Phosphorylation of Murine Caspase-9 by the Protein Kinase Casein Kinase 2 Regulates Its Cleavage by Caspase-8/*

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Previous studies from our laboratory had indicated that cytochrome c-independent processing and activation of caspase-9 by caspase-8 contributed to early amplification of the caspase cascade in tumor necrosis factor (TNF)-α-treated murine cells. Here we show that murine caspase-9 is phosphorylated by casein kinase 2 (CK2) on a serine near the site of caspase-8 cleavage. CK2 has been shown to regulate cleavage of the pro-apoptotic Bid protein by phosphorylating serine residues near its caspase-8 cleavage site. Similarly, CK2 modification of Ser348 on caspase-9 appears to render the protease refractory to cleavage by active caspase-8. This phosphorylation did not affect the ability of caspase-9 to autoprocess. Substitution of Ser348 abolished phosphorylation but not cleavage, and a phospho-site mutant promoted apoptosis in TNF-α-treated caspase-9 knock-out mouse embryonic fibroblasts. Furthermore, inhibition of CK2 activity and RNA interference-mediated knockdown of the kinase accelerated caspase-9 activation, whereas phosphatase inhibition delayed both caspase-9 activation and death in response to TNF receptor occupation. Taken together, these studies show that TNF receptor cross-linking promotes dephosphorylation of caspase-9, rendering it susceptible to processing by activated caspase-8 protein. Thus, our data suggest that modification of pro-caspase-9 to protect it from inappropriate cleavage and activation is yet another mechanism by which the oncogenic kinase CK2 promotes survival.

Activation of mammalian initiator caspase-9 in intrinsic apoptotic pathways occurs by “induced proximity” or dimerization and is dependent on mitochondrial cyt c release (1, 2). However, cyt c-independent mechanisms of activation of this caspase have also been demonstrated. Caspase-12 was able to process and activate caspase-9 in response to endoplasmic reticulum stress, whereas Sendai virus infection triggered a novel pathway of caspase-9 cleavage (3–5). Furthermore, early amplification of the caspase cascade in response to TNF receptor cross-linking on Bcl-xL-expressing murine pro B lymphoma cells, in which the mitochondrial amplification loop is essentially unavailable, occurred via cyt c-independent activation of caspase-9 (6). This study did not rule out dimerization of caspase-9 in this pathway but implicated active caspase-8 in its processing and activation. The involvement of caspase-8 in apoptosome-independent activation of caspase-9 in murine cells has recently been demonstrated in another study (7). The observation that caspase-8 could directly activate an initiator caspase of the intrinsic pathway without the involvement of mitochondria in death receptor-induced apoptosis suggested that some murine cells harbor both Type I and II characteristics (8) and implied that additional regulatory mechanisms could protect caspase-9 from inappropriate activation.

Both human and murine caspase-9 harbor a number of serines, threonines, and tyrosines, and a variety of kinases including ERK2, protein kinase A, protein kinase Cζ, and c-Abl tyrosine kinase have been implicated in regulating the activation of this protease (9–12). Phosphorylation of Ser196 on human caspase-9 by the oncogenic kinase Akt is believed to regulate its apoptotic activity, although the murine form lacks this serine (13, 14). Protein kinase CK2 (formerly casein kinase II), is a serine/threonine kinase that affects several signaling pathways (15, 16). Unlike the majority of protein kinases, CK2 is constitutively active and independent of second messengers or phosphorylation events. It phosphorylates a variety of substrates involved in cell cycle control, differentiation and proliferation (17, 18). It is a highly conserved, ubiquitous, and pleiotropic protein kinase comprising two catalytic subunits, α and/or α′, and two regulatory (β) subunits that form a heterotetrameric holoenzyme (19). The high constitutive activity of CK2 in cancer cells is believed to underlie its oncogenic potential (15, 16, 20). CK2 recognizes phospho-acceptor sites that are specified by clusters of acidic residues, with the one at position n + 3 relative to the target amino acid playing a particularly crucial role (21). The frequency of aspartyl and glutamyl residues in its phosphoacceptor sites suggested that phosphorylation by CK2 could regulate caspase cleavage, which is
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known to occur at the carboxyl terminus of the acidic consensus E/DXD (17).

In the present study we show that CK2 modifies a serine in the vicinity of the primary processing site of murine caspase-9 in proliferating cells, protecting the protease from cleavage and activation by other caspases.

EXPERIMENTAL PROCEDURES

Cell Lines, Antibodies, and Plasmid Constructs—FL5.12 murine pro B lymphoma cells were cultured as described earlier (22). The generation of FL5.12-Neo and FL5.12-Bcl-xL cells has also been described previously (23). Electroporation of FL5.12 Bcl-xL cells with pGIPZ shRNA mir vectors was carried out under similar conditions, and transfectedants were selected in the presence of 2 µg/ml puromycin. Caspase-9−/−/− MEFs (a gift from Richard Flavell, Yale University) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin sulfate, and 10 mM β-mercaptoethanol. Caspase-9 monoclonal antibodies were purchased from Stressgen Biotechnologies and NeoMarkers, Inc., anti-FLAG M2 antibody from Sigma, anti-actin monoclonal from EMD Biosciences, anti-CK2 from Upstate/Millipore, and anti-vinculin from Abcam, Inc. The caspase-8 antibody was a gift from Idun Biotechnologies, and polyclonal phosphoserine-specific antibodies were from Qiagen. Active recombinant caspase-3 and caspase-8 were purchased from Pharmingen. The pGIPZ shRNA mir CK2 vectors and nonsilencing control were purchased from Open Biosystems (CK2-α clone identification code V2LMM_188343 and CK2-β clone identification code V2LMM_66668). Construction of full-length murine caspase-9 (mC-9) cDNA and the Myc-tagged version, mC-9myc has been previously described (6). Point mutants of mC-9, substituting Ser348 with alanine, glycine, or valine or Ser350 with alanine, were generated by a two-step PCR method. Inserts were ligated into HindIII/SalI sites of the pFLAG-CTC vector.

In Vitro Caspase Cleavage Assay—Radiolabeled mC-9 and mutant proteins were synthesized in vitro using the TnT T7 transcription/translation system (Promega Biotech) and 15 µCi of [35S]methionine (Amersham Biosciences) per reaction. Translated proteins were cleaved at 37 °C for 60 min using 5 µl of in vitro translation mix as substrate in the presence of active human recombinant caspase-3 (15 milliunits/µl), or caspase-8 (150 milliunits/µl), in a 25 µl of total reaction volume. The reactions were attenuated with an equal volume of 2× Laemmli buffer containing reducing agent and resolved by SDS-PAGE, and the gels were fixed, dried, and autoradiographed.

In Vitro Kinase Assays—in vitro translated products were immunoprecipitated with either anti-Myc (Santa Cruz) or caspase-9 (NeoMarkers) antibodies and incubated at 30 °C for 1 h in kinase buffer (50 mM Tris/HCl, pH 7.5, 10 mM MgCl2, 1 mM dithiothreitol, 10 µM β-glycerophosphate, 10 mM ATP, and 5 µCi of [γ-32P]ATP) with or without 0.05 milliunit purified CK2, in the presence or absence of inhibitors, DRB (200 µM) heparin (28 µg/ml), and apigenin (400 µM). Kinase reactions were attenuated in Laemmli buffer and resolved by SDS-PAGE, and the gels were fixed, dried, and autoradiographed. For kinase assays with untagged human and mouse caspase-9, in vitro translated products were immunoprecipitated with caspase-9 antibody prior to the kinase reaction. FL5.12 cell lysates (5 µg of protein), prepared as described by Desagher et al. (25), were incubated with 5 µg of purified recombinant mC-9 for 20 min at 30 °C in 50 µl of kinase buffer containing 5 µCi of [γ-32P]ATP with or without inhibitors. The inhibitors used were as follows: 60 µM emodin, 400 µM apigenin, 1 µM wortmannin, 100 µM cold GTP, or 7 µg/ml heparin. The protein was then immunoprecipitated with an anti-FLAG antibody, resolved by SDS-PAGE, and visualized by autoradiography.

To determine caspase-9 autoprocessing in a cell-free system, 5 µl of a 25-µl cold kinase reaction were incubated alone, with caspase-9−/− cytosolic S100 extract (2) (50 µg) or with extract, plus 1 mM ATP and 0.2 µg of cyt c in Buffer A (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride) in a total volume of 35 µl. The reactions were carried out for 30 min at 30 °C attenuated with Laemmli Buffer and Western blotted using anti-caspase-9 antibody (Stressgen). Phosphorylation of caspase-9 was confirmed in Western blots using anti-phosphoserine antibodies (Qiagen).

Cell Viability Assays—FL5.12 cells were harvested at regular intervals following the addition of TNF-α (5 ng/ml) and CHX (20 µg/ml) to the medium, and viability was determined by flow cytometric analysis of annexin V-fluorescein isothiocyanate and propidium iodide uptake. Inhibitors such as okadaic acid (1 µM), DRB (50 µM), or TBB (25 µM) were added 30 min to 3 h prior to the addition of TNF-α as indicated. Viability of shRNA transfectants was determined 24 h after withdrawal from IL-3-containing medium or exposure to 100 µM etoposide.

Western Blotting, Metabolic Labeling, and Immunoprecipitation—Cell pellets were washed in phosphate-buffered saline and lysed in radioimmunoprecipitation assay buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with pro-
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Results

CK2 Phosphorylates Murine Caspase-9 on Ser348 in Vitro—Previous studies from our laboratory had determined that activated caspase-9 contributed to early amplification of the death receptor pathway in type II murine cells (6), independent of cyt c release. This likely involved cleavage at the auto-processing motif, SEPD (residues 350–353 in murine caspase-9; Fig. 1A), which serves as the primary substrate site for processing by active caspase-8 in vitro. We investigated the possibility that regulatory mechanisms were in place to prevent the inappropriate activation of this caspase in healthy proliferating cells.

A scan of the sequence using NetPhos, a sequence- and structure-based prediction program of eukaryotic protein phosphorylation sites (24), revealed the first serine residue, Ser348, within the motif TLDSDSE in murine caspase-9, CK2 (Fig. 1A). CK2 is known to phosphorylate Bid on serine residues in the vicinity of the caspase-8 recognition site, rendering it resistant to cleavage by active caspase-8 in the absence of death receptor trimerization (25). In vitro kinase reactions using purified recombinant CK2 and in vitro translated murine caspase-9 as substrate (Fig. 1B) showed that caspase-9 was indeed a CK2 target. This phosphorylation was diminished in the presence of CK2 inhibitors. Auto-phosphorylation of the CK2 β-subunit (26) was also markedly reduced in the presence of the inhibitors. Fig. 1C shows that phosphorylation was reduced at least 4-fold in the LDAD mutant (Ser348 to Ala), relative to wild type, whereas auto-phosphorylation of the CK2 β-subunit in these reactions was unchanged in the absence of the inhibitor, DRB. The immunoprecipitation/Western in the lower panel indicates that the reduced levels of phosphorylation of the LDAD mutant are not the result of decreased translation relative to caspase-9 or a failure of the immunoprecipitating caspase-9 antibody to recognize the mutant protein. The kinase assay in Fig. 1D, using the AEPD (Ser350 to Ala) mutant and an additional mutant LDVD (Ser348 to Val), demonstrated that, at least in vitro, Ser350 was not a CK2 target. We had previously shown that activated caspase-8 could process caspase-9 efficiently at the SEPD motif (Ref. 6; Fig. 1A, left arrowhead). We tested the sensitivity of some of the serine mutants in cleavage assays to determine whether Ser348 affects the ability of active caspase-8 to process caspase-9 at Asp353 in vitro. Supplemental Fig. S1 shows that S348G (LDGD), S348V (LDVD), S348A (or LDAD, not shown) mutants, as well as S350A (AEPD) are all cleaved efficiently by the active initiator caspase.

Murine Caspase-9 Is Phosphorylated by Endogenous CK2—Having established that Ser348 within the LDSD motif could be efficiently phosphorylated by purified recombinant CK2 enzyme in vitro, we determined whether endogenous CK2 phosphorylated the caspase-9 protein. Lysates prepared from FL5.12 pro B lymphoma cells were incubated with purified recombinant mC-9-FLAG in the absence or presence of inhibitors and immunoprecipitated with an anti-FLAG antibody. Caspase-9 phosphorylation was unaffected by the phosphatidylinositol 3-kinase inhibitor, wortmannin, whereas specific inhibitors of CK2, such as emodin, apigenin, and GTP (27), as well as heparin, which exhibits a broader specificity, all inhibited phosphorylation of caspase-9. Significant reduction in caspase-9 phosphorylation observed in the presence of DRB indicates that the protein is also a substrate for CK2 in vivo. The presence of the 20-kDa fragment in the immu-
noprecipitation/Western of DRB-treated lysates may suggest an increased susceptibility of the hypophosphorylated caspase to processing.

CK2-mediated Phosphorylation of Ser\textsuperscript{348} on Caspase-9 Interferes with Its Cleavage by Caspase-8—Caspase-9 is a tightly regulated initiator caspase in mammalian cells. It is auto-activated by induced proximity or dimerization following the release of mitochondrial cyt\textsubscript{c} and the assembly of the apoptosome in the cytosol (1, 2, 18, 28). However, a number of groups have determined that cyt\textsubscript{c}-independent mechanisms could also contribute to activation of this caspase (3, 5, 6). Previous studies in our laboratory had suggested that initiator caspase-8 cleaves and activates murine caspase-9 early during TNF receptor-induced apoptosis in murine cells. It was possible that the presence of a modified Ser\textsuperscript{348} on caspase-9 proximal to its cleavage site (see Fig. 1) inhibited the cleavage in a manner similar to that observed with pro-apoptotic protein, Bid (25). To test this possibility, purified recombinant mC-9-FLAG was incubated in kinase buffer containing \([\gamma\textsuperscript{32P}]\)ATP in the presence or absence of purified CK2. B, phosphorylation of mC-9 in the presence of CK2 inhibitors (lanes 3–5): DRB, heparin, and apigenin. C, top panel, CK2 phosphorylation of mC-9 and the LDAD mutant in the presence or absence of DRB. Lower panel, immunoprecipitation (IP)/Western of cold in vitro translated mC-9 and LDAD. D, CK2 phosphorylation of mC-9 and mutants AEPD and LDVD in vitro.

products detected by chemiluminescence. Phosphorylation clearly resulted in a reduction in the levels of the 38-kDa cleavage product.

Next, in vitro translated \textsuperscript{35}S-labeled caspase-9 was phosphorylated by CK2 in vitro in the presence of ATP and then subjected to an active caspase-8 cleavage assay. The attenuated cleavage reactions were resolved by SDS-PAGE, and visualized by autoradiography. B, FL5.12 cells were cultured and harvested, first by autoradiography (top panel) and then by Western blotting (bottom panel).
increase its molecular mass only by 0.1–0.2 kDa, the modification did not cause a detectable shift in the mobility of caspase-9 in either 10 or 14% polyacrylamide gels. However, two-dimensional resolution of in vitro translated 35S-labeled, unphosphorylated, and phosphorylated caspase-9 (Fig. 3C) showed two major spots corresponding to isoelectric points of 5.8 and 5.9 (bottom panel). Densitometric analyses of a number of kinase assays determined that 30–60% of the recombinant caspase-9 was phosphorylated in the presence of purified recombinant CK2 in vitro (although the levels were much lower in whole cell lysates), and activated caspase-8 was able to cleave 30–35% of the unphosphorylated translated or purified recombinant mC-9 (not shown) within 30 min in the cleavage assays.

The observation that caspase-9 phosphorylated on Ser348 was refractory to processing at the SEPD motif strongly suggested that dephosphorylation of endogenous caspase-9 precedes its activation by caspase-8 in the death receptor pathway. To determine whether phosphorylation of murine caspase-9 affected its ability to autoprocess in the intrinsic apoptotic pathway following its recruitment to the apoptosome, CK2 phosphorylated caspase-9 or a control was incubated with extracts from caspase-9−/− MEFs in the presence or absence of ATP and cyt c (2). The results (supplemental Fig. S2) indicate that the autoprocessing ability of the unphosphorylated caspase is similar to that of CK2 phosphorylated form, suggesting that phosphorylation at Ser348 does not affect its activation through the mitochondrial pathway.

Caspase-9 Dephosphorylation and Cleavage Are Both Necessary and Sufficient for an Apoptotic Response to TNF-α in Murine pro B Cells and Embryo Fibroblasts—If endogenous caspase-9 could be processed and activated by a cyt c-independent mechanism early in the death receptor pathway (6), then TNF receptor trimerization could be expected to promote its dephosphorylation prior to cleavage. To test this, phosphate-starved FL5.12-Bcl-xL cells were labeled with [32P]orthophosphate (0.5 mCi) for 6 h. The overexpressed Bcl-xL in these cells prevents mitochondrial cyt c release (29, 30), allowing detection of the alternative pathway. Three hours following the addition of radiolabel, the culture medium was supplemented with TNF-α/CHX or CHX alone. Fig. 4A shows that a decrease in levels of the phosphorylated form following TNF-α/CHX exposure (upper panel) was accompanied by processing of the dephosphorylated form (lower panel).

We had previously identified SEPD, the autoprocessing site on mC-9, as the primary cleavage site for activated caspase-8 in vitro (6). These data and the result in Fig. 4A suggested that both dephosphorylation and processing at this site were critical for the TNF response. To determine this, we expressed caspase-9 and relevant point mutants in knock-out cells. Caspase-9 knock-out MEFs are resistant to low doses (5 ng/ml) of TNF-α, although they do mount an apoptotic response in 24 h to concentrations about 10 times higher (31). The MEFs were transiently transfected with empty vector or plasmids encoding mC-9, LDAD, or SEPD. The results (supplemental Fig. S3) indicate that dephosphorylation and processing at SEPD, as predicted by our in vitro experiments, were critical for the TNF response. As shown in supplemental Fig. S3A, the SEPD mutants were refractory to processing in both HeLa and MEFs. Fig. 4B shows that expression of the noncleavable mutant (SEPD) or the empty vector continued to be unresponsive. Fig. 4C shows a quantification of the data, expressed as the percentage of transfected

![FIGURE 3. Phosphorylation of murine caspase-9 CK2 decreases its susceptibility to cleavage by active caspase-8.](image-url)
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**FIGURE 4.** Caspase-9 dephosphorylation and cleavage are both necessary and sufficient for an apoptotic response to TNF-α. A, Bcl-xL FL5.12 cells were labeled with 32P orthophosphate (0.5 μCi) for 6 h. Three hours following the addition of radiolabel, the culture medium was supplemented with TNF-α/CHX or CHX alone. Endogenous caspase-9 was immunoprecipitated, SDS-PAGE-resolved, transferred to nitrocellulose, and visualized, first by autoradiography (top panel), and then by Western blotting (lower panel) with an antibody against caspase-9 (AAM-139, Stressgen) that recognizes both full-length and processed protein. B, caspase-9−/− fibroblasts were transiently transfected with empty vector, wild type caspase-9, LDAD, or SEPA constructs in chamber slides and either incubated with TNF-α/CHX (or CHX alone, not shown) 36 h post-transfection. The cells were fixed and stained 4 h later and observed using an Olympus Fluoview 1000 multiphoton confocal microscope. C, quantification of data from the transfection experiments. The y axis indicates percent caspase-9 expressing green fluorescent cells with condensed (apoptotic) nuclei (mean and standard error, n = 3).

**TNF-α-induced Caspase-9 Processing and Activation in FL5.12 Cells Is Accelerated by CK2-specific Inhibitors and Inhibited by the Serine Phosphatase Inhibitor Okadaic Acid**—It has been reported that CK2 inhibits Fas ligand and TNF-α-induced apoptosis (25, 32, 33). We determined whether inhibition of endogenous CK2 would enhance the rate of cell death induced by TNF receptor cross-linking in FL5.12 cells. The results in Fig. 5A show that preincubation with the CK2-specific inhibitor TBB accelerated the onset of apoptosis in TNF-α-treated FL5.12 cells as assessed by flow cytometry. The acceleration in control cells could be attributed to enhanced cleavage of Bid and early release of mitochondrial cyt c. However, the mitochondrial route is largely blocked in Bcl-xL-overexpressing cells at early time points, suggesting that CK2 inhibition might also accelerate caspase-9 cleavage and activation. Caspase-9 activity was measured in cell pellets collected at 2-h intervals following the addition of ligand (Fig. 5B). The results indicate that the accelerated response to TNF-α in cells preincubated with CK2 inhibitor TBB is due in large part to the early activation of caspase-9, particularly in Bcl-xL-overexpressing cells.

The studies described above strongly suggest that dephosphorylation of murine caspase-9 precedes its cleavage and activation in TNF receptor-induced cell death. The dephosphorylation of CK2 targets that occurs in response to a death stimulus is crucial for the normal control of cell signaling and is accomplished in part by the activation of protein phosphatases such as protein phosphatase 2A (34, 35). However, the naturally occurring protein phosphatase 2A inhibitor okadaic acid (OA) appears not to abrogate, and may even enhance, caspase-8 activation in response to Fas ligand (36). Okadaic acid delayed the onset of TNF-induced apoptosis in both controls and Bcl-xL-overexpressing cells (supplemental Fig. S3). Keeping in perspective the possibility that OA could affect a number of phosphatase dependent pathways, we asked whether the protection of TNF-treated FL5.12 by OA, at least in part, included caspase-8 or its downstream targets. Western blots of cell lysates were tested for the presence of cleaved caspases-8 and 9 (Fig. 5C) following TNF cross-linking in the presence or absence of the phosphatase inhibitor. The presence of the small (p10) cleavage product of caspase-8 in cells treated with TNF, both in the absence or in the presence of OA, suggested that the inhibitory effect was downstream of this initiator caspase.
Although initiator caspases (caspases-8 and -9) undergo auto-activation in intrinsic apoptotic pathways, effector caspases (caspases-3, -6, and -7) are activated following processing at specific cleavage sites by other activated caspases. In the intrinsic apoptotic pathway auto-activation of initiator caspase-9 by induced proximity or homodimerization is dependent on the release of mitochondrial cyt c and occurs following its recruitment to the apoptosome (2, 40). However, growing evidence points to alternate mechanisms of activation for this caspase that involve neither cyt c release nor Apaf-1 activation (3, 5–7). Previous studies from our laboratory had suggested that murine caspase-9 could be directly cleaved and activated by initiator caspase-8 during death receptor-induced apoptosis, implying that caspase-9 was functioning not as an initiator but as an effector caspase early in this apoptotic pathway (Ref. 6 and Fig. 5). In the current study we have described a mechanism by which the sensitivity of caspase-9 to cleavage by caspase-8 is controlled by phosphorylation. We show that residue Ser153 on murine caspase-9 is specifically phosphorylated by CK2 and that the phosphorylation protects it from cleavage at Asp353 in the processing motif.

To our knowledge, this is the first example of phospho-regulation of caspase-9 activation in an extrinsic apoptotic pathway. A variety of kinases, including ERK2, protein kinase A, protein kinase Cζ and c-Abl tyrosine kinase, have been implicated in the regulation of mammalian caspase-9 function in the intrinsic pathway. For instance, ERK/mitogen-activated protein kinase (MAPK)-mediated phosphorylation of human caspase-9 at a conserved threonine is sufficient to block its processing (9), and protein kinase A inhibits cyt c-dependent recruitment of pro-caspase-9 to the apoptosome (11). Phosphorylation of Ser153 on caspase-9 by protein kinase Cζ during hyperosmotic stress also prevents the activation of the intrinsic apoptotic pathway (10), whereas c-Abl-mediated tyrosine phosphorylation of caspase-9 on residue 153 contributes to its DNA damage-induced autoprocessing and, subsequently, to apoptosis (12). It is interesting to note that human caspase-9 is not phosphorylated by CK2 in vitro (supplemental Fig. S4A).

Early indications that human and murine caspase-9 may be regulated differently came from a study showing that the Akt phosphorylation site (at Ser196), presumed to be involved in the inactivation of human caspase-9, is absent in the murine form (13, 14). Furthermore, the 46-kDa human caspase-9 protein lacks residues 99–131 of its murine counterpart, a 50-kDa protein. Although human protein harbors a serine residue at a similar position, flanking residues do not conform to the CK2 requirement for acidic residue clusters (21) and thus constitute a weak consensus for the kinase. Alignment of a few caspase-9 sequences in the vicinity of the CK2 motif shows that rat and dog caspase-9 also harbor strong CK2 consensi (supplemental Fig. S4B). The absence of a strong CK2 target consensus in the human protein is not surprising for another reason. Human cells have been classified as cells of type I or type II based on the nature of their response to death receptor activation by ligands such as Fas and TNF-α. Survival members of the Bcl-2 family, by virtue of their ability to prevent cyt c release, therefore, impart almost total protection against apoptosis induced via Fas receptor cross-linking to type II cells. The activation of caspase-9 in the presence of Bcl-\textsubscript{xL} and in the absence of cyt c release during TNF-induced apoptosis precludes classification by an upstream initiator caspase but does not affect its ability to auto-activate in intrinsic apoptotic pathways.

**DISCUSSION**

Caspases, a family of cysteiny l aspartate-specific proteases, are the executioners of apoptotic cell death (38). Caspases are synthesized aszymogens with a prodomain of variable length followed by a large subunit and a small subunit and are activated through proteolysis at the carboxyl-terminal aspartic acid residues in specific tetrapeptide processing motifs (39). Although initiator caspases (caspases-8 and -9) undergo auto-
of some murine cells as Type II cells (6). In vitro cleavage assays had previously shown that human caspase-9 is cleaved less efficiently than the murine protein by active caspase-8 and that DQLD (Asp330), rather than PEPD (Asp315), was the preferred processing site (6). This difference in susceptibility between human and murine caspase-9 to both processing by active caspase-8 as well as to phosphomodification of a proximal serine may help explain the difference in response to death receptor activation between human cells of type II and murine cells that elude strict classification as Type II cells.

CK2 is a ubiquitous and constitutively active kinase with oncogenic potential. In addition to modifying hundreds of known substrates with cell growth, differentiation, and cell cycle functions (17, 18), this kinase counters apoptosis promoted by DNA damaging agents as well as death receptors (33, 41). A number of proteins have been reported to become refractory to caspase cleavage upon CK2-mediated phosphorylation (42–45). ARC, a protein that inhibits caspase-8 activity when phosphorylated, has also been identified as a CK2 target (46). Recently procaspase-2 has emerged as a CK2 target and shown to be activated by dimerization independent of the PIDDosome (a complex comprising death domain-containing protein, PIDD, and the adaptor protein, RAIDD) following dephosphorylation (47). In the current study, we have identified yet another pro-apoptotic protein that is rendered resistant to caspase-8 by CK2-mediated modification. Thus, CK2 appears to exert its survival role by controlling the activity of pro-apoptotic caspase substrates in specific signaling pathways. We suggest, furthermore, that constitutive activation of this kinase ensures that the CK2 target site remains modified and protected unless specifically dephosphorylated in response to an apoptotic stimulus. The appearance of a 20-kDa (late) processing product of caspase-9 in cells incubated with the CK2 inhibitor, DRB (Fig. 2B), and the presence of the 38-kDa cleavage product in untreated CK2 negative cells (Fig. 6C) suggests that the dephosphorylated protein may be susceptible to low levels of active caspasas in the cell. Studies have shown that

![Figure 6](image-url)
caspase-8 is active at a low basal level in a number of cells (48). The absence of the 38- and 40-kDa intermediates in Fig. 2B is currently unclear, although it is tempting to speculate that in the absence of a death stimulus the 38-kDa cleavage product of caspase-8 activity may be the only intermediate generated and that even this is quickly processed down to the 20-kDa active subunit in FL5.12 cells.

Exposure of murine FL5.12 pro B lymphoma cells to TNF-α promotes dephosphorylation of caspase-9, rendering it susceptible to proteolytic processing. Inhibition of CK2 activity in the presence of the apoptotic trigger accelerates both caspase-9 activity and cell death. Although the dephosphorylation of caspase-9 during death receptor activation is likely to be the combined result of activated phosphatase(s) and CK2 down-regulation or inhibition, it is significant that in protecting FL5.12 cells, a naturally occurring inhibitor of serine/threonine protein phosphatases (okadaic acid) specifically inhibits caspase-9 cleavage without affecting auto-processing and activation of caspase-8, the initiator caspase (Fig. 5). This experiment also confirms that activated caspase-9 is a major contributor to death induced by TNF-α in these cells. The inability of caspase-9-negative MEF transfectants expressing the noncleavable SEPA mutant to activate apoptosis in response to TNF treatment (unlike wild type caspase and LDAD expressing MEFs) is further evidence that processing of murine caspase-9 is essential for the death response (Fig. 4B).

In conclusion, we have shown that a serine residue adjacent to the primary site of caspase-8 cleavage on murine caspase-9 is modified by the survival protein kinase, CK2, in vitro and in vivo. We have also shown that this modification lowers the susceptibility of caspase-9 to cleavage but does not affect caspase-9 auto-processing (model in Fig. 7). Phosphatase inhibitors delay TNF-induced apoptosis in control cells as well as in cells over-expressing the Bcl-xL protein. Inhibition of intracellular CK2 or loss of the kinase accelerates apoptosis and promotes early caspase-9 activation in response to TNF-α and cycloheximide but does not affect the ability of anti-apoptotic protein Bcl-xL to protect against inducers of mitochondrial apoptotic pathways. These studies strongly suggest that modification of murine procaspase-9 in the vicinity of its processing motif protects the protease from mistimed or inappropriate activation by other caspases and further emphasize the role of the ubiquitous kinase CK2 in promoting cell survival.

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