Induction of Apoptosis by Antisense CK2 in Human Prostate Cancer Xenograft Model

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Abstract

Protein serine/threonine kinase CK2 (formerly casein kinase 2) is a ubiquitous protein kinase that plays key roles in cell growth, proliferation, and survival. We have shown previously that its molecular down-regulation induces apoptosis in cancer cells in culture. Here, we have employed a xenograft model of prostate cancer to extend these studies to determine whether antisense CK2α evokes a similar response *in vivo*. A single dose of antisense CK2α oligodeoxynucleotide given directly into the PC3-LN4 xenograft tumor in nude mouse induced a dose- and time-dependent tumor cell death *in vivo*. The tumor was completely resolved at the higher tested dose of the antisense. Cell death was due to apoptosis and correlated with a potent down-regulation of the CK2α message and loss of CK2 from the nuclear matrix in the xenograft tissue as well as in cancer cells in culture. These observations accorded with several of the earlier studies indicating that loss of CK2 from the nuclear matrix is associated with induction of apoptosis. Comparison of the effects of antisense CK2α oligodeoxynucleotide on cancer versus normal or noncancer cells showed that the concentration of antisense CK2α that elicited extensive apoptosis in tumor cells in culture or xenograft tumors *in vivo* had a relatively small or minimal effect on noncancer cells in culture or on normal prostate gland subjected to orthotopic injection of antisense oligodeoxynucleotide *in vivo*. The basis for the difference in sensitivity of cancer versus noncancer cells to antisense CK2α is unknown at this time; however, this differential response under similar conditions of treatment may be significant in considering the potential feasibility of targeting the CK2 signal for induction of apoptosis in cancer cells *in vivo*. Although much further work will be needed to establish the feasibility of targeting CK2 for cancer therapy, to our knowledge, this is the first report to provide important new evidence as an initial "proof of principle" for the potential application of antisense CK2α in cancer therapy, paving the way for future detailed studies of approaches to targeting CK2 *in vivo* to induce cancer cell death. (Mol Cancer Res 2004; 2(12):712–21)

Introduction

Protein kinase CK2 (formerly casein kinase 2) is a most highly conserved and ubiquitous protein serine/threonine kinase localized in both the cytoplasm and the nucleus where it engages in many functions, including roles in normal and abnormal cell growth and proliferation (1-6). Its heterotetrameric structure consists of two catalytic subunits (42 kDa α and 38 kDa α′) and two regulatory subunits (28 kDa β) existing as $\alpha_2\beta_2$, $\alpha\alpha'\beta_2$, or $\alpha'\beta_2$ configurations. The growth-related functions of CK2 are reinforced by its involvement in the phosphorylation of numerous substrates in the cell, many of which are nuclear associated and are involved in gene expression and cell growth (1-6). A key feature of CK2 biology is that it is essential for cell survival (e.g., ref. 7), and it is noteworthy that attempts to produce CK2 knockout mice have been unsuccessful (8).

Protein kinase CK2 undergoes nucleocytoplasmic shuttling in response to a variety of signals. For example, our studies on the prostate have shown that growth and survival signals (androgens and growth factors) promote trafficking of the kinase to the nucleus where nuclear matrix and chromatin are the differential loci for its signaling. On the other hand, the removal of these growth or survival factors promotes translocation of the kinase from the nucleus to the cytoplasm as an early event that precedes or accords with cessation of cell growth and promotion of apoptosis in the prostate epithelial cells. Further in this context, it is of particular note that the nucleocytoplasmic shuttling occurs prior to actual changes in the total cellular CK2 (reviewed in refs. 2, 3). The kinase has been implicated in multiple functions in the cells. For example, recent work has suggested that it has a role in chromatin remodeling (9, 10). Further, besides its involvement in cell growth, CK2 has been shown to exert a strong protective effect on cells against apoptosis (3, 11). Because CK2 is a ubiquitous kinase, the importance of this function of CK2 is underscored by the observed dysregulation of CK2 in the cancer cells, as it has been observed to be consistently elevated by 3- to 7-fold in the various cancer types examined (e.g., refs. 2, 5). The up-regulation of CK2 in cancer cells is related to the state of dysplasia as well as proliferation and that the characteristics of intracellular distribution of CK2 in normal versus cancer cells

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are distinct (12). Likewise, the suppression of apoptosis by CK2 is of particular note because dysregulation of apoptosis is a key feature of the cancer cell phenotype with the consequent imbalance in cell growth (e.g., ref. 13). Our studies of the human squamous cell carcinoma (SCC) of the head and neck and of prostate cancer have suggested that elevated CK2 may be associated with aggressive tumor behavior (14, 15). Likewise, studies based on transgenic models of cancer have provided strong evidence that CK2 overexpression in the transgene imparts a potent oncogenic potential resulting in markedly enhanced tumorigenesis by various oncogenes (16-18).

Considering that CK2 is essential for cell survival (3, 7, 8, 11), we investigated the effects of molecular down-regulation of CK2 on cell viability. Our studies showed that treatment of a variety of prostate carcinoma and SCC of head and neck cells in culture with antisense CK2 oligodeoxynucleotide evoked a strong apoptotic response, with antisense CK2α oligodeoxynucleotide being more potent than antisense CK2β oligodeoxynucleotide (19, 20). These results prompted us to further explore the effects of antisense CK2α oligodeoxynucleotide in xenograft models of prostate cancer. Given that CK2 is present in all cells and is essential for cell survival, a concern in targeting this kinase *in vivo* would be that significant toxicity might occur as a result of concomitant damage in local or distant normal tissue. Therefore, we also assessed the effects of antisense CK2α oligodeoxynucleotide on various noncancer cells *in vitro* and on normal murine prostate tissue *in vivo*. Here, we report that the antisense CK2α oligodeoxynucleotide treatment of the prostate cancer xenograft resulted in a substantial down-regulation of CK2α message and extensive apoptosis in the cells and destruction of the tumor. By comparison, under the same conditions, the normal (and noncancer) cells seemed to be relatively resistant to the antisense CK2α treatment. The basis of the latter difference is unclear at present; however, the results presented here represent the initial "proof of principle" that CK2 may serve as a potential target for cancer therapy, setting the stage for future extensive studies aimed at this goal.

Results

Effect of Antisense CK2α Oligodeoxynucleotide on PC3-LN4 Xenograft in Mice

In previous work, by employing the androgen-sensitive ALVA-41 and LNCaP and androgen-insensitive PC-3 prostate cancer cells, we showed that antisense CK2α and antisense CK2β oligodeoxynucleotides profoundly influenced cell viability of these cancer cells by inducing apoptosis in a dose- and time-dependent manner. Antisense CK2α was somewhat more effective than antisense CK2β in most of the cells tested (19). These studies prompted us to examine whether similar responses could be obtained by employing an *in vivo* model of prostate cancer subjected to antisense CK2α oligodeoxynucleotide treatment. Nude mice bearing PC3-LN4 tumors growing in the subcutis (3-5 mm in diameter) were treated with a single intratumor injection of either the nonsense oligodeoxynucleotide or increasing doses of antisense CK2α oligodeoxynucleotide. Tumor size was assessed over time and the final weight was measured. Results shown in Fig. 1A show the effects of intratumoral treatment with a single increasing dose of the antisense CK2α on the tumor size. Treatment with the lower dose (5 μg per mouse) resulted in a 30% to 40% reduction in tumor size by 7 days. The reduction in tumor size in the animals treated with 10 μg antisense was ~85%, whereas at a dose of 20 μg of the antisense, no tumor was detected at 7 days following the treatment. By contrast, a similar treatment with the control nonsense oligodeoxynucleotide at a dose of 20 μg resulted in continued growth of the xenograft tumor. No obvious toxic response was noted in the mice receiving 20 μg dose of antisense CK2α oligodeoxynucleotide.

To further confirm the observations in Fig. 1A, paraffin-embedded tumor sections were stained with H&E and for terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) to assess the effects of antisense CK2α on apoptosis (Fig. 1B). The results clearly showed the appearance of extensive apoptosis in the cells exposed to the antisense CK2α. In a parallel experiment, we undertook a quantitative estimation of the number of apoptotic cells at a given time point following treatment of the xenograft tumor with the nonsense or antisense CK2α oligodeoxynucleotide. The tumor tissue corresponding to 6-day time point in Fig. 1A for 10 μg/mL dose of antisense CK2α or nonsense oligodeoxynucleotide was subjected to TUNEL staining. Visual examination of the apoptotic cells in multiple fields gave 9 ± 5 and 56 ± 22 (percentage ± SD) apoptotic cells in nonsense versus antisense-treated xenograft tumor, respectively. Together, these data suggest that relatively low doses of the antisense CK2α are effective in inducing apoptosis in this model of prostate cancer.

Expression of CK2α Message in Prostate Cancer Xenograft Treated with Antisense CK2α

To establish the specificity of the action of the antisense CK2α oligodeoxynucleotide in the prostate cancer xenograft, we examined the tissue from the treated xenograft by fluorescence *in situ* hybridization (FISH) analysis. Results in Fig. 1C show that the tissue treated with antisense CK2α oligodeoxynucleotide and hybridized with a CK2α probe has a marked reduction in the expression of CK2α message compared with that treated with the nonsense oligodeoxynucleotide (A compared with B). Corresponding analysis by bisbenzamide staining indicated the presence of damaged nuclei (C). The specificity of the probes was shown by *in situ* hybridization with the nonsense oligodeoxynucleotide probe (D).

Further examination of the effect of the antisense CK2α on CK2α message expression was undertaken by performing reverse transcription-PCR (RT-PCR) analysis on the RNA extracted from the treated and control xenograft tissues. Results in Fig. 1D show that antisense treatment, which promotes extensive apoptosis and tumor ablation, is associated with a profound down-regulation of the CK2α message. These results provide the evidence that the antisense CK2α oligodeoxynucleotide treatment of the xenograft produced its apoptotic effect by specifically affecting the CK2α message expression. Corresponding changes in CK2α protein and activity in the cytoplasmic and nuclear matrix fractions were also determined. Results in Fig. 1E show that immunoblot for CK2α did not reflect a change in the cell CK2 activity or protein but that there was a significant alteration in the CK2 activity and CK2α protein associated with the nuclear matrix. These results accord with our

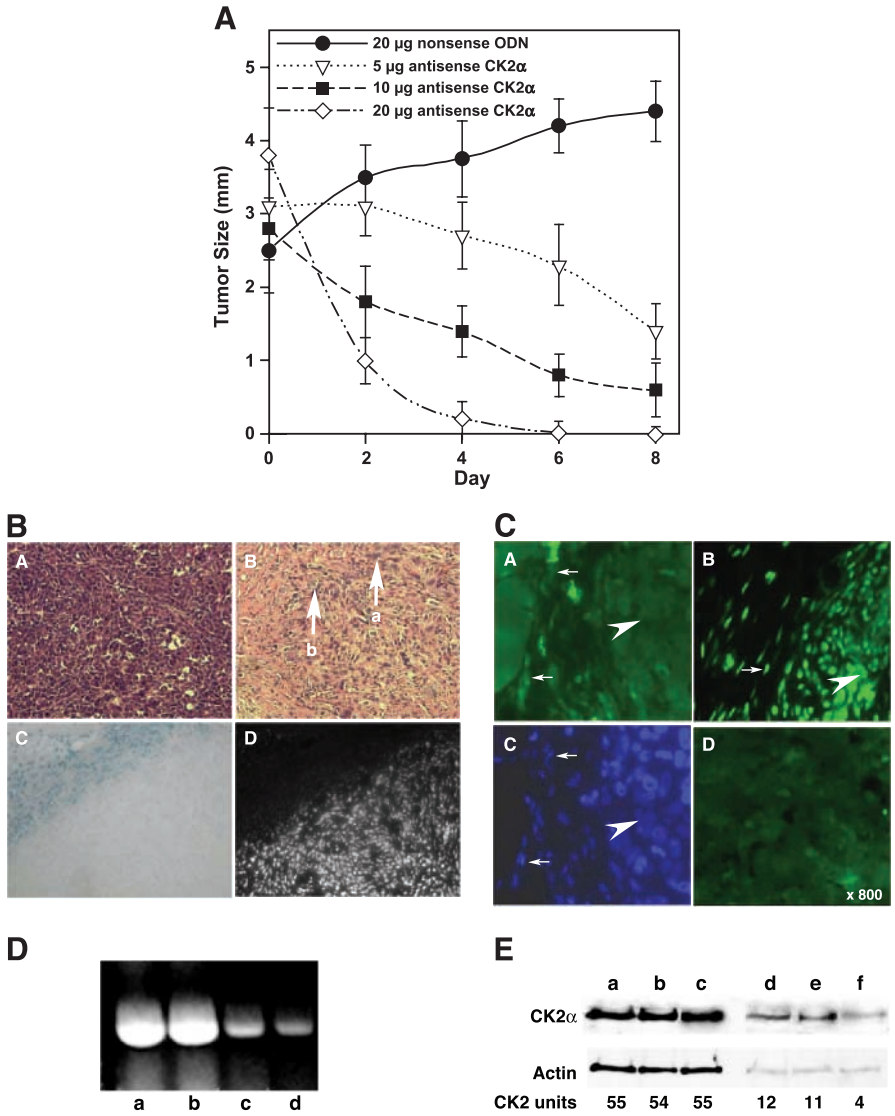

FIGURE 1. Effects of direct administration of antisense CK2α oligodeoxynucleotide in prostate cancer xenograft. **A.** Dose-dependent decrease in PC3-LN4 xenograft tumor size by a single injection of antisense CK2α. Groups of athymic nude mice (n = 5 for each group) were injected with PC3-LN4 (2×10^6 cells) into the subcutis. Once the tumors reached ~3-5 mm in diameter, therapy was begun with a single dose of naked antisense CK2α oligodeoxynucleotide as phosphorothioate (5-20 μg in 100 μL PBS per tumor) delivered in and around each tumor. Tumor size was measured every second day. Treatment with 20 μg antisense CK2α caused a significantly greater and more rapid decrease in tumor size compared with 5 μg dose (P < 0.05, log rank); the differences from the control were significant at day 4 for the 5 μg dose and at day 2 for the 10 and 20 μg dose. Treatment with a 20 μg dose of the control nonsense oligodeoxynucleotide did not affect the progression of the tumor growth. *Points,* five tumors. In a parallel experiment, tumor tissue corresponding to 6-day time point with 10 μg/mL dose of antisense CK2α or nonsense oligodeoxynucleotide was subjected to TUNEL staining for quantitative analysis of the percentage apoptosis. Visual examination of the apoptotic cells in five different fields gave the values of 9 ± 5 and 56 ± 22 (% apoptotic cells ± SD) in nonsense versus antisense-treated xenograft tumor, respectively. **B.** Evidence of induction of apoptosis by antisense CK2α in the prostate cancer xenograft model. Animals were treated with 5 μg antisense CK2α as under **A**. At the end of 7 days, tissue sections of control (*A*) and treated (*B*) mice were stained for histology (H&E stain) and for apoptosis (TUNEL stain). Note individual cancer cells (*B*, *arrow a*) interspersed throughout necrotic and apoptotic stroma (*B*, *arrow b*). *C,* hematoxylin stain of the antisense CK2α-treated tumor tissue section. *D,* same section subjected to TUNEL staining. (Please note the nontumor tissue adjacent to the tumor at the *top left* of *C* and *D*.) **C.** FISH analysis of CK2α message in PC3-LN4 xenograft following treatment with antisense CK2α oligodeoxynucleotide. The xenograft was treated with 5 μg antisense CK2α oligodeoxynucleotide for 7 days as under **A**. Tissue sections were probed for the CK2α mRNA as described in Materials and Methods. *A,* antisense CK2α oligodeoxynucleotide treated and hybridized with CK2α probe; *B,* nonsense oligodeoxynucleotide control treated and hybridized with CK2α probe; *C,* same section as *A* but stained with bisbenzamide to identify cell nuclei; *D,* antisense CK2α oligodeoxynucleotide treated and hybridized with a nonsense probe. *Large arrows,* tumor nest interiors; *small arrows,* marginal cells. **D.** RT-PCR analysis of CK2α message in PC3-LN4 xenograft following treatment with antisense CK2 oligodeoxynucleotide. The xenograft was treated with 10 μg antisense CK2α oligodeoxynucleotide as under **A**, and tissue was harvested at day 7. RNA was isolated from the fresh tissue and subjected to RT-PCR analysis as described in Materials and Methods. *Lanes a* and *b,* control nonsense oligodeoxynucleotide treated; *lanes c* and *d,* antisense oligodeoxynucleotide treated. β-actin control reference gene is also shown for the same DNA templates. **E.** Immunoblot for CK2α and the associated CK2 activity in the cytoplasm and nuclear matrix fractions of PC3-LN4 tumor xenograft tissue corresponding to the 6-day time point and 10 μg/mL antisense or nonsense oligodeoxynucleotide. *Lanes a-c,* cytosolic fraction; *lanes d-f,* nuclear matrix fraction; *lanes a* and *d,* saline-injected controls; *lanes b* and *e,* nonsense oligodeoxynucleotide injected; *lanes c* and *f,* antisense CK2α oligodeoxynucleotide injected. The corresponding CK2 activity is shown as CK2 units (nmol ^{32}P/mg protein/h); the variation in each of the activity assays was <5%. Actin immunoblot is shown to confirm that the sample loading was uniform for each of the fractions.

several previous observations showing that the early changes in CK2 activity in relation to the induction of apoptosis are predominantly in the nuclear fractions (refs. 15, 19; see also Discussion).

Comparison of the Effects of Antisense CK2α Oligodeoxynucleotide on CK2α mRNA, CK2α Protein, and Apoptosis in Nonprostate Cancer Cells

In previous work, we showed that the induction of apoptosis by antisense CK2α was qualitatively similar in a variety of cell types (19). Further, the apoptotic response became apparent at a modest down-regulation of the nuclear matrix–associated CK2. Here, we further show by employing a different cancer (SCC of head and neck) cell line (SCC-15) that the induction of apoptosis by antisense CK2α oligodeoxynucleotide is associated with down-regulation of the CK2α mRNA and nuclear-associated CK2 (Fig. 2). The antisense oligodeoxynucleotide employed in this case was as phosphodiester and was delivered via the tenascin nanocapsules (20).

Cells treated with the antisense CK2α oligodeoxynucleotide showed extensive changes such as the extensive apoptosis (Fig. 2F), which was concordant with the down-regulation of CK2α mRNA (Fig. 2C) and nuclear CK2α protein (Fig. 2I). By comparison, the control untreated cells (Fig. 2A, D, and G) and the cells treated with control sense CK2α oligodeoxynucleotide (Fig. 2B, E, and H) did not show any significant effect in either the FISH, the TUNEL assay, or the immunostaining for CK2α. These various data further indicate that induction of apoptosis in various cancer cell types mediated by antisense CK2α is most likely related to the down-regulation of CK2α message.

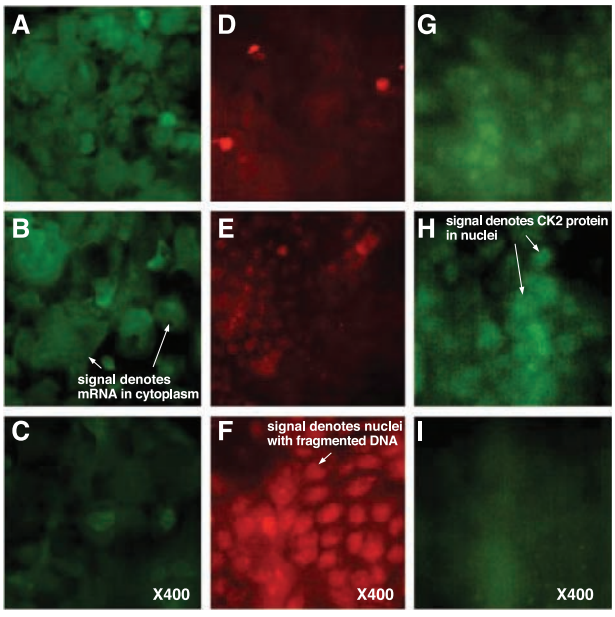

FIGURE 2. FISH analysis for CK2α mRNA, TUNEL assay, and immunostaining for CK2α following treatment of SCC-15 cells with antisense CK2α. Cells were grown in glass chamber slides to prepare them for treatment with 2 μg/mL phosphodiester CK2α oligodeoxynucleotides packaged in s-50 tenascin nanocapsules as the delivery vehicle (20). After a 12-hour incubation with the antisense CK2α oligodeoxynucleotide in tenascin nanocapsules, the cells were subjected to FISH analysis to detect the CK2α mRNA, TUNEL staining to determine the extent of apoptosis, and immunostaining for CK2α protein. **A**, **D**, and **G.** Control untreated cells. **B**, **E**, and **H.** Cells treated with control sense CK2α oligodeoxynucleotide. **C**, **F**, and **I.** Cells treated with antisense CK2α oligodeoxynucleotide. **A**, **B**, and **C.** FISH results. **D**, **E**, and **F.** TUNEL results. **G**, **H**, and **I.** Immunostaining for CK2α. In this experiment, the TUNEL analysis was carried out as described in Materials and Methods, except for the modification that monoclonal anti-BrdUrd (1:10, BD Biosciences) was detected by indirect immunofluorescence employing biotinylated donkey anti-mouse immunoglobulin G (1:200) and Cy3-conjugated streptavidin (1:800, Jackson ImmunoResearch) as secondary antibodies. Immunostaining for CK2α was as described in Materials and Methods.

Effects of Antisense CK2α Oligodeoxynucleotide on Normal Prostate Cells

Antisense CK2α is a potent inducer of apoptosis in tumor cells *in vitro* (19) and in the *in vivo* xenograft model as shown here. Because of the ubiquitous nature of the CK2 signal, we decided to examine the effect of antisense CK2α on certain noncancer human cells in culture with the goal of determining whether there were differences in the response of these cells in comparison with neoplastic cells under similar experimental conditions. Cells were transfected with antisense CK2α oligodeoxynucleotide at 2 and 4 μg/mL (i.e., at concentrations that were determined previously to be fatal for PC-3 and other related cancer cell lines) using *N*-[1-(2,3-dioleoyloxyl)propyl]-*N*,*N*,*N*-trimethylammoniummethyl sulfate as a transfection agent. After 6 hours, the medium was replaced with standard growth medium (without the antisense oligodeoxynucleotide) and cells were allowed to grow for 24 hours. Cell viability was then assessed using the WST-1 reagent as described in Materials and Methods. The results in Table 1 show that, although the expected dramatic reduction occurred in the viability of PC3-LN4 and ALVA-41 cancer cells after exposure to antisense CK2α oligodeoxynucleotide, there was only minimal or no reduction in the viability of the noncancer prostate BPH-1 and PrEC cells and other nonprostate cells (normal human dermal fibroblasts and normal human epidermal keratinocytes) treated under the same conditions.

Corresponding assessment of apoptosis induced by the presence of antisense CK2α was undertaken in different cells under similar experimental conditions. A large number of apoptotic cells were found in the PC3-LN4 lines, whereas minimal apoptosis was detected in the normal or noncancer prostate epithelial cells at equivalent doses of the antisense CK2α oligodeoxynucleotide (Fig. 3). A quantitative TUNEL analysis in multiple fields on BPH-1 and PC3-LN4 cells as the representative prostate noncancer and cancer cells treated with antisense CK2α was also undertaken. The result showed that percentage apoptotic cells (mean ± SD) in BPH-1 cells treated with antisense CK2α oligodeoxynucleotide was 6.9 ± 1.5, whereas it was 80.3 ± 5.6 in the PC3-LN4 cells treated with the antisense CK2α under the same conditions. These observations further corroborated that noncancer type of cells compared with cancer cells were relatively resistant to antisense CK2α oligodeoxynucleotide under the experimental conditions employed. Thus, these results show that antisense CK2α oligodeoxynucleotide have a differential effect on normal versus cancer cells in culture and that much higher concentrations of the antisense may be required to affect cell growth in normal or noncancer cells than the cancer cells.

Table 1. Effect of Antisense CK2α Oligodeoxynucleotide on Cell Viability of Cancer and Noncancer Cells in Culture

Antisense CK2α Oligodeoxynucleotide	Time (h)	% Cell Viability ± SE*					
		ALVA-41	PC3-LN4	BPH-1	PrEC	Normal Human Dermal Fibroblasts	Normal Human Epidermal Keratinocytes
2 μg/mL Nonsense	24	100 ± 8	100 ± 16	100 ± 3	100 ± 10	100 ± 8	100 ± 14
2 μg/mL Antisense	24	74 ± 10	80 ± 12	96 ± 5	100 ± 12	100 ± 12	100 ± 7
5 μg/mL Nonsense	24	100 ± 16	100 ± 14	100 ± 11	100 ± 8	100 ± 9	100 ± 12
5 μg/mL Antisense	24	59 ± 14	69 ± 11	94 ± 8	100 ± 14	98 ± 11	100 ± 6
2 μg/mL Nonsense	48	100 ± 14	100 ± 18	100 ± 10	100 ± 16	100 ± 9	100 ± 17
2 μg/mL Antisense	48	68 ± 6	70 ± 16	94 ± 9	100 ± 9	90 ± 9	100 ± 12
5 μg/mL Nonsense	48	100 ± 5	100 ± 16	100 ± 6	100 ± 17	100 ± 11	100 ± 15
5 μg/mL Antisense	48	42 ± 4	64 ± 11	83 ± 2	100 ± 14	90 ± 5	100 ± 16

*Cells were plated in 96-well plates and transfected with the antisense CK2α oligodeoxynucleotide or the nonsense oligodeoxynucleotide control at the concentrations indicated and for the times shown. Cell viability was determined using WST-1 reagent as described in Materials and Methods. Data are expressed as percentage ± SE cell viability and are based on three experiments.

Effect of Antisense CK2α Oligodeoxynucleotide on Normal Mouse Prostate In vivo

Considering that antisense CK2α oligodeoxynucleotide treatment of prostate cancer xenograft in the mouse *in vivo* had a potent induction of apoptosis, and in view of the results shown in Table 1 and Fig. 3, we decided to also determine the effects of *in vivo* treatment of normal murine prostate gland with the nonsense and antisense CK2α oligodeoxynucleotide. The results in Fig. 4 show the effects of intraprostatic injection of the nonsense and antisense CK2α oligodeoxynucleotide into nude mice. At 7 days, the mice were sacrificed and the prostates were examined for histologic damage and apoptosis. As also shown in Fig. 4, there was minimal evidence of necrosis or apoptosis in the normal mice treated with antisense CK2α oligodeoxynucleotide. Further, we examined the effect of the antisense CK2α on CK2α immunostaining in normal murine prostate. The tissue section corresponding to the control (A) in Fig. 4 showed the expected immunostaining for CK2α (E); however, the tissue section corresponding to the antisense CK2α treated prostate (B) did not show a significant effect on the immunostaining for CK2α (F), suggesting resistance of CK2 message to down-regulation by antisense CK2α in these cells. Taken together with the data in Table 1, these results further suggest the relative lack of sensitivity of the normal tissue to the action of the antisense CK2α (see also Discussion).

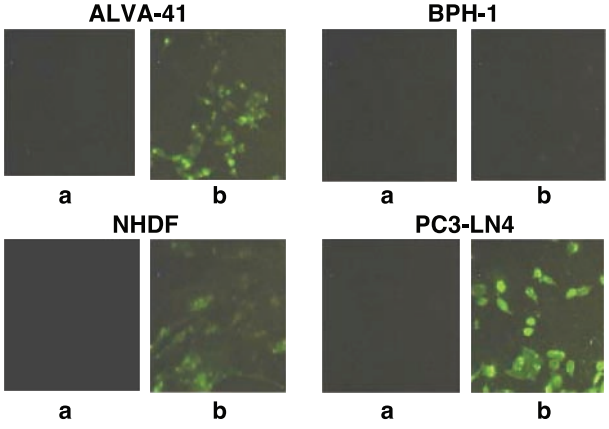

FIGURE 3. Apoptosis inducing activity of antisense CK2α oligodeoxynucleotide toward cancer versus noncancer cells in culture. Cells were grown on a chambered culture slide and treated with 3 μg/mL nonsense (*a*) or antisense CK2α (*b*) oligodeoxynucleotide (as phosphorothioate) for 24 hours. *N*-[1-(2,3-dioleoyloxyl)propyl]-*N*,*N*,*N*-trimethylammonium-methyl sulfate was used as the transfection agent at 1 μg oligodeoxynucleotide/*N*-[1-(2,3-dioleoyloxyl)propyl]-*N*,*N*,*N*-trimethylammonium-methyl sulfate (5 μL). Cells were fixed in TBS containing 4% paraformaldehyde before TUNEL staining employing *In situ* Cell Death Detection kit with fluorescein dUTP. Digital fluorescence images were captured using a Zeiss Axiovert microscope system and imported into Adobe Photoshop. Responses of prostate cancer cells PC3-LN4 and ALVA-41, benign prostate epithelial cells BPH-1, and normal human dermal fibroblasts (*NHDF*). Representative quantitative TUNEL analysis in multiple fields on BPH-1 and PC3-LN4 cells treated with antisense CK2α showed that percentage apoptotic cells in BPH-1 cells treated with antisense CK2α oligodeoxynucleotide was 6.9 ± 1.5 (mean ± SD), whereas it was 80.3 ± 5.6 (mean ± SD) in the PC3-LN4 cells treated with the antisense CK2α under the same conditions.

Discussion

Molecular approaches to treatment of cancer, including prostate cancer, continue to evolve despite challenges that include design of delivery vectors and choice of the target gene for induction or ablation (e.g., refs. 20, 21). Despite certain concerns, the use of antisense oligodeoxynucleotide to down-regulate the activity of specific genes remains a highly promising area of research. A few examples of this approach in prostate cancer cells are antisense insulin-like growth factor receptor-I (22), antisense L-plastin (23), antisense FGF8 (24), and antisense Bcl-2 (25), the latter entered the human trial for prostate cancer therapy (25). The currently known features of CK2 biology, such as its functional roles in cell growth, proliferation, and cell survival coupled with its uniformly observed dysregulation in a variety of the examined cancers, point to a reasonably strong rationale for its potential usefulness as a target for cancer therapy. To that end, our work is the first to examine the effects of antisense CK2 in an *in vivo* model of prostate cancer and on normal murine prostate. Our results show that antisense CK2α oligodeoxynucleotide can evoke a potent apoptotic response in both *in vitro* and *in vivo* models of prostate cancer as well as in other types of cancers such as the SCC of the head and neck as shown in Fig. 2 and also documented previously (19, 20).

Recent studies have shown that down-regulation of the CK2 by 4,5,6,7-tetrabromobenzotriazole, an inhibitor of CK2 activity,

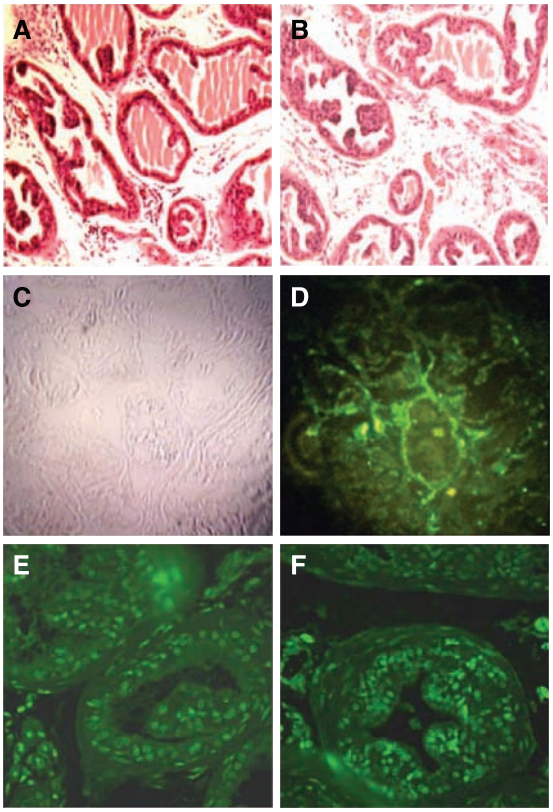

FIGURE 4. Effect of antisense CK2α oligodeoxynucleotide in normal murine prostate. Prostates of mice were injected orthotopically with either 20 μg nonsense oligodeoxynucleotide (control) or 20 μg antisense CK2α oligodeoxynucleotide phosphorothioates (treated) as described in Materials and Methods. Mice were sacrificed at 7 days. Tissue sections of control (**A**) and treated (**B**) mice were stained for histology (H&E stain). Note intact glandular architecture in H&E staining of normal prostate treated with antisense CK2α compared with nonsense CK2α. **C.** Bright-field image of the antisense CK2α-treated murine prostate gland. **D.** Same section subjected to TUNEL staining showing minimal evidence of apoptosis. **E** and **F.** Immunostaining for CK2α in sections of normal murine prostate (**E**, nonsense oligodeoxynucleotide-treated control; **F**, antisense CK2α treated as above).

induces apoptosis in Jurkat cells in culture (26). The present results accord with these observations and provide further evidence that the apoptosis induced by antisense CK2α is associated with a potent down-regulation of the CK2α message (Fig. 1C and D). Concomitant with this observation is the significant loss of nuclear (nuclear matrix)–associated CK2 protein and activity (Fig. 1C and E). Our previous data also showed that cells treated with antisense CK2α undergo extensive apoptosis when the CK2 activity in the nuclear matrix or chromatin is reduced by only ~30% to 40% in different types of cancer cells (e.g., ref. 19). Thus, it seems that the loss of CK2 from the nuclear structures may be particularly relevant to the induction of apoptosis (for reviews, see refs. 1, 2). Given the substantial down-regulation of the CK2α mRNA, the lack of significant change in cytoplasmic CK2 reflects the relatively slow rate of its turnover as has also been observed previously (27). Previous studies have shown that significant modulation can occur in CK2 signaling to the nuclear matrix without a significant change in overall level of CK2 such as in various phases of cell cycle (1, 2, 28). Clearly, an interruption of this signal would have a profound effect on cell cycle progression (28). Germane to these considerations are the various observations suggesting that CK2 signaling to nuclear structures, such as chromatin and nuclear matrix, is associated with cell proliferation and survival, whereas loss of CK2 from these structures is associated with cessation of cell growth and induction of apoptosis (1, 3, 27, 29). It must be noted that aggregate knowledge on CK2 has suggested that normally the cells resist changes in its level, and modest alterations by either up-regulation or down-regulation can have profound effects on the biological behavior of cells (2, 16-18). Thus, cancer cells in which CK2 is elevated (i.e., dysregulated) seem to be highly susceptible to an even relatively modest forced down-regulation of the CK2 protein and activity leading to an extensive induction of apoptosis. The rapid regression of *in vivo* prostate tumor xenografts after a single intratumoral dose of the antisense CK2α oligodeoxynucleotide accords with this notion.

The remarkable *in vivo* apoptotic response of the xenograft tumors to a direct single injection of the antisense CK2α shown in Fig. 1A merits further discussion. Because the phosphorothioate formulation of the antisense was employed, it may be argued that the profound effect on the tumor may be in response to elicitation of an immune response in the nude mouse (30). However, in the present case, only a single injection of the antisense was given, and such a concern would be valid if multiple doses of the antisense over a prolonged period were employed as has been the case in other studies (30). Further, in other work in progress in our laboratory aimed at in vivo targeting of CK2 in tumor cells by delivering the antisense via a nanocapsule method (as also employed in Fig. 2), the initial results show that the phosphodiester formulation of antisense CK2α was equally effective in inducing apoptosis *in vivo* (e.g., ref. 20).[5] Based on the emerging knowledge on CK2 functional biology, we propose that the remarkable sensitivity to antisense CK2α may relate to its impact on the diverse range of functional biological activities mediated by CK2 and as such the interruption of this signal would have profound consequences. Examples of potentially susceptible functions of CK2 are the dynamic association of cellular CK2 subunits with the potential of their integration into different multimolecular functional assemblies (31), a global role of CK2 in chromatin remodeling (9, 10, 28), a role in cell cycle progression (28), and suppression of apoptosis by CK2 (3, 11). It seems that the latter function of CK2 may involve its impact on diverse pathways of the apoptosis machinery.[6] Of note is the recent observation that CK2 facilitates repair of chromosomal DNA single-strand breaks (32). Taken together, it would stand to reason that down-regulation of CK2 would produce a catastrophic concatenation of events precluding cell survival.

Our observations on the eradication of the tumor by a single appropriately high dose of the antisense CK2α oligodeoxynucleotide imply that repeated treatment regimens or prolonged tumor exposure to antisense oligodeoxynucleotide may not be required to achieve a therapeutic response *in vivo*. Considering the aforementioned wide range of critical functional activities of

[5] Unpublished data.
[6] G. Wang, K.A. Ahmad, J. Slaton, G. Unger, A. Davis, and K. Ahmed, unpublished data.

protein kinase CK2, it is reasonable to propose that CK2 would make a particularly appealing target for investigation of the antisense approach to induce death in cancer cells. However, one of the chief concerns regarding targeting the multifunctional protein kinases with antisense strategies is the potential for collateral damage in normal tissue. This could be a special concern for the CK2 signal because of its ubiquitous nature and critical requirement in cell survival. In this context, particularly noteworthy is our observation that nonneoplastic or normal cells may be able to tolerate reductions in CK2 expression to a greater extent than the cancer cells. The resistance to injury in the murine prostate after injection of tumoricidal doses of antisense CK2α is concordant with our *in vitro* findings on normal cells. At present, the basis of the differences in the response of the cancer versus normal cells to antisense CK2α is unclear. With respect to the lack of effect on normal murine prostate it may be argued that the cells in this case may be in a quiescent state and thus resist effect of antisense CK2α oligodeoxynucleotide; however, this would not be the case for the noncancer cells in culture, which were treated under the same conditions as the cancer cells. Our initial studies on the transfection of BPH-1 (noncancer cells) with the antisense CK2α oligodeoxynucleotide compared with that of PC3-LN4 cells have found no difference in the uptake of the oligodeoxynucleotide in the two cases (data not shown). Accordingly, the lack of entry of the antisense oligodeoxynucleotide into the noncancer cells is not the basis of the differential sensitivity of the cancer versus noncancer cells to antisense CK2α oligodeoxynucleotide. Rather, the differential response may conceivably relate to inherent biological differences in cancer versus noncancer cells. Regardless of the mechanism, the differential sensitivity of cancer versus normal cells observed under the present experimental conditions is of interest, as it might bode well for potential sparing of surrounding normal prostate (and other tissues) if this type of therapy were to be eventually translated into the human. Although the present studies employed the administration of the antisense oligodeoxynucleotide directly into the xenograft tumor for the primary purpose of establishing that the antisense CK2α can induce apoptosis *in vivo*, it will be important to undertake these studies with the view of systemic delivery of the antisense preferably to target the tumor *in vivo*. We have initiated such studies aiming at delivering the phosphodiester antisense CK2α oligodeoxynucleotide engulfed in nanocapsules to the tumor cells *in vivo* (20).

It is well known that prostate disease progresses from androgen-sensitive cancer to the androgen-insensitive phenotype. The growth in the latter is primarily mediated by cytokines and growth factors. For an effective anticancer agent, an important consideration would be its equal effectiveness in inducing apoptosis in androgen-dependent and androgen-independent cells. The ability of antisense CK2α oligodeoxynucleotide to induce apoptosis in diverse type of cancer cells in culture, including androgen-sensitive and androgen-insensitive prostate cancer cells (19), is of particular significance in the context of prostate cancer. We have documented previously that, regardless of the nature of growth stimuli (androgens or growth factors) that act on the responsive prostate cancer cells, the dynamics of the CK2 signal in the cell are similar, suggesting that CK2 is a common downstream response to diverse growth stimuli (33). Thus, in accord with the present observations, the antisense CK2α-mediated down-regulation of CK2 would be expected to be equally effective in inducing apoptosis in androgen-dependent and androgen-independent cells *in vivo* as was documented in our *in vitro* studies (19).

While the present article was in review, another group reported on a small peptide (P15) that was capable of binding to the acidic domain of CK2α and blocking its activity. This peptide when conjugated with a cell penetrating protein derived from HIV-Tat (P15-Tat) could induce apoptosis in cells in culture and in TC-1 (transformed mouse lung) tumor model *in vivo* (34). Repeated injections of the P15-Tat over a period of 5 days into the tumor were required (34), whereas the data that we provided show the profound antineoplastic effect of the antisense CK2α by administration of a single injection *in vivo*. Together, these observations provide further support to our proposal (19, 20, 35) that CK2 may serve as a target for anticancer therapy.

In summary, to our knowledge, this is the first report to document that antisense CK2α can evoke a potent apoptotic response in prostate cancer xenografts and that normal cells *in vitro* and *in vivo* seem to be relatively resistant to this treatment. These observations are a promising first step warranting further work to determine the effectiveness of the systemically delivered antisense CK2α oligodeoxynucleotide as a prelude to its potential application in cancer therapy. We are currently investigating novel approaches to systemic delivery of the antisense CK2α for its specific targeting to the tumor cells *in vivo* (20).

Materials and Methods

Cell Lines

The highly metastatic prostate cancer cell line PC3-LN4 was selected by orthotopic implantation within the prostate of athymic nude mice. These cells were maintained by growing in a monolayer culture in modified Eagle's MEM supplemented with 10% fetal bovine serum, vitamins, sodium pyruvate, L-glutamine, nonessential amino acids, and penicillin-streptomycin as described previously (36). PC3-LN4 grows as a subcutis xenograft and was used for these experiments to facilitate the evaluation of response to therapy without the need for repeated laparotomy. The immortalized BPH-1 cells were obtained form Dr. Simon Hayward (Vanderbilt University, Nashville, TN). These cells were grown in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 2 mmol/L L-glutamine. Human cells PrEC, normal human dermal fibroblasts, and normal human epidermal keratinocytes were obtained along with their specific medium from Cambrex (Walkersville, MD). ALVA-41 cells were obtained from Dr. Richard C. Ostenson (University of Washington, Seattle, WA). They were grown in RPMI 1640 supplemented with 6% fetal bovine serum and 2 mmol/L L-glutamine. Human head and neck carcinoma SCC-15 cells were purchased from American Type Culture Collection (Manassas, VA) and were cultured in Ham's F-12 medium supplemented with 1% amino acids, 2.5 mmol/L L-glutamine, and 0.4 μg/mL hydrocortisone.

Oligodeoxynucleotides

The sequences of the CK2α antisense oligodeoxynucleotide were based on the cDNA sequences of the α subunit of CK2 (19) and were evaluated for sequence specificity using Blast

search. The sequence for the antisense CK2α oligodeoxynucleotide was 5′-CCTGCTTGGCACGGGTCCCGACAT-3′. The corresponding nonsense sequence used as a control was 5′-CTCGAGGTCGACGGTATCGATCCG-3′. The nonsense sequence was a noncoding sequence synthesized to maintain the overall A + T/G + C composition of the antisense CK2α oligodeoxynucleotide. Phosphodiester and phosphorothioate oligodeoxynucleotide were obtained from MWG Biotech (High Point, NC) and were employed as indicated in the text.

Cell Viability and Proliferation Assay

The cell proliferation reagent WST-1 (Roche, Indianapolis, IN), a tetrazolium salt that is cleaved by mitochondrial dehydrogenases in viable cells, was employed to determine cell viability and proliferation in cells treated with various antisense oligodeoxynucleotide. An aliquot of cell suspension (200 μL containing 2-5 × 10^3 cells) was placed in each well of a 96-well plate, and cells were allowed to reattach over a period of 24 hours. Various oligodeoxynucleotides were added to the culture medium to achieve the desired concentration using the *N*-[1-(2,3-dioleoyloxyl)propyl]-*N*,*N*,*N*-trimethylammoniummethyl sulfate (Roche) transfection method. Following the treatment of cells with various oligodeoxynucleotides for the indicated periods of time, medium in each well was replaced with 100 μL fresh medium containing 100 μL WST-1/mL, and incubation was carried out at 37°C for an additional 60 minutes. An automated plate reader was employed to measure $A_{450\ nm}$.

Animals

Male athymic BALB/c nude mice (obtained from the Frederick Cancer Research Facility, National Cancer Institute, Frederick, MD) were maintained in a laminar airflow cabinet under pathogen-free conditions and used at 8 to 12 weeks of age. All facilities were approved by the American Association for Accreditation of Laboratory Animal Care in accordance with the current regulations and standards of the U.S. Department of Agriculture, U.S. Department of Health and Human Services, and NIH.

In Vivo *Therapy of Xenograft Human Prostate Cancer in Athymic Nude Mice*

Cultured PC3-LN4 cells (60-70% confluent) were prepared for injection as described previously (36). Mice were anesthetized with phenobarbital. Viable tumor cells (2 × 10^6 per 0.2 mL) in PBS were implanted subcutaneously into the flank. Formation of a bulla indicated a satisfactory injection. Therapy began when the tumors reached a diameter of 3 to 5 mm (designated day 0). Groups of mice (n = 5) were subjected to intratumor treatment with a single dose of antisense CK2α (varying from 5 to 20 μg in 100 μL PBS per mouse) or a single dose of nonsense oligodeoxynucleotide as a control. Tumor size and volume were assessed every 2 days. Mice were subjected to necropsy at 7 to 10 days after initiation of therapy. The tumors were removed and weighed and the presence of tumor was determined grossly and microscopically. The tumors were quickly frozen in liquid nitrogen for mRNA extraction, fixed in 10% buffered formalin, or placed in OCT compound (Miles Laboratories, Elkhart, IN).

In Vivo *Instillation of Antisense CK2α into Normal Prostate of Athymic Nude Mice*

Athymic nude mice were anesthetized with phenobarbital. A midline abdominal incision was made and the dorsal surface of the prostate was exposed for injection. Antisense CK2α and nonsense oligodeoxynucleotide (100 μL of 200 μg/mL in PBS) were injected into the prostate (n = 3 each). Necropsy was done on mice at 7 days after initiation of therapy. The prostates were removed and weighed, and injury was determined grossly and microscopically. The prostates were then quickly frozen in liquid nitrogen for mRNA extraction, fixed in 10% buffered formalin, or placed in OCT compound.

TUNEL Analysis for Detection of Apoptosis

Apoptosis in cells following treatments with various oligodeoxynucleotide was determined by using the Cell Death Detection kit (TUNEL staining, Roche). The sections were initially counterstained by dipping the slides in Mayer's hematoxylin solution for 30 seconds. They were then rinsed twice with TBS [consisting of 10 mmol/L Tris-HCl, 0.9% NaCl (pH 7.4)], dipped in 0.1% NH_4OH for 2 seconds, and finally washed several times with TBS. TUNEL staining was then done according to the instructions supplied by the manufacturer. The stained cells were mounted with Universal Mount obtained from Invitrogen. Digital images of the cells were obtained using a Zeiss Axiovert 25 CFL microscope equipped with a color digital CCD camera. The images were prepared for presentation using Adobe Photoshop 7.0 software.

FISH Analysis

FISH analysis on residual tumor sections was carried out using as probes the same antisense CK2α oligodeoxynucleotide and random oligodeoxynucleotide as employed in various experiments, except that the oligodeoxynucleotides used as probes were fluorescein-labeled (IDT, Coralville, IA). Paraffin-embedded tumor tissue sections (4 μm) mounted on silanized slides were deparaffinized, rehydrated through graded alcohol solutions, and washed in PBS. These tissue samples were subjected to Pronase digestion [2.5 mg Pronase/mL containing 5 mmol/L EDTA, 50 mmol/L Tris-HCl (pH 7.6)] for 4 minutes, following which they were fixed in 1% paraformaldehyde in PBS for 5 minutes. The sections were washed twice in PBS and acetylated for 10 minutes at room temperature, following which they were dehydrated sequentially in 35%, 70%, and 100% ethanol. They were then delipidated in chloroform and were air dried. The dried sections were preheated for 1 hour at 42°C in 1:1 formamide/2× SSC before applying the probes (50 μg/mL) in hybridization buffer (Sigma H-7140, St. Louis, MO), formamide, 50% dextran sulfate (50-kDa size, 2:2:1) and kept in a humidified chamber at 42°C for 18 hours. Following hybridization, sections were washed in prehybridization buffer followed by 2× and 1× SSC at 54°C over 2 hours and then 1× SSC at 37°C. Slides were blocked for probe detection using an avidin-biotin blocking kit (Vector Laboratories, Burlingame, CA) in combination with 50 μg/mL goat anti-mouse $F(ab')_2$ fragments (Jackson ImmunoResearch, West Grove, PA) and then rinsed briefly in PBS-0.05% NP40 (PBS-NP). Probes were detected by incubation at room temperature for 30 minutes with

5 μg/mL biotinylated mouse anti-FITC (Jackson ImmunoResearch) diluted in fish serum (Seablock from Pierce, Rockford, IL) and PBS-NP (1:1). After rinsing in PBS-NP, sections were incubated with 40 μmol/L Qdot 525 streptavidin conjugate (Quantum Dot Corp., Hayward, CA) in 100 mmol/L borate and fish serum (1:1) for 30 minutes at room temperature. Counterstaining was done with 10 μg/mL bisbenzamide followed by rinses in PBS-NP and mounting in 90% glycerol in PBS. Sections were viewed with a fluorescence microscope (Zeiss Axioplan 2). Images were collected using a digital color CCD camera (SPOT Diagnostic Instruments, Ann Arbor, MI). The procedure for analysis of cultured cell monolayers was essentially similar to that for the tissue sections. The digestion with Pronase was for only 2 minutes followed by fixation in a medium consisting of 4% paraformaldehyde, 5 mmol/L $MgCl_2$, 10% acetic acid in PBS for 20 minutes. In this case, the probe was detected using the antibody series of goat anti-FITC (1:25, Molecular Probes, Eugene, OR), biotinylated donkey anti-goat (1:200), streptavidin-FITC (1:800, Jackson ImmunoResearch), and Alexa 488 goat anti-FITC (1:1,000, Molecular Probes). Buffers and diluents were similar to those used for tissue samples described above.

Assay of CK2 Activity and Immunodetection of CK2α

Measurement of CK2 activity in the cytosolic and nuclear matrix fractions was carried out on these fractions isolated from the tissue by employing method "C" as described previously (37). Immunodetection of the CK2α protein was carried out on paraffin sections with two different CK2α primary antibodies, a rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and a mouse immunoglobulin M monoclonal antibody (12), using standard immunofluorescence microscopy employing a Qdot streptavidin-biotin conjugate method. Briefly, slides were deparaffinized and rehydrated through a series of ethanol solutions before antigen retrieval. The latter was carried out by incubating the sections for 9 minutes in a medium consisting of 0.1% trypsin, 0.1% $CaCl_2$, and 20 mmol/L Tris-HCl (pH 7.6) at 37°C. This process was stopped by chilling the sections on ice followed by washing twice in PBS and incubation for 30 minutes at 37°C in 10 mmol/L citrate buffer (pH 3). Sections were then blocked with fish serum using an avidin-biotin blocking kit in combination with 70 μg/mL goat anti-mouse $F(ab')_2$ fragments, rinsed briefly in PBS-NP, and incubated overnight at 4°C with primary antibodies (1:400) or buffer diluted in fish serum/PBS-NP (1:1). An omission control was included on every slide. Sections were then rinsed and sequentially incubated with biotinylated donkey $F(ab')_2$ fragments of either anti-rabbit immunoglobulin G or anti-mouse immunoglobulin M (1:200; Jackson ImmunoResearch) followed by 40 μmol/L Qdot 525 streptavidin conjugate in 100 mmol/L borate/fish serum (1:1). Sections were then counterstained in 10 μg/mL bisbenzamide, washed, and mounted in 90% glycerol in PBS. For detection of CK2α in the paraformaldehyde-fixed cell monolayers, the Santa Cruz Biotechnology antibody in concert with 5 μg/mL FITC-conjugated donkey anti-rabbit and 1 μg/mL Alexa 488–conjugated goat anti-FITC antibodies was employed. Sections were viewed with a Zeiss Axioplan 2 fluorescence microscope equipped with a digital color CCD camera. The images were prepared for presentation using Adobe Photoshop.

For detection of CK2α in the Western blots of cytosolic and nuclear matrix fractions isolated from PC3-LN4 xenograft tumor tissue, mouse anti-CK2α antibody (BD Biosciences, San Diego, CA) was employed in conjunction with a goat anti-mouse immunoglobulin G/horseradish peroxidase–conjugated antibody (Pierce). After stripping the blot, actin was detected using mouse anti-actin antibody followed by goat anti-mouse immunoglobulin M/horseradish peroxidase–conjugated secondary antibody (Pierce). For both assays, SuperSignal WestPico chemiluminescence reagent (Pierce) was used with Kodak Biomax ML film to detect peroxidase activity.

RT-PCR Analysis

Total RNA was prepared from fresh tumor tissue. RT-PCR was carried out using RETROscript kit (Ambion, Austin, TX) for first-strand DNA synthesis, and SuperTaq Plus for the PCR. The forward and reverse primers used were 5′-TCCTGGCCAAGAATAATAATGTCCG-3′ and 5′-TTACTGC-TAGAGCGCCAGCGGCAGC-3′, respectively. The template DNAs used for the CK2α RT-PCR experiment were also used to test for the level of β-actin message. The PCR forward and reverse primers specific for β-actin were 5′-ACAAAACC-TAACTTGCGCAG-3′ and 5′-TCCTGTAACAACG-CATCTCA-3′, respectively.

Statistics

The difference in the tumor sizes was assessed by Mann-Whitney U test, with $P < 0.05$ being the cutoff for significance.

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