Casein kinase 2 (CK2) increases survivin expression via enhanced β-catenin–T cell factor/lymphoid enhancer binding factor-dependent transcription

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Increased expression of casein kinase 2 (CK2) is associated with hyperproliferation and suppression of apoptosis in cancer. Mutations in the tumor suppressor APC (adenomatous polyposis coli) are frequent in colon cancer and often augment β-catenin–T cell factor (Tcf)/lymphoid enhancer binding factor (Lef)-dependent transcription of genes such as *c-myc* and *cyclin-D1*. CK2 has also been implicated recently in the regulation of β-catenin stability. To identify mechanisms by which CK2 promotes survival, effects of the specific CK2 inhibitors 4,5,6,7-tetrabromobenzotriazole (TBB) and 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole were assessed. TBB and 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole significantly decreased proliferation and increased apoptosis of HT29(US) colon cancer cells. RT-PCR and immunoblot analysis revealed that both inhibitors decreased survivin mRNA and protein levels in HT29(US) cells. Similar effects were observed with TBB in human DLD-1 and SW-480 colorectal cells as well as ZR-75 breast cancer cells and HEK-293T embryonic kidney cells. Expression of GFP–CK2α in HEK-293T cells resulted in β-catenin–Tcf/Lef-dependent up-regulation of survivin and increased resistance to anticancer drugs. Augmented β-catenin–Tcf/Lef-dependent transcription and resistance to apoptosis observed upon GFP–CK2α expression were abolished by TBB. Alternatively, HEK-293T cells expressing GFP–survivin were resistant to TBB-induced apoptosis. Finally, siRNA-mediated down-regulation of CK2α in HEK-293T cells coincided with reduced β-catenin and survivin levels. Taken together, these results suggest that CK2 kinase activity promotes survival by increasing survivin expression via β-catenin–Tcf/Lef-mediated transcription. Hence, selective CK2 inhibition or down-regulation in tumors may provide an attractive opportunity for the development of novel cancer therapies.

apoptosis | cancer | cell cycle

Protein kinase casein kinase 2 (CK2) is a ubiquitous eukaryotic kinase composed of catalytic (α or α′) and regulatory (β) subunits that form the holoenzyme tetramer combinations $\alpha_2\beta_2$, $\alpha'_2\beta_2$, or $\alpha\alpha'\beta_2$ (for reviews see refs. 1 and 2). CK2 phosphorylates >300 proteins that participate in many cellular processes including replication, transcription, translation, signal transduction, and cell death (3–5). When overexpressed CK2 behaves as an oncogene and induces neoplastic growth (6–8). Furthermore, increased CK2 activity and protein levels are frequently observed in tumor cells and are associated with hyperproliferation and suppression of apoptosis. Molecular mechanisms accounting for the ability of CK2 to promote proliferation include phosphorylation of cell-cycle proteins such as p53, MDM-2, $p34^{cdc2}$, $p21^{WAF1}$, and $p27^{KIP1}$, among others (3, 5, 9–11). Evidence linking CK2 activity to inhibition of apoptosis includes experiments showing that the CK2 inhibitor 4,5,6,7-tetrabromobenzotriazole (TBB) decreases cell viability and induces apoptosis in human leukemia Jurkat T cells (12). The antiapoptotic role of CK2 has been attributed to phosphorylation of specific proteins, like HS1, Max, Bid, and connexin-45.6, at sites that inhibit caspase-mediated cleavage (13–15). Furthermore, degradation of IκB by calpain, inhibition of the proapoptotic transcription factor CHOP, and activation of the caspase-inhibiting protein ARC, as well as the cochaperone for antiapoptotic kinases CDC-37, have been attributed to phosphorylation by CK2 (16–19).

CK2 is also implicated in Wnt signaling, where it may act as a positive regulator by phosphorylating β-catenin and thereby precluding degradation mediated by the proteasome (20). The Wnt signaling pathway plays a central role in the etiology of inherited and sporadic colorectal cancer. A large number of studies associate deletions in the C-terminal region of the adenomatous polyposis coli protein, APC, with the state of the disease. Resulting increased β-catenin levels in the nucleus promote, together with T cell factor (Tcf)/lymphoid enhancer binding factor (Lef), enhanced transcription of genes such as *c-myc* and *cyclin-D1* (21, 22). Interestingly, the same region of APC deleted in colorectal cancers was proposed to contain a basic domain that suppresses CK2 activity (23). However, whether CK2 functions alone or in a complex with other Wnt signaling proteins is still controversial.

On the other hand, APC has been shown to control survivin expression via β-catenin–Tcf4 signaling in colon cancer cells (24). Survivin is a member of the inhibitor of apoptosis protein (IAP) family (25, 26) that modulates cell death and controls cell cycle progression, particularly in G_2/M (27–29). This protein is generally expressed during embryonic development but becomes undetectable in most healthy adult tissues. Interestingly, augmented survivin expression is observed in many human cancers and during angiogenesis (30–33). Its presence in tumors is generally associated with unfavorable patient prognosis because of reduced apoptosis through a variety of mechanisms including inhibition of caspases, sequestration of Smac/Diablo, or stabilization of XIAP (34–36).

In the present report we provide evidence linking the antiapoptotic role of CK2 to enhanced transcription of the β-catenin–Tcf/Lef target gene *survivin*. We show that CK2 inhibition reduced survivin levels in human cancer as well as embryonic kidney cells. Moreover, loss of survivin was attributed to reduced β-catenin–Tcf/Lef-dependent transcription and correlated with decreased viability and augmented apoptosis, as well as a reduced number of cells in the G_2/M phase. These results identify a mechanism to explain the ability of CK2 to promote survival and preclude apoptosis.

Results

Inhibition of CK2 by TBB in Human Cancer Cells. Initially, the effect of TBB (37) on the viability of several human cancer cell lines was assessed. At 100 μM, a concentration previously used to

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Abbreviations: CK2, casein kinase 2; Tcf, T cell factor; Lef, lymphoid enhancer binding factor; TBB, 4,5,6,7-tetrabromobenzotriazole; DMAT, 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole.

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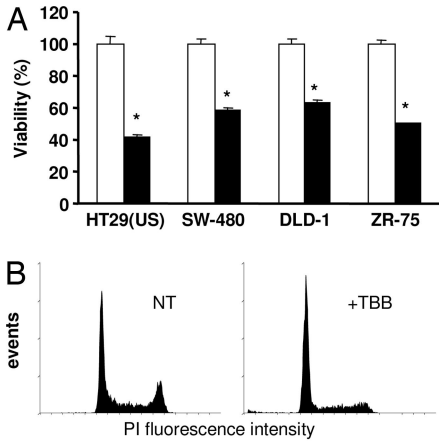

TBB (μM)	Sub-G_0/G_1 (%)	G_0/G_1 (%)	S (%)	G_2/M (%)
0	0.1±0.1	44.2±1.8	40.4±2.4	15.6±0.9
40	1.8±0.5	44.4±2.1	41.2±2.3	13.1±0.6
80	*4.7±1.4	*58.3±2.1	*30.7±1.5	*9.5±0.8

Fig. 1. TBB reduces viability, augments cell death, and alters the cell cycle in human cancer cell lines. (*A*) Viability was determined by the MTS assay in cells either untreated (white bars) or treated with 100 μM TBB for 24 h (black bars). Reductions in viability were significant in all cases (*, $P \leq 0.05$). (*B*) Cell cycle distribution of HT29(US) cells in the absence (NT) or presence of 40 or 80 μM TBB was determined by flow cytometry. TBB indicates the presence of this inhibitor (80 μM) during the 24-h treatment period. Results averaged from three independent experiments are shown (mean ± SE). Significant changes with respect to controls are indicated (*, $P \leq 0.05$).

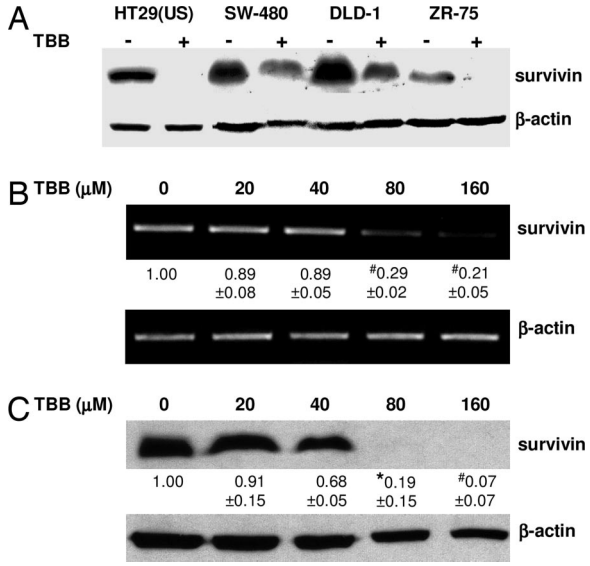

Fig. 2. TBB reduces survivin mRNA and protein levels in human cancer cell lines. (*A*) Western blot analysis of survivin and β-actin levels in lysates (25 μg) from human colon [HT29(US), DLD-1, and SW-480] cancer cells and breast cancer cells (ZR-75) grown for 24 h in the absence (−) or presence (+) of 100 μM TBB. One experiment representative of three with similar outcomes is shown. (*B*) RT-PCR analysis of survivin and actin mRNA levels in HT29(US) cells treated for 24 h with increasing concentrations of TBB. (*C*) Western blot showing survivin and β-actin protein levels in HT29(US) cells treated as in *B*. Values shown for survivin mRNA and protein levels in the respective lanes were averaged from three independent experiments after standardization to actin (mean ± SE). Significant changes with respect to controls are indicated (*, $P \leq 0.05$; #, $P \leq 0.01$).

inhibit CK2 in cells (17, 38), TBB significantly decreased viability of three human colon cancer cell lines [HT29(US), SW-480, and DLD-1] and one breast cancer cell line (ZR-75) (Fig. 1*A*). Also, TBB increased sensitivity of these cells to chemotherapeutic drugs like doxorubicin, etoposide, and taxol (data not shown). In HT29(US) cells, TBB induced apoptosis in a dose-dependent manner, whereby the effects became significant at TBB concentrations of 80 μM or higher (Fig. 6*A*, which is published as supporting information on the PNAS web site). The extent of apoptosis observed with 80–160 μM TBB was comparable to that induced by Fas ligand (100 ng/ml). Notably, treatment of HT29(US) cells with 80 μM TBB diminished substantially CK2 activity in lysates from these cells (Fig. 7*A*, which is published as supporting information on the PNAS web site). Interestingly, cell cycle analysis revealed that TBB decreased by ≈40% the number of cells in G_2/M and increased the sub-G_0/G_1 cell population (Fig. 1*B*). Thus, TBB, used at concentrations that significantly inhibit CK2 *in situ*, reduced cancer cell viability, augmented apoptosis, and reduced the number of cells in G_2/M.

TBB Reduces Survivin Levels in Cancer Cells. These results suggested that CK2 may regulate expression of an inhibitor of apoptosis protein (IAP) like survivin. Indeed, decreased viability observed for the different colorectal and breast cancer cell lines (Fig. 1*A*) was paralleled by reduced expression of survivin protein (Fig. 2*A*). In HT29(US) cells highly significant reductions in survivin mRNA levels were apparent by using TBB at or beyond 80 μM (Fig. 2*B*), and the reduction in survivin mRNA was paralleled by similar decreases in survivin protein levels (Fig. 2*C*). Taken together, these results linked TBB-induced losses in cell viability and changes in the cell cycle to down-regulation of survivin by a transcriptional mechanism.

TBB Blocks the β-Catenin–Tcf/Lef Pathway. Survivin is strongly up-regulated in cancer and is also expressed in early development. Thus, we investigated whether TBB promoted down-regulation of survivin mRNA and protein levels in HEK-293T human embryonic kidney cells. A dose-dependent reduction in both survivin mRNA and protein levels was readily detected in response to TBB in these cells (Fig. 3 *A* and *B*). Considering that (*i*) activity of the β-catenin–Tcf/Lef pathway is frequently altered in several human cancers (39), (*ii*) CK2 may promote signaling through this pathway by enhancing β-catenin stability (20), and (*iii*) *survivin* has been described as a β-catenin–Tcf/Lef target gene (40), we then investigated whether CK2 promoted signaling through this pathway and thereby augmented survivin levels. β-Catenin–Tcf/Lef reporter activity after transfection with a construct encoding HA-tagged wild-type CK2α was assessed in HEK-293T cells. As anticipated, reporter activity increased in a dose-dependent fashion with increasing amounts of DNA encoding HA–CK2α. Moreover, CK2α-induced transcriptional activity was blocked by the presence of 100 μM TBB (Fig. 3*C*). These results suggested that CK2 increases survivin expression via enhanced β-catenin–Tcf/Lef-dependent transcription.

CK2α and Survivin Promote Viability of HEK-293T Cells. Next, GFP–CK2α and GFP–survivin fusion proteins were expressed in HEK-293T cells (Fig. 8*A*, which is published as supporting information on the PNAS web site), and their effect on viability was compared with that of GFP alone. Overexpression of either GFP–CK2α or GFP–survivin promoted viability in the absence of drugs; however, the protective effect of these proteins was most apparent in the presence of several cytotoxic drugs (Fig. 8*B*). Importantly, protection against these drugs offered by CK2α coincided with augmented survivin expression (data not

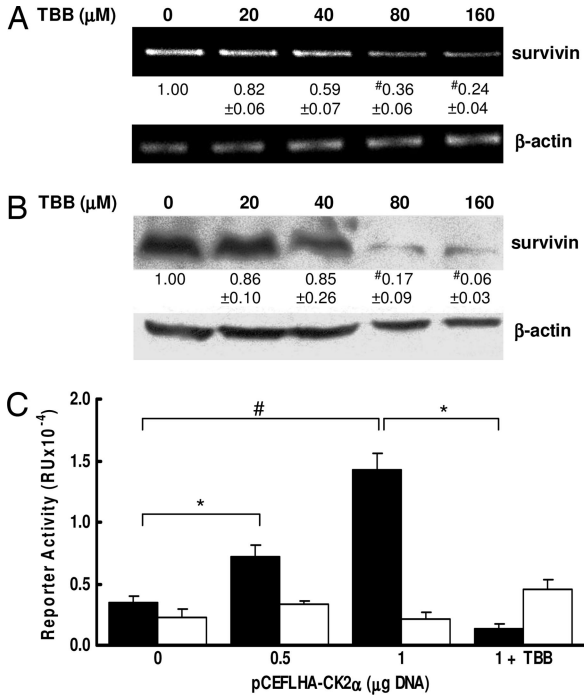

Fig. 3. TBB reduces survivin levels and blocks CK2α-induced β-catenin–Tcf/Lef transcriptional activity in HEK-293T cells. (*A*) RT-PCR analysis of survivin and actin mRNAs in HEK-293T cells treated for 24 h with increasing concentrations of TBB. (*B*) Western blot showing survivin and β-actin protein levels in HEK-293T cells treated as mentioned in *A*. Values shown for survivin mRNA and protein levels were standardized to actin and averaged from three independent experiments (mean ± SE). (*C*) β-Catenin–Tcf/Lef-dependent luciferase reporter activity expressed in relative units (RU) in HEK-293T cells cotransfected with increasing amounts of cDNA encoding HA–CK2α (pCEFL-HA–CK2α) and wild-type Tcf/Lef (TOP-FLASH, black bars) or mutated Tcf/Lef (FOP-FLASH, white bars) luciferase reporter plasmids. TBB indicates the presence of 100 μM TBB for 24 h after transfection. A β-galactosidase-encoding plasmid was cotransfected in all experiments to standardize results. Values in *A–C* were averaged from three independent experiments, each in triplicate (mean ± SE) (*, $P \leq 0.05$; #, $P \leq 0.01$).

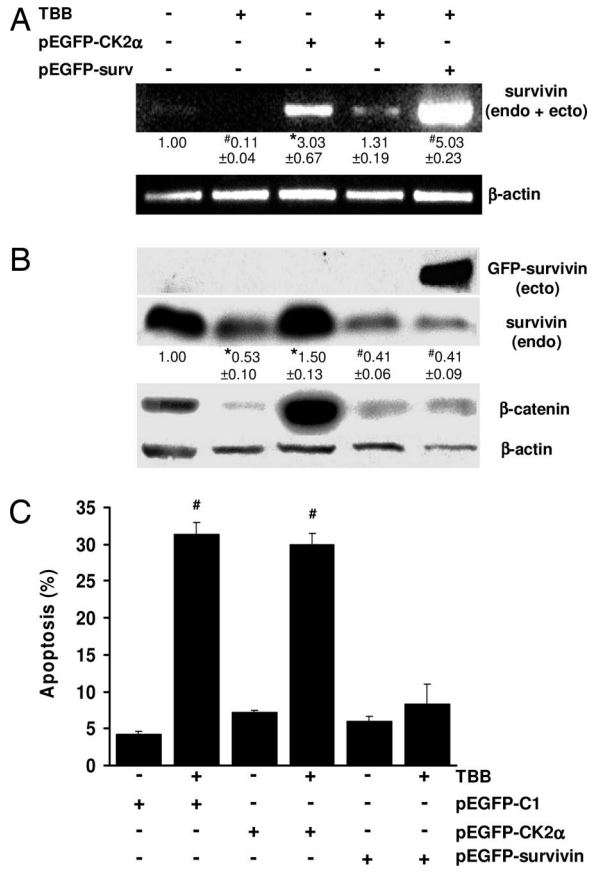

Fig. 4. Ectopic GFP–survivin, but not GFP–CK2α, precludes TBB-induced apoptosis in HEK-293T cells. HEK-293T cells transfected with 2 μg of the plasmids pEGFP–C1, pEGFP–CK2α, or pEGFP–survivin were subsequently treated with either 100 μM TBB (+) or DMSO control (−) for 24 h. Survivin and actin mRNA (*A*) and protein (*B*) levels were determined by RT-PCR or Western blotting, respectively. For survivin mRNA, endogenous and pEGFP–survivin-derived (endo + ecto) levels combined are shown. At the protein level, GFP–survivin (ecto) and endogenous survivin (endo) are shown separately. Quantitative data for survivin standardized to actin averaged from three independent experiments are shown in the respective lanes (mean ± SE) (*, $P \leq 0.05$; #, $P \leq 0.01$). (*C*) Apoptosis was analyzed by FACS in HEK-293T cells for the conditions indicated. Results averaged from three experiments are shown (mean ± SE). Values obtained in mock-transfected cells were compared with those of the other experimental conditions by using ANOVA (*, $P \leq 0.05$). Note that increments in TBB-induced apoptosis were significant only in the two cases indicated.

shown) as illustrated subsequently in experiments using TBB (see Fig. 4). Taken together, these results establish a putative antiapoptotic role for CK2 by positively regulating β-catenin–Tcf/Lef-dependent expression of survivin.

Survivin Precludes TBB-Triggered Apoptosis in HEK-293T Cells. Experiments were designed to link directly augmented CK2 activity to increased expression of survivin and resistance to apoptosis. As expected, in HEK-293T cells TBB reduced survivin mRNA (Fig. 4*A*) and protein (Fig. 4*B*) levels whereas expression of GFP–CK2α augmented both. Also, increased β-catenin protein levels were observed in the presence of GFP–CK2α (Fig. 4*B*). TBB blocked CK2α-induced increases in survivin mRNA and protein levels; however, expression of GFP–survivin in the presence of TBB resulted in a significant increase in total survivin mRNA (Fig. 4*A*, endo + ecto) and GFP–survivin protein (Fig. 4*B*), whereas endogenous survivin protein was low (Fig. 4*B*). Importantly, ectopic GFP–survivin but not GFP–CK2α protected cells from TBB-induced apoptosis (Fig. 4*C*).

2-Dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT) and CK2α siRNAs Elicit Similar Responses in HT29(US) and HEK-293T Cells. Results described so far relied heavily on the use of the inhibitor TBB. Thus, alternative approaches were included to strengthen our conclusions. In preliminary studies, the effects of a number of other CK2 inhibitors including apigenin, 5,6-dichlorobenzimidazol riboside, and emodin (0–40 μM), generally considered less specific than TBB, yielded results similar to those shown for TBB in HT29(US) cells (data not shown). In addition, DMAT, a more recently developed CK2 inhibitor (41), was evaluated. As expected, treatment of HT29(US) cells with DMAT reduced β-catenin and survivin levels in a concentration-dependent manner (Fig. 5*A*). Furthermore, this inhibitor reduced CK2 kinase activity *in situ* in HT29(US) cells to 10% of the control levels and induced changes in the cell cycle similar to those observed with TBB (Fig. 7*B*) whereas apoptosis was more pronounced (Fig. 6*B*). In HEK-293T cells, increments in β-catenin–Tcf/Lef reporter activity observed upon expression of HA–CK2α were efficiently blocked by DMAT (Fig. 5*B*). Additionally, expression in HEK-293T cells of an inactive CK2α variant (KI, CK2α^{D156A}) did not augment reporter activity (Fig. 5*B*). Finally, the siRNA approach was used to specifically reduce endogenous

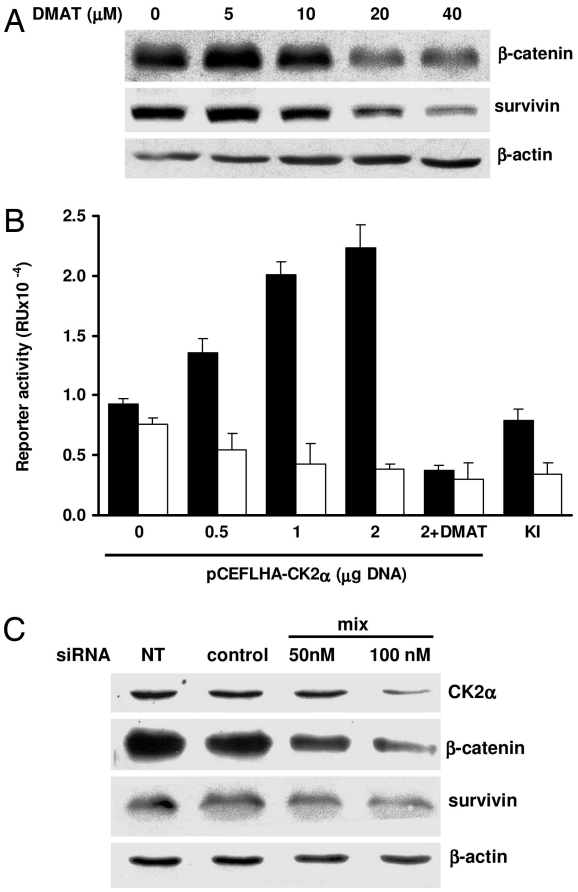

Fig. 5. DMAT or CK2α silencing using siRNAs has effects similar to those observed with TBB in HT29(US) and HEK-293T cells. (*A*) Western blot showing survivin and β-catenin protein levels in HT29(US) cells treated with increasing concentrations of DMAT. An experiment representative of two is shown. (*B*) Luciferase reporter assay of β-catenin–Tcf/Lef activity in HEK-293T cells co-transfected with increasing amounts of cDNA encoding HA–CK2α (pCEFLHA–CK2α) and the TOP-FLASH (black bars) or FOP-FLASH (white bars) vectors. DMAT indicates the presence of this inhibitor (40 μM) during the 24-h period after transfection. As an additional control, transfection with a plasmid (2 μg) encoding a kinase-inactive mutant HA–CK2α^{D156A} (KI) was used under the same conditions. Data shown were averaged from two experiments in triplicate (mean $\pm$ SE). (*C*) Western blot showing CK2α, β-catenin, and survivin protein levels in HEK-293T cells transfected with either control siRNA (100 nM) or an equimolar mixture (mix) of two CK2α-specific siRNAs (α-siRNA and α10-siRNA) at the indicated final concentrations. An experiment representative of two is shown.

CK2α expression in HEK-293T cells. A combination of two CK2α-specific siRNAs that have been described in the literature (see *Materials and Methods*) substantially reduced CK2α and, concomitantly, β-catenin and survivin protein levels (Fig. 5*C*).

Discussion

CK2 is a pleiotropic kinase with a large number of protein substrates, including β-catenin. CK2-mediated phosphorylation of β-catenin at residues Ser-29, Thr-102, and Thr-112 was initially postulated to play a negative role in controlling β-catenin stability (42). To the contrary, others showed that CK2-mediated β-catenin phosphorylation at Thr-393 stabilized β-catenin and enhanced downstream transcriptional activity (20, 43). Our results agree with the latter reports (Figs. 3 and 5) in that CK2 activity favors β-catenin–Tcf/Lef-dependent transcription. Furthermore, our findings suggest that CK2-mediated changes in the cell cycle and resistance to apoptosis involve transcriptional up-regulation of the IAP survivin via a β-catenin–Tcf/Lef-dependent mechanism.

Clearly, CK2α expression augmented β-catenin–Tcf/Lef reporter activity (Figs. 3*C* and 5*B*), yet the potential significance of these changes may appear difficult to appreciate. In this context, we have previously shown that treatment of HEK-293T cells with 20 mM LiCl, which is used to block GSK3β activity, increases reporter activity $\approx$3-fold. Also, the changes in β-catenin and survivin protein levels elicited by lithium are comparable to those seen here (44). Alternatively, stimulation of HEK-293T cells with supernatants from cells expressing Wnt3a increases survivin levels to an extent similar to that reported here with CK2α (44). Thus, changes in β-catenin and survivin levels, as well as β-catenin–Tcf/Lef reporter activity due to ectopic expression of CK2α in HEK-293T cells, are comparable to what is observed by blocking GSK3β-mediated β-catenin degradation either pharmacologically (lithium) or by using a physiological ligand (Wnt3a).

Our results do not investigate in detail how CK2 affects the canonical Wnt signaling pathway and whether CK2α is involved in the stabilization of β-catenin. However, they do show that CK2α promotes β-catenin–Tcf-dependent transcriptional activity via a mechanism requiring CK2 kinase activity, because both TBB and DMAT block the increase in reporter activity (Figs. 3*C* and 5*B*). These results were confirmed by expressing an inactive CK2α^{D156A} variant (Fig. 5*B*).

A remaining issue is the role of the regulatory CK2β subunit in this process. CK2 is suggested to function as a tetrameric enzyme composed of two catalytic α-subunits and two regulatory β-subunits, whereby β-subunits are thought to promote α activity. Recently, however, cases have been discussed whereby CK2α may function differently in the absence of CK2β (10). Indeed, transfection with pEGFP–CK2β in HEK-293T cells augmented survivin mRNA and protein levels and generally promoted viability in the presence of cytotoxic drugs. However, the increments in survivin expression and viability were neither as pronounced nor as reproducible as with CK2α (data not shown). Thus, only the latter results with ectopic CK2α are reported here. Although the results with CK2β may suggest that a functional tetrameric enzyme, rather than CK2α alone, is required, additional experiments will be necessary to solve this issue.

TBB was used at relatively high concentrations in our experiments, suggesting that it may also have affected the activity of molecules other than CK2. Although we cannot exclude non-specific effects, results obtained with DMAT and using alternative approaches to modulate CK2 activity and protein levels indicate that CK2 represents the major target. First, TBB concentrations used here were similar to those reported by others to inhibit CK2 in cells (17, 38). Second, CK2 kinase activity was substantially reduced in lysates from HT29(US) cells treated with TBB or DMAT at the concentrations used here (Fig. 7*A*). Third, both TBB and DMAT reduced survivin levels and viability of HT29(US) cells (Figs. 1, 2, and 5). Fourth, TBB-induced apoptosis in HEK-293T cells was not overcome by pEGFP–CK2α at the concentrations tested (Fig. 4*C*); however, when the amount of pEGFP–CK2α was increased, viability also increased, even in the presence of TBB (data not shown). Fifth, CK2α-mediated increments in TOP-FLASH reporter activity were blocked by TBB (Fig. 3*C*) and DMAT (Fig. 5*B*). Furthermore, kinase-inactive HA–CK2α^{D156A} did not augment reporter activity (Fig. 5*B*). Finally, down-regulation of CK2α using siRNA reduced survivin and β-catenin protein levels (Fig. 5*C*), as predicted from experiments with TBB and DMAT. Thus, all data presented here sustain the conclusion that CK2α activity promotes β-catenin–Tcf/Lef-dependent transcription of the target *survivin* and in doing so promotes survival by inhibiting apoptosis.

Preliminary microarray data obtained by comparing HT29(US) cells in the presence or absence of TBB showed that this inhibitor reduced expression of several β-catenin–Tcf/Lef target genes, including cyclin-D1 and c-myc. Indeed, the most significant changes in expression observed in response to TBB were all known targets of the β-catenin–Tcf/Lef pathway (data not shown). Thus, the canonical β-catenin–Tcf/Lef pathway appears to represent a prime target for CK2-mediated transcriptional changes, at least in HT29(US) cells. Even taking these observations into consideration, the fact that reconstitution of survivin alone in HEK-293T cells (Fig. 4) was sufficient to inhibit TBB-induced apoptosis is somewhat surprising. A possible interpretation is that survivin represents a common effector relevant to survival downstream of β-catenin–Tcf/Lef target genes. Hence, CK2 may control survivin levels both directly by regulating transcription of the gene itself and indirectly by regulating the transcription of other genes that use pathways involving survivin.

In summary, the results presented here establish a mechanism by which CK2 promotes survival and precludes apoptosis that involves enhanced transcription of β-catenin–Tcf/Lef-dependent genes. Loss of CK2 activity due to inhibitors reduced viability and the number of cells in G_2/M as well as increased apoptosis. These changes were linked to reduced β-catenin–Tcf/Lef-dependent transcription and loss of survivin, a protein that is increased in essentially all human tumors and is required for tumor survival. Given the emerging importance of survivin in tumor biology, our findings identifying this protein as a crucial target downstream of CK2 may open up a new window of therapeutical opportunity involving selective inhibition of CK2.

Materials and Methods

Materials. Cell medium and antibiotics were from Gibco/BRL (Paisley, Scotland, U.K.). FBS was from HyClone (Logan, UT). The CK2 inhibitors TBB and DMAT were purchased from Calbiochem (San Diego, CA). siRNA directed against CK2α and TRIzol were obtained from Invitrogen (Carlsbad, CA). The MTS Proliferation Assay Kit was from Promega (Madison, WI). The monoclonal anti-β-catenin antibody was from Transduction Laboratories (Lexington, KY). The monoclonal antibodies anti-CK2α and anti-GFP were from Calbiochem. Polyclonal anti-survivin and anti-actin antibodies were purchased from R & D Systems (Minneapolis, MN) and Sigma (St. Louis, MO), respectively. Anti-rabbit IgG and anti-mouse IgG antibodies coupled to HRP were from Bio-Rad Laboratories (Hercules, CA) and Sigma, respectively. Protran membrane was from PerkinElmer (Boston, MA). The BCA protein determination kit was from Pierce (Rockford, IL). The Superfect and the Plasmid Midi kit were from Qiagen (Valencia, CA). The EZ-ECL Chemiluminescence kit was purchased from Biological Industries (Kibbutz Beit Haemek, Israel). Luciferin and 2-nitrophenyl-β-D-galactopyranoside were from Sigma and Roche Molecular Biochemicals (Mannheim, Germany), respectively. Doxorubicin and etoposide were obtained from Calbiochem, and Taxol was purchased from Molecular Probes (Eugene, OR). Buffers and all other reagents used but not specified were from Sigma or were of the highest grade available.

Cell Culture. HT29(US) human colon adenocarcinoma cells (provided by Bernhard Sordat, Swiss Institute for Experimental Cancer Research, University of Lausanne, Lausanne, Switzerland) correspond to a subpopulation of HT29(ATCC) cells selected for higher metastatic potential by several passages in nude mice whereby cells were injected dorsally and recovered from lung metastases. The SW-480 and DLD-1 human colon cancer cell lines were described previously (45). ZR-75 breast cancer cells were from Gareth Owen (Catholic University of Chile, Santiago, Chile), and HEK-293T human embryonic kidney cells were provided by Pascal Schneider (Institute of Biochemistry, University of Lausanne, Lausanne, Switzerland). HT29(US), SW-480, DLD-1, and HEK-293T cells were cultured at 37°C and 5% CO_2 in DMEM supplemented with 10% FBS and antibiotics (10,000 units/ml penicillin and 10 μg/ml streptomycin). ZR-75 breast cancer cells were cultured in DMEM-F12 supplemented with 10% FBS and antibiotics. For transfection experiments, Superfect reagent was used following instructions provided by the manufacturer. Transfection efficiency was ≈50% in HEK-293T cells as assessed by flow cytometric analysis after transfections with pEGFP–C1 (data not shown).

Plasmids for Transfection Experiments. The plasmids pCEFLHA–CK2α and pCEFLHA–CK2α^{D156A} encoding HA-tagged wild-type CK2α and inactive CK2α^{D156A}, respectively, were kindly provided by Jorge Allende (Institute of Biomedical Sciences, Cellular and Molecular Biology Program, Faculty of Medicine, University of Chile) and have been described elsewhere (46, 47). The construct pEGFP–CK2α was obtained by double digestion of pT7HX-CK2α (48) with BglII and SalI to release a cDNA encoding CK2α that was ligated in-frame into pEGFP–C1 (Clontech, Palo Alto, CA) previously digested with the same enzymes. The construct pEGFP–survivin was described previously (49). The reporter plasmids pTOP-FLASH (containing wild-type Tcf/Lef binding sites fused to luciferase reporter gene) and pFOP-FLASH (containing mutated Tcf/Lef binding sites) previously described (50) were kindly provided by Hans Clevers (Hubrecht Laboratory, Uppsalalaan, The Netherlands). The plasmid pON260 encoding β-galactosidase for standardization in reporter assays was provided by Sergio Lavandero (Institute of Biomedical Sciences, Cell and Molecular Biology Program, Faculty of Medicine, University of Chile) and has been described elsewhere (51).

Viability Assay. HT29(US), DLD-1, SW-480, and ZR-75 cells were seeded on 96-well plates at a density of 1×10^4 cells per well. After 24 h cells were treated with 100 μM TBB for an additional 16–24 h and viability was determined. Alternatively, HEK-293T cells were transfected with 2 μg of pEGFP–C1, pEGFP–CK2α, or pEGFP–survivin for 24 h and subsequently replated on 96-well plates at a density of 1×10^4 cells per well. Cells were treated 24 h later with 250 nM doxorubicin, 25 μM etoposide, or 250 nM taxol and maintained in culture for an additional 24 h. Cell viability was measured by the MTS assay according to the manufacturer's instructions.

Apoptosis and Cell Cycle Analysis. Apoptosis was analyzed by flow cytometry after propidium iodide staining, essentially as described (52). Apoptosis was determined by plotting propidium iodide fluorescence versus the forward scatter parameter by using the CellQuest program. Human recombinant Fas ligand (Apotech, Epalinges, Switzerland) at a concentration of 100 ng/ml was used as a positive control. Alternatively, HEK-293T transfected cells were gated based on the presence of GFP, and then apoptosis was assessed in this population as described (52). To quantify cell cycle distribution (DNA content analysis), cells were previously permeabilized in methanol and stained with propidium iodide. Samples containing $\approx 2 \times 10^4$ cells were analyzed.

Silencing of CK2α by siRNAs. The siRNAs used correspond to the sequences α-siRNA (sense, 5′-GAUGACUACCAGCUGGUUCUU-3′; antisense, 5′-GAACCAGCUGGUAGUCAUCUU-3′) and α10-siRNA (sense, 5′-UCAAGAUGACUACCAGCUGUU-3′; antisense, 5′-CAGCUGGUAGUCAUCUUGAUU-3′) described elsewhere (53) but containing two uridines instead of deoxythymidine at the 3′ end as originally described. To generate siRNA duplexes, sense and antisense siRNA strands

were mixed at a final concentration of 100 μM, incubated for 5 min at 65°C, and then maintained at room temperature for 15 min. Duplex siRNAs were used at 50 and 100 nM final concentrations. For transfection, 5×10^5 HEK-293T cells were seeded on six-well plates and maintained in culture for 24 h. Cells were transfected with either the individual siRNAs (α-siRNA or α10-siRNA at 50 nM) or a mixture of both (mix) at 50 and 100 nM by using the siPORT Amine reagent (Ambion, Austin, TX) following the manufacturer's instructions. The same concentration of Silencer Negative Control #2 siRNA (100 nM) from Ambion was used as a control. Cells were harvested 48 h after transfection, and extracts were prepared for protein analysis by Western blotting as described. Only results obtained by using equimolar mixtures of siRNAs are shown (Fig. 5*C*, mix).

Analysis of mRNA Levels by RT-PCR. Total RNA was isolated with the reagent TRIzol following instructions provided by the manufacturer. RNA templates were used to generate cDNAs for survivin (sense, 5′-CCGACGTTGCCCCCTGC-3′; antisense, 5′-TCGATGGCACGGCGCAC-3′) and actin (sense, 5′-GCATTGTAACCAACTGGGACG-3′; antisense, 5′-CATGAGGTAGTCTGTCAGGTC-3′) by PCR. All reaction products were analyzed after 28–30 amplification cycles in 2% agarose gels containing ethidium bromide as described (44).

Tcf/Lef–Luciferase Reporter Assay. HEK-293T cells were transfected with 15 μg of total DNA by using 5 μg of each plasmid in the combinations pTOP-FLASH or pFOP-FLASH plus pON260 plus pCEFLHA and pCEFLHA–CK2α or pCEFLHA–CK2α^{D156A} as indicated. Cells were lysed 24 h after transfection in a buffer containing 0.1 M KH_2PO_4 (pH 7.9) and 0.5% Triton X-100. Supernatants obtained after extraction and centrifugation were either analyzed by SDS/PAGE or used to measure luciferase and β-galactosidase activities with the substrates luciferin and 2-nitrophenyl-β-D-galactopyranoside, respectively, following instructions provided by the manufacturers. The values reported for luciferase activity were standardized to β-galactosidase activity.

Statistical Analysis. Where indicated, results were generally compared by using the unpaired *t* test or, on one occasion, using an ANOVA (Fig. 4*C*), with values from at least three independent experiments. $P \leq 0.05$ was considered significant.

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