Apoptosis 2005; **10**: 875–885

Validation of protein kinase CK2 as oncological target

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Protein kinase CK2 is a highly conserved enzyme composed of two catalytic subunits α and/or α' and two regulatory subunits β whose activity is elevated in diverse tumour types as well as in highly proliferating tissues. Several results suggest that the overexpression of either CK2 catalytic subunits or the CK2 holoenzyme contributes to cellular transformation. In a similar vein, experiments performed compromising the intracellular expression of CK2 has led to somehow contradictory results with respect to the ability of this enzyme to control survival and apoptosis. To better elucidate the role of CK2 in programmed cell death, we have depleted cells of CK2 catalytic subunits by the application of antisense oligodeoxynucleotides and siRNAs techniques, respectively. Our results indicate that protein kinase CK2 is characterized by an extremely high stability that might be due to its association with other intracellular proteins, enhanced half-life or lower vulnerability towards proteolytic degradation. In addition, we show that despite the effectiveness of the methods applied in lowering CK2 kinase activity in all cells investigated, CK2 might not by itself be sufficient to trigger enhanced drug-induced apoptosis in cells.

Keywords: antisense oligodeoxynucleotide; apoptosis; protein kinase CK2; siRNA duplex.

Introduction

Antisense oligodeoxynucleotides (ASO) are designed for binding specifically and efficiently to a target messenger RNA (mRNA) interfering with the synthesis of the corresponding protein. Unmodified oligodeoxynucleotides are substrates for ubiquitous nucleases causing their rapid degradation. To prevent for being degraded, the ASO are therefore chemically modified prior to application in intact cells.[1] Beside targeting genes by antisense techniques, gene function has been determined by the application of other methods *e.g.* deletion of murine genes and/or introduction of transgenes. More recently, the ability of small interfering RNAs duplex (siRNAs) to silence gene expression has been used as a new approach for studying gene function in cultured mammalian cells. In this latter case, the mechanism responsible for the gene silencing induced by siRNAs is a multi-step process that ultimately leads to the degradation of the target mRNA.[2] No matter which kind of approach one decides to follow, an efficient silencing of gene expression is dependent on the efficiency of delivery of small molecules (which in turn is dependent on an effective transfection reagent and the endocytotic activity of the selected cell line), on the turnover of the target protein and, last but not least, on the design of an optimal oligodeoxynucleotide/ siRNA duplex sequence.

In this report, we have followed these two different approaches *i.e.* ASO and siRNAs delivery for silencing the expression of protein kinase CK2 and study the effect on apoptosis induced by DNA damaging agents. Protein kinase CK2 is a serine/threonine protein kinase highly conserved and ubiquitously distributed in lower as well as in higher eukaryotic organisms so far investigated.[3] The protein is composed of two catalytic subunits α and/or α' and two regulatory subunits β. Although the β-subunit of CK2 modulates the enzyme activity conferring specificity towards some substrates,[4] a second messenger-mediated mechanism able to regulate the activity of protein kinase CK2 is not known to date. The catalytic subunits of CK2 (*i.e.* CK2α and CK2α') although translational products of different genes[5,6] share a high sequence homology between species (for a review see[7]). In yeast, the disruption of either CKA1 or CKA2 leads to a viable phenotype while the disruption of both CKA1 and CKA2 is lethal indicating that in lower eukaryotes these two isoforms can compensate each other.[8] In mice, although the knockout of CK2α has not yet been reported, the knockout of the CK2α' gene leads to a viable offspring although the males are unfertile indicating that in mice the ability of CK2α to functionally compensate CK2α' is only partial.[9] An extensive body of work has indicated that in cells the CK2β subunits are synthesized in excess and that this event precedes the incorporation of the catalytic subunits into the tetrameric complexes.[10] Moreover, structural and functional studies conducted *in vitro* under different salt

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conditions have revealed a correlation between the molecular forms of CK2 and its optimal kinase activity.[11] On a more physiological front, CK2 has been detected within the nucleus and the cytoplasm of different cell lines as well as specifically associated with organelles and intracellular structures (reviewed by[12]). Although the physiological role of CK2 remains poorly understood, its enhanced expression correlates with rapid proliferating cells found for instance in the cryptae of intestinal villi or during embryo development or as a result of uncontrolled tumour cell proliferation.[3] In the case of colorectal polyps this tendency has been confirmed inasmuch a clear correlation has been established between progression of the tumour and increased CK2 activity.[13,14] The screening of a large variety of solid tumours and cell lines for CK2 expression and activity suggested that the dysregulation of CK2 expression, in cooperation with other proteins, might augment the oncogenic potential of cells. In line with this hypothesis are studies conducted by Seldin *et al.*[15] which demonstrated that the expression of CK2 catalytic subunit in lymphocytes of transgenic mice leads to a stochastic tendency to develop lymphoma and that the co-expression of c-myc protein results in neonatal leukemia. The oncogenic potential of CK2α has also been observed in the case of ectopic expression of Tal-1 (whose oncogenic potential seems to be accelerated with the concomitant expression of CK2α[16]) and p53 down regulation a condition which favours the development of thymic lymphoma in mice missexpressing CK2α.[17] The ectopic expression of CK2α' and H-Ras was shown to induce transformation in rat primary embryo fibroblasts.[18] Furthermore, the critical role of CK2 catalytic subunits has also been indicated by studies conducted with ASO targeting the CK2α and α' mRNAs for protein down regulation in various cell lines.[19–27] A careful analysis of the literature published on this topic reveals some discrepancies concerning the effect that down regulated CK2 exerts in cells undergoing programmed cell death. Studies conducted by Wang *et al.*[24] demonstrated that the reduced expression of CK2α by ASO results in a potent induction of apoptosis in a human prostate cell adenocarcinoma as well as in a human head and neck squamous cell carcinoma. Moreover, Li *et al.*[26] showed that the CK2-catalyzed phosphorylation of ARC, apoptosis repressor with caspase recruitment domain, targets ARC to mitochondria where it binds and inhibits the pro-apoptotic caspase 8. On the other hand, studies by Sayed *et al.*[25] demonstrated that cells transfected with CK2 ASO show a significant reduction in apoptosis even greater in cells expressing wild-type p53. Based on these data, we set out to thoroughly investigate a set of different mammalian cell lines with respect to CK2α and α' protein expression and activity after cell transfection with ASO or siRNAs targetting CK2 catalytic subunits to shed light on the controversial role of CK2 in controlling cell survival and apoptosis. Our studies reveal that CK2 modulates apoptosis in a cell-type specific manner and emphasize the complex nature of this protein kinase with respect to its regulation and function.

Material and methods

Cell lines culture conditions and treatments

HeLa and HCT116 ($p53^{+/+}$) cell lines were grown in Dulbecco's modified Eagles' medium (Gibco) supplemented with 10% fetal bovine serum (FBS) and 1 mM L-glutamine. Jurkat and HCC1937 cell lines were cultured in Roswell Park Memorial Institute medium (RPMI, Gibco) supplemented with 10% fetal bovine serum (FBS) and 1 mM L-glutamine. All cell lines were grown at 37°C with 5% CO_2. The inhibition of new protein synthesis was performed by incubating the cells, after 24 h from seeding, with 80 μg/ml cycloheximide (CHX, Sigma) for variable amounts of time as indicated in the figure legends. Apoptosis was induced in HeLa and HCT116 cell lines incubating cells with 400 ng/ml nocodazole (Calbiochem) for 24 h. Jurkat cells were incubated with 68 μM etoposide (Calbiochem) for 6 h while HCC1937 were exposed to 40 μM cisplatin (Sigma) for 24 h prior cell harvesting. In all cases, cells were incubated with ASO or siRNAs for 24 h prior to any treatment.

Transfection assays with ASO or siRNAs

Phosphorothioated oligodeoxynucleotides were obtained from DNA Technology A/S (Denmark) while siRNAs duplex were purchased from Dharmacon (USA). Mirus TransIT-Oligo (USA) was used as transfection reagent when cells were transfected with oligodeoxynucleotides (5 μg/ml) twice at 12 h interval following the manufacturer's guideline. The transfection of siRNAs (300 nM, twice at 12 h interval) was performed using Mirus TransIT-TKO (USA) following the manufacturer's protocol. The transfection experiments were performed in triplicate for each cell line. Control experiments where the above-mentioned transfection reagents were tested for the determination of transfection efficiency, provided similar results.

Protein analysis

Protein extracts from cells treated as indicated in the figure legends were prepared by resuspending cell pellets in extraction buffer containing 25 mM Tris/HCl pH 8.5, 1 mM DTT, 300 mM NaCl and protease inhibitors cocktail (Roche). Disruption of cells was carried out by sonication followed by a 4000 × g centrifugation step for 30 min at 4°C. 80 μg of the supernatant were analysed

by SDS-PAGE which was followed by Western blotting. PVDF membranes were probed with either mouse monoclonal anti CK2α antibody (1AD9, Calbiochem), mouse monoclonal anti CK2β antibody (6D5, Calbiochem), rabbit polyclonal anti CK2α' antibody obtained immunizing rabbits with a specific peptide sequence (SQPCADNAVLSSGTAAR) of human CK2α', mouse monoclonal anti p53 antibody (Ab-2, Calbiochem) or mouse monoclonal anti β-actin antibody (AC-15, Sigma). After probing the membranes with the indicated antibodies, proteins were visualized by a chemiluminescence Western blotting detection system following the manufacturer's guideline (CDP-Star, Applied Biosystem).

Protein kinase assay

Protein kinase CK2 activity was performed essentially as described previously.[28] Protein extracts (10 μg) were tested in a reaction mixture containing 25 mM Tris-HCl pH 8.5, 1 mM DTT, 20 mM NaCl, 5 mM $MgCl_2$, 100 μM sodium orthovanadate, 50 μM [$\gamma-^{32}$P]ATP, 190 μM synthetic peptide in a final volume of 50 μl. The kinase assay was performed at 37°C for 5 min. Reactions were stopped by cooling the samples on ice. 30 μl of each sample were then transferred onto phosphocellulose filter-paper P81. After an extensive washing of the filters, the radioactivity incorporated into the substrate peptide (RRRDDDSDDD) was determined by scintillation counting (Hewlett-Packard). The values shown in the diagrams are the mean ($\pm$ S.D.) of measurements done in duplicate from three independent experiments. 1U of CK2 activity is defined as the amount of activity necessary to transfer 1 μmol phosphate/min into the substrate peptide at 37°C.

Apoptosis assay and flow cytometry analysis

Apoptosis was determined by propidium iodide (Sigma)-stained nuclei of control and treated cells. Cells were trypsinized, combined with floating cells present in the medium and washed with phosphate-buffered saline (PBS) prior fixation in 70% ethanol overnight at -20°C. Fixed cells were then resuspended in PBS containing 40 μg/ml RNAse and 20 μg/ml propidium iodide for 30 min at room temperature prior analysis. The DNA content was analysed by a FACSCalibur Flow Cytometer (Becton Dickinson Biosciences) and the acquired data processed by Cell Quest Pro analysis software. Alternatively, apoptosis was determined monitoring the externalization of phosphatidylserine (PS) by the Annexin V-FITC stained method (Oncogene). Cells were collected, washed twice with PBS and double stained with Annexin V-FITC/ propidium iodide according to the manufacturer's instructions. Quantitation of apoptosis was then performed by flow cytometry analysis.

Results

Effectiveness of ASO and siRNAs in targeting CK2 catalytic subunits mRNA in various mammalian cell lines

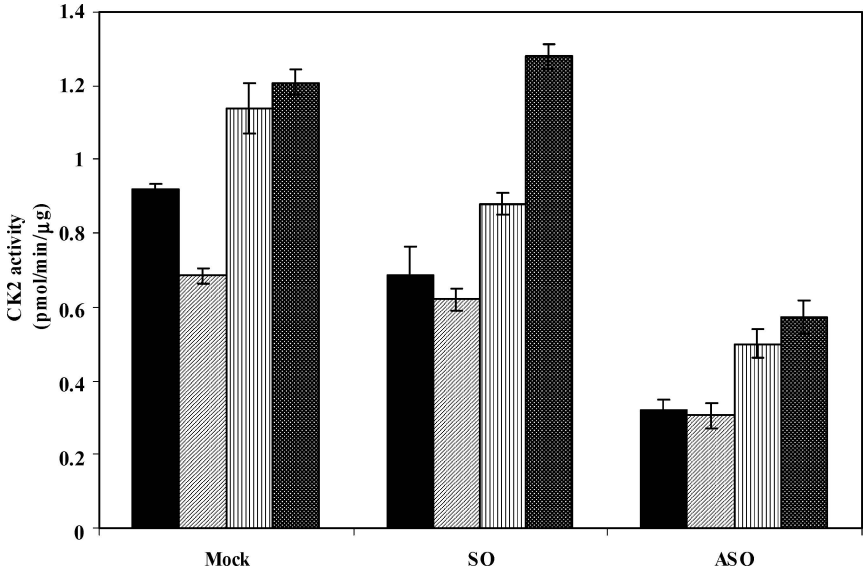
We have initially screened a panel of four mammalian cell lines which have been transfected with sense (SO) and an antisense oligodeoxynucleotide sequence (ASO), respectively, (Table 1) and verified their ability to prevent the expression of CK2 catalytic subunits measuring protein kinase CK2 enzymatic activity. As it is shown in Figure 1, it has been possible to achieve in all cell lines investigated an average 50% reduction of CK2 activity although in the case of HeLa cells the treatment with ASO has sometimes led to a higher reduction (results not shown). Control experiments performed transfecting cells with random nonsense oligodeoxynucleotides gave the same results as with SO (results not shown). As mentioned above, one of the most important prerequisite for preventing the expression of a protein is the fact that the ASO should hybridise with a target mRNA in an efficient way. What we noticed during our studies was the requirement of a

Table 1. Oligodeoxynucleotide and siRNA duplex sequences used for CK2α/α' cell depletion.

Target protein	**Oligodeoxynucleotide sequence**	**Target sequence**
CK2α/CK2α'	5′-GTAATCATCTTGATTACCCCA-3′[20] (antisense)	5′-TGGGGAAATCAAGATGACTAC-3′ (CK2α) 5′-TGGGGTAATCAAGATGATTAC-3′ (CK2α')
	5′-CCTGTCTGACATGTCGGGAC-3′ (sense)	
Target protein	**siRNA Duplex**	**Target sequence**
CK2α	Duplex #1 Smart pool Q-003480-00-09	nd
CK2α'	5′-GAAGAUAAAACGAGAGGUUdTdT-3′ 3′-dTdTCUUCUAUUUUGCUCUCCAA-5′	5′-AAGAAGAUAAAACGAGAGGUU-3′

The antisense oligodeoxynucleotide sequence employed in this study was designed as described by[20] and have been shown to effectively target CK2 catalytic subunit mRNAs. The CK2α' siRNA duplex sequence was based on the CK2α' cDNA sequence. Cells were transfected with indicated molecules as described in the material and methods section.

Figure 1. Effect of ASO treatment on protein kinase CK2. Bar graph showing CK2 kinase activity performed with a specific CK2 peptide substrate in cell extracts after cell treatment with transfection reagent only (Mock), sense oligodeoxynucleotide (SO), and antisense oligodeoxynucleotide (ASO), respectively, as specified in the material and methods section. Black bars refer to HeLa cells analysis, oblique-lines bars refer to HCT116 cells analysis, vertical-lines bars refer to Jurkat while white-dots bars refer to HCC1937 cells analysis. Averages (± S.D.) of three independent experiments are shown.

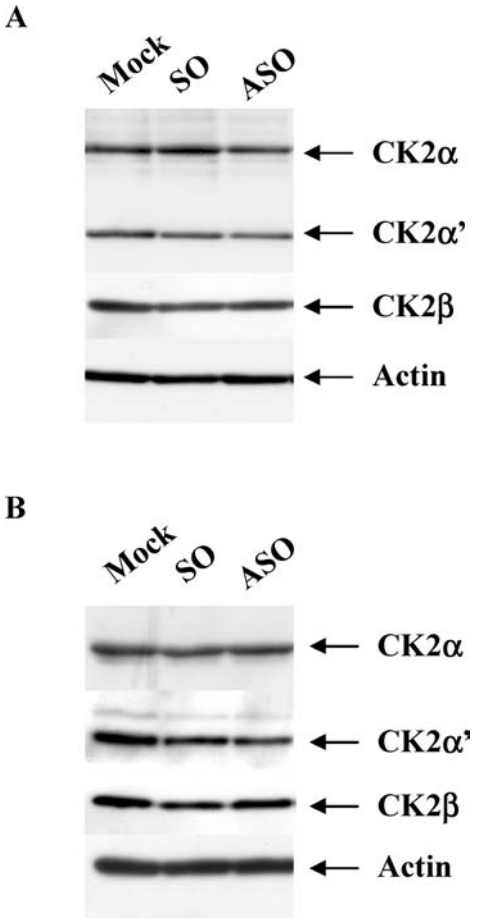


high amount of ASO, added to the culture medium, to achieve the results shown in Figure 1. Because of the usage of phosphorothioate oligodeoxynucleotides, we exclude that the high amount of ASO employed in these studies was needed because of degradation of the oligodeoxynucleotides by ubiquitous nucleases. To further confirm our results, we performed a Western blotting analysis to investigate, at the protein level, the depletion of CK2 catalytic subunits. As it is shown in Figure 2, protein extracts from HeLa (Figure 2A) and HCT116 (Figure 2B) cells treated with various oligodeoxynucleotides as indicated in the figure legends were analysed probing the Western blotting membrane with antibodies recognizing either CK2α or CK2α'. Surprisingly, despite the fact that the treatment applied led to a consistent reduction of CK2 kinase activity, a concomitant decrease of CK2α/α' protein expression in both cell lines investigated was not observed. These somehow unexpected results are in contrast with those obtained by others which showed that cells treated with ASO leads to a decrease of CK2 catalytic subunits protein expression.[20,21,24–26] Since in eukaryotic cells the regulatory CK2 β-subunit plays a central role in the assembly of the CK2 tetrameric enzyme it could not be excluded that the down-regulation of the α/α'-subunit could lead to changes in the CK2β levels. Therefore we have probed the Western blotting membranes also with an antibody directed against CK2β. As shown in Figure 2A and B, the treatment of both cell lines with ASO did not affected the expression of the CK2 regulatory subunit. Next, we analysed the effect on CK2 catalytic subunits both at the protein and activity levels after the cells have been transfected with siRNAs targeting CK2α/α' mRNAs (Figure 3). Figure 3A shows that siRNAs are effective in reducing CK2 kinase activity in both HeLa and HCT116 cell lines. The Western blotting analysis of the crude extracts from both cell lines shows that the decreased catalytic activity of CK2 after cell incubation with siRNAs, is accompanied by a concomitant slight decrease in the amount of CK2α/α' proteins (Figure 3B and C). Unexpectedly, the analysis by Western blotting of CK2β expression before and after cell treatment with siRNAs targeting CK2α/α' revealed a lower CK2β protein expression in both cell lines investigated (Figure 3B and C). Moreover, when the individual CK2 catalytic subunits were silenced (*i.e.* CK2α or CK2α'), the protein expression level of CK2β changed only in cells depleted of CK2α. The knockout of CK2α' did not affect the expression of CK2β (Figure 3D).

Rate of synthesis and degradation of CK2 catalytic subunits

The fact that for reaching a consistent decrease in CK2 kinase activity cells had to be transfected with a high amount of either ASO or siRNAs, prompted us to determine the rate of synthesis and degradation of CK2α/α' subunits in the presence of CHX. Cells were incubated for the indicated amounts of time with 80 μg/ml CHX to block new protein synthesis (Figure 4). Western blotting analysis of

Figure 2. Western blotting analysis of CK2α, CK2α' and CK2β protein expressions before (Mock) and after treatment of the cells with sense oligodeoxynucleotide (SO) and antisense oligodeoxynucleotide (ASO), respectively. A, shows the Western blotting analysis of HeLa cells protein extract while B, shows the analysis of HCT116 cells protein extracts. Equal cell lysate loading onto SDS-PAGE gel was verified by probing the Western blotting membranes with mouse monoclonal anti β-actin antibody.



crude extracts from untreated or drug-treated cells for up to 72 h shows that the CK2 catalytic subunits-half-life is greater than 72 h. As a control, Western blotting membranes from lysates from HeLa (Figure 4A) and HCT116 (Figure 4B) were probed with an antibody against the tumour suppressor gene product p53 known to be a protein with a rather short half-life.[28,29] As expected, p53 protein is detected in untreated cells (Figure 4A and B lane C) but not after 24 h and for longer incubation times with CHX. We have analysed several cell lines and verified CK2 catalytic subunits-protein level before and after treatment with CHX. In all cell lines investigated included HeLa and HCT116, CK2α/α' showed to be a rather stable protein and only after 72 h incubation (cells were monitored for up to 120 h) with CHX we could observe a decrease in the protein expression level which was more pronounced in the case of CK2α' (data not shown).

The apoptotic response of cells incubated with ASO against CK2 catalytic subunits α/α' varies among the cell lines investigated

Studies conducted in different eukaryotic organisms support the notion that CK2 beside being associated with cell proliferation and viability, seems to play an important role in pathways that control apoptosis (for reviews see[3,30,31]). However, despite the application of many strategies for lowering the basal level and/or the activity of CK2 in cells (*e.g.* with the usage of inhibitors, ASO, the expression of dominant negative of CK2) the results obtained by different research groups are not fully in agreement. While, in fact, studies by Faust *et al*.[23] showed that treatment of cancer cells with ASO against the α subunit of CK2 results in a strong induction of apoptosis, Sayed *et al*.,[25] showed that CK2-depleted, nocodazole treated cells undergo a dramatic reduction in the apoptotic cell fraction. Based on these observations, we set out to investigate the apoptotic response of four different cell lines treated with ASO directed against the CK2 catalytic subunits. As it is shown in Table 2, the percentage of apoptosis in the investigated cell lines varies considerably. The treatment of HeLa cells with 400 ng/ml nocodazole for 24 h, induces apoptosis (Figure 5B and Table 2) compared to control cells (Figure 5A and Table 2) in agreement with previous studies showing that cells persistently exposed to nocodazole undergo apoptosis.[25] However, the treatment of HeLa cells with CK2α/α' ASO followed by apoptosis induction in the presence of nocodazole leads to an increased percentage of apoptotic cells (*i.e.* 13.2%, Figure 5F and Table 2) compared to cells treated with nocodazole only and expressing CK2α/α' (Figure 5B and Table 2). Although these results differ from those previously reported,[25] we could confirm that HeLa cells CK2α/α'-depleted and

Table 2. Flow cytometry analysis of the percentage of apoptotic cells of the indicated cell lines.

Cell line	Mock	T	SO	SO+T	ASO	ASO+T
HeLa	1.92	4.88	2.85	6.70	4.62	13.20
HCT116	0.34	1.47	0.95	4.45	1.63	3.53
	2.34*	8.75*	8.38*	15.36*	7.87*	14.12*
Jurkat	3.06	5.51	4.06	5.45	2.27	4.50
HCC1937	8.17	16.00	13.32	18.87	14.00	23.37

Cells were mock-treated (Mock), treated with a drug inducing apoptosis (T) as indicated under material and methods, with the sense oligodeoxynucleotide only (SO), with the SO and then with a drug inducing apoptosis (SO+T), with the antisense oligodeoxynucleotide only (ASO) and with the ASO followed by induction of programmed cell death (ASO+T), respectively. Indicated values (% of cells in subG1) relative to each condition were determined by Cell Quest Pro analysis software. Number with an asterisk refer to values obtained by flow cytometry analysis applying the Annexin V-FITC staining method. Experiments were performed three times obtaining similar results.

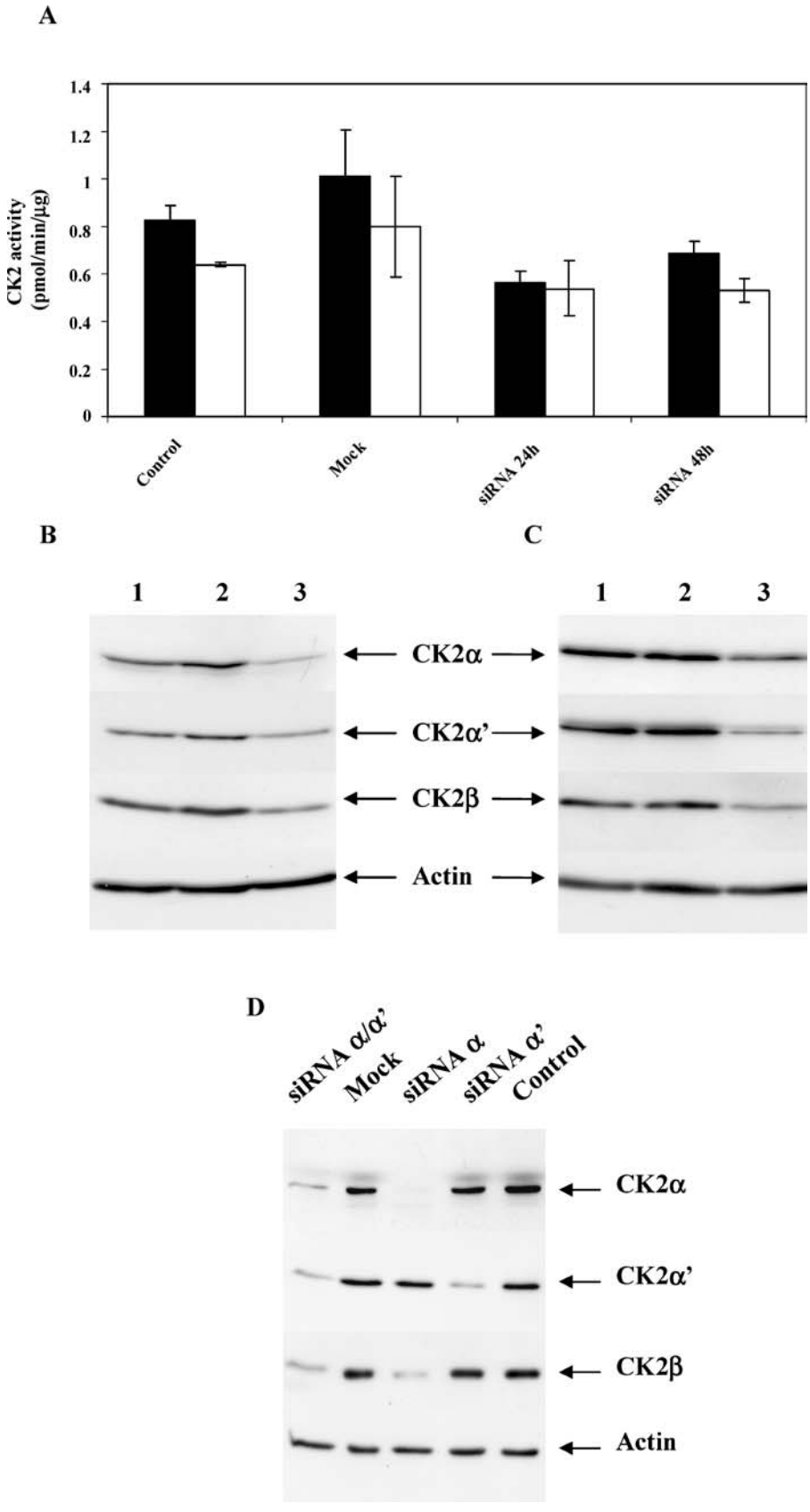
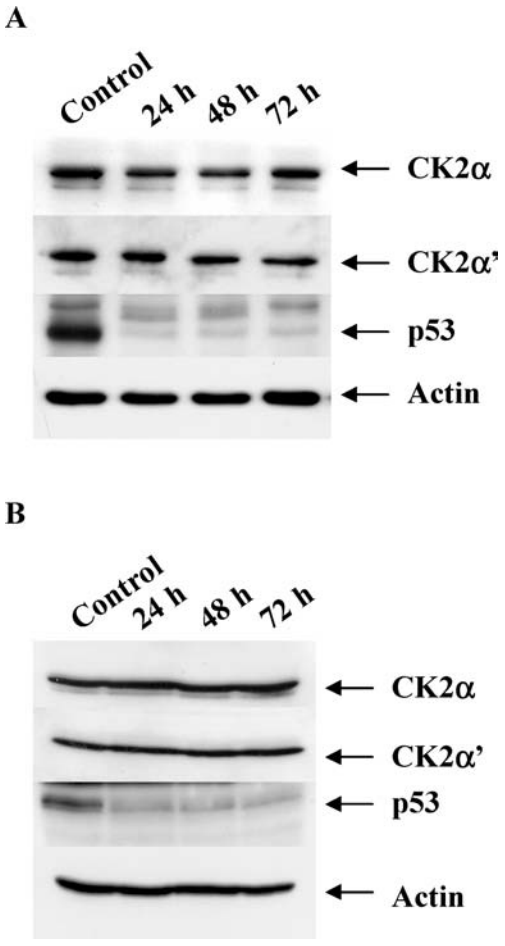

A
CK2 activity (pmol/min/µg)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
Mock
siRNA 24h
siRNA 48h
B
C
1 2 3
1 2 3
CK2α
CK2α'
CK2β
Actin
D
siRNA α/α'
Mock
siRNA α
siRNA α'
Control
CK2α
CK2α'
CK2β
Actin

Figure 3. Effect of siRNAs duplex treatment on CK2 kinase activity and protein expression level. A, bar graph showing CK2 kinase activity present in cell extracts after cells have been incubated with transfection reagent only (Mock), siRNAs directed against CK2α/α' mRNA and harvested after 24 h and 48 h, respectively, after the last transfection. The graph shows also CK2 kinase activity in lysates from cells that did not receive any treatment (Control). Black bars refer to HeLa cells analysis while white bars refer to HCT116 cells analysis. Mean values ($\pm$ S.D.) resulting from three independent experiments are shown. B, Western blotting detection of CK2α/α' and CK2β proteins in lysates from HeLa cells untreated (lane 1), treated only with transfection reagent (lane 2) and with siRNA and harvested 48 h after the last transfection (lane 3). C, Western blotting analysis of CK2α/α' and CK2β protein expressions in lysates from HCT116 cells treated as indicated in B. D, Western blotting analysis of the expression of the indicated proteins relative to HCT116 cells left untreated (Control), incubated with transfection reagent only (Mock) or with siRNAs directed against CK2 catalytic subunits as indicated in the figure. Equal loading of protein extracts was verified probing the immunoblot with a mouse monoclonal anti-actin antibody.

Figure 4. Rate of protein kinase CK2 α/α'-subunits synthesis and degradation. A, Western blotting analysis of lysates from HeLa cells. B, Western blotting analysis of lysates from HCT116 cells. For both cell lines, cell lysates from untreated (Control) or incubated with CHX for the indicated amounts of time were analysed. One representative experiment out of three is shown. Western blotting membranes were probed with anti β-actin mouse monoclonal antibody for loading control.

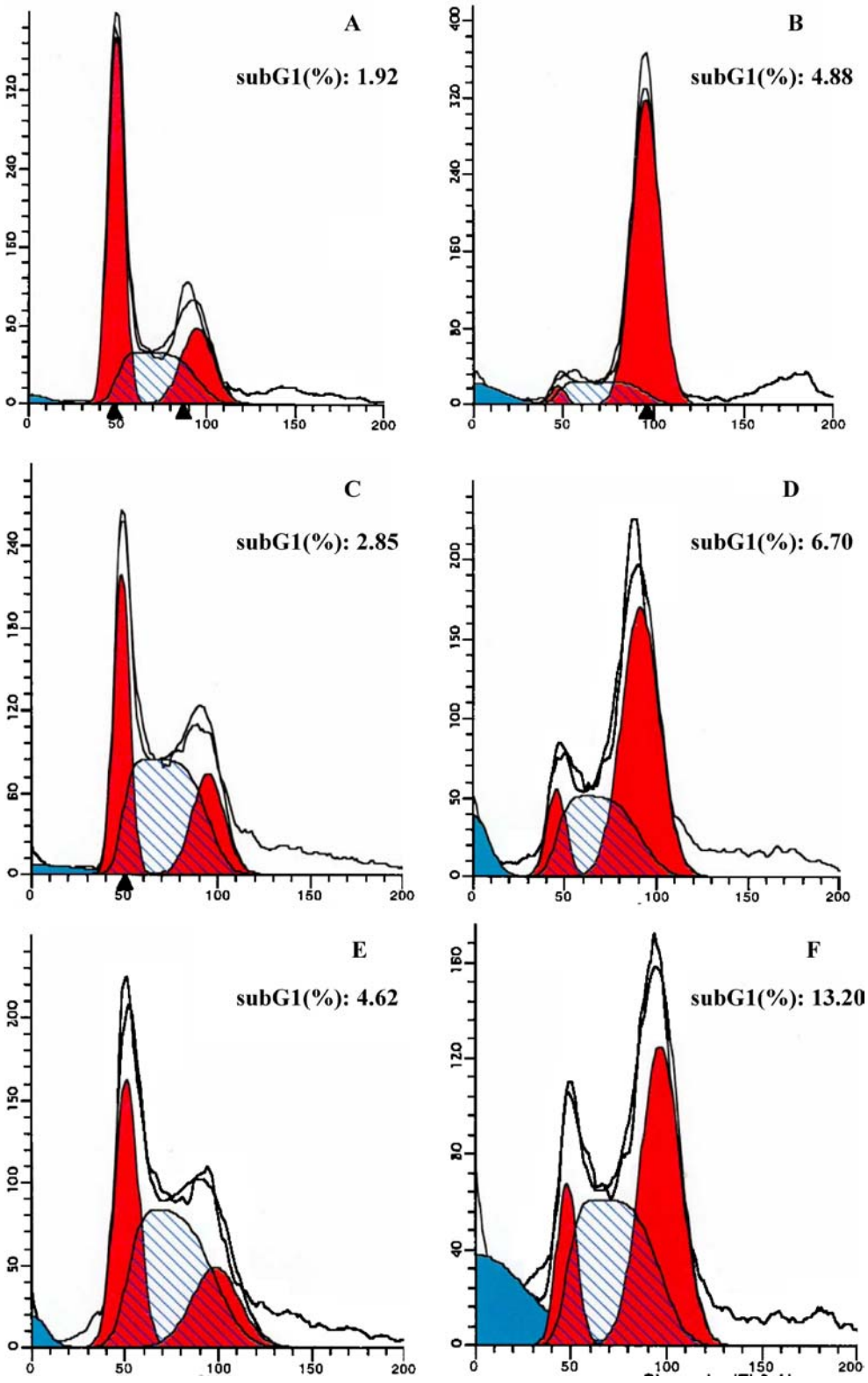


exposed to nocodazole for 24 h, appear to progress into the cell cycle because of a compromised mitotic arrest (Figure 5F). Programmed cell death was induced in HCT116 cells under the same conditions as for HeLa cells. As shown in Table 2, despite the fact that the depletion of CK2α/α' and the subsequent incubation with nocodazole lead to an increased percentage of apoptotic cells (*i.e.* 3.53%) in comparison with cells treated with nocodazole only (*i.e.* 1.47%), the incubation of HCT116 cells with SO, the same used in the experiments performed with HeLa cells, contributes to the induction of apoptosis (*i.e.* 4.45%). As reported in Figure 1, the incubation of HCT116 cells with SO did not exert a significant effect on CK2 kinase activity. Because of the low amplitude of the apoptotic response of nocodazole-treated HCT116 cells (Table 2), exponentially growing HCT116 cells were treated with nocodazole after CK2α/α' antisense transfection as described above. The quantitation of apoptosis was performed by double staining with Annexin V-FITC and propidium iodide. As shown in Table 2, the higher sensitivity of the applied method permitted to determine with higher accuracy the percentage of cells undergoing apoptosis but confirmed the results reported above obtained by flow cytometry analysis of the subG1 population of cells stained with propidium iodide. Apoptosis was induced in Jurkat cells by incubation with etoposide. As shown in Table 2, the incubation of Jurkat cells with 68 μM etoposide for 6 h leads to an increased level of apoptosis between control cells and etoposide-treated cells of 2.5% but the induction of apoptosis which followed cell depletion of CK2α/α', did not lead to a higher percentage of apoptosis. The induction of apoptosis in HCC1937 cell line was induced successfully incubating the cells with 40 μM cisplatin (cisPt) for 24 h. The data shown in Table 2, indicate that the depletion of CK2 catalytic subunits prior induction of programmed cell death leads to a significant increase in apoptosis as it is observed in the case of HeLa cells.

Discussion

In the last few years protein kinase CK2 has gained increasing importance because of its remarkable involvement in many intracellular pathways in particular in those controlling cell proliferation and apoptosis. Mounting evidence indicates distinct roles exerted by CK2 subunits when not present in the tetrameric holoenzyme complex. Studies conducted in yeast have indicated that the regulatory β-subunit of CK2 plays a role in

Figure 5. CK2 α/α'-subunit depletion by ASO enhances apoptosis induced by nocodazole in HeLa cells. ModiFit LT representation of cell cycle profile of HeLa cells: A, untreated; B, treated with nocodazole; C, with ASO; D, with SO; E, with SO and subsequently with nocodazole; F, with ASO; G, with ASO then with nocodazole. The experiment was performed as described under material and methods section. The percentage of apoptotic cells relative to each experimental condition was determined using Cell Quest Pro analysis software.



growth and cytokinesis[32] and it is required in checkpoint adaptation.[33] The involvement of CK2β in many intracellular processes has also been supported by studies conducted with mammalian cells although the influence of CK2β on cell proliferation seems to vary between different cell lines. As already mentioned, although CK2 has been considered for a long time an enzyme assembled in a tetrameric form, several lines of evidence indicate functional specializations of CK2α and CK2α' at the cellular level distinct from those involving the CK2 β-subunit. Because in many cell lines CK2 is detected at high level it may not be possible to observe a consistent effect when cells are forced to overexpressed CK2 subunits. In this respect, a more effective approach would be to employ techniques that interfere with the expression of endogenous CK2 subunits. In our study, we have induced the down-regulation of CK2α/α' subunits by the ASO and siRNAs techniques, respectively. The depletion of cells of CK2 α/α'-subunits has been achieved with the usage of a consistent amount of RNA-interfering molecules which may indicate a long apparent half-life of CK2, as previously reported,[34] and/or a highly expressed gene. The analysis of CK2α/α' turnover from cells incubated with CHX, indicates that CK2 is indeed a highly stable protein and that CK2 stability (*i.e.* its protection from degradation) might be due to its association, in a dynamic equilibrium, with other intracellular proteins which contribute to the formation of high molecular mass complexes as observed previously.[28] The treatment of cells with ASO or siRNAs leads to an average 50% decrease of CK2 kinase activity but we have obtained incongruous results concerning CK2α/α' protein expression level. One possible reason for this discrepancy is the fact that these methods induce gene silencing by different mechanisms being the latter (*i.e.* the siRNA technique) perhaps more efficient in depleting cells of CK2α/α'subunits. The finding that the incubation of cells with ASO directed against CK2α/α' leads to a decrease in protein kinase activity but not in protein expression level, further emphasizes the complex nature of CK2 and the regulation of its expression. One could speculate that in cells would exist a dynamic equilibrium between the active CK2 tetrameric form (*i.e.* composed of two regulatory and two catalytic subunits) and the individual subunits (*i.e.* 2α, 2α', or $\alpha\alpha'$ and β_2) the latter characterized by a lower activity. It is conceivable that in untreated cells the newly synthesised CK2 catalytic subunits would be incorporated into the nascent CK2 holoenzyme molecules. Upon cell treatment with ASO, being the cells depleted of free CK2 catalytic subunits, CK2 holoenzyme would "release" free catalytic subunits from the pre-existing pool of CK2 molecules. In both situations we would detect the same CK2 protein level but after treatment with ASO, we would observe a decrease in CK2 kinase activity. In support of our data, Hui *et al*.[35] have observed an increase in the CK2 activity during the replication cycle of influenza virus although Western blotting analysis did not reveal increased expression of CK2 protein in virus-infected cells. This indicates that changes in kinase activity are not necessarily connected with alteration in protein expression level analyzed by Western blotting that is a semi-quantitative detection method. The finding that the cellular depletion of CK2 catalytic subunits by siRNAs affects negatively the expression of CK2 regulatory β-subunit, reinforces the notion that the stability of CK2β is related to its ability to rapidly assemble with CK2α/α' in stable tetrameric complexes. Indeed, it has been previously demonstrated that if CK2β fails to form complexes with catalytic CK2 subunits, it undergoes rapid degradation with a half-life of less than 1 h.[36] Mounting evidence suggests that in mammalian cells CK2α/α' subunits are functionally independent isozymes with respect to cell proliferation, subcellular localization and interacting proteins (for a review see[30]). In this study, we have demonstrated for the first time that only the depletion of CK2α and not CK2α' affects the expression, thus the stability, of CK2β further emphasizing the functional specialization of CK2 catalytic subunits in mammalian cells.

In the second part of this study, we have focused on the role exerted by protein kinase CK2 in oncogenesis in particular in protecting cancer cells from apoptosis. Numerous evidence supports this notion: there are studies indicating that CK2 protects BID from Apo2L/TRAIL-induced caspase 8-mediated degradation[37] as well as that CK2-catalyzed ARC protein phosphorylation is essential for ARC to prevent apoptosis by binding caspase 8 in the mitochondria.[26,38] Additional support for a role of CK2 in controlling cell survival and death comes from data showing that CK2 targets for phosphorylation several proteins related to apoptosis *e.g.* the tumour suppressor gene product p53,[39] NF-κB,[40] IkB[41] as well as c-Myc.[42]

Collectively, our results suggest that CK2 indeed plays a role in pathways that control cell survival and apoptosis confirming numerous previous results. Moreover, the screening of different cell lines depleted of CK2 catalytic subunits by the ASO technique reveals the complex nature of this protein kinase inasmuch we did not observe enhanced drug-induced apoptosis in all cell lines investigated. We do not have a precise explanation concerning the apoptotic response of HCT116 and Jurkat cell lines depleted of CK2 catalytic subunits since in both cell lines the treatment with ASO leads to a 50% decrease in CK2 activity. Nevertheless, our finding further emphasizes the complexity of the apoptotic machinery and that, in agreement with the results recently reported by Hessenauer *et al*.,[43] CK2 might be dispensable for protecting cells against apoptosis. Further investigations to identify the precise role exerted by CK2 in apoptosis through a large

screening of mammalian cell lines will certainly provide more insights on the regulation of this complex process.

Conclusion

In summary, the application of ASO and/or siRNAs techniques for gene silencing is a useful method to study the function of a selected protein in relation to a specific cellular event. The present study, shows that CK2 is characterized by a long half-life which might be due to its binding with specific intracellular proteins and/or reduced exposure to proteolytic degradation. Cell depletion of CK2 catalytic subunits by the siRNA technique reveals that the stability of the CK2 regulatory β-subunit depends on the presence of an intact CK2α and not CK2α' further emphasizing the functional specialization of the individual CK2 catalytic subunits.

The apoptotic response of cells depleted of CK2α/α' confirms findings previously reported about the role that CK2 exerts in protecting cells from apoptosis in addition, it indicates that CK2 might be a dispensable protein in the regulation of programmed cell death.

Acknowledgments

This work was supported by the Danish Cancer Society (grant No. DP 03 093 to BG), the Danish Research Council (grant No. 21-03-0508 to BG) and ROCHE Diagnostics GmbH.

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