Oncogene (2008) 27, 2513–2524

www.nature.com/onc

ORIGINAL ARTICLE

CK2 controls TRAIL and Fas sensitivity by regulating FLIP levels in endometrial carcinoma cells

D Llobet[1], N Eritja[1], M Encinas[2], N Llecha[1], A Yeramian[1], J Pallares[1], A Sorolla[1], FJ Gonzalez-Tallada[3], X Matias-Guiu[1,4] and X Dolcet[1,4]

[1]*Oncologic Pathology Group, Institut de Recerca Biomèdica de Lleida, IRBLleida, Lleida, Spain;* [2]*Cell signalling and Apoptosis Group, Institut de Recerca Biomèdica de Lleida, IRBLleida, Lleida, Spain and* [3]*Department of Gynecology, Institut de Recerca Biomèdica de Lleida, IRBLleida, Lleida, Spain*

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has emerged as a promising antineoplastic agent because of its ability to selectively kill tumoral cells. However, some cancer cells are resistant to TRAIL-induced apoptosis. We have previously demonstrated that in endometrial carcinoma cells such resistance is caused by elevated FLICE-inhibitory protein (FLIP) levels. The present study focuses on the mechanisms by which FLIP could be modulated to sensitize endometrial carcinoma cells to TRAIL-induced apoptosis. We find that inhibition of casein kinase (CK2) sensitizes endometrial carcinoma cells to TRAIL- and Fas-induced apoptosis. CK2 inhibition correlates with a reduction of FLIP protein, suggesting that CK2 regulates resistance to TRAIL and Fas by controlling FLIP levels. FLIP downregulation correlates with a reduction of mRNA and is prevented by addition of the MG-132, suggesting that CK2 inhibition results in a proteasome-mediated degradation of FLIP. Consistently, forced expression of FLIP restores resistance to TRAIL and Fas. Moreover, knockdown of either FADD or caspase-8 abrogates apoptosis triggered by inhibition of CK2, indicating that CK2 sensitization requires formation of functional DISC. Finally, because of the possible role of both TRAIL and CK2 in cancer therapy, we demonstrate that CK2 inhibition sensitizes primary endometrial carcinoma explants to TRAIL apoptosis. In conclusion, we demonstrate that CK2 regulates endometrial carcinoma cell sensitivity to TRAIL and Fas by regulating FLIP levels.
Oncogene (2008) **27,** 2513–2524; doi:10.1038/sj.onc.1210924; published online 5 November 2007

Keywords: endometrial carcinoma; apoptosis; death receptor; casein kinase 2; FLIP

Correspondence: Dr X Dolcet, Laboratori de Recerca Biomèdica, Hospital University Arnau de Vilanova, Institut de Recerca Biomèdica de Lleida, IRBLleida, Av Rovira Roure 80, Lleida 25198, Spain.
E-mail: dolcet@cmb.udl.es
[4]These are the senior co-authors.
Received 21 August 2007; revised 15 October 2007; accepted 17 October 2007; published online 5 November 2007

Introduction

The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and Fas Ligand (FasL) belong to the pro-apoptotic cytokines of the tumor necrosis factor (TNF) superfamily. TRAIL induces apoptosis in many types of cancer with limited cytotoxicity on normal cells (Ashkenazi *et al*., 1999; Walczak *et al*., 1999) indicating that it may become a promising anticancer agent (Srivastava, 2001; Takeda *et al*., 2007). However, some neoplastic cells are resistant to TRAIL and recent evidences show that both cytokines can even induce proliferation of normal or neoplastic cells. TRAIL can bind four different receptors DR4/TRAIL-R1 (MacFarlane *et al*., 1997; Pan *et al*., 1997b), DR5/TRAIL-R2 (Sheridan *et al*., 1997; Walczak *et al*., 1997), DcR1/TRAIL-R3 (Pan *et al*., 1997a) and DcR2/TRAIL-R4 (Pan *et al*., 1998). DR4 and DR5 are functional receptors that induce apoptosis upon ligation with TRAIL. DcR1 and DcR2, known as decoy receptors, lack the intracellular domains required to induce apoptosis (LeBlanc and Ashkenazi, 2003).

Both FasL and TRAIL trigger similar intracellular signaling pathways. Engagement of Fas or TRAIL receptors leads to the formation of a death-inducing signaling complex (DISC). The intracellular death domain (DD) of these receptors recruits Fas Associated DD-containing protein (FADD) which in turn binds procaspase-8. After recruitment to the DISC, procaspase-8 is activated by autoproteolytic cleavage resulting in the initiation of apoptotic signaling (Bodmer *et al*., 2000; Kischkel *et al*., 2000; Sprick *et al*., 2000). One of the critical regulators of apoptosis triggered by FasL and TRAIL is the FLICE-inhibitory protein (FLIP) (Thome *et al*., 1997; Irmler *et al*., 2000). High levels of FLIP are found in many tumoral tissues including endometrial carcinoma. We have recently demonstrated that FLIP plays a key role in the regulation of sensitivity of endometrial carcinoma cells (ECCs) to TRAIL-induced apoptosis. In this previous work, we show that inhibition of FLIP expression is enough to sensitize endometrial cancer cells to TRAIL-induced apoptosis (Dolcet *et al*., 2005). FLIP shares a high degree of homology with caspase-8, and contain two death effector domains (DED) and a defective caspase-like

domain that lacks proteolytic activity. Thus, high levels of FLIP compete with caspase-8 and displace its binding to FADD, which results in inhibition of apoptosis.

Because of the importance of FLIP in sensitization of ECC to TRAIL-induced apoptosis, we focused in the search for possible mechanisms by which FLIP expression could be modulated. Increasing evidences in the literature suggest that the protein casein kinase (CK2) may play an important role in the regulation of TRAIL-induced apoptosis in many cell types. CK2 is a ubiquitous, highly conserved tetrameric serine threonine/kinase integrated by two regulatory subunits called CK2β and two catalytic subunits which can be two of either the subunits CK2α or CK2α′ (Padmanabha *et al*., 1990; Kikkawa *et al*., 1992; Allende and Allende, 1995). It is well established that increased CK2 activity is associated with cell growth and proliferation and many types of tumors display aberrant or increased CK2 activity (Tawfic *et al*., 2001; Ahmed *et al*., 2002; Litchfield, 2003). Importantly, transgenic mice expressing CK2α in lymphocytes or mammary gland display an increased incidence of lymphomas and breast carcinomas (Seldin and Leder, 1995; Landesman-Bollag *et al*., 2001). Recent evidences point to CK2 as an important regulator of apoptosis by both intrinsic/mitochondrial and extrinsic/death receptor apoptotic pathways. Inhibition of CK2 results in sensitization of resistant prostate (Wang *et al*., 2005a, b, 2006), esophageal (Shin *et al*., 2005), colon (Ravi and Bedi, 2002; Izeradjene *et al*., 2005) and rhabdomyosarcoma cancer cells (Izeradjene *et al*., 2004) to apoptosis induced by TRAIL treatment. Such evidences have pointed CK2 as a possible target for cancer therapy (Ahmad *et al*., 2005; Wang *et al*., 2005b).

Here, we provide evidence that CK2 inhibition sensitizes ECC to TRAIL and agonistic Fas antibodies (aFas) by regulating FLIP protein levels. Importantly, CK2 inhibition correlates with reduction of endogenous FLIP levels. Such reduction is caused by both transcriptional downregulation of FLIP expression and increased FLIP protein proteasomal degradation Consistently, in ECC treated with CK2 inhibitors or CK2β short hairpin RNAs (shRNAs), overexpression of FLIP completely abolishes caspase activation and restores resistance to both ligands. Accordingly, downregulation of FADD or caspase-8 by specific shRNA also blocks apoptosis triggered by TRAIL or aFas. Finally, we show that inhibition of CK2 sensitizes primary endometrial carcinoma explants to TRAIL-induced apoptosis, suggesting that CK2 might be an important target for cancer therapy.

Results

CK2 regulates TRAIL- and Fas-induced apoptosis in endometrial carcinoma cells

First, we explored the viability of ECC after TRAIL or aFas treatment by an 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay (MTT) cytotoxicity assay. The Ishikawa 3-H-12 cell line (IK) cell line displayed no reduction of viability after 24 or 48 h treatment with doses up to 100 ng ml^{-1} of either aFas or TRAIL. To ascertain whether CK2 could regulate cytotoxicity induced by such death ligands, we treated IK with three pharmacological inhibitors of CK2, namely 5,6-dichloro-ribifuranosylbenzimidazole (DRB), apigenin and emodin in presence or absence of increasing doses of aFas or TRAIL. Both aFas and TRAIL together with any of all three CK2 inhibitors significantly reduced cell viability of IK after 24 or 48 h (Figure 1). In contrast, the RL-95 cell line showed a significant reduction of cell viability upon aFas or TRAIL stimulation after 24 h treatment which was further increased at 48 h (Figure 1). Despite the sensitivity of RL-95 cells, addition of CK2 inhibitors caused further decrease on viability induced by both ligands. These results suggest that CK2 regulates sensitivity to both TRAIL and aFas.

To ascertain whether the decrease in cell viability was specifically caused by apoptotic cell death, we quantified the number of nuclei displaying apoptotic morphology by Hoechst staining and we assessed caspase processing and activation by western blot to initiator caspases-8, -9 and -2 and the effector caspase-3. Treatment of IK cells with DRB, apigenin or emodin plus either aFas or TRAIL caused a marked increase in the number of nuclei showing apoptotic morphology (Figure 2a). As we show in Figure 2b, treatment of IK cells with either TRAIL or aFas plus CK2 inhibitors resulted in processing of the initiator caspases-8, -9 and -2, and the effector caspase-3.

Next, we used lentiviral delivery of shRNA to achieve a more specific inhibition of CK2. To avoid problems associated with possible redundancy of CK2α and CK2α′ catalytic subunits, we decided to target the regulatory subunit CK2β for shRNA design. One of the shRNAs designed significantly reduced the expression of the CK2β subunits and was chosen for subsequent experiments (data not shown). IK cells were infected with lentiviruses carrying shRNA to CK2β. After 3–4 days to allow gene silencing, cells were treated with 50 ng ml^{-1} of TRAIL. Apoptotic cell death was also assessed by Hoechst staining and caspase activation. Knockdown of CK2β by itself caused a slightly increase in the number of apoptotic cells (10–12%), which was dramatically increased after addition of TRAIL (Figure 2c). In line with the results obtained with pharmacological CK2 inhibitors, IK cells in which CK2β was silenced, addition of TRAIL or aFas resulted in processing of the initiator caspase-8, -9 and -2, and the effector caspase-3 (Figure 2d). It is worth to mention that downregulation of CK2β caused a remarkable increase in the amount caspase-8 fragment p43/p41 and residual activation of caspases. This activation is compatible with the slight increase in the number of apoptotic cells observed by Hoechst staining. Taken together, these results strongly support a key role in regulation of sensitivity to apoptosis triggered by aFas and TRAIL in ECC.

Inhibition of CK2 depletes endogenous FLIP

Having demonstrated that CK2 is critical in the regulation of cell death induced by either TRAIL or

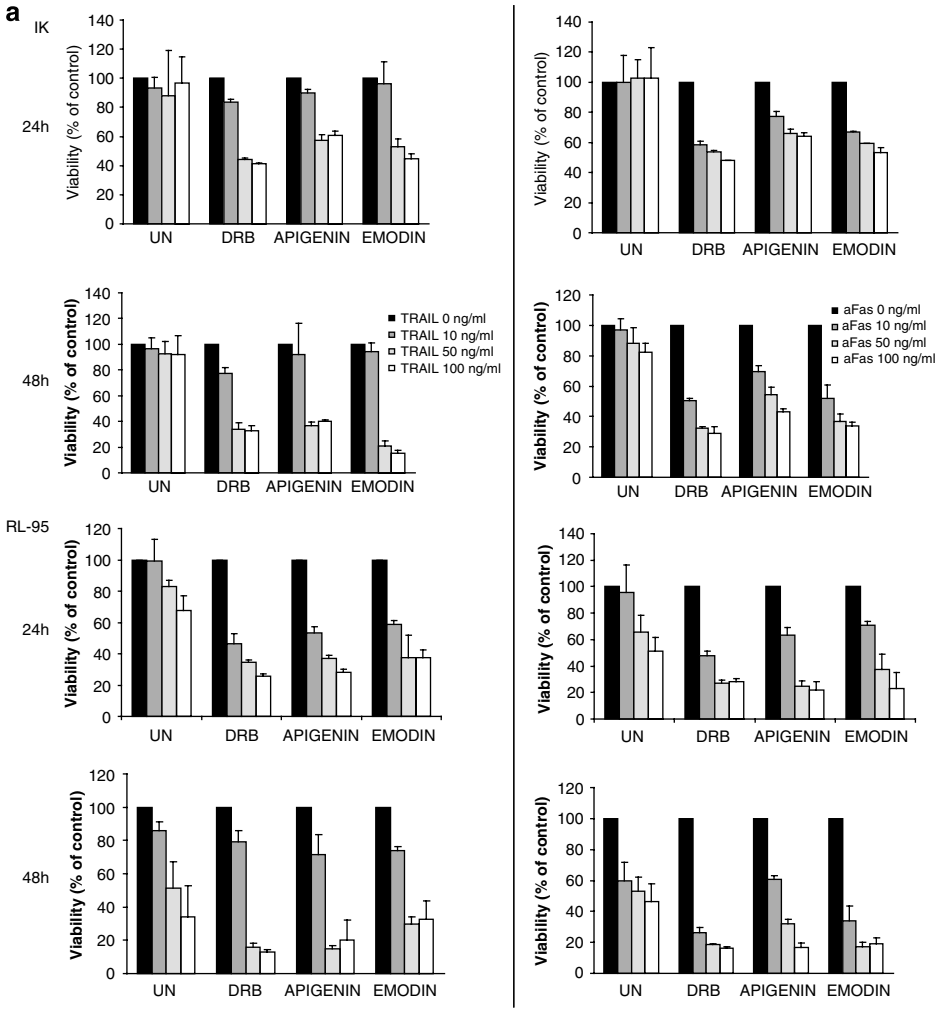

Figure 1 CK2 inhibitors plus tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) or agonistic Fas antibodies (aFas) reduce cell viability of ECC. The ECC lines IK and RL-95 were pretreated for 2 h with 5,6-dichloro-ribifuranosylbenzimidazole (DRB) 50 μM, apigenin 50 μM or emodin 150 μM and then left untreated (UN) or treated with 10, 50 or 100 ng ml^{-1} of either TRAIL or agonistic antibody (aFas). Cell viability was measured 24 or 48 h later. Results are presented as percentage of survival of TRAIL or aFas conditions over each control condition.

aFas on ECC, we focused our interest in investigating the mechanism of such regulation. FLIP is a key regulator of both TRAIL- and FasL-induced of apoptosis in many cell types, and we have recently demonstrated that FLIP is necessary and sufficient to prevent TRAIL-induced apoptosis of ECC (Dolcet *et al*., 2005). In this scenario, we postulated that FLIP protein levels might be regulated by CK2. To test this hypothesis, we analysed FLIP expression under different experimental conditions. First, we pretreated IK cells with DRB for 6–8 h and then treated IK cells with TRAIL for the indicated times (Figure 3a). FLIP levels decreased after treatment with DRB alone, but addition of TRAIL further decreased FLIP levels as soon as 3 h after its addition. Such reduction was also observed when IK and RL-95 cells were treated with DRB plus either TRAIL (Figure 3b). It is worth to mention that in RL-95 cells, treatment with TRAIL without DRB, reduced the levels of FLIP, which correlated with the sensitivity of these cells to TRAIL apoptosis after 24 h treatment (Figure 3b). Similar results were obtained with IK treated with apigenin or DRB plus either TRAIL or aFas. Treatment of IK cells with DRB or apigenin reduced FLIP levels which were even increased after addition of either TRAIL or aFas (Figure 3c). In agreement with the data obtained with CK2 inhibitors, silencing of CK2β by shRNA reduced the levels of FLIP, which were further diminished after addition of TRAIL (Figure 3d). All these data support the hypothesis that CK2 sensitizes ECCs to TRAIL-induced apoptosis by diminishing FLIP protein levels.

Next, we investigated the mechanisms by which CK2 regulates FLIP levels. The levels of endogenous FLIP protein can be controlled transcriptionally but recent evidences also suggest that endogenous FLIP protein levels may be regulated by the ubiquitin proteasome system. To ascertain whether FLIP levels are transcriptionally regulated, we performed real-time PCR on mRNA extracted from IK cells treated with apigenin or CK2β shRNA. Both apigenin and shRNA cause a significant decrease of the

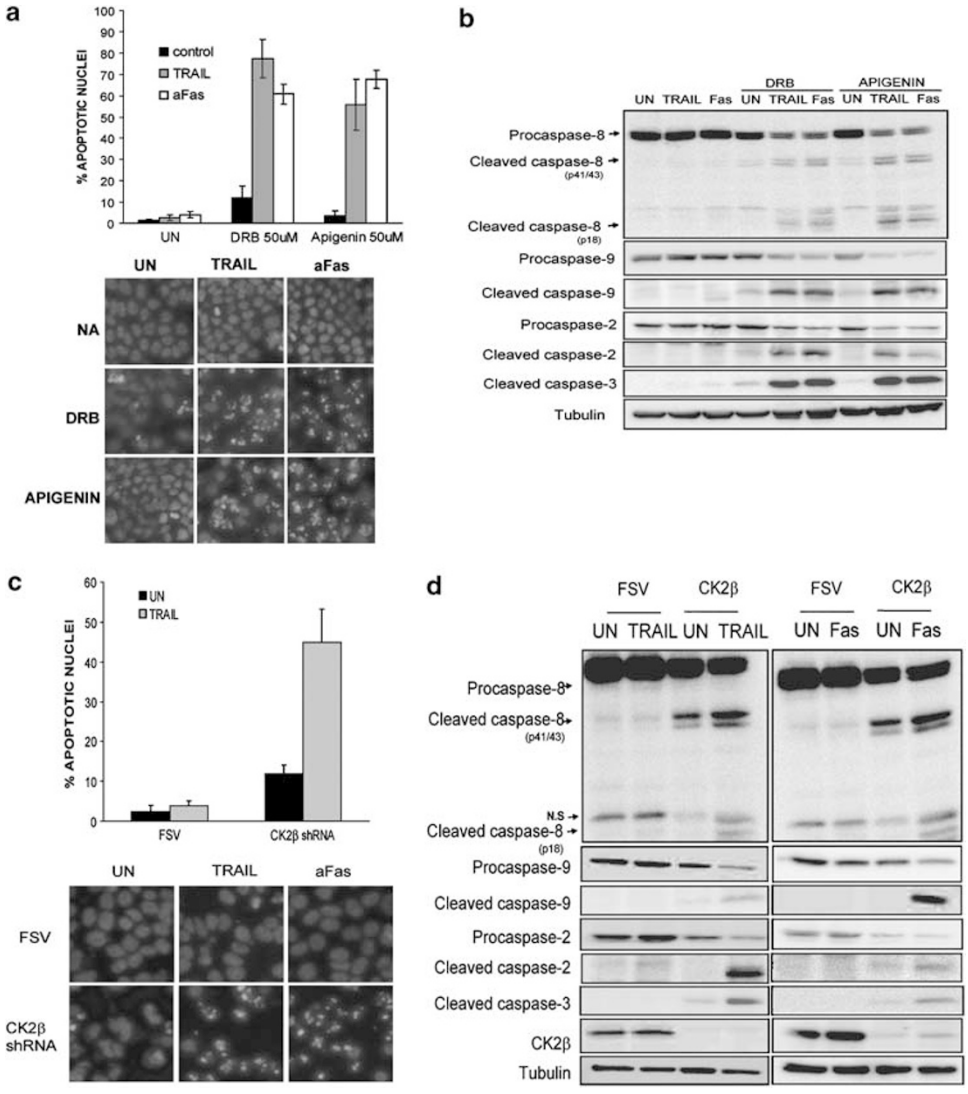

Figure 2 Inhibition of casein kinase (CK2) sensitize ECC to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and agonistic Fas antibodies (aFas)-induced apoptosis. (**a**) Treatment of ECC with pharmacological inhibitors of CK2 plus either TRAIL or aFas increase the number of apoptotic nuclei. Top, IK cells were pretreated for 2 h with 5,6-dichloro-ribifuranosylbenzimidazole (DRB) 50 μM, apigenin 50 μM and 50 ng ml^{-1} of TRAIL or aFas were added to the culture. Nuclei displaying apoptotic morphology were visualized by Hoechst staining counted 24 h later. Results are presented as percentage of apoptotic nuclei compared to the total number of nuclei. Bottom, representative micrographs of IK cells treated as indicated. (**b**). Pharmacological inhibitors of CK2 plus 50 ng ml^{-1} of either TRAIL or aFas activate caspases-8, -9, -2 and -3. IK cells were pretreated for 2 h with DRB 50 μM, apigenin 50 μM and stimulated with 50 ng ml^{-1} TRAIL or aFas or left untreated (UN). After 18 h, cells were lysed and protein extracts were analysed by western blot with antibodies to the indicated caspases. Membranes were reprobed with tubulin to ensure equal protein amounts. NS (nonspecific bands). (**c**) Treatment of ECC with short hairpin RNAs (shRNA) to CK2β plus either TRAIL or aFas increases the number of apoptotic nuclei. IK cells were infected with lentiviruses carrying shRNA to CK2β subunit. After 3 days to allow downregulation of the protein, cells were treated with 50 ng ml^{-1} TRAIL. Top, nuclei displaying apoptotic morphology were visualized by Hoechst staining and counted 24 h later. Results are presented as percentage of apoptotic nuclei compared to the total number of nuclei. Bottom, representative micrographs of IK cells treated as indicated. (**d**) shRNA to CK2β plus 50 ng ml^{-1} of either TRAIL or aFas activate caspases-8, -9, -2 and -3. IK cells were infected with lentiviruses carrying shRNA to CK2β subunit. After 3 days to allow downregulation of the protein, cells were treated with 50 ng ml^{-1} of TRAIL or aFas. After 8 h, cells were lysed and protein extracts were analysed by western blot with antibodies to the indicated caspases. Membranes were reproved with tubulin to ensure equal protein amounts and antibodies to CK2β subunit to check inhibition of CK2β expression by the shRNA. NS (nonspecific bands).

mRNA levels compared to the untreated cells (Figure 3e). As a control of FLIP downregulation, we analysed FLIP mRNA from IK cells infected with FLIP shRNA.

FLIP levels are also regulated by ubiquitin–proteasome-mediated degradation (Kim *et al*., 2002; Poukkula *et al*., 2005; Chang *et al*., 2006). To determine whether proteasomal degradation was also involved in downregulation of FLIP protein, we treated IK cells with apigenin in presence or absence of the proteasome inhibitor MG-132. As we show in Figure 3f, addition of MG-132 completely inhibits the reduction in FLIP protein caused by either DRB or apigenin. These results suggest that inhibition of CK2 triggers FLIP degradation through the proteasome.

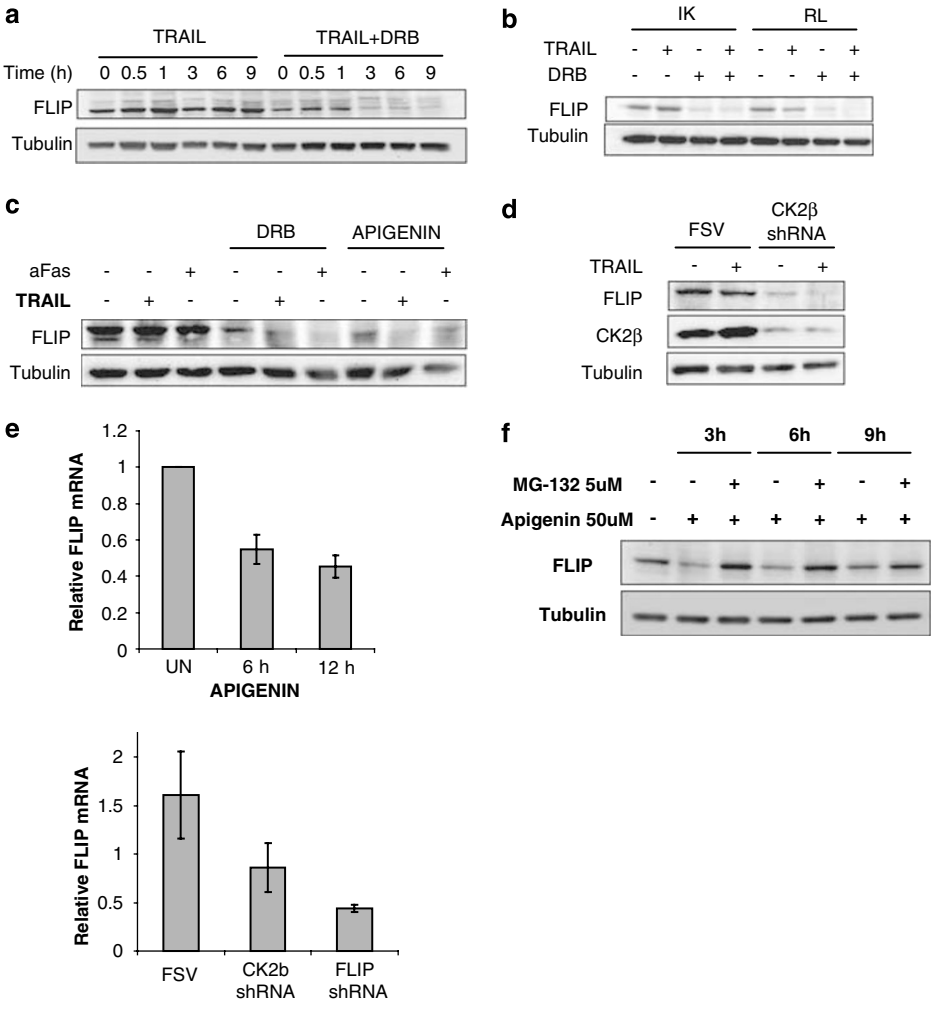

Figure 3 Inhibition of casein kinase (CK2) reduces FLICE-inhibitory protein (FLIP) levels. (**a**) FLIP protein levels are reduced by 5,6-dichloro-ribifuranosylbenzimidazole (DRB) and further decreased after addition of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Ishikawa 3-H-12 cell line (IK) cells were pretreated for 3 h with DRB 50 μM and then stimulated for the indicated times with 50 ng ml^{-1} of TRAIL. Cell lysates were analysed by western blot with antibodies to FLIP. Membranes were reprobed with tubulin to ensure equal protein amounts. (**b**) IK and RL-95 cells were pretreated for 3 h with DRB 50 μM and then stimulated for the indicated times with 50 ng ml^{-1} of TRAIL. Cells lysates were analysed by western blot with antibodies to FLIP. Membranes were reprobed with tubulin to ensure equal protein amounts. (**c**) IK cells were pretreated for 3 h with DRB 50 μM or apigenin 50 μM and then stimulated for the indicated times with 50 ng ml^{-1} of TRAIL or 50 ng ml^{-1} agonistic Fas antibodies (aFas). Cell lysates were analysed by western blot with antibodies to FLIP. Membranes were reprobed with tubulin to ensure equal protein amounts (**d**) IK cells were infected with lentiviruses carrying short hairpin RNAs (shRNA) to CK2β subunit. After 3 days to allow downregulation of the protein, cells were treated with 50 ng ml^{-1} of TRAIL. After 8 h, cells were lysed and protein extracts were analysed by western blot with antibodies to FLIP. Membranes were reproved with tubulin to ensure equal protein amounts and antibodies to CK2β subunit to check inhibition of CK2β expression by the shRNA. (**e**) Apigenin and CK2β shRNA downregulate FLIP mRNA levels. IK cells were treated with apigenin for 6 or 12 h (top graph) or transfected with lentiviruses carrying CK2β or FLIP shRNAs. mRNA was extracted and subjected to reverse transcription. mRNA relative levels were analysed by real-time PCR. Results are expressed as relative mRNA levels compared to untreated (UN) cells (**f**) Proteasome inhibition block degradation of FLIP. IK cells were pretreated in presence or absence of 5 μM MG-132 for 30 min and then apigenin was added to the medium for the indicated periods of time (3, 6, 9 h). Cell lysates were analysed by western blot with antibodies to FLIP. Membranes were reproved with tubulin to ensure equal protein amounts.

Altogether, our results suggest that CK2 regulate both the transcription of FLIP mRNA and FLIP protein degradation through the proteasome.

Overexpression of FLIP overcomes CK2 sensitization to TRAIL and aFas

To ascertain whether high levels of FLIP blocked TRAIL- and aFas-induced apoptosis in conditions where CK2 was inhibited, we infected IK cells with a plasmid coding for a Flag-tagged mouse FLIP. After 3–4 days to allow FLIP expression, cells were treated with aFas or TRAIL in presence or absence of the CK2 inhibitors DRB or apigenin. Apoptotic nuclei were then visualized by Hoechst staining and caspase processing by western blotting. As shown in Figure 4a, overexpression of FLIP resulted in a dramatic reduction of apoptotic nuclei caused by CK2 inhibitors plus either TRAIL or

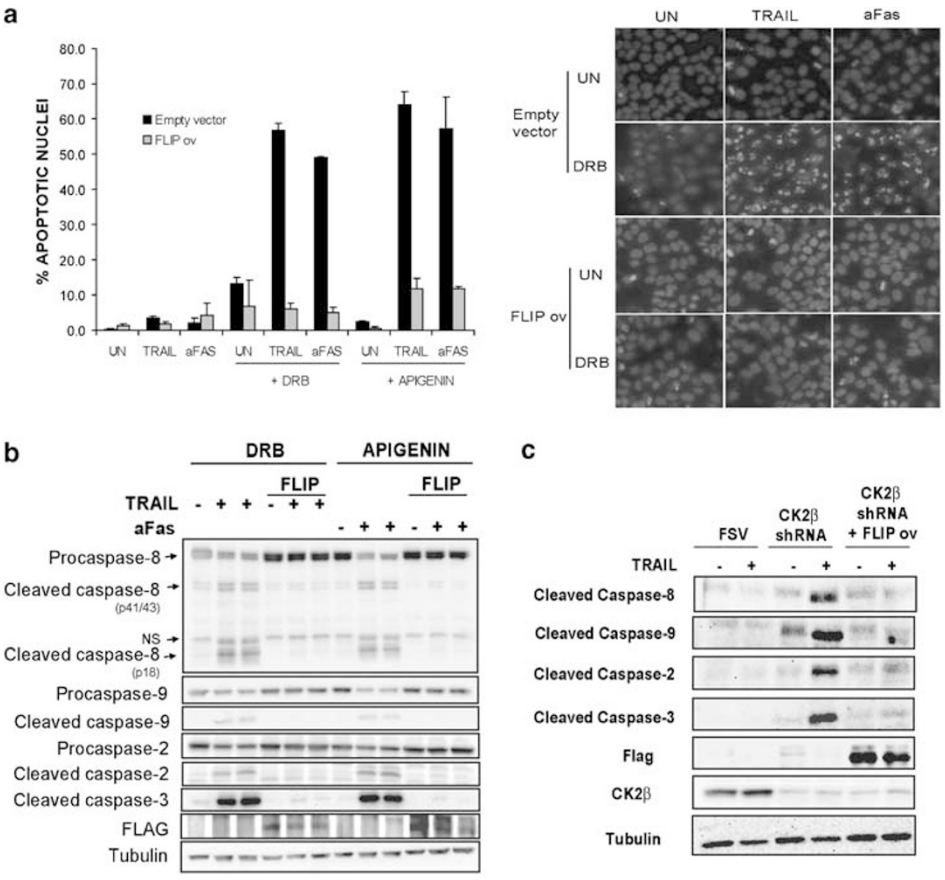

Figure 4 FLICE-inhibitory protein (FLIP) overexpression overrides sensitization to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and agonistic Fas antibodies (aFas) caused by casein kinase (CK2) inhibition. (**a**) FLIP overexpression reduces the number of apoptotic nuclei caused by CK2 inhibitors plus TRAIL or aFas treatment. IK cells were infected with lentiviruses carrying a FLIP expression plasmid. After 3 days, cells were pretreated for 2 h with 5,6-dichloro-ribifuranosylbenzimidazole (DRB) 50 μM, apigenin 50 μM and 50 ng ml^{-1} of TRAIL or aFas were added to the culture. Nuclei displaying apoptotic morphology were visualized by Hoechst staining counted 24 h later. Left: results of apoptotic nuclei quantification are presented as percentage of apoptotic nuclei compared to the total number of nuclei. Right: representative micrographs of IK infected with the empty vector or plasmid encoding FLIP (FLIP ov) and cells treated as indicated. (**b**) FLIP overexpression inhibits activation of caspase-8, -9, -2 and -3. IK cells were infected with lentiviruses carrying a FLIP expression plasmid. After 3 days, cells were pretreated for 2 h with DRB 50 μM, apigenin 50 μM and then stimulated with 50 ng ml^{-1} of TRAIL or aFas. After 18 h, cells were lysed and protein extracts were analysed by western blot with antibodies to the indicated caspases. Membranes were reprobed with tubulin to ensure equal protein amounts. NS (nonspecific bands). (**c**) FLIP overexpression suppresses caspase activation caused by downregulation of CK2β. IK cells were infected with lentiviruses carrying shRNA to CK2β subunit, lentiviruses carrying FLIP overexpression plasmid (FLIP ov) or co-infected with both viruses. After 3 days, cells were treated with 50 ng ml^{-1} of TRAIL or aFas. After 18 h, cells were lysed and protein extracts were analysed by western blot with antibodies to the indicated caspases. Membranes were reproved with tubulin to ensure equal protein amounts and antibodies to CK2β subunit to check inhibition of CK2β expression by the shRNA.

aFas. Consistent with this observation, FLIP overexpression inhibited processing of the caspases-8, -9, -2 and 3 caused by TRAIL or aFas in presence of either DRB or apigenin (Figure 4b). Similar results were obtained using shRNA to silence CK2β (Figure 4c).

Downregulation of endogenous FADD or caspase-8 expression restores TRAIL and aFas resistance to apoptosis

The results obtained suggested that CK2 is modulating apoptosis triggered after engagement of TRAIL and Fas receptors at the DISC level, since apoptosis induced by TRAIL or Fas requires the formation of a functional DISC. The adaptor protein FADD directly binds TRAIL or Fas receptor through its DD. FADD in turn binds caspase-8/10 which initiate caspase processing of apoptosis. To determine whether CK2 requires formation of DISC to regulate the sensitivity of ECC to either TRAIL or aFas, we infected IK cells with viruses carrying shRNAs to the two main components of Fas and TRAIL receptor DISC, FADD and caspase-8. After 3 days of infection, cells were pretreated with DRB or apigenin for 2 h and then treated with TRAIL or aFas, and apoptotic morphology and caspase activation were analysed. Both FADD and caspase-8 shRNA markedly reduced the number of apoptotic nuclei observed in condition containing any of the two CK2 inhibitors or CK2β shRNA plus either TRAIL or aFas (Figures 5a and c). Accordingly, caspase-8 and FADD shRNA

reduced activation of caspase-8, 2 and -3. We would like to point out that caspase-8 shRNA, which strongly inhibited caspase-8 expression, completely blocked caspase-2 activation, indicating that the activation of the later caspase-2 requires caspase-8 (Figure 5b).

DRB and apigenin sensitize primary endometrial carcinoma explants to TRAIL-induced apoptosis

Among all the members of TNF superfamily, TRAIL has raised some interest as a potential anti-cancer agent, because of its ability to trigger apoptosis in cancer cells without affecting normal cells. However, an increasing number of tumor types show mechanisms of TRAIL resistance. Such resistance has increased the interest of combinatorial therapies (Takeda *et al*., 2007). Since CK2 seems to be critical in the regulation of TRAIL apoptosis, we decided to test whether pharmacological inhibition of CK2 could be effective to kill primary endometrial carcinoma explants treated with TRAIL. We cultured two different endometrial carcinoma explants obtained from biopsies of patients with endometrial carcinoma. We have previously characterized these explant cultures to be of epithelial origin by means of cytokeratin and β-catenin expression (Dolcet *et al*., 2006). Both DRB and apigenin lead to a dramatic increase of nuclei displaying apoptotic morphology (Figure 6a). Accordingly, DRB or apigenin plus TRAIL treatment activate caspases-8, -9, -2 and -3 (Figure 6b). In agreement with the results observed in ECC lines, both DRB and apigenin dramatically decrease the levels of endogenous FLIP protein in primary endometrial explants.

Discussion

In the present study, we have assessed the mechanisms by which FLIP could be modulated to sensitize ECCs to TRAIL-induced apoptosis. Among the regulators of death receptor-induced apoptosis, recent evidences point to CK2 as an important regulator of apoptosis. Here, we demonstrate that CK2 is an important determinant in sensitivity to TRAIL and aFas. Blockade of CK2 activity by different pharmacological inhibitors or lentiviral-mediated transduction of shRNA targeting the regulatory CK2β subunit, sensitizes resistant ECC to both TRAIL- and aFas-induced apoptosis. Such sensitization correlates with a reduction of endogenous FLIP, which is a key regulator of apoptosis triggered by death ligands in ECC. To determine the contribution of FLIP downregulation to CK2 sensitization, we overexpressed of FLIP in presence of shRNA or CK2 inhibitors. FLIP overexpression overcomes CK2 sensibilization to TRAIL and aFas. Importantly, downregulation of endogenous FADD or caspase-8 expression restores TRAIL and aFas resistance to apoptosis.

First, we assessed the effects of CK2 inhibitors in sensitization of ECC to TRAIL or aFas. All of three inhibitors of CK2 activity used in this study (DRB, apigenin or emodin), sensitized ECC to death ligand-induced apoptosis, suggesting that such sensitization

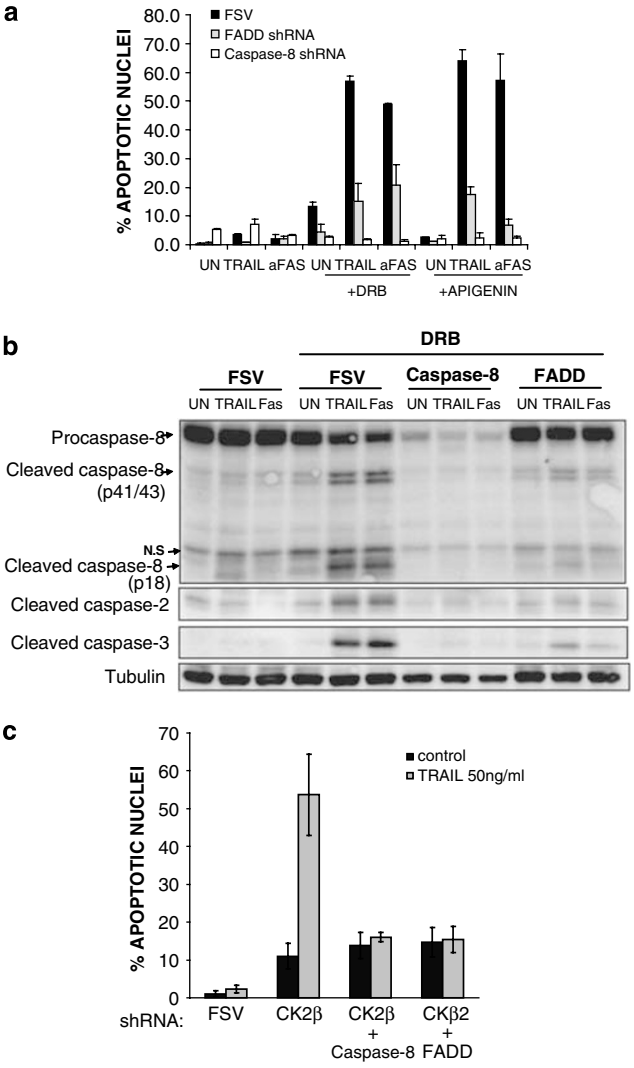

Figure 5 Silencing of Fas associated DD-containing protein (FADD) and caspase-8 restores tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and agonistic Fas antibodies (aFas) resistance. (**a**) IK cells were infected with lentiviruses carrying FADD or caspase-8 short hairpin RNAs (shRNAs). After 3 days, cells were pretreated for 2 h with 5,6-dichloro-ribifurano-sylbenzimidazole (DRB) 50 μM, apigenin 50 μM and 50 ng ml^{-1} of TRAIL or aFas were added to the culture. Nuclei displaying apoptotic morphology were visualized by Hoechst staining counted 24 h later. Results of apoptotic nuclei quantification are presented as percentage of apoptotic nuclei compared to the total number of nuclei. (**b**) Downregulation of caspase-8 or FADD inhibit activation of caspase-8, -9, -2 and -3. IK cells were infected with lentiviruses carrying shRNA to FADD or caspase-8. After 3 days, cells were pretreated for 2 h with DRB 50 μM, and then stimulated with 50 ng ml^{-1} of TRAIL or aFas. After 18 h, cells were lysed and protein extracts were analysed by western blot with antibodies to the indicated caspases. Membranes were reprobed with tubulin to ensure equal protein amounts. NS (nonspecific bands). (**c**) Caspase-8 and FADD shRNA reduces apoptotic nuclei caused downregulation of CK2β. IK cells were co-infected with lentiviruses carrying the empty vector (FSV) or shRNA to CK2β subunit together with shRNA to either caspase-8 or FADD. After 3 days, cells were treated with 50 ng ml^{-1} of TRAIL. Nuclei displaying apoptotic morphology were visualized by Hoechst staining counted 24 h later. Results of apoptotic nuclei quantification are presented as percentage of apoptotic nuclei compared to the total number of nuclei.

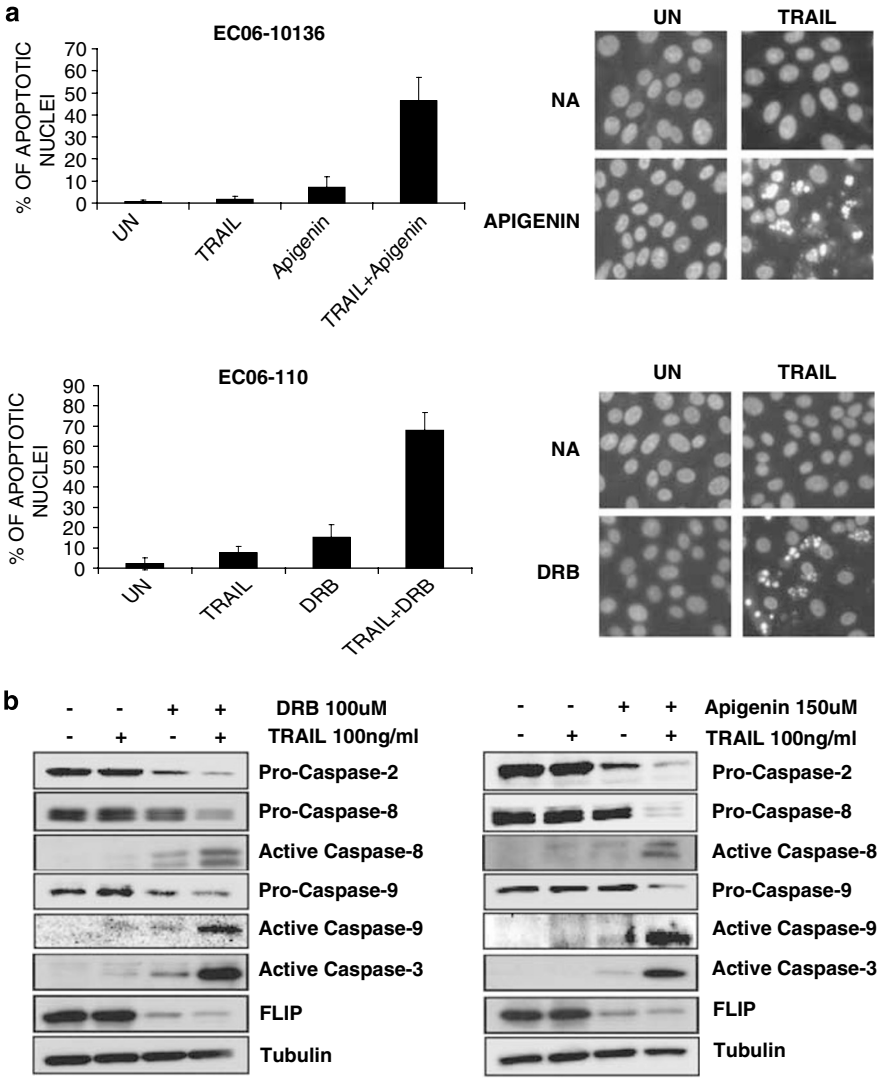

Figure 6 Casein kinase (CK2) inhibitors sensitize primary endometrial explants to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis. Primary explants were established from two different biopsies (top panel EC06-10136 and bottom panel EC06-110) as described in Materials and methods. Explants were treated with the indicated doses of 5,6-dichloro-ribifuranosylbenzimidazole (DRB) or apigenin or with no additives (NA) and left untreated (UN) of treated with TRAIL. (**a**) Nuclei displaying apoptotic morphology were visualized by Hoechst staining counted 24 h later. Left: results of apoptotic nuclei quantification are presented as percentage of apoptotic nuclei compared to the total number cells. Right: representative micrographs of primary explants treated as indicated. (**b**) Parallel wells of the two explants were lysed and protein extracts were analysed by western blot with antibodies to caspases and FLICE-inhibitory protein (FLIP). Membranes were reprobed with tubulin to ensure equal protein amounts.

depends on the kinase activity of the CK2. There are some evidences that CK2 catalytic subunits may have compensatory effects in some cell types. To preclude the problem of redundancy between subunits (Tawfic *et al*., 2001; Ahmed *et al*., 2002; Litchfield, 2003), we targeted CK2β regulatory subunit activity. We found that shRNA to CK2β had the same effects that pharmacological inhibition of CK2 kinase activity, suggesting that downregulation of CK2 regulatory subunit is effective to disrupt CK2 activity.

FLIP is a well-established regulator of TRAIL- and FasL-triggered apoptosis in many cell types. FLIP is constitutively or highly expressed in some tumors such as prostate cancer (Zhang *et al*., 2004), Hodgkin's lymphoma (Dutton *et al*., 2004), gastric cancer (Lee *et al*., 2003), bladder carcinoma (Korkolopoulou *et al*., 2004) and malignant mesothelial cell lines (Rippo *et al*., 2004). Increased levels of FLIP inhibit caspase-8 activation and apoptosis. In a previous study, we demonstrated that siRNA to FLIP is enough to sensitize IK cells to TRAIL-induced apoptosis (Dolcet *et al*., 2005), suggesting that FLIP levels are critical in sensitization to TRAIL-induced apoptosis. Although increasing evidence suggest an important role for CK2, there are few and controversial evidences concerning the role of FLIP in regulation of CK2 sensitization to death ligands. Shin *et al*. (2005) showed that overexpression of FLIP does not prevent caspase activation of the FLIP-L negative HCE4 cells after treatment with TRAIL plus DRB. The authors postulated that FLIP might not be

an essential regulator of TRAIL apoptosis in these cells. In contrast to the observations of Shin and collaborators, our results demonstrate that FLIP overexpression completely blocks caspase activation and apoptosis triggered by aFas or TRAIL plus either DRB, apigenin or downregulation of CK2β subunits by specific shRNA. Another recent report also suggested a role for FLIP in regulation of CK2 sensitization to TRAIL (Wang *et al*., 2006). The authors demonstrated in the prostate carcinoma lines PC-3 and ALVA-41 that treatment with suboptimal TRAIL plus the CK2 inhibitor TBB leads to downregulation of FLIP expression and concomitant caspase-8 activation. They also showed that overexpression of CK2α restores FLIP expression and TRAIL resistance. Accordingly, we have found that in ECC both pharmacological inhibition and CK2β knockdown reduce the levels of FLIP.

Next, we investigated the mechanism by which CK2 may regulate FLIP levels. Our data suggest that CK2 regulates FLIP levels by both transcriptional and post-transcriptional mechanisms. It is well known that FLIP can be transcriptionally downregulated by some antineoplastic drugs such as 5-fluorouracil, oxaliplatin and irinotecan in colon carcinoma cells (Galligan *et al*., 2005). Such FLIP mRNA downregulation has been shown to sensitize these cells to TRAIL-induced apoptosis. FLIP levels are also regulated by ubiquitin-proteasome-mediated degradation (Kim *et al*., 2002; Poukkula *et al*., 2005; Chang *et al*., 2006). Moreover, recent findings suggest that some anticancer drugs such as the cyclooxygenase-2 inhibitor celecoxib (Liu *et al*., 2006) or the flavonoids flavopiridol (Son *et al*., 2007) and flavopiridol (Palacios *et al*., 2006) can sensitize cancer cells to TRAIL-induced apoptosis by inducing a proteasome-mediated degradation of FLIP. In agreement with these previous reports, addition of proteasome inhibitors such as MG-132 restores FLIP levels in IK cells treated with CK2 inhibitors. Thus, in our model, CK2 maintains FLIP levels by controlling FLIP expression and degradation.

We showed that inhibition of CK2 plus addition of either TRAIL- or aFas-activated caspase-8 as initiator caspase of the extrinsic pathway; however, it also activated caspase-9 and -2 and the effector caspase-3. These data suggest that sensitization to TRAIL and aFas requires mitochondrial amplification. That is, caspase-8 cleaves the 'BH3 only' protein Bid. Truncated Bid translocates to the mitochondria, which ultimately results in the release of cytochrome c and activation of caspase-9 (Li *et al*., 1998; Luo *et al*., 1998). Previous reports have demonstrated that inhibition of CK2 results in Bid processing (Ravi and Bedi, 2002; Izeradjene *et al*., 2005). Among the caspases activated by CK2 inhibition, the role caspase-2 has been recently studied in death receptor signaling. Caspase-2 is shown to be activated by both stress-induced (Lassus *et al*., 2002) and death receptor apoptosis (Droin *et al*., 2001; Wagner *et al*., 2004; Lavrik *et al*., 2006). However, its role as initiator caspase in death receptor signaling is controversial. A recent publication suggested that caspase-2 prime cancer cells to TRAIL-induced apoptosis by processing caspase-8 (Shin *et al*., 2005). In this work, the authors demonstrated that CK2 directly phosphorylates and inhibits caspase-2. Thus, inhibition of CK2 results in dephosphorylation and processing of procaspase-2 which in turn processes caspase-8. The authors postulated that the inhibition of CK2 is enough to induce a caspase-2 dependent processing of procaspase-8 to the p41/p43 fragments and the addition of TRAIL triggers the second processing of caspase-8 that generates the active form. In contradiction to these data, Lavrik and collaborators demonstrated that in T- and B-cell lines, procaspase-2 is recruited and activated at the DISC after Fas engagement. Importantly, authors showed that caspase-2 failed to initiate apoptosis in caspase-8 deficient cells (Droin *et al*., 2001; Wagner *et al*., 2004; Lavrik *et al*., 2006). We have found that CK2 inhibition generates p41/43 fragment of caspase-8 even in absence of TRAIL and, addition of TRAIL or Fas, gives rise to the p18 active fragments and apoptosis. However caspase-2 active fragment is only activated when we add TRAIL or Fas, suggesting that activation of caspase-2 depends on the addition of the ligand. Moreover, FADD and caspase-8 shRNA or FLIP overexpression block caspase-2 activation and CK2 sensitization to TRAIL and Fas apoptosis, which suggest that in our cell system caspase-2, although activated, might not be an initiator caspase.

Finally, we demonstrated that pharmacological inhibition of CK2 sensitizes primary endometrial carcinoma explants to TRAIL-induced apoptosis. TRAIL has emerged as a promising antineoplastic agent, these data suggest that pharmacological inhibition of CK2 may be an interesting target for combinatorial therapies for endometrial carcinomas.

In summary, we provide evidence that CK2 is an important regulator of TRAIL- and FasL-induced apoptosis. We demonstrate that the mechanism used by CK2 requires downregulation of FLIP, enhancing the importance of this protein in TRAIL and Fas signaling and, ultimately, the cell fate.

Materials and methods

Reagents, plasmids and antibodies

MTT and monoclonal antibody to Tubulin and anti-Flag M2 were from Sigma (St Louis, MO, USA). CK2 inhibitors DRB, apigenin and emodin and monoclonal antibody to caspase-8 was from Calbiochem (La Jolla, CA, USA). Antibody to caspase-9 and cleaved caspase-3 were obtained from Cell Signalling (Beverly, MA, USA). Monoclonal antibody to FLIP (NF6) was purchased from Alexis Corp (Lausen, Switzerland). Anti-CK2β antibody was from SantaCruz Biotechnology Inc. (Santa Cruz, CA, USA). Antibody to caspase-2 was from BD Biosciences (San Jose, CA, USA). Peroxidase-conjugated anti-mouse and anti-rabbit antibodies were from Amersham-Pharmacia (Uppsala, Sweden).

Lentiviral vector containing Flag-tagged mouse FLIP complementary DNA (cDNA) was a gift from Dr Joan Comella.

Cell lines, culture conditions and transfection

IK was obtained from the American Type Culture Collection (Manassas, VA, USA). RL-95 cell line was a gift from

Dr Reventos (Hospital Vall d'Hebron, Barcelona). All cell lines were grown in Dulbecco's modified Eagle's Medium (DMEM) (Sigma) supplemented with 10% Fetal Bovine Serum (Invitrogen Inc., Carlsbad, CA, USA), 1 mM HEPES (Sigma), 1 mM sodium pyruvate (Sigma), 2 mM L-glutamine (Sigma) and 1% of penicillin/streptomycin (Sigma) at 37 °C with saturating humidity and 5% CO_2.

When indicated, transfections plasmids constructs were performed by calcium phosphate or Lipofectamine 2000 reagent (Invitrogen) following the manufacturers instructions.

Explant culture of endometrial adenocarcinoma

Endometrial carcinoma samples were collected in the operating room of the Department of Gynecology, Hospital Universitari Arnau de Vilanova of Lleida, by a pathologist (JP). A specific informed consent was obtained from each patient, and the study was approved by the local Ethic Committee. Tissue was collected in DMEM, chopped in 1 mm pieces and incubated with collagenase in DMEM for 1.5 h at 37 °C with periodic mixing. Digested tissue was mechanically dissociated through a 10 ml pipette and a 1 ml blue tip and resuspended in 2 ml of fresh DMEM medium. To separate endometrial epithelial cells from the stromal fraction, the dissociated tissue was seeded on top of 8 ml of DMEM medium and tissue was allowed to sediment by gravity for 5 min. This step was repeated three times. Finally, tissue explants were resuspended in DMEM supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 2 mM L-glutamine and 1% of penicillin/streptomycin (Sigma) and seeded on M24 multiwell plates. Explant cultures were incubated at 37 °C with saturating humidity and 5% CO_2.

Lentiviral production and infection

Oligonucleotides to produce plasmid-based shRNA were cloned into the FSV vector using *Age*I-*Bam*HI restriction sites. shRNA target sequence were CK2β, TGGTTTCCC TCACATGCTCT; FADD, CATGGAACTCAGACGC ATCT; caspase-8, GAATCACAGACTTTGGACAA. To produce infective lentiviral particles, 293T cells were co-transfected by calcium phosphate method with the virion packaging elements (VSV-G and Δ8.9) and the shRNA producing vector (FSV) or the expression vector (FCIV) on 293T human embryonic kidney. 293T cells were allowed to produce lentiviral particles during 3–4 days in same culture medium used for endometrial cell lines and explants. Culture medium was collected, centrifuged for 5 min at 1000 r.p.m. and filtered through a 0.45 μM filter (Millipore, Bedford, MA, USA). The medium was diluted 1:2–1:4 with fresh medium, and added to growing cell lines or primary explants. Cells were incubated for 24–48 h in presence of medium containing lentiviral particles. After this period, medium was replaced for fresh medium and cells were incubated for 2 additional days to allow endogenous protein knockdown or protein overexpression.

Cell viability assays and assessment of apoptosis

Cell viability was determined by MTT assay. Endometrial adenocarcinoma cells were plated on M96 well plates at 15×10^3 cells per well. After the indicated treatments, the cells were incubated for 2–3 h with 0.5 mg ml^{-1} of MTT reagent and lysed with dimethyl sulfoxide. Absorbance was measured at 595 nm in a microplate reader (Bio-Rad, Richmond, CA, USA).

Hoechst staining was performed by adding Hoechst dye to a final concentration of 0.5 mg ml^{-1} to each M96 well. Cells were counted under epifluorescence microscope (Leica Microsystems, Wetzlar, Germany).

Western blot analysis

Endometrial adenocarcinoma cell lines were washed with cold phosphate-buffered saline and lysed with lysis buffer (2% sodium dodecyl sulfate (SDS), 125 mM Tris–HCl pH 6.8). Protein concentrations were determined with the Protein assay Kit (Bio-Rad). Equal amounts of proteins were subjected to SDS–PAGE and transferred to polyvinylidene difluoride membranes (Millipore). Nonspecific binding was blocked by incubation with Tris-buffered saline/Tween (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Tween-20) plus 5% of non-fat milk. Membranes were incubated with the primary antibodies overnight at 4 °C. Signal was detected with ECL Advance (Amersham-Pharmacia, Buckinghamshire, UK).

RNA extraction, reverse transcription and real-time PCR

Total RNA was extracted from IK cells treated as indicated using Trizol reagent (Invitrogen). One microgram of total RNA was converted into cDNA using TaqMan Reverse Transcription Reagents (Applied Biosystems P/N N808-0234) according to the manufacturer's recommendations. A total of 2 μl of the reverse transcription reaction were used as template for a for real-time detection of human FLIP expression using TaqMan Technology on an Applied Biosystems 7000 sequence detection system. Gene expression quantitation was performed in separate tubes (singleplex) for both target gene and endogenous control gene using the primer and probe sequences for human FLIP and β-glucuronidase (GUSB) obtained commercially from Applied Biosystems Assay-on-demand Gene (ABI P/N 4331182: Hs00236002_m1 and ABI P/N 4326320E). The reaction was performed with 10 μl TaqMan Universal PCR Master Mix, No AmpErase UNG 2X (P/N 4324018), 1 μl 20 × Assay-on-demand Gene and 2 μl of cDNA diluted in RNase-free water adjusted to 20 μl volume reaction. The thermal cycler conditions was UNG activation 2 min at 50 °C, AmpliTaq activation 95 °C for 10 min, denaturation 95 °C for 15 s, and annealing/extension 60 °C for 1 min (repeat 40 times) on ABI7000. Triplicate cycle threshold (CT) values were analysed with Quantitative Relative software using de comparative CT (ΔΔCT) method as described by the manufacturer. The amount of target($2^{-\Delta\Delta CT}$) was obtained by normalizing to an endogenous reference gene (GUSB). Results are presented as a relative mRNA amount compared to the untreated samples.

Acknowledgements

This study was supported by grants FIS070304, FIS070276, FIS PI020227, SAF2002-10529-E, SAF2004-05250, Marató de TV3 2005-47, and 2004XT00090, AECC, Catalunya contra el cancer and programa de intensificación de la investigación, Instituto Carlos III. XD holds a postdoctoral fellowship from the Fondo de Investigaciones Sanitarias, Ministerio de Sanidad y Consumo (CP05/00028). DLL holds a predoctoral fellowship from the Fondo de Investigaciones Sanitarias, Ministerio de Sanidad y Consumo (FI05/00191) and ME holds a Ramon y Cajal Fellowship from Ministerio de Educación y Ciencia.

References

Ahmad KA, Wang G, Slaton J, Unger G, Ahmed K. (2005). Targeting CK2 for cancer therapy. *Anticancer Drugs* **16**: 1037–1043.

Ahmed K, Gerber DA, Cochet C. (2002). Joining the cell survival squad: an emerging role for protein kinase CK2. *Trends Cell Biol* **12**: 226–230.

Allende JE, Allende CC. (1995). Protein kinases. 4. Protein kinase CK2: an enzyme with multiple substrates and a puzzling regulation. *FASEB J* **9**: 313–323.

Ashkenazi A, Pai RC, Fong S, Leung S, Lawrence DA, Marsters SA *et al*. (1999). Safety and antitumor activity of recombinant soluble Apo2 ligand. *J Clin Invest* **104**: 155–162.

Bodmer JL, Holler N, Reynard S, Vinciguerra P, Schneider P, Juo P *et al*. (2000). TRAIL receptor-2 signals apoptosis through FADD and caspase-8. *Nat Cell Biol* **2**: 241–243.

Chang L, Kamata H, Solinas G, Luo JL, Maeda S, Venuprasad K *et al*. (2006). The E3 ubiquitin ligase itch couples JNK activation to TNFalpha-induced cell death by inducing c-FLIP(L) turnover. *Cell* **124**: 601–613.

Dolcet X, Llobet D, Encinas M, Pallares J, Cabero A, Schoenenberger JA *et al*. (2006). Proteasome inhibitors induce death but activate NF-kappaB on endometrial carcinoma cell lines and primary culture explants. *J Biol Chem* **281**: 22118–22130.

Dolcet X, Llobet D, Pallares J, Rue M, Comella JX, Matias-Guiu X. (2005). FLIP is frequently expressed in endometrial carcinoma and has a role in resistance to TRAIL-induced apoptosis. *Lab Invest* **85**: 885–894.

Droin N, Bichat F, Rebe C, Wotawa A, Sordet O, Hammann A *et al*. (2001). Involvement of caspase-2 long isoform in Fas-mediated cell death of human leukemic cells. *Blood* **97**: 1835–1844.

Dutton A, O'Neil JD, Milner AE, Reynolds GM, Starczynski J, Crocker J *et al*. (2004). Expression of the cellular FLICE-inhibitory protein (c-FLIP) protects Hodgkin's lymphoma cells from autonomous Fas-mediated death. *Proc Natl Acad Sci USA* **101**: 6611–6616.

Galligan L, Longley DB, McEwan M, Wilson TR, McLaughlin K, Johnston PG. (2005). Chemotherapy and TRAIL-mediated colon cancer cell death: the roles of p53, TRAIL receptors, and c-FLIP. *Mol Cancer Ther* **4**: 2026–2036.

Irmler M, Steiner V, Ruegg C, Wajant H, Tschopp J. (2000). Caspase-induced inactivation of the anti-apoptotic TRAF1 during Fas ligand-mediated apoptosis. *FEBS Lett* **468**: 129–133.

Izeradjene K, Douglas L, Delaney A, Houghton JA. (2004). Influence of casein kinase II in tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in human rhabdomyosarcoma cells. *Clin Cancer Res* **10**: 6650–6660.

Izeradjene K, Douglas L, Delaney A, Houghton JA. (2005). Casein kinase II (CK2) enhances death-inducing signaling complex (DISC) activity in TRAIL-induced apoptosis in human colon carcinoma cell lines. *Oncogene* **24**: 2050–2058.

Kikkawa U, Mann SK, Firtel RA, Hunter T. (1992). Molecular cloning of casein kinase II alpha subunit from *Dictyostelium discoideum* and its expression in the life cycle. *Mol Cell Biol* **12**: 5711–5723.

Kim Y, Suh N, Sporn M, Reed JC. (2002). An inducible pathway for degradation of FLIP protein sensitizes tumor cells to TRAIL-induced apoptosis. *J Biol Chem* **277**: 22320–22329.

Kischkel FC, Lawrence DA, Chuntharapai A, Schow P, Kim KJ, Ashkenazi A. (2000). Apo2L/TRAIL-dependent recruitment of endogenous FADD and caspase-8 to death receptors 4 and 5. *Immunity* **12**: 611–620.

Korkolopoulou P, Goudopoulou A, Voutsinas G, Thomas-Tsagli E, Kapralos P, Patsouris E *et al*. (2004). c-FLIP expression in bladder urothelial carcinomas: its role in resistance to Fas-mediated apoptosis and clinicopathologic correlations. *Urology* **63**: 1198–1204.

Landesman-Bollag E, Romieu-Mourez R, Song DH, Sonenshein GE, Cardiff RD, Seldin DC. (2001). Protein kinase CK2 in mammary gland tumorigenesis. *Oncogene* **20**: 3247–3257.

Lassus P, Opitz-Araya X, Lazebnik Y. (2002). Requirement for caspase-2 in stress-induced apoptosis before mitochondrial permeabilization. *Science* **297**: 1352–1354.

Lavrik IN, Golks A, Baumann S, Krammer PH. (2006). Caspase-2 is activated at the CD95 death-inducing signaling complex in the course of CD95-induced apoptosis. *Blood* **108**: 559–565.

LeBlanc HN, Ashkenazi A. (2003). Apo2L/TRAIL and its death and decoy receptors. *Cell Death Differ* **10**: 66–75.

Lee SH, Kim HS, Kim SY, Lee YS, Park WS, Kim SH *et al*. (2003). Increased expression of FLIP, an inhibitor of Fas-mediated apoptosis, in stomach cancer. *APMIS* **111**: 309–314.

Li H, Zhu H, Xu CJ, Yuan J. (1998). Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* **94**: 491–501.

Litchfield DW. (2003). Protein kinase CK2: structure, regulation and role in cellular decisions of life and death. *Biochem J* **369**: 1–15.

Liu X, Yue P, Schonthal AH, Khuri FR, Sun SY. (2006). Cellular FLICE-inhibitory protein down-regulation contributes to celecoxib-induced apoptosis in human lung cancer cells. *Cancer Res* **66**: 11115–11119.

Luo X, Budihardjo I, Zou H, Slaughter C, Wang X. (1998). Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell* **94**: 481–490.

MacFarlane M, Ahmad M, Srinivasula SM, Fernandes-Alnemri T, Cohen GM, Alnemri ES. (1997). Identification and molecular cloning of two novel receptors for the cytotoxic ligand TRAIL. *J Biol Chem* **272**: 25417–25420.

Padmanabha R, Chen-Wu JL, Hanna DE, Glover CV. (1990). Isolation, sequencing, and disruption of the yeast CKA2 gene: casein kinase II is essential for viability in *Saccharomyces cerevisiae*. *Mol Cell Biol* **10**: 4089–4099.

Palacios C, Yerbes R, Lopez-Rivas A. (2006). Flavopiridol induces cellular FLICE-inhibitory protein degradation by the proteasome and promotes TRAIL-induced early signaling and apoptosis in breast tumor cells. *Cancer Res* **66**: 8858–8869.

Pan G, Ni J, Wei YF, Yu G, Gentz R, Dixit VM. (1997a). An antagonist decoy receptor and a death domain-containing receptor for TRAIL. *Science* **277**: 815–818.

Pan G, Ni J, Yu G, Wei YF, Dixit VM. (1998). TRUNDD, a new member of the TRAIL receptor family that antagonizes TRAIL signalling. *FEBS Lett* **424**: 41–45.

Pan G, O'Rourke K, Chinnaiyan AM, Gentz R, Ebner R, Ni J *et al*. (1997b). The receptor for the cytotoxic ligand TRAIL. *Science* **276**: 111–113.

Poukkula M, Kaunisto A, Hietakangas V, Denessiouk K, Katajamaki T, Johnson MS *et al*. (2005). Rapid turnover of c-FLIPshort is determined by its unique C-terminal tail. *J Biol Chem* **280**: 27345–27355.

Ravi R, Bedi A. (2002). Sensitization of tumor cells to Apo2 ligand/TRAIL-induced apoptosis by inhibition of casein kinase II. *Cancer Res* **62**: 4180–4185.

Rippo MR, Moretti S, Vescovi S, Tomasetti M, Orecchia S, Amici G *et al*. (2004). FLIP overexpression inhibits death receptor-induced apoptosis in malignant mesothelial cells. *Oncogene* **23**: 7753–7760.

Seldin DC, Leder P. (1995). Casein kinase II alpha transgene-induced murine lymphoma: relation to theileriosis in cattle. *Science* **267**: 894–897.

Sheridan JP, Marsters SA, Pitti RM, Gurney A, Skubatch M, Baldwin D *et al*. (1997). Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. *Science* **277**: 818–821.

Shin S, Lee Y, Kim W, Ko H, Choi H, Kim K. (2005). Caspase-2 primes cancer cells for TRAIL-mediated apoptosis by processing procaspase-8. *EMBO J* **24**: 3532–3542.

Son YG, Kim EH, Kim JY, Kim SU, Kwon TK, Yoon AR *et al*. (2007). Silibinin sensitizes human glioma cells to TRAIL-mediated apoptosis via DR5 up-regulation and down-regulation of c-FLIP and survivin. *Cancer Res* **67**: 8274–8284.

Sprick MR, Weigand MA, Rieser E, Rauch CT, Juo P, Blenis J *et al*. (2000). FADD/MORT1 and caspase-8 are recruited to TRAIL receptors 1 and 2 and are essential for apoptosis mediated by TRAIL receptor 2. *Immunity* **12**: 599–609.

Srivastava RK. (2001). TRAIL/Apo-2L: mechanisms and clinical applications in cancer. *Neoplasia* **3**: 535–546.

Takeda K, Stagg J, Yagita H, Okumura K, Smyth MJ. (2007). Targeting death-inducing receptors in cancer therapy. *Oncogene* **26**: 3745–3757.

Tawfic S, Yu S, Wang H, Faust R, Davis A, Ahmed K. (2001). Protein kinase CK2 signal in neoplasia. *Histol Histopathol* **16**: 573–582.

Thome M, Schneider P, Hofmann K, Fickenscher H, Meinl E, Neipel F *et al*. (1997). Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. *Nature* **386**: 517–521.

Wagner KW, Engels IH, Deveraux QL. (2004). Caspase-2 can function upstream of bid cleavage in the TRAIL apoptosis pathway. *J Biol Chem* **279**: 35047–35052.

Walczak H, Degli-Esposti MA, Johnson RS, Smolak PJ, Waugh JY, Boiani N *et al*. (1997). TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. *EMBO J* **16**: 5386–5397.

Walczak H, Miller RE, Ariail K, Gliniak B, Griffith TS, Kubin M *et al*. (1999). Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand *in vivo*. *Nat Med* **5**: 157–163.

Wang G, Ahmad KA, Ahmed K. (2005a). Modulation of death receptor-mediated apoptosis by CK2. *Mol Cell Biochem* **274**: 201–205.

Wang G, Ahmad KA, Ahmed K. (2006). Role of protein kinase CK2 in the regulation of tumor necrosis factor-related apoptosis inducing ligand-induced apoptosis in prostate cancer cells. *Cancer Res* **66**: 2242–2249.

Wang G, Unger G, Ahmad KA, Slaton JW, Ahmed K. (2005b). Downregulation of CK2 induces apoptosis in cancer cells—a potential approach to cancer therapy. *Mol Cell Biochem* **274**: 77–84.

Zhang X, Jin TG, Yang H, DeWolf WC, Khosravi-Far R, Olumi AF. (2004). Persistent c-FLIP(L) expression is necessary and sufficient to maintain resistance to tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in prostate cancer. *Cancer Res* **64**: 7086–7091.