mRNA. This observation accounts for a lack of any apparent alteration of the cell cycle progression under these conditions compared with control cells. Finally, the present results, taken together with the previous observations, suggest that cancer cells compared with normal cells demonstrate a higher concentration of CK2 in the nuclear matrix which may have a role in the oncogenic process.

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