Influence of Casein Kinase II in Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand–Induced Apoptosis in Human Rhabdomyosarcoma Cells

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ABSTRACT

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces apoptosis via the death receptors DR4 and DR5 in transformed cells *in vitro* and exhibits potent antitumor activity *in vivo* with minor side effects. Protein kinase casein kinase II (CK2) is increased in response to diverse growth stimuli and is aberrantly elevated in a variety of human cancers. Rhabdomyosarcoma tumors are the most common soft-tissue sarcoma in childhood. In this investigation, we demonstrate that CK2 is a key survival factor that protects tumor cells from TRAIL-induced apoptosis. We have demonstrated that inhibition of CK2 phosphorylation events by 5,6-dichlorobenzimidazole (DRB) resulted in dramatic sensitization of tumor cells to TRAIL-induced apoptosis. CK2 inhibition also induced rapid cleavage of caspase-8, -9, and -3, as well as the caspase substrate poly(ADP-ribose) polymerase after TRAIL treatment. Overexpression of Bcl-2 protected cells from TRAIL-induced apoptosis in the presence of the CK2 inhibitor. Death signaling by TRAIL in these cells was Fas-associated death domain and caspase dependent because dominant negative Fas-associated death domain or the cowpox interleukin 1β-converting enzyme inhibitor protein cytokine response modifier A prevented apoptosis in the presence of DRB. Analysis of death-inducing signaling complex (DISC) formation demonstrated that inhibition of CK2 by DRB increased the level of recruitment of procaspase-8 to the DISC and enhanced caspase-8–mediated cleavage of Bid, thereby increasing the release of the proapoptotic factors cytochrome *c*, HtrA2/Omi, Smac/DIABLO, and apoptosis inducing factor (AIF) from the mitochondria, with subsequent degradation of X-linked inhibitor of apoptosis protein (XIAP). To further interfere with CK2 function, JR1 and Rh30 cells were transfected with either short hairpin RNA targeted to CK2α or kinase-inactive CK2α (K68M) or CK2α′ (K69M). Data show that the CK2 kinase activity was abrogated and that TRAIL sensitivity in both cell lines was increased. Silencing of CK2α expression with short hairpin RNA was also associated with degradation of XIAP. These findings suggest that CK2 regulates TRAIL signaling in rhabdomyosarcoma by modulating TRAIL-induced DISC formation and XIAP expression.

INTRODUCTION

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand [TRAIL (Apo2L)] is a member of the TNF family and, like TNF-α and Fas ligand, is a type II membrane protein that can induce apoptotic cell death in a variety of transformed cell types (1–3). However, unlike other members of this family, TRAIL does not appear cytotoxic to normal cells *in vitro* (1, 2, 4). The potential importance of TRAIL as an anticancer agent has been supported by studies in animal models that demonstrate selective toxicity to transplanted human tumors but not to normal tissues (4–6).

TRAIL binds to the apoptosis-inducing receptors DR4 and DR5, which are type I transmembrane receptors, expressed at the cell surface. TRAIL also binds to non–apoptosis-inducing decoy receptors, which compete with death receptors for the ligand and suppress apoptosis. These include DcR1, DcR2, and osteoprotegerin (4, 7) and may constitute one mechanism by which normal cells can evade the induction of apoptosis by TRAIL. The mechanism of induction of apoptosis by TRAIL is believed to be similar to that of TNF-α and Fas ligand and to be initiated by ligand-induced aggregation of DR4 and DR5 and their death domains on the cytoplasmic side of the receptors (3, 8). The death domains in turn orchestrate the assembly of adaptor proteins such as Fas-associated death domain (FADD), which activate caspases after interaction of caspase recruitment domains of the adaptor proteins with the prodomains of the caspases (9). The adaptor proteins involved in TRAIL-induced apoptosis have been controversial, with some reports suggesting that FADD and TNF receptor-associated death domain were not involved (10), whereas others have demonstrated direct binding of FADD and TNF receptor-associated death domain protein to the TRAIL receptor (11, 12). Fibroblasts from FADD knockout mice were shown to undergo TRAIL-induced apoptosis, suggesting that FADD was not essential in TRAIL signaling (13). The caspases involved also appear to be similar to those activated by Fas ligand, with activation of caspase-8 being an early event that eventually leads to activation of effector caspases including caspase-3 (3, 8). Ectopic expression of the cowpox

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virus gene cytokine response modifier A (CrmA) was also shown to inhibit TRAIL-induced apoptosis, consistent with involvement of caspase-1 and/or -8 (10).

Rhabdommyosarcoma (RMS) is a family of soft-tissue tumors that generally occur in the pediatric population. This tumor consists of several different subtypes, depending primarily on the characteristic histology, with the alveolar RMS (ARMS) and embryonal RMS (ERMS) subtypes being the most common forms. We have shown previously that in pediatric RMS cell lines that demonstrate either sensitivity or innate resistance to TRAIL, a death-inducing signaling complex (DISC) is formed in both TRAIL-sensitive and -resistant RMS cell lines with recruitment of FADD and procaspase-8 to the receptor complexes (14). Overexpression of Bcl-2 or Bcl-xL inhibited TRAIL-induced apoptosis and loss in clonogenic survival (14).

Protein kinase casein kinase II (CK2) is a ubiquitously distributed and highly conserved protein serine/threonine kinase in eukaryotes (15, 16). It comprises two catalytic α or α' subunits and two regulatory β subunits that can form a holoenzyme (17). In all human cancers that have been examined, as well as experimental tumors, CK2 activity has been found to be consistently enhanced (18–20). Although a mechanistic understanding of how CK2 supports cellular viability remains far from complete, recent evidence linking CK2 to apoptosis has yielded certain insights (reviewed in ref. 21). In response to apoptotic stimuli, Max, the transcriptional partner of the c-Myc proto-oncogene, has undergone caspase-mediated degradation subsequent to an apparent dephosphorylation at CK2 phosphorylation sites (22). Parallel experiments performed *in vitro* demonstrated that phosphorylation of Max by CK2 *in vitro* protected it from caspase-mediated cleavage. Recent studies have shown that CK2 can phosphorylate Bid, a proapoptotic member of the Bcl-2 family, close to the recognition site for caspase-8 cleavage, thereby preventing cells from undergoing apoptosis (23). In this study, a mutant of Bid that cannot be phosphorylated was found to be highly sensitive to caspase-8–induced cleavage (23). An analogous role for CK2 in modulation of caspase susceptibility has also been observed with the gap junction protein connexin 45.6 in the lens (24) and with the hematopoietic lineage cell-specific protein 1 (25). In all cases, phosphorylation by CK2 protects these proteins from caspase-mediated degradation. A complementary mechanism for the regulation of caspases by CK2 has recently emerged with the demonstration that phosphorylation by CK2 is required for the apoptotic protein ARC to exert its inhibitory activity toward caspase-8 (25). Together with the ability of CK2 to protect individual proteins from caspase-mediated cleavage, this latter observation suggests that CK2 may have general antiapoptotic function. This latter conjecture is supported by the observations that increased expression of CK2 has protected cells from Fas- and drug-induced apoptosis (23, 26). In a similar manner, inhibitors of CK2 have been reported to trigger apoptosis and increase the susceptibility of cancer cells to chemotherapeutic agents or apoptotic stimuli (27–29). A further implication of these results is that the increased expression of CK2, which is frequently observed in cancer (30), may result in enhanced survival of these cells resulting from the potential antiapoptotic function of CK2.

In the present study, we have focused on the role of CK2 in RMS cellular resistance to TRAIL-induced apoptosis. We demonstrate that inhibition of CK2 function effectively sensitizes RMS cell lines to TRAIL at the level of the DISC by enhancing the recruitment of procaspase-8 to the receptor complex. Enhancement of DISC formation is followed by acceleration of TRAIL-induced apoptosis. Apoptosis does not proceed in JR1 cells treated with TRAIL, even though executioner procaspase-6 and XIAP are cleaved at early time points. If such apoptosis does not occur, the transient death receptor signaling obtained may terminate formation of the active initiator caspase, thereby enhancing the opportunity for survival. Using isogenic derivatives of the RMS cell line JR1 that differ only in the presence or expression level of dominant negative (DN)-FADD, Bcl-2, or CrmA, respectively, we further demonstrate that sensitization to TRAIL-induced apoptosis in the presence of the specific inhibitor of CK2 [5,6-dichlorobenzimidazole (DRB)] required the recruitment of procaspase-8 and FADD to the DISC and activation of mitochondrial signaling pathways necessary for inhibition of XIAP. Expression of kinase-inactive CK2α (K68M), CK2α' (K69M), or short hairpin RNA (shRNA) targeted to CK2α disrupted CK2 kinase activity and/or enhanced TRAIL-induced apoptosis in RMS cells. Silencing of CK2α was also associated with XIAP degradation. These findings demonstrate that inhibition of CK2 function augments TRAIL-induced cell death by modulating TRAIL-induced DISC formation, XIAP degradation, and activation of TRAIL mitochondrial pathways (intrinsic pathway).

MATERIALS AND METHODS

Cell Lines. JR1, derived from a human RMS, has been described previously (31). RMS cell line RD was obtained from American Type Culture Collection (Manassas, VA), and Rh30 was established at St. Jude Children's Research Hospital [Memphis, TN (32)]. Cell lines were maintained in RPMI 1640 (Gibco, Gaithersburg, MD) supplemented with 2 mmol/L glutamine and 10% fetal calf serum.

Kinase Assays. The kinase assay is based on phosphorylation of a CK2-specific peptide substrate using the transfer of the γ-phosphate of [γ-^{32}P]GTP by CK2 kinase. Whole-cell extracts from JR1 cells (20 μg) were assayed for CK2 kinase activity at 30°C for 15 minutes in kinase buffer [250 mmol/L Tris-HCl (pH 7.5), 250 mmol/L NaCl, 250 mmol/L KCl, 50 mmol/L $MgCl_2$, 250 μmol/L Na_3VO_4, and 5 μCi of [γ-^{32}P]GTP] containing 1 mmol/L peptide as substrate. The phosphorylated substrate was separated from residual [γ-^{32}P]GTP using P81 phosphocellulose paper. Incorporation of ^{32}P into the substrate was measured using a scintillation counter. CK2-specific peptide substrate RRREEETEEE was synthesized in the Hartwell Center (St. Jude Children's Research Hospital). Inhibition of CK2 activity was achieved by incubation of cells with DRB (40 μmol/L; Calbiochem, La Jolla, CA; ref. 33) or apigenin (40 μmol/L; Sigma-Aldrich, St. Louis, MO; ref. 34).

Production of Recombinant Human TRAIL. The cDNA of the extracellular domain of TRAIL corresponding to amino acids 114 to 281 was subcloned into the pET17/b (Novagen, Madison, WI) bacterial expression vector and expressed in the BL21(DE3)pLysE (Novagen) bacterial host. After induction of TRAIL expression using isopropyl-1-thio-$\beta\beta$-D-galacto-

pyranoside (1 mmol/L), bacterial pellets were harvested, and TRAIL was purified after passage through a nickel column (Ni-NTA) followed by a size-exclusion column Amersham Pharmacia Biotech (Piscataway, NJ), according to previously published procedures (35).

Reverse Transcription-Polymerase Chain Reaction. Expression of CrmA and β-actin was evaluated using the following primers: CrmA, 5′-GAAAGGAGAGAATGTATTC-ATTTC and 5′-GTTGTTGGAGAGCAATATCTACC; and β-actin, 5′-GTGGGGCGCCCCAGGCACCA and 5′-CTCCT-TAATGTCACGCACGATTTC. Total RNA was extracted from cells using the RNeasy mini kit (Qiagen, Valencia, CA), and cDNA was prepared using an oligo(dT) primer and Moloney leukemia virus reverse transcriptase as described previously (36). After reverse transcription, the cDNA product was amplified by polymerase chain reaction with Taq DNA polymerase using standard protocols (36). The amplified products (25 μL) were separated on 1% agarose gels, stained with ethidium bromide, and photographed using ultraviolet illumination.

Analysis of Cell Death. Cells were plated at a density of 100,000 cells per well in 12-well plates, and after overnight attachment, the cells were treated with TRAIL (5–50 ng/mL) in either the absence or presence of the CK2 inhibitors for up to 24 hours. Apoptotic cells were determined by annexin V-propidium iodide staining. Cells were stained with 10 μL of annexin V-APC (Becton Dickinson, San Jose, CA) and 10 μL of propidium iodide (50 ng/mL) according to the manufacturer's instructions, incubated for 15 minutes at room temperature in the dark, and analyzed immediately. JR1 cells were also pretreated with the pan-caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk) (50 μmol/L; Enzyme Systems Products, Livermore, CA) for 1 hour before TRAIL treatment. In some experiments, apoptotic cells were also determined by forward side scatter after propidium iodide staining (37).

Analysis of the Death-Inducing Signaling Complex. Immunoprecipitation of receptor complexes after cell treatment for up to 120 minutes with 1 μg/mL FLAG-tagged TRAIL (Upstate Biotechnology, Lake Placid, NY) and analysis of components of the DISC were conducted as described previously (14).

Western Blot Analysis. Western blot analyses were conducted as described previously (35, 38). Primary antibodies to detect caspase-8, caspase-9, caspase-6, and XIAP were from MBL (Woburn, MA). Anti-c-IAP1 was purchased from Alexis Biochemicals (San Diego, CA), and primary antibodies for caspase-3, Bid, and poly(ADP-ribose) polymerase (PARP) were purchased from BD Pharmingen (San Diego, CA). The cytochrome *c* monoclonal antibody was obtained from Clontech (Palo Alto, CA), anti-AIF was from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-HtrA2/Omi antibody was kindly provided by Dr. Emad Alnemri (Kimmel Cancer Institute, Philadelphia, PA). The secondary antibody used was horseradish peroxidase-conjugated sheep antimouse IgG1 (Amersham Pharmacia Biotech).

Plasmid Vectors and Transfection. The retroviral expression vectors pMSCV-I-GFP [expressing green fluorescent protein (GFP)] and pMSCV-DN-FADD (expressing the death domain only of FADD) were kind gifts from Drs. Jill M. Lahti and Vincent J. Kidd (St. Jude Children's Research Hospital). The retroviral expression vector pMSCV-Bcl-2 (expressing human Bcl-2 protein and GFP, separated by an internal ribosome entry site (IRES) sequence) was kindly provided by Dr. John Cleveland (St. Jude Children's Research Hospital). Kinase-inactive CK2α (K68M) and CK2α′ (K69M), generous gifts from Dr. David W. Litchfield (University of Western Ontario, London, Ontario, Cananda), and CrmA (a small pox protein, kindly provided by Drs. Jill M. Lahti and Vincent J. Kidd), were subcloned into the pMSCV-I-GFP vector. Retroviral supernatants were prepared as described previously (39). The U6 promoter vector pSHAG-1 and the recipient vector MSCV-I-GFP were provided by Dr. Gregory J. Hannon (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Cells were incubated overnight in a 50% mixture of RPMI 1640 and retroviral supernatants in the presence of Polybrene (8 μg/mL) and sorted 48 hours later for GFP expression using fluorescence-activated cell sorting. The expression of Bcl-2 and DN-FADD was confirmed by Western blotting. Expression of CrmA was confirmed by reverse transcription-polymerase chain reaction.

Plasmids Expressing Short Hairpin RNAs and Transfection. The shRNA sequences were designed using designated software found on the OligoRetriever Database and en-

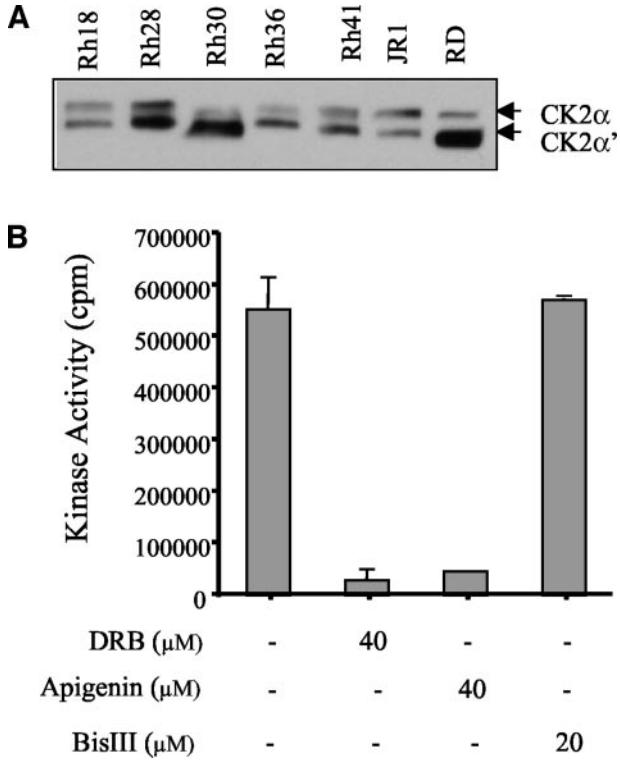

Fig. 1 *A*, expression of CK2α and CK2α′ isoforms in human RMS cell lines determined by Western analysis. *B*, effect of DRB, apigenin, or bisindoylmaleimide III on CK2 kinase activity in JR1 cells. Cells were either untreated or treated with the indicated concentrations of each agent for 2 hours and harvested, and the phosphotransferase activity of CK2 was determined using a CK2-specific substrate (RRREEETEEE). Data represent ^{32}P incorporated (cpm) into the CK2 substrate peptide (mean ± SD of two determinations per point).

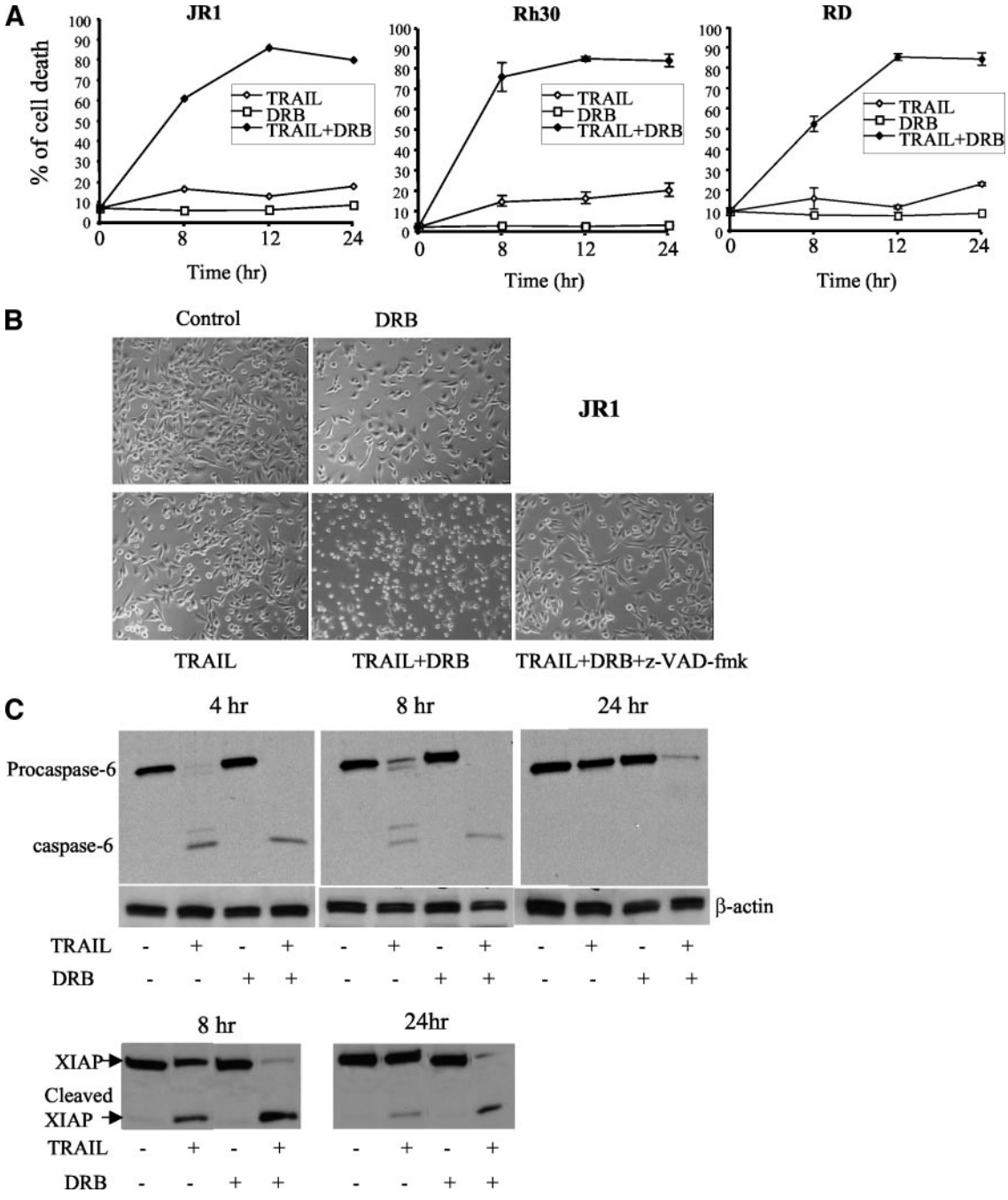

Fig. 2 DRB sensitizes human RMS cell lines to TRAIL-induced cell death. *A*, Cells were treated with TRAIL (JR1, 50 ng/mL; Rh30, 5 ng/mL; RD, 5 ng/mL) in either the absence or presence of DRB (40 μmol/L, added 2 hours before and concurrently with TRAIL) for up to 24 hours. *B*, Caspase-dependent cytotoxicity of TRAIL was only detectable in JR1 cells in the presence of the CK2 inhibitor DRB (40 μmol/L), added for 2 hours before and during exposure to TRAIL. The pan-caspase inhibitor z-VAD-fmk (50 μmol/L) completely abolished TRAIL-induced apoptosis in JR1 when coincubated with TRAIL. Data represent the mean ± SD of two determinations per point. *C*, procaspase-6 and XIAP processing in TRAIL-resistant JR1 cells. Cells were treated with DRB (40 μmol/L) 2 hours before and during TRAIL (50 ng/mL) treatment. After 4, 8, or 24 hours, lysates were prepared and analyzed by SDS-PAGE using specific antibody as described in Materials and Methods.

coded inverted repeats of 27 to 29 bp separated by an 8-nucleotide spacer that corresponded to nucleotides 447 to 475 (sh447), 723 to 751 (sh723), or 1137 to 1165 (sh1137) of the CK2α cDNA. The sequences were prepared by the Hartwell Center and subsequently ligated to the U6 promoter vector (pSHAg-1), compatible with the GATEWAY system (Invitrogen, Carlsbad, CA) that was used to transport the shRNA expression cassette into the recipient MSCV-I-GFP retroviral

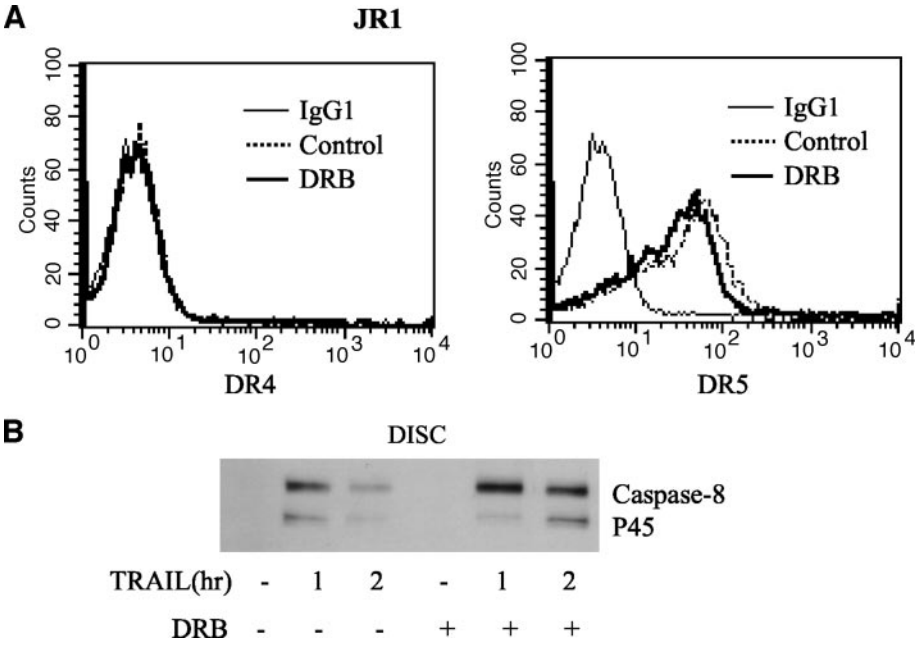

Fig. 3 Influence of DRB on DR5 expression and on the formation of the TRAIL receptor complex. *A*, Inhibition of CK2 did not alter expression levels of DR4 or DR5. Cells were treated with DRB (40 μmol/L) for 2 hours, harvested, and stained with IgG1 or monoclonal antibodies against DR4 and DR5. The expression of both receptors was assessed by phycoerythrin fluorescence using flow cytometry. *B*, JR1 cells were treated for up to 2 hours with FLAG-TRAIL precomplexed with anti-FLAG antibodies (clone M2; 2 μg/mL) in the presence or absence of DRB (40 μmol/L). The resulting protein complexes were separated by SDS-PAGE and analyzed by Western blot for components of the TRAIL-induced DISC.

vector. JR1 cells were incubated overnight in a 50% mixture of RPMI 1640 and retroviral supernatants in the presence of Polybrene (8 μg/mL). After an additional 48 hours, cells were evaluated for GFP expression using fluorescence-activated cell sorting. Design of shRNA primers from gene accession numbers was conducted from the on-line RNAi OligoRetriever Database.[1]

Cellular Fractionation. Cytosolic extracts were prepared using the ApoAlert kit (Clontech) with a Dounce homogenizer and subjected to centrifugation at 700 × *g* to pellet nuclei. The postnuclear supernatant was centrifuged at 10,000 × *g* to pellet the mitochondria-enriched heavy membrane fraction, and the resulting supernatant was further centrifuged at 100,000 × *g* to obtain the cytosolic fraction. Total proteins (15 μg) were subjected to immunoblot analysis. Design of shRNA primers from gene accession numbers used the on-line RNAi OligoRetriever Database.[1]

RESULTS

Expression of CK2 Isoforms and Kinase Activity. To facilitate investigation of the potential role of CK2 in attenuating TRAIL-mediated apoptosis in RMS cell lines, the expression of CK2 isoforms α or α′ was determined in a panel of seven human RMS cell lines. Rh30 expressed the CK2 α′ isoform only, and Rh18 and JR1 expressed both isoforms, whereas Rh28, Rh36, Rh41, and RD expressed predominantly CK2α′ with low-level expression of CK2α (Fig. 1*A*).

JR1 cells were examined further for CK2 kinase activity (Fig. 1*B*). CK2 was constitutively activated in these cells, as demonstrated by a high level of activity of the endogenous kinase. Exposure of JR1 to the classic CK2 inhibitor DRB (40 μmol/L) resulted in inhibition of phosphorylation of the CK2-specific peptide substrate (RRREEETEEE; Fig. 1*B*). Inhibition of CK2 activity was also achieved by treatment with the plant flavonoid apigenin (40 μmol/L), but not by treatment with the protein kinase C inhibitor bisindoylmaleimide III (20 μmol/L; Fig. 1*B*).

CK2 Attenuates TRAIL-Induced Cell Death. DRB, an inhibitor of CK2, was subsequently evaluated for the ability to sensitize human RMS cell lines to TRAIL-induced apoptosis during 24-hour coincubation. Treatment with DRB (40 μmol/L) 2 hours before and during incubation with TRAIL (5–50 ng/mL) resulted in dramatic sensitization of JR1, Rh30, and RD to TRAIL-induced cell death, increasing maximally to >80% (Fig. 2*A*). The effect was completely inhibited by the broad spectrum caspase inhibitor z-VAD-fmk, (Fig. 2*B*), confirming the critical role of caspases in this process. Treatment with DRB alone, at the concentration required for sensitization (40 μmol/L), was devoid of any significant cytotoxic activity.

Transient Processing of Caspase-6 and XIAP in TRAIL-Treated, TRAIL-Resistant RMS Cells. Caspase-6 is an example of an executioner caspase that functions downstream of the mitochondria in the induction of apoptosis (40). Treatment of TRAIL-resistant JR1 cells with TRAIL (50 ng/mL) demonstrated an enhancement of the processing of procaspase-6 within 4 hours of TRAIL treatment in cells treated with TRAIL in the absence or presence of DRB (Fig. 2*C*). By 8 hours, cleavage of procaspase-6 was reduced in JR1 cells treated with TRAIL alone, and by 24 hours, this procaspase was no longer cleaved under these conditions. In contrast, treatment of JR1 cells with TRAIL in the presence of DRB enhanced the cleavage of procaspase-6 at all times, even at 24 hours (Fig. 2*C*). Similar results were obtained with XIAP, the most widely expressed member of the inhibitor of apoptosis protein family

[1] http://www.cshl.org/public/science/hannon.html.

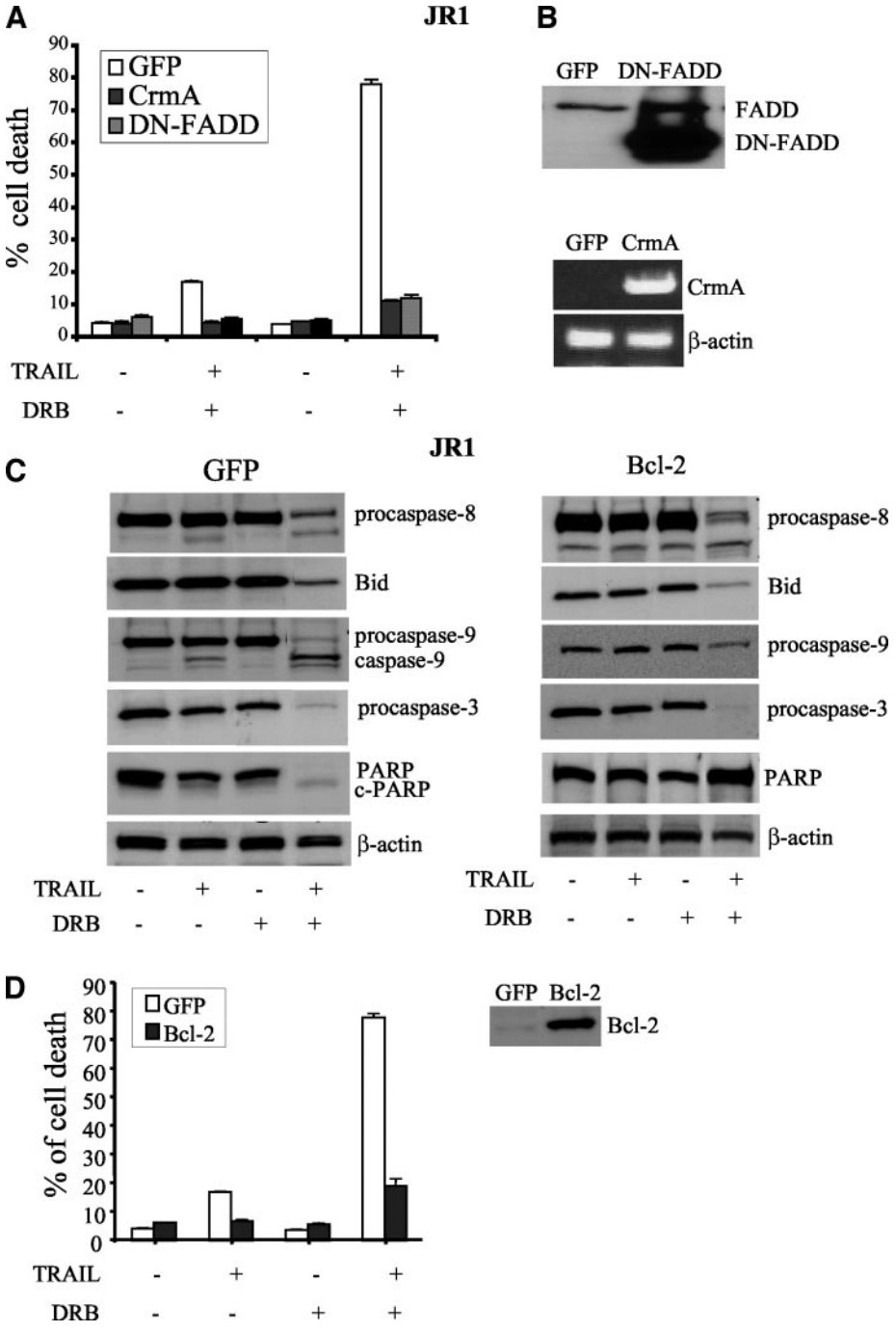

Fig. 4 *A*, JR1 cells were stably transfected with plasmids encoding GFP alone or GFP and DN-FADD or CrmA separated by an IRES sequence and sorted for GFP expression by fluorescence-activated cell-sorting analysis. Cells were treated with DRB (40 μmol/L) 2 hours before and during TRAIL (50 ng/mL) stimulation. After 24 hours, cell death was determined as described in Materials and Methods. *B*, Lysates were prepared and analyzed by SDS-PAGE using FADD antibody. Expression of CrmA and β-actin was determined by (reverse transcription) polymerase chain reaction using specific primers described in Materials and Methods. *C*, JR1 cells were stably transfected with plasmids encoding GFP or GFP and Bcl-2 separated by an IRES sequence and sorted for GFP expression by fluorescence-activated cell-sorting analysis. Cells were treated with DRB (40 μmol/L) 2 hours before and during TRAIL (50 ng/mL) treatment. After 8 hours, lysates were prepared and analyzed by SDS-PAGE using specific antibodies as described in Materials and Methods. *D*, Cells were treated with TRAIL (50 ng/mL) for 24 hours in the presence or absence of DRB (40 μmol/L), and cell death was determined as described in Materials and Methods. Expression of Bcl-2 was determined by Western analysis after transfection and sorting for GFP expression.

and the most potent inhibitor of caspases (41, 42). In JR1 cells treated with TRAIL (50 ng/mL) alone for 8 hours, a moderate decrease in XIAP levels was observed, with detection of a cleaved fragment of XIAP (30 kDa; ref. 43; Fig. 2*C*). However, by 24 hours, cleavage of XIAP was substantially reduced, and the level of expression was restored to normal. In contrast, treatment of cells with the combination of TRAIL (50 ng/mL) and DRB (40 μmol/L) resulted in almost complete cleavage of XIAP at 8 hours and even at 24 hours, whereas at this time, XIAP was no longer degraded in JR1 cells treated with TRAIL alone (Fig. 2*C*). Data suggest that TRAIL receptor signaling may be transient in TRAIL-resistant JR1 cells, thereby terminating the formation of active initiator caspases and increasing the chance of survival, and that CK2 may be involved in this process.

CK2 Regulates TRAIL Signaling at the Level of the Receptor. To determine whether the constitutive expression of CK2 in JR1 cells demonstrated a role in regulating the

expression of the TRAIL receptors or the formation of the TRAIL-induced DISC, JR1 cells were treated with TRAIL (50 ng/mL) in the absence or presence of DRB (40 μmol/L). DR4 was not expressed at significant levels at the cell surface of JR1 cells, which expressed predominantly DR5 (Fig. 3*A*). Treatment with DRB (40 μmol/L; 2 hours) had no effect on the level of DR5 expression at the cell surface in JR1 cells, as determined by flow cytometry. However, immunoprecipitation of receptor complexes for DR5 followed by Western analysis demonstrated that treatment of JR1 cells with DRB (40 μmol/L) increased the recruitment of procaspase-8 to the DISC, determined at 1 hour and 2 hours after the initiation of TRAIL treatment (Fig. 3*B*). In JR1 cells treated with DRB + TRAIL, the DISC remained stable at 2 hours, whereas in cells treated with TRAIL alone, caspase-8 was no longer recruited and activated at the DISC. FADD was below the level of detection within the DISC, even in the presence of DRB.

TRAIL-Induced Apoptosis in the Presence of DRB Depends on Intact FADD/Caspase-8 Signaling. To further dissect the TRAIL-induced pathway leading to apoptosis of JR1 cells in the presence of CK2 inhibition, the effect of the FADD/caspase-8 pathway was determined using JR1 cells in which FADD and caspase-8 signaling was blocked by stable transfection of a DN-FADD or CrmA (a small pox protein that potently inhibits caspase-8 and caspase-1; Fig. 4*B*). TRAIL-induced apoptosis was inhibited in JR1 cell lines overexpressing either DN-FADD or CrmA (Fig. 4*A*), indicating the importance of FADD and caspase-8 in the mechanism of sensitization to TRAIL-induced apoptosis by DRB.

Influence of Bcl-2 Overexpression. Using isogenic JR1 cell lines transfected with vector alone (GFP) or overexpressing Bcl-2 (JR1-Bcl-2), TRAIL (50 ng/mL) combined with DRB (40 μmol/L) significantly enhanced the cleavage of caspase-8, Bid, caspase-9, caspase-3, and PARP in JR1-GFP control cells (Fig. 4*C*). In JR1-Bcl-2 (Fig. 4*C* and *D*), overexpression of Bcl-2 resulted in inhibition of TRAIL-induced cell death. However, whereas Bcl-2 blocked the cleavage of PARP in cells treated with the combination of TRAIL and DRB, the cleavage of caspase-8, caspase-9, caspase-3, and Bid was not inhibited.

Inhibition of CK2 Facilitates TRAIL-Induced Release of Proapoptotic Factors from the Mitochondria. The kinetics of release of mitochondrial proteins into the cytosol was subsequently examined during DRB-induced sensitization of JR1 cells to TRAIL-induced apoptosis (Fig. 5). In cells treated with TRAIL alone (50 ng/mL), small amounts of apoptosis inducing factor (AIF), HtrA2/Omi, and Smac/DIABLO could be detected in the cytosol. In contrast, in cells treated with TRAIL in the presence of DRB (40 μmol/L), the release of proapoptotic factors into the cytosol was increased by 8 hours after treatment, including the release of cytochrome *c*. Exposure to DRB alone had no effect on the release of proapoptotic factors (Fig. 5). Inhibition or degradation of XIAP but not c-IAP1 was observed only in cells treated with the combination TRAIL and DRB.

Short Hairpin RNAs Targeted to CK2α Modulate XIAP Expression and TRAIL Sensitivity in ERMS JR1 Cells. To determine whether shRNA could modulate the expression of CK2α, several retroviral vectors were generated that contained shRNA targeting CK2α (designated sh447, sh723, and sh1137). Transfection of shRNAs into JR1 cells resulted in

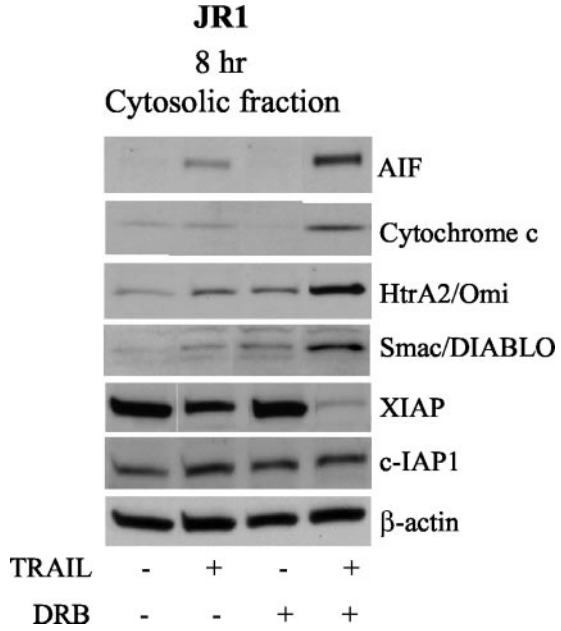

Fig. 5 Inhibition of CK2 facilitates TRAIL-induced release of proapoptotic factors from the mitochondria. JR1 cells were treated with DRB (40 μmol/L) 2 hours before and during TRAIL (50 ng/mL) exposure. After 8 hours, cytoplasmic lysates were subsequently prepared as described in Materials and Methods and analyzed for their content of cytochrome *c*, HtrA2/Omi, Smac/DAIBLO, AIF, XIAP, c-IAP1, and β-actin by Western analysis.

suppression of CK2α expression, with sh723 and sh1137 achieving the greatest reduction in CK2α levels (Fig. 6*A*). No effect was observed with sh447 on the expression of CK2α, and all shRNAs were without effect on the expression of CK2α′ or β-actin. Inhibitor of apoptosis proteins are a family of related proteins that suppress cell death by inhibiting both upstream and terminal caspases (41, 42, 44, 45). The chemical CK2 inhibitor DRB, when combined with TRAIL, converted TRAIL-resistant JR1 cells to TRAIL sensitivity, with simultaneous and enhanced release of proapoptotic factors from the mitochondria, and reduced expression of XIAP. It was therefore determined whether a specific reduction in CK2α expression levels could convert TRAIL-resistant JR1 cells to TRAIL sensitivity and simultaneously down-regulate the expression of XIAP. Transfected cells were treated for 24 hours with increasing concentrations of TRAIL (5–50 ng/mL). Transfection of JR1 with sh723 or sh1137 resulted in dramatic sensitization of JR1 cells to TRAIL-induced apoptosis approaching 70%, whereas transfection with sh447 had no effect (Fig. 6*A*). Furthermore, expression of kinase-inactive CK2α (K68M) increased JR1 sensitivity to TRAIL-induced apoptosis (Fig. 6*B*). Of particular interest was that XIAP expression was down-regulated in JR1 cells transfected with sh723 and sh1137 alone at 72 hours (whereas transfection with sh447 demonstrated no effect on XIAP expression; Fig. 6*A*), suggesting that the function of CK2α and XIAP may be directly linked. Of interest was that both approaches of inhibiting CK2 activity were also associated with increased spontaneous cell death (Fig. 6*A* and *B*), suggesting that CK2α has a potential role in cell viability and survival.

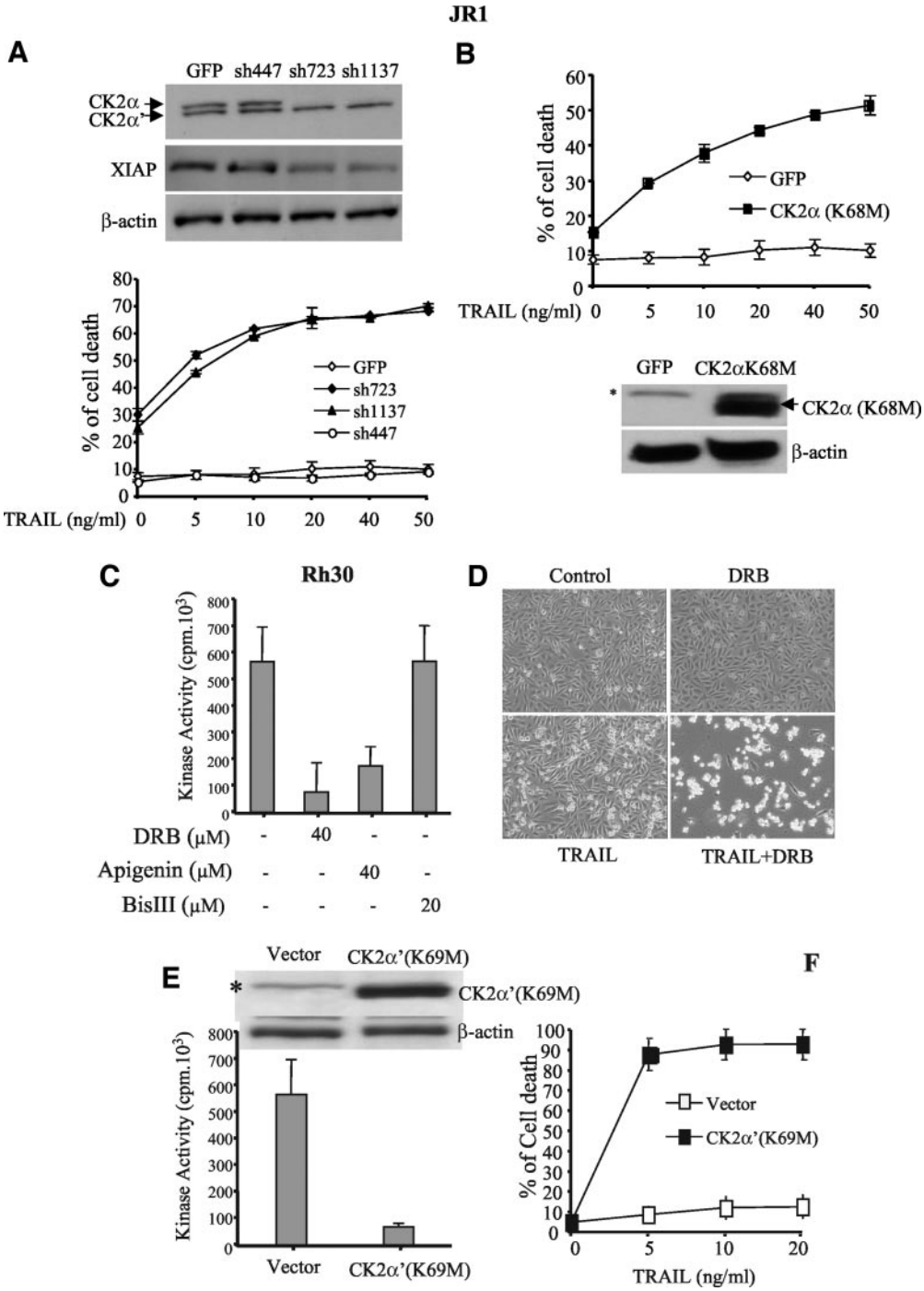

Fig. 6 JR1 cells were stably transfected with MSCV-I-GFP or CK2αshRNA. *A*, Cell lysates were subsequently prepared and analyzed for expression of CK2α, XIAP, and β-actin. JR1 cells transfected with shRNA were treated with varied concentrations of TRAIL (5–50 ng/mL) for 24 hours, and the percentage of cell death was determined as described in Materials and Methods. *B*, Cells transfected with MSCV-I-GFP or kinase-inactive CK2α (K68M) were treated with various concentrations of TRAIL (5–50 ng/mL) for 24 hours, and the percentage of cell death was determined as described in Materials and Methods. Data represent the mean ± SD of two determinations per point. *, nonspecific band. *C*, Rh30 cells were untreated or treated with the indicated concentrations of each agent for 2 hours and harvested, and the phosphotransferase activity of CK2 was determined using a CK2-specific substrate (RRREEETEEE). Data represent ^{32}P incorporated (cpm) into the CK2 substrate peptide (mean ± SD of two determinations per point). *D*, Rh30 cells were treated with DRB (40 μmol/L) for 2 hours before and during 24-hour treatment with TRAIL (10 ng/mL). *E*, Rh30 cells transfected with CK2α′ (K69/M) were harvested, and the kinase activity of CK2 was determined using a CK2-specific substrate (RRREEETEEE). Data represent ^{32}P incorporated (cpm) into the CK2 substrate peptide (mean ± SD of two determinations per point). *F*, Rh30 cells transfected with either vector control or CK2α′ (K69/M) were treated with varying concentrations of TRAIL (10–20 ng/mL) for 24 hours (mean ± SD of two determinations per point).

Expression of a Protein Kinase-Inactive CK2α′ Subunit Promotes TRAIL-Induced Apoptosis in ARMS Rh30 Cells. To elucidate whether the CK2α′ subunit can also play a role in regulating TRAIL-induced cell death, we used Rh30 RMS cells that express only the CK2α′ subunit (Fig. 1*A*). CK2 was found to be constitutively activated in Rh30 and inhibitable by DRB (40 μmol/L) or apigenin (40 μmol/L), as determined by phosphorylation of a CK2-specific peptide, but was not inhibited by bisindoylmaleimide III (20 μmol/L; Fig. 6*C*). Pretreatment of Rh30 cells with DRB enhanced TRAIL-induced apoptosis, and DRB alone was not toxic (Fig. 6*D*). To further characterize the role of the CK2α′ subunit in TRAIL sensitivity, Rh30 cells were transfected with either empty vector (GFP) or kinase-inactive CK2α′ (K69M). In control-transfected cells, substantial kinase activity was determined (Fig. 6*E*). As expected, no kinase activity was detected after transfection of the kinase-inactive CK2α′ (K69M; Fig. 6*E*), which resulted in complete inhibition of CK2 activity in Rh30 cells. Treatment of Rh30 expressing CK2α′ (K69M) with varied concentrations of TRAIL (5–20 ng/mL) resulted in dramatic sensitization to TRAIL-induced apoptosis (>90%; Fig. 6*F*). These data suggest that the CK2α′ subunit also plays a major role in regulating TRAIL-induced tumor cell death.

DISCUSSION

The ability of death receptors to induce apoptosis is critical in several disease processes and has been the focus of most work to date. The principal findings of the present study have brought forth more direct evidence for an antiapoptotic function for CK2. We have demonstrated that inhibition of CK2 activity leads to sensitization of tumor cells derived from human rhabdomyosarcomas to TRAIL-induced apoptosis by controlling the most apical point in the TRAIL signaling pathway, involving the recruitment of procaspase-8 to the DISC at the level of the TRAIL receptors. In view of the fact that phosphorylation by CK2 can protect specific proteins from caspase-mediated degradation, it is noteworthy that there is a striking similarity between the recognition sequence for caspase degradation (46, 47) and the consensus motif for phosphorylation by CK2. Thus, it is conceivable that CK2 functions to some degree as a sensor of cell integrity that exerts a general cell survival or antiapoptotic function through its ability to phosphorylate numerous proteins that would be destined for caspase-mediated degradation during apoptosis. We have demonstrated that inhibition of CK2 results in enhanced recruitment of procaspase-8 to the DISC after TRAIL stimulation, enhanced caspase-8 cleavage, and significantly increased TRAIL-induced apoptosis. After activation of caspase-8 at the DISC, the cytosolic substrate Bid is rapidly cleaved, leading to the generation of a truncated form of Bid, tBid (48). Cleaved/truncated Bid translocates to the mitochondria (49) on its myristoylation (50), where it triggers initiation of mitochondrial disruption and further amplification of the death signaling cascade. Phosphorylation of Bid by CK2 has also been reported to render Bid resistant to caspase-8 cleavage in cell-free systems (23). Recent studies have shown that CK2 can phosphorylate Bid in the vicinity of the recognition site for caspase-8 and that this phosphorylation renders Bid resistant to cleavage by caspase-8 and protects cells from Fas-induced apoptosis (23). This has been shown to contribute to CK2-induced inhibition of TRAIL-induced apoptosis in malignant cells (28) and, in the current investigation, may also contribute to the acceleration of both caspase-8–mediated Bid cleavage and TRAIL-induced apoptosis in cells treated with the CK2 inhibitor DRB. According to such a model, events that compromise the expression of CK2 or its activity would lead to decreased phosphorylation of its targets, which in turn would lead to a release of caspase inhibition as well as increased susceptibility of proteins to caspase-mediated degradation (22, 27, 30). However, we have demonstrated that additional sites also exist upstream of caspase activation, at the level of DISC formation, which does not involve elevated levels of expression of the death receptors themselves.

In the current study, we have demonstrated that the activity of caspases in TRAIL-treated JR1 cells persists in the absence of CK2 activity and that caspase activation is only transient when CK2 is functional. This was demonstrated by TRAIL-induced activation of effector caspase-6 and cleavage of XIAP in TRAIL-resistant JR1 RMS cells at early time points. However by 24 hours, apoptosis did not proceed as depicted by the absence of cleavage of both XIAP and caspases. Only the combination of TRAIL and DRB was associated with persistent caspase activity and apoptosis. These data suggest that RMS cells rely on CK2 activity to increase their chances of survival in response to TRAIL treatment. Overexpression of DN-FADD or CrmA in JR1 cells abrogated TRAIL-induced cell death in the presence of DRB, confirming that events upstream of the mitochondria were involved in the mechanism of DRB-induced sensitization to TRAIL-induced apoptosis. The role of the mitochondria in TRAIL-induced cell death has been evaluated in type I and II cells (51–53). In some cell lines, TRAIL-induced apoptosis was shown to be largely independent of mitochondrial pathways (54, 55); however, others have demonstrated that Bcl-xL protected pancreatic carcinoma cells from TRAIL-induced cell death (56). Taken together, data support the view that cell type-specific differences downstream of active initiator caspases are important in the fate of a cell after ligation of the TRAIL receptors. In JR1 cells overexpressing Bcl-2, TRAIL-induced apoptosis in the presence of DRB was completely inhibited. We have also demonstrated that inhibition of CK2 enhances TRAIL-induced release of cytochrome *c*, AIF, Smac/DIABLO, and HtrA2/Omi with concomitant inhibition of the function of XIAP. Therefore, activation of the mitochondrial apoptotic pathway is required for the execution of TRAIL-induced apoptosis in RMS cells.

The demonstration that shRNA can induce sequence-specific silencing in mammalian cells has begun to revolutionize the manner in which gene function is examined in cultured mammalian cells. These siRNAs (57) can be used to examine the consequences of reducing the function of virtually any protein-coding gene and have proved effective in provoking relevant phenotypes in numerous somatic cell types from both humans and mice. We have demonstrated that introduction of a shRNA duplex targeting CK2α decreases the expression of the CK2α subunit, thereby restoring TRAIL sensitivity in JR1 RMS cells, unequivocally demonstrating the importance of CK2 function in attenuating TRAIL-induced apoptosis. In addition, shRNA alone, targeting CK2α, also decreased XIAP protein

levels, suggesting an additional CK2 target in the TRAIL signaling pathway. Reducing the activity of CK2 using kinase-inactive CK2α (K68M) or CK2α′ (K69M) was also associated with increased sensitivity of RMS to TRAIL-induced apoptosis. It is apparent that a relatively small reduction in the expression level of CK2 can result in potent induction of apoptosis (58), indicating the important function of CK2 in promotion of cellular survival.

In summary, we propose additional mechanisms promoted by CK2 upstream of the function of both caspase-8 and Bid (at the level of the receptor and DISC formation) and downstream of the mitochondria (at the level of XIAP) in mediating TRAIL resistance. Decreased Bid processing found in TRAIL-stimulated, TRAIL-resistant cells may arise, at least in part, from the limited levels of procaspase-8 recruited to the level of the DISC and the active caspase-8 generated at the level of the DISC. Furthermore, we have demonstrated the role of CK2 as an antiapoptotic factor in cells derived from both ERMS (JR1) and ARMS (Rh30) and that inhibition of the function of this protein dramatically sensitized both subtypes of RMS to TRAIL-induced cell death. This may have future implications for therapy of RMS with the development of ligands against the TRAIL receptors for the treatment of human cancers, including pediatric cancers.

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