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MADD Is a Downstream Target of PTEN in Triggering Apoptosis

Shankar Jayarama,[1] Liang-Cheng Li,[1] Lakshmy Ganesh,[1] David Mardi,[1] Prasad Kanteti,[1] Nissim Hay,[2] Peifeng Li,[1] and Bellur S. Prabhakar[1]*

[1]*Department of Microbiology and Immunology, College of Medicine, University of Illinois at Chicago, Chicago, Illinois 60612*

[2]*Department of Biochemistry and Molecular Genetics, College of Medicine, University of Illinois at Chicago, Chicago, Illinois 60612*

ABSTRACT

Mitogen-activated kinase activating death domain containing protein (MADD) is abundantly expressed in cancer cells and necessary for maintaining cancer cell survival. However, this survival function of MADD is dependent upon its phosphorylation by protein kinase B (Akt). The tumour suppressor PTEN (phosphatase and tensin homolog deleted on chromosome 10) is a lipid phosphatase that negatively regulates the phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathway. The downstream targets of PTEN in triggering apoptosis have not yet been completely identified. Here, we report that MADD can act as a pro-apoptotic factor to initiate TRAIL-induced apoptosis when its phosphorylation is attenuated by PTEN. Our data show that tumor necrosis factor α-related apoptosis-inducing ligand (TRAIL) induced a reduction in MADD phosphorylation with a concomitant up-regulation of PTEN. Knock down of PTEN using a specific siRNA prevented TRAIL-induced reduction in pMADD levels. Surprisingly, Akt non-phopshorylated MADD translocated from the plasma membrane to cytoplasm where it bound to 14-3-3 and displaced 14-3-3 associated Bax, which translocated to mitochondria resulting in cytochrome *c* release. Taken together, our data reveal that PTEN can convey the death signal by preventing MADD phosphorylation by Akt. J. Cell. Biochem. 115: 261–270, 2014.

KEY WORDS: APOPTOSIS; MADD; PTEN; AKT; PI3K

The mitogen-activated kinase activating death domain protein (MADD) is expressed at very low levels in a variety of tissues and organs under physiological conditions. However, it is over-expressed in many types of human tumors and tumor cell lines [Chow and Lee, 1996; Al-Zoubi et al., 2001]. Enforced expression of exogenous MADD has no apparent effect on cell survival, but knockdown of endogenous MADD can lead to spontaneous as well as enhanced tumor necrosis factor α-related apoptosis-inducing ligand (TRAIL) induced apoptosis, indicating that MADD is necessary for cancer cell survival [Mulherkar et al., 2006, 2007; Li et al., 2008a]. Furthermore, MADD contributes to the resistance of several different types of cancer cells to TRAIL-induced apoptosis [Efimova et al., 2004; Li et al., 2008a, 2011, 2013; Subramanian et al., 2009; Turner et al., 2013]. These features strongly suggested a role for MADD in tumor pathophysiology.

Protein kinase B (i.e., Akt) promotes cell survival by phosphorylating a variety of apoptosis-related factors. For example, it phosphorylates mouse double minute 2 (mdm2) and enhances its ability to degrade p53 [Mayo and Donner, 2001; Zhou et al., 2001]. It can phosphorylate caspase-9, Bad, IKKα, Fork head transcription factor and Yap [Bhaskar and Hay, 2007; Manning and Cantley, 2007]. Earlier work from our laboratory demonstrated that MADD can be

Abbreviations: caAkt, constitutively activated form of Akt; DN-Akt, dominant negative form of Akt; MADD, mitogen-activated kinase activating death domain protein; pMADD, Akt phosphorylated MADD; non-pMADD, non-phosphorylated MADD; MADD3A, mutant MADD in which serine-70, threonine-173 and threonine-1041 were mutant to alanine; shRNA, small hairpin RNA; PI3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homolog deleted on chromosome 10; TRAIL, tumor necrosis factor α-related apoptosis-inducing ligand.

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Shankar Jayarama, Liang-Cheng Li, and Lakshmy Ganesh are contributed equally to this work.

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*Correspondence to: Bellur S. Prabhakar, PhD, Professor Head, Department of Microbiology and Immunology, College of Medicine, University of Illinois at Chicago, Chicago, IL 60612. E-mail: bprabhak@uic.edu

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phosphorylated at serine-70, threonine-173 and threonine-1041 by Akt, and such phosphorylation is necessary for MADD to exert its anti-apoptotic function [Li et al., 2010]. In spite of the abundant expression of MADD, TRAIL is able to induce cell death [Mulherkar et al., 2007]. Such a discrepancy can be related to the reduction in Akt and the consequent MADD phosphorylation levels in response to TRAIL treatment [Li et al., 2013]. However, it remains unknown as to the molecular mechanism by which TRAIL induces the reduction in Akt and MADD phosphorylation levels in TRAIL susceptible cells.

The tumor suppressor phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a lipid phosphatase that negatively regulates the phosphatidylinositol 3-kinase (PI3K) signaling pathway [Cully et al., 2006]. It removes the 3′ phosphate of PIP3, thereby regenerating PIP2 and abolishing the downstream events regulated by PI3K [Dillon et al., 2007]. Akt is phosphorylated and thus activated by PI3K. It is not yet clear whether PTEN contributes to the observed reduction of MADD phosphorylation upon TRAIL treatment.

The intrinsic apoptotic pathway is initiated when a death signal induces the release of mitochondrial pro-apoptotic proteins such as cytochrome *c* [Li et al., 1997], mitochondrial apoptosis-inducing factor [Susin et al., 1999] and Smac/Diablo [Du et al., 2000; Verhagen et al., 2000]. Cytochrome *c* forms a complex with Apaf-1 and procaspase-9 resulting in the activation of caspase-9. Smac/Diablo can associate with inhibitor of apoptosis proteins (IAPs) and counteract their caspase inhibitory effects. The intrinsic pathway is regulated by the Bcl-2 family members. For example, in response to pro-apoptotic stimuli, the cytosolic Bax and Bad translocate to mitochondria to permeabilize the outer mitochondrial membrane leading to cytochrome *c* release into the cytosol. In contrast, Bcl-2 and Bcl-xL can associate with Bax and Bad thereby preventing them from inducing death [Antignani and Youle, 2006]. Although earlier studies have implicated a role for MADD in regulating intrinsic apoptotic pathway [Efimova et al., 2003], it is not clear how MADD exerts its influence in this apoptosis pathway.

Our present work demonstrated that TRAIL treatment caused elevation in the levels of PTEN and resulted in a reduction in the levels of pAkt. Consequently, Akt-phosphorylated MADD (pMADD) levels were also reduced. Surprisingly, Akt non-pMADD (non-phosphorylated MADD) bound to 14-3-3. Such an association released Bax, which translocated to mitochondria thereby causing cytochrome *c* release. Our data provide a novel model in which TRAIL induces apoptosis by up-regulating PTEN. Moreover, they also show one of the mechanisms by which PTEN can facilitate apoptosis.

MATERIALS AND METHODS

CELL CULTURE AND VIABILITY ASSAY

HEK293 and HeLa cells were cultured as we have described earlier [Mulherkar et al., 2007]. In brief, cells were cultured in Dulbecco′s modified Eagle′s medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine with 5% CO_2. Cell death was determined by Trypan Blue exclusion and the numbers of Trypan Blue-positive and -negative cells were counted using a haemocytometer.

CONSTRUCTION OF MADD3A MUTANT

The serine-70, threonine-173 and threonine-1041 residues at the Akt phosphorylation sites of MADD were changed to the alanine residue using QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer′s instructions and as reported in our earlier study [Li et al., 2010]. The protein encoded by the mutated construct could not be phosphorylated by Akt and was designated as MADD3A. All constructs were sequenced to ensure that only the desired mutations had been introduced.

VIRUS INFECTION AND TRANSFECTION OF CELLS

Adenovirus harboring PTEN cDNA was obtained from Vector Biolabs. Adenovirus β-galactosidase (β-gal) has been described elsewhere [Li et al., 2008b]. All viruses were amplified in HEK293 cells. Cells were infected at the indicated multiplicity of infection (moi) for 60 min. After washing with phosphate buffered saline (PBS), culture medium was added and cells were cultured for the indicated duration. For delivering siRNA constructs of PTEN (Santa Cruz Biotechnology, Inc.), the cells were transfected using an Effectene Transfection Kit (Qiagen) according to the kit instruction.

TRAIL TREATMENT AND WESTERN BLOT

HeLa cells were treated with 50 ng/ml TRAIL, and then harvested at the indicated time for the analysis of MADD and Akt phosphorylation levels. Cells were lysed for 1 h at 4°C in a lysis buffer (20 mM Tris pH 7.5, 2 mM EDTA, 3 mM EGTA, 2 mM dithiothreitol (DTT), 250 mM sucrose, 0.1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100) containing a protease inhibitor cocktail (Sigma). Equal protein loading was controlled by Ponceau Red staining of membranes. Western blots were probed using corresponding primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies, and the protein bands visualized by enhanced chemiluminescence. The anti-MADD and anti-phosphorylated MADD antibodies were produced by Eurogentec [Li et al., 2010]. The anti-PTEN antibody was purchased from Millipore. The anti-Akt antibody and the anti-phosphorylated Akt antibody were purchased from Cell signaling.

DETECTION OF ACTIVE CASPASE-3

Active caspase-3 was detected using a commercial assay kit as per the manufacturer′s instructions (R&D Systems). In brief, cells were collected by centrifugation at 250*g* for 10 min. The supernatant was gently removed and discarded while the cell pellet was lysed by adding 25 μl of cold lysis buffer provided in the kit. The cell lysate was incubated on ice for 10 min and then centrifuged at 10,000*g* for 1 min. Protein concentration was determined using commercial assay kit (Bio-Rad protein assay kit). Fifty microliters of 2× reaction buffer was added; and this was followed by the addition of 5 μl of caspase-3 colorimetric substrate (both provided in the kit). The reaction mixture was incubated at 37°C for 1 h and the samples were read at wavelength of 405 nm.

PTEN KNOCKDOWN

To see if knockdown of PTEN attenuates the decrease in Akt and MADD phosphorylation levels induced by TRAIL, we transfected HeLa cells with the PTEN siRNA or the siRNA control. Forty-eight

hours after transfection, cells were treated with 50 ng/ml TRAIL. Cells were harvested 2 h after TRAIL treatment for the analysis of Akt or MADD phosphorylation levels by immunoblot.

PREPARATION OF SUBCELLULAR FRACTIONS

Mitochondrial fractions were prepared as described before [Li et al., 1999]. Briefly, cells were washed twice with PBS and the pellet was suspended in 0.2 ml of buffer A (20 mM HEPES pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 250 mM sucrose) containing a protease inhibitor cocktail. The cells were homogenized by 12 strokes in a homogenizer. The homogenates were centrifuged twice at 750*g* for 5 min at 4°C. The supernatants were centrifuged at 10,000*g* for 15 min at 4°C to collect mitochondria-enriched heavy membranes.

IMMUNOFLUORESCENCE STAINING

Total of 3×10^5 cells were placed into 60-mm culture dishes with cover glasses, and cultured overnight. The cells were left alone or transduced with Ad-β-Gal or Ad-PTEN adenovirus. Thirty-six hours later, the cells were fixed with 4% paraformaldehyde and permeabilized with 0.05% Triton X-100 and subsequently blocked with 1% BSA for 1 h, and the cells were incubated with rabbit anti-pMADD antibody [Li et al., 2010] at 4°C, washed and probed with a biotinylated anti-rabbit antibody (Caltag Laboratories, CA, USA) and streptavidin-FITC (BD PharMingen). Normal rabbit anti-serum was used as a negative control. The image was visualized and captured with a Zeiss LSM 710 META confocal microscope.

IMMUNOPRECIPITATION OF MADD- AND MADD3A-GST

We expressed GST-tagged wtMADD and MADD3A in bacteria (b-wtMADD-GST, or b-MADD3A-GST, respectively), purified the proteins and then incubated them with HeLa cell cytosol. To determine MADD- and MADD3A-GST binding to 14-3-3, the mixture was immunoprecipitated using an anti-14-3-3 antibody. Subsequently, the immunoprecipitate was subjected to Western blotting using an anti-GST or an anti-14-3-3 antibody.

PLASMA MEMBRANE PREPARATIONS

Plasma membrane and cytosolic fractions were prepared as previously described [Barber et al., 2007]. Cells were washed in an ice cold buffer (160 mM NaCl, 38 mM HEPES, pH 7.4, 1 mM $MgCl_2$, 1 mM EGTA) containing a protease inhibitor cocktail (Roche). They were sonicated (with setting at 5 and 20 V) with three pulses of 15 s with 15 s pauses on ice, subjected to low speed centrifugation at 400*g*, for 10 min at 4°C, to remove unbroken cells, debris, and nuclei. Further the lysates were separated by ultracentrifugation at 117,000*g*, 60 min at 4°C into cytosol and membrane fractions. Membrane pellets were re-suspended in 2× Laemmli sample buffer and boiled for 5 min. All samples were frozen at −80°C. Equal loading for membrane and cytosolic fractions were monitored by re-probing the membrane with anti-caveolin-1 antibody or anti-β-actin antibody, respectively.

IMMUNOPRECIPITATION

Immunoprecipitations were performed as described earlier [Mulherkar et al., 2007]. The samples were pre-cleared with 10% (v/v) Protein-A agarose (Roche) for 1 h on a rocking platform. Specific antibodies were added and rocked for 1 h. Immunoprecipitates were captured by incubating with 10% (v/v) Protein-A agarose for an hour. The agarose beads were spun down and washed three times with NET/NP40 buffer (150 mM NaCl, 2 mM EDTA, 50 mM Tris-HCl pH 7.5, 0.1% NP-40). The antigens were released and denatured by adding SDS sample buffer. Immunoblots were prepared and analyzed as described above.

IN VITRO BINDING ASSAY

In vitro protein binding assay was performed as described elsewhere [Moreau et al., 2003; Cartron et al., 2004]. In brief, GST-tagged wtMADD and MADD3A were expressed in bacteria. wtMADD or MADD3A was incubated with 50 μl HeLa cell cytosol (2 μg/μl) in binding buffer (142 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES (pH 7.4), 0.5 mM DTT, 1 mM EGTA, 0.1% NP-40 and a protease inhibitor cocktail) at 4°C for 2 h. Immunoprecipitation with the anti-14-3-3 antibody was performed as described above. To perform binding assay using YFP-tagged proteins, HEK293 cells were transfected with the constructs of YFP-wtMADD or YFP-MADD3A. Forty-eight hours after transfection, cells were harvested and plasma membranes and cytosol were prepared. The plasma membranes were lysed with a lysis buffer. The plasma membrane lysates (100 μl, 2 μg/μl) and cytosol (100 μl, 2 μg/μl) were then incubated with HeLa cell cytosol (100 μl, 2 μg/μl) at 4°C for 10 h. Immunoprecipitation with the anti-14-3-3 antibody was performed as described above.

CONSTRUCTION OF MADD siRNA

Construction of lentiviruses containing siRNA of MADD or the scramble siRNA is described elsewhere [Mulherkar et al., 2006].

STATISTICAL ANALYSIS

All results are expressed as mean ± SEM of at least three independent experiments. Data were evaluated by Student's *t*-test. A 1-way ANOVA was used for multiple comparisons. A value of $P < 0.05$ was considered significant.

RESULTS

TRAIL INDUCED REDUCTION IN pMADD LEVELS IS DEPENDENT ON PTEN

MADD is a survival factor and contributes to cancer resistance to TRAIL treatment. Nevertheless, some types of cancer cells readily undergo apoptosis even in the presence of MADD [Mulherkar et al., 2006, 2007; Subramanian et al., 2009; Li et al., 2011, 2013; Turner et al., 2013]. We explored the molecular mechanism by which TRAIL induces apoptosis in cancer cells with abundant MADD expression. MADD is expressed in HeLa cells. However, TRAIL treatment could induce a reduction in pMADD levels without reducing the total MADD levels (Fig. 1A). Since our recent work revealed that MADD is phosphorylated by Akt [Li et al., 2010], we determined if Akt expression or phosphorylation was altered upon TRAIL treatment. Akt expression levels remained unchanged, but its phosphorylation levels were reduced in response to TRAIL treatment (Fig. 1A). Because PTEN can regulate Akt activation via PI3K [LoPiccolo et al., 2007; Carracedo and Pandolfi, 2008], we tested whether PTEN is related to the reduction in MADD phosphorylation

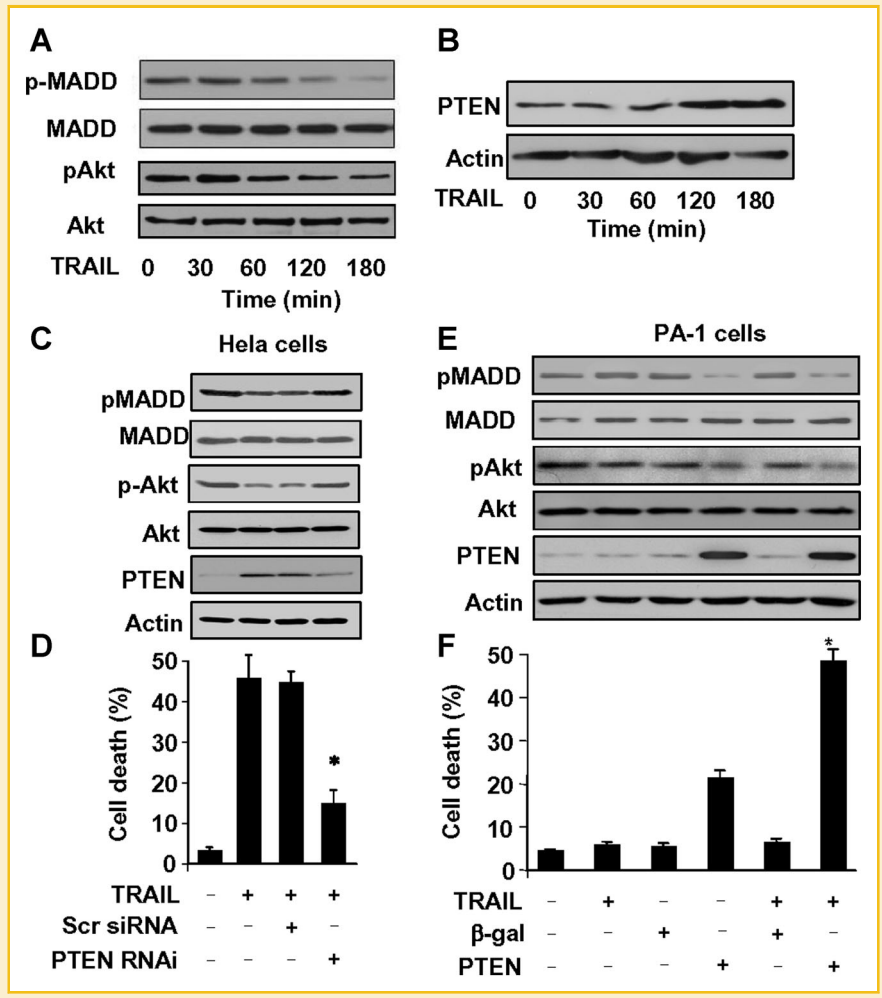

Fig. 1. TRAIL induces a reduction in MADD phosphorylation levels dependent on PTEN. A: TRAIL treatment induces a reduction in MADD and Akt phosphorylation levels. HeLa cells were treated with 50 ng/ml TRAIL, and then harvested at the indicated time for the analysis of MADD and Akt phosphorylation levels by immunoblot using the anti-phospho MADD or the anti-phospho Akt antibody. Loaded MADD and Akt were visualized by staining the same membrane with an anti-MADD or the anti-Akt antibody. B: PTEN is elevated in response to TRAIL treatment. Cells were treated as described for (A). PTEN levels were detected by immunoblot using the anti-PTEN antibody. C: Knockdown of PTEN attenuates the decrease in Akt and MADD phosphorylation levels induced by TRAIL. HeLa cells were transfected with the PTEN siRNA or the siRNA control. 48 h after transfection, cells were treated with 50 ng/ml TRAIL. Cells were harvested 2 h after TRAIL treatment for the analysis of Akt or MADD phosphorylation levels by immunoblot. D: Knockdown of PTEN attenuates cell death induced by TRAIL. HeLa cells were treated as described for (C). Cell death was analyzed 12 h after TRAIL treatment. $^{*}P < 0.05$ versus TRAIL alone. Various treatment groups are indicated under panel D and the same holds true for the corresponding lanes in panel C. Data are expressed as the mean $\pm$ SEM of three independent experiments. Unlike HeLa cells which are TRAIL sensitive, the PA-1 ovarian carcinoma cells are TRAIL resistant and the PTEN levels are not up regulated upon TRAIL treatment. Therefore, we determined if TRAIL treatment upon expression of exogenous PTEN can increase TRAIL sensitivity. E: Shows that treatment with TRAIL upon PTEN expression can reduce the levels of pAkt and pMADD, and (F) shows concomitant increase in TRAIL sensitivity. Various treatment groups are indicated under panel F and same holds true for the corresponding lanes in panel E.

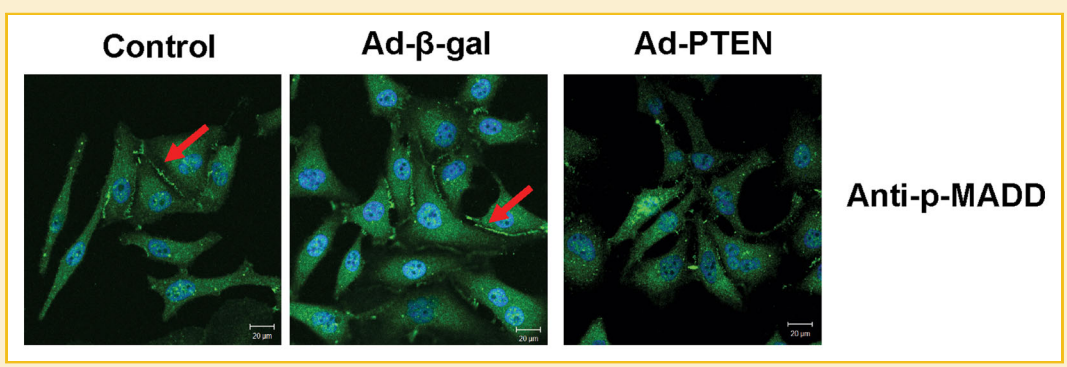

Fig. 2. MADD redistributes from the plasma membrane to cytoplasm upon modulation of Akt. HeLa cells were transduced with a control (Ad-β-Gal) or PTEN expressing (Ad-PTEN) adenovirus. Expression of phosphorylated MADD was detected by immunofluorescence staining with the anti-phospho MADD antibody. Arrows point to FITC staining of MADD protein localized to the plasma membrane in the control and Ad-β-Gal transfected cells, which is absent in Ad-PTEN transfected cells.

levels. The expression levels of PTEN were elevated upon TRAIL treatment (Fig. 1B). These data suggested that TRAIL can target Akt, MADD, and PTEN.

To understand whether PTEN plays a functional role, we tested whether knockdown of PTEN can influence cell fate in response to TRAIL treatment. Knockdown of PTEN could attenuate the decrease in the levels of pAkt and pMADD (Fig. 1C). Concomitantly, cell death induced by TRAIL could be attenuated upon PTEN knockdown (Fig. 1D).

Earlier we had noted that PA-1 ovarian carcinoma cells are resistant to TRAIL induced apoptosis, and the resistance was related to the inability of TRAIL to increase the levels of PTEN and thus reduce the levels of pAkt and pMADD in these cells [Li et al., 2010]. Therefore, we wanted to determine if expression of exogenous PTEN could suppress the levels of pAkt and pMADD and as a consequence render these cells TRAIL sensitive. As shown in Figure 1E, the levels of both pAkt and pMADD were attenuated in PTEN expressing cells. Similarly cells expressing PTEN showed increased sensitivity to TRAIL (Fig. 1F). Taken together, it appears that PTEN can induce a reduction in pMADD levels and enhance susceptibility to TRAIL treatment.

NON-pMADD IS RELEASED FROM THE PLASMA MEMBRANE TO CYTOPLASM

In the following experiments, we explored the molecular mechanism by which non-pMADD triggers apoptosis. Our previous work showed that MADD is constitutively bound to DR4 in cells [Li et al., 2010]. However, upon treatment with TRAIL, a PI3K inhibitor or a dominant negative Akt (DN-Akt), the levels of pMADD was significantly reduced with concomitant loss of MADD biding to DR4. In contrast, in cells treated with a constitutively activated Akt Ca-AKT increased pMADD binding to DR4 and accumulation in the plasma membrane [Li et al., 2010]. These results are further confirmed by immunofluorescence staining (Fig. 2). In control cells and cells transfected with an empty vector (Ad-β-Gal) pMADD could be readily detected on the

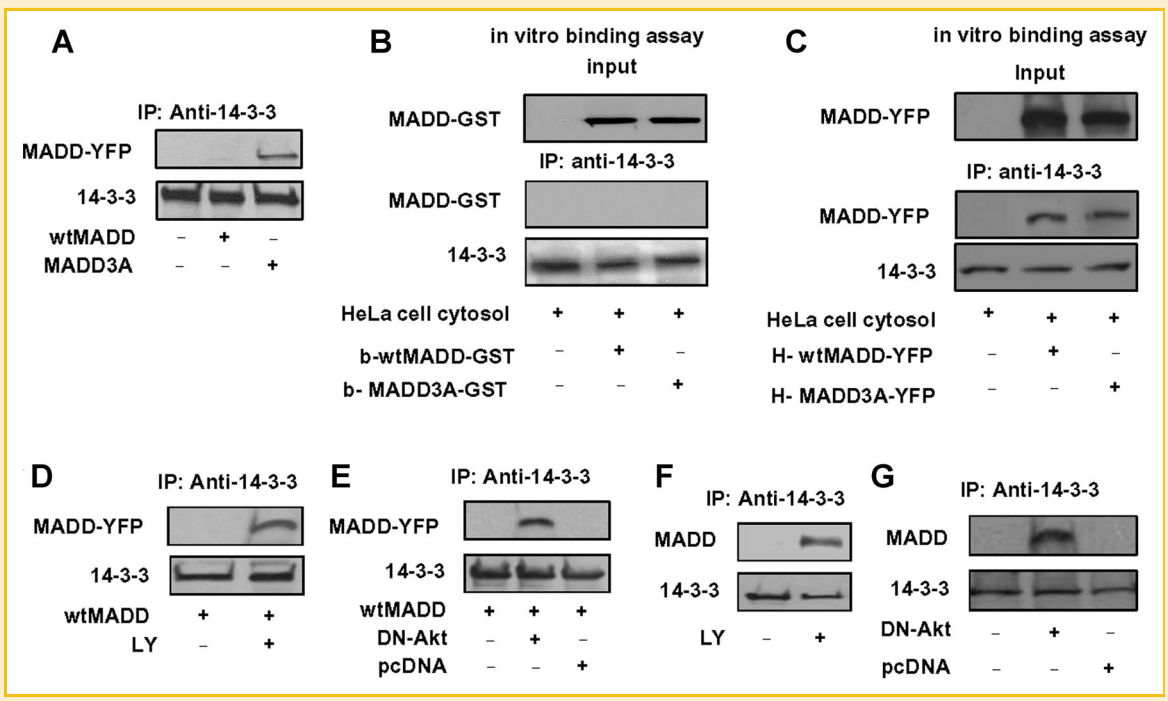

Fig. 3. Lack of phosphorylation at the Akt sites allows MADD binding to 14-3-3. A: MADD3A but not wtMADD binds to 14-3-3. HEK293 cells were transfected with wtMADD or MADD3A. Thirty-six hours after transfection, the cells were harvested for immunoprecipitation using an anti-14-3-3 antibody followed by immunoblotting using an anti-YFP antibody for detecting exogenous MADD. The membrane was re-probed with an anti-14-3-3 antibody to show loading. B: WtMADD and MADD3A produced in bacteria are unable to bind to 14-3-3 in vitro. GST-tagged wtMADD and MADD3A were expressed in bacteria (b-wtMADD-GST, or b-MADD3A-GST, respectively), purified and then incubated with HeLa cell cytosol. Immunoprecipitation with the anti-14-3-3 antibody was followed by immunoblot using an anti-GST or an anti-14-3-3 antibody. C: WtMADD and MADD3A produced from HEK293 cells are able to bind to 14-3-3 in vitro. YFP-tagged wtMADD and MADD3A were expressed in HEK293 cells (H-wtMADD-YFP, or H-MADD3A-YFP, respectively). In vitro binding assay was performed as described in Experimental Procedures Section. Immunoprecipitation with the anti-14-3-3 antibody was followed by immunoblot using an anti-YFP or an anti-14-3-3 antibody. D: Inhibition of PI3K can lead to the association of wtMADD with 14-3-3. HEK293 cells were transfected with wtMADD. Cells were serum starved for 20 h and treated with LY (10 μM) for 1 h. Immunoprecipitation was performed using an anti-14-3-3 antibody, followed by immunoblotting using an anti-YFP or an anti-14-3-3 antibody. E: Inhibition of Akt can result in the association of wtMADD with 14-3-3. HEK293 cells were co-transfected with wtMADD along with DN-Akt or pcDNA. Cells were harvested for immunoprecipitation with an anti-14-3-3 antibody followed by immunoblotting using the anti-YFP or anti-14-3-3 antibodies. F: Inhibition of PI3K leads to the association of endogenous MADD with 14-3-3. HeLa cells were serum starved for 20 h, then treated with LY (10 μM) for 1 h. Immunoprecipitation with the anti-14-3-3 antibody was followed by immunoblotting using anti-MADD or anti-14-3-3 antibodies. G: Inhibition of Akt results in the association of endogenous MADD with 14-3-3. HeLa cells were transfected with DN-Akt or pcDNA. Immunoprecipitation with the anti-14-3-3 antibody was followed by immunoblot using an anti-MADD or an anti-14-3-3 antibody. Representative results of three independent experiments are shown.

plasma membrane. In contrast, in cells treated with PTEN (Ad-PTEN) there was little or no membrane staining. These results further suggested that MADD distribution within the cell is determined by its Akt mediated phosphorylation status.

NON-pMADD BINDS TO 14-3-3

It is known that 14-3-3 participates in the regulation of a variety of signaling processes including apoptosis by interacting with phosphorylated and non-phosphorylated intracellular signaling molecules [Manning and Cantley, 2007]. We tested whether MADD can interact with 14-3-3. Surprisingly, our data showed a significant association between 14-3-3 and non-phosphorylatable MADD3A but not phosphorylatable wtMADD, indicating that MADD3A can associate with 14-3-3 in cells (Fig. 3A).

14-3-3 is known to predominantly interact with the phosphorylated proteins. Therefore, we wondered as to why MADD3A and not the wtMADD could bind to 14-3-3 in cells. MADD is a large protein consisting of 1,588 amino acids. It is possible that MADD3A undergoes post-translational modifications including phosphorylation of other serine or threonine residues, and such modifications are necessary for the association of MADD3A and 14-3-3. To test this hypothesis, we employed the in vitro binding assay to compare the ability of MADD produced in bacteria (i.e., b-MADD-GST and b-MADD3A-GST) with that produced in mammalian cells (i.e., H-WtMADD-YFP and H-MADD3A-YFP) for their ability to associate with 14-3-3. Both recombinant wtMADD and MADD3A produced in bacteria were unable to bind to 14-3-3 (Fig. 3B). In contrast, both wtMADD and MADD3A produced in HEK293 cells were able to bind to 14-3-3 in vitro (Fig. 3C). The ability of wtMADD to bind to 14-3-3 in vitro but not in cells suggested that wtMADD in the whole cell is sequestered from 14-3-3 because of its binding to DRs.

To further confirm the in vivo results, we tested whether MADD could interact with 14-3-3 under conditions where PI3K or Akt was inhibited. The exogenous wtMADD in the presence, but not in the

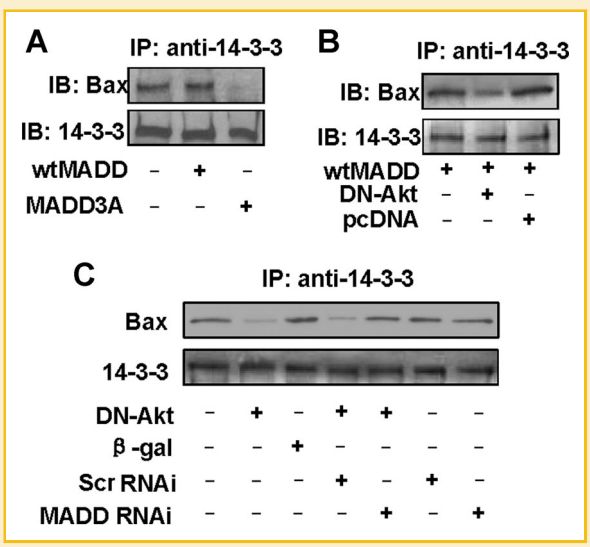

Fig. 4. Phosphorylation status of MADD can influence the binding of 14-3-3 to Bax. A: MADD3A but not wtMADD can cause Bax dissociation from 14-3-3. HEK293 cells were transfected with wtMADD or MADD3A, and harvested 36 h after transfection. Immunoprecipitation using an anti-14-3-3 antibody was followed by immunoblotting using an anti-Bax antibody. Protein loading was illustrated using anti-14-3-3 antibody. B: Inhibition of Akt influences the binding of 14-3-3 to Bax. HEK293 cells were transfected with cDNAs encoding wtMADD along with DN-Akt or the empty vector pcDNA3.1. Immunoprecipitation using an anti-14-3-3 antibody was followed by immunoblotting using an anti-Bax antibody. C: Knockdown of MADD can influence the binding of Bax to 14-3-3. HeLa cells were transduced with MADD siRNA, the scrambled siRNA, DN-Akt or β-galactosidase (β-gal). Immunoprecipitation using an anti-14-3-3 antibody was followed by immunoblottings using an anti-Bax antibody. Representative results of three independent experiments are shown.

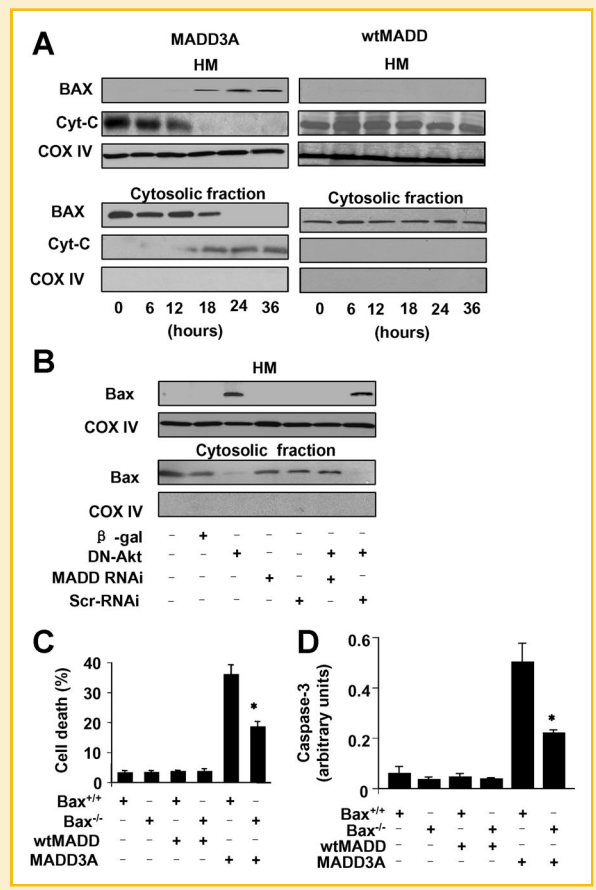

Fig. 5. MADD not phosphorylated at the Akt sites triggers Bax translocation to the mitochondria. A: MADD3A but not wtMADD is able to induce Bax translocation and cytochrome c release. HEK293 cells were transfected with MADD3A or wtMADD. The distributions of Bax and cytochrome c in mitochondria-enriched heavy membrane (HM) and cytosolic fractions were analyzed by immunoblotting using antibodies against Bax or cytochrome c. Cytochrome oxidase subunit 4 (COX IV) served as a mitochondrial marker. B: Endogenous MADD is required for Bax translocation. HeLa cells were transduced with β-galactosidase (β-gal), DN-Akt, MADD siRNA or scrambled siRNA and 24 h later the cells were harvested and Bax distribution in mitochondria and cytosol was determined by immunoblotting using an anti-Bax antibody. C,D: Bax is required for MADD3A-induced apoptosis. HCT116Bax$^{-/-}$ and HCT116Bax$^{+/+}$ cells were transfected with the wtMADD or MADD3A constructs, and 48 h later the cell death was determined by trypan blue exclusion (C) and apoptotic cell death was assessed by measuring caspase-3 activity. $^{*}P < 0.05$ versus Bax$^{+/+}$ + MADD3A in both experiments.

absence, of LY (LY294002, aPI3K inhibitor, EMD Millipore, Billerica, MA, Fig. 3D) or DN-Akt (Fig. 3E) was able to bind to 14-3-3. Similarly, an interaction between endogenous MADD and 14-3-3 could be detected only in the presence, but not in the absence, of LY (Fig. 3F) or DN-Akt (Fig. 3G). These data indicated that lack of phosphorylation by Akt is necessary for MADD to bind to 14-3-3.

BINDING OF MADD TO 14-3-3 RELEASES BAX

What is the molecular consequence of MADD association with 14-3-3 in the apoptotic cascade? Bax has been shown to bind to 14-3-3 [Nomura et al., 2003] and is also necessary for TRAIL-induced apoptosis [Kim et al., 2004]. Therefore, we investigated to see if MADD binding to 14-3-3 was of consequence to Bax association with 14-3-3 in cells expressing wtMADD and MADD3A. We observed that enforced expression of MADD3A but not wtMADD led to the dissociation of Bax from 14-3-3 (Fig. 4A). The wtMADD in the presence, but not in the absence, of DN-Akt was able to reduce Bax association with 14-3-3 (Fig. 4B). We tested whether this holds true for endogenous MADD. As shown in Figure 4C, in the presence of DN-Akt there was a decreased association between Bax and 14-3-3. Because Akt has a variety of substrates, we tested whether the disassociation of Bax from 14-3-3 required non-pMADD. DN-Akt could reduce Bax-14-3-3 interaction only in the presence of MADD, but not when MADD was knocked down. These data indicated that non-pMADD can cause Bax release from 14-3-3.

NON-pMADD TRIGGERS BAX TRANSLOCATION TO THE MITOCHONDRIA

To test whether Bax is the downstream mediator of apoptosis noted in the presence of non-pMADD, we tested for Bax localization. Bax was translocated to the mitochondria in cells expressing MADD3A but not wtMADD. Concomitantly, cytochrome *c* was released from the mitochondria into the cytosol (Fig. 5A). These data suggested that upon displacement from 14-3-3 by non-pMADD, Bax translocates to mitochondria.

To determine whether endogenous non-pMADD is able to induce Bax translocation, DN-Akt was employed to inhibit Akt activity. As shown in Figure 5B, Bax translocation to the mitochondria could be observed in cells expressing DN-Akt. To test whether Bax translocation induced by DN-Akt is related to MADD, MADD RNAi was employed to knock down endogenous MADD. DN-Akt in the presence, but not in the absence, of endogenous MADD was able to induce Bax translocation to mitochondria.

Further, we tested whether Bax is required for MADD3A-induced apoptosis. MADD3A induced significantly less cell death (Fig. 5C) and apoptosis (Fig. 5D) in HCT116Bax$^{-/-}$ cells than in HCT116Bax$^{+/+}$ cells. Taken together, these data suggested that non-pMADD can provoke the intrinsic apoptotic pathway.

TRAIL-INDUCED BAX TRANSLOCATION TO MITOCHONDRIA IS RELATED TO Akt AND PTEN

The ability of non-pMADD to induce Bax translocation led us to consider whether TRAIL mediated death also involves Bax translocation to mitochondria. As shown in Figure 6A, upon TRAIL treatment, Bax translocated from the cytoplasm to the mitochondria. To determine if Akt can influence Bax translocation, we expressed caAkt and found that it could block Bax translocation to the mitochondria (Fig. 6B). Furthermore, we observed that knockdown of PTEN could attenuate Bax translocation to mitochondria (Fig. 6C). Taken together, these results indicated that Akt and PTEN, the upstream regulators of MADD function, are involved in TRAIL-induced Bax translocation.

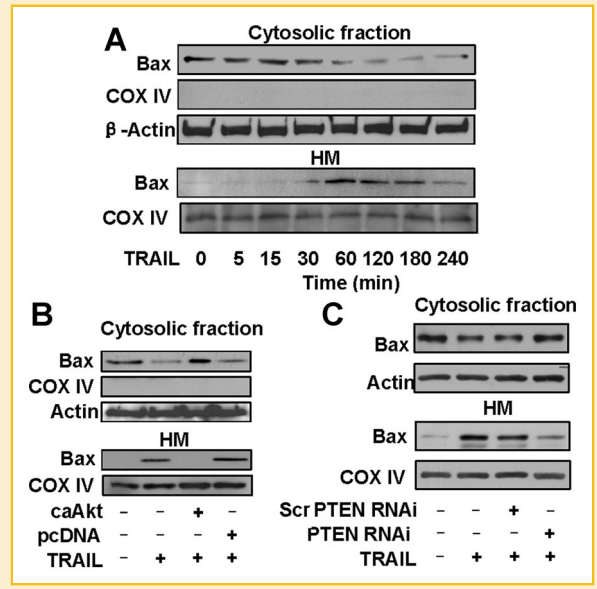

Fig. 6. TRAIL-induced Bax translocation is dependent on PTEN. A: TRAIL induces Bax translocation to mitochondria. HeLa cells were treated with TRAIL (50 ng/ml). The Bax distribution in cytoplasm and HM was analyzed by immunoblotting using an anti-Bax antibody. COX IV served as a mitochondria marker. B: Bax translocation to mitochondria can be inhibited by the constitutively active form of Akt (caAkt). HeLa cells were transfected with the caAkt or pcDNA3.1 empty vector. Cells were treated with TRAIL (50 ng/ml) for 1 h. Distribution of Bax in cytoplasm and HM was analyzed as described for (A). C: Knockdown of PTEN reduces Bax redistribution to mitochondria. HeLa cells were transfected with the PTEN siRNA or the siRNA control. Forty eight hours after transfection, cells were treated with 50 ng/ml TRAIL. Cells were harvested 1 h after TRAIL treatment for the analysis of Bax distributions as described for (A). Representative result from three independent experiments are shown.

DISCUSSION

Our present work reveals that MADD function is controlled by PTEN-PI3K-Akt signaling pathway. TRAIL treatment leads to the up-regulation of PTEN which reduces the level of pAkt with a concomitant reduction in the levels of Akt pMADD. The non-pMADD activates the intrinsic pathway by displacing Bax from 14-3-3. Subsequently, Bax translocates to mitochondria leading to cytochrome *c* release. Our data clearly demonstrate a critical role for MADD as a downstream mediator of PTEN in regulating the intrinsic apoptotic pathway (Fig. 7).

Akt participates in regulating apoptosis by phosphorylating a variety of apoptotic factors. For example, it can phosphorylate the pro-apoptotic protein Bad and facilitate its binding to 14-3-3 in the

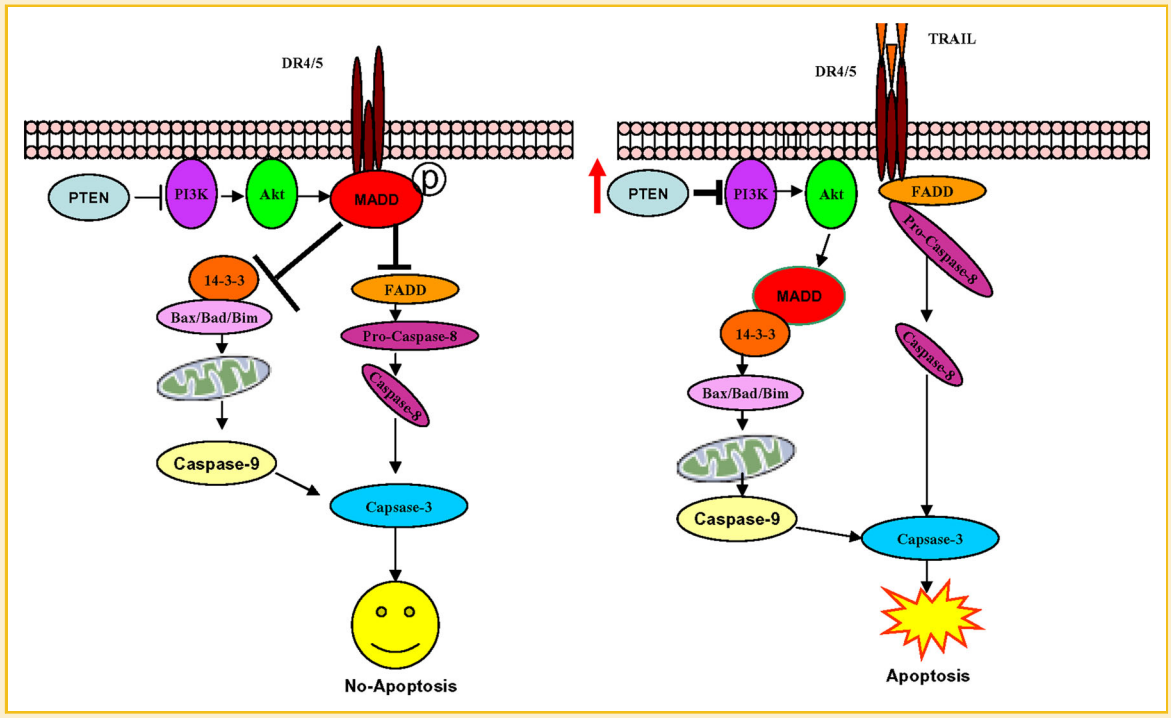

Fig. 7. Diagram illustrates how, under normal condition, Akt-phosphorylated MADD (pMADD) remains bound to death receptor (DR) and prevents death inducing signaling complex (DISC) formation (i.e., recruitment of FADD and caspase-8 to DR) and initiation of extrinsic apoptosis. pMADD binding to DRs also sequesters it from interacting with 14-3-3 and allows Bax to remain bound to 14-3-3 and prevents its translocation to mitochondria where it can initiate intrinsic apoptosis. In TRAIL susceptible cells, TRAIL binding to DR causes up-regulation of PTEN, which inactivates Akt and thus allows accumulation of non-pMADD. Akt non-pMADD loses it binding to DRs and allows DISC formation and activation of extrinsic apoptosis. Moreover, the Akt non-pMADD can now bind to 14-3-3 and displace Bax, which can translocate to mitochondria and initiate intrinsic apoptosis. Therefore, preventing Akt phosphorylation of MADD might render cancer cells more susceptible to the induction of both extrinsic and intrinsic apoptosis.

cytosol, and prevent its translocation to mitochondria where it can exert its pro-apoptotic effect [Zha et al., 1996; Datta et al., 1997]. Bax is also regulated by Akt signaling pathway in that Akt phosphorylates Bax on Ser184 and allows it to remain in the cytosol and prevents its translocation to mitochondria [Gardai et al., 2004; Xin and Deng, 2005]. Thus, these proteins are sequestered upon phosphorylation by Akt and prevented from exerting their pro-apoptotic functions. In contrast, phosphorylation by Akt allows MADD to bind to DRs and thereby sequesters it close to the plasma membrane and prevents its binding to 14-3-3 and thus from exerting its pro-apoptotic function.

Our previous work showed that TRAIL at a low dose is unable to induce cell death. In contrast, the same low dose of TRAIL can trigger cell death when MADD phosphorylation is inhibited [Li et al., 2010]. In the present study, we found that TRAIL at a higher dose is able to induce cell death even in the presence of abundant MADD expression. We further observed that a high dose of TRAIL stimulates the expression of PTEN, which is required for TRAIL to induce a reduction in the levels of pMADD. Although PTEN participates in regulating apoptosis through PI3K-Akt pathway [Cully et al., 2006], its downstream apoptotic mediators have not been fully delineated. Our present work identified MADD as a mediator of apoptotic signal emanating from PTEN.

Mitochondria play a pivotal role in apoptosis by releasing various apoptotic molecules into the cytoplasm, which is triggered by the translocation of pro-apoptotic proteins sequestered in the cytosol into mitochondria. For example, Bax resides predominantly in the cytoplasm of healthy cells, but upon treatment with apoptotic stimuli it translocates to mitochondria where it forms oligomers and causes cytochrome *c* release from mitochondria into cytosol. 14-3-3 has been shown to interact directly with Bax and negatively regulate its function [Nomura et al., 2003]. Some pro-apoptotic proteins can interact with 14-3-3 thereby allowing Bax to dissociate from 14-3-3 and translocate to mitochondria where it can initiate apoptosis. For example, interaction of hepatitis C virus core protein with 14-3-3 causes Bax dissociation from 14-3-3 in the cytosol, causing free Bax to move to mitochondria and provoke apoptosis [Lee et al., 2007]. Interestingly, 14-3-3 family of proteins is known to interact with specific phosphorylated as well as non-phosphorylated signaling molecules [Zhai et al., 2001; Manning and Cantley, 2007; Ottmann et al., 2007]. Our present work shows that the non-pMADD translocates from the plasma membrane to cytosol where it interacts with 14-3-3 causing release of Bax from Bax/14-3-3 complex.

How can non-phosphorylated MADD at the Akt sites influence the association of 14-3-3 with Bax? It has been shown that the c-Jun NH2-terminal kinase (JNK) promotes Bax translocation to mitochondria through phosphorylation of 14-3-3 [Tsuruta et al., 2004]. A C-terminal fragment of MADD (amino acids 1,396–1,588) without the three phosphorylation sites is able to activate JNK, whereas the intact MADD containing the three Akt phosphorylation sites is unable to

activate JNK [Schievella et al., 1997; Zhang et al., 1998]. This could, at least in part, explain why the non-pMADD can influence the association of 14-3-3 with Bax.

It is of note that MADD consists of 1,588 amino acids, and among them about 250 are serine and threonine residues. Therefore, it is possible that Akt non-pMADD binds to 14-3-3 because phosphorylation at other sites might control the association of MADD with 14-3-3. Future studies are required to fully resolve this.

TRAIL is able to induce apoptosis in a wide variety of cancer cells with minimal cytotoxicity to most normal cells and tissues. However, resistance to TRAIL-mediated apoptosis induction in cancer cells remains a challenge for the successful application of TRAIL in cancer therapy [Zhang and Fang, 2005]. Our previous work shows that knockdown of MADD can increase the susceptibility of cells to TRAIL-induced apoptosis. Interestingly, our present work reveals that TRAIL treatment could lead to a reduction in the activity of Akt and phosphorylation of its substrate-MADD resulting in cell death, while maintenance of MADD phosphorylation levels by knocking down PTEN or expressing caAkt could inhibit TRAIL-induced apoptosis. These results suggest that PTEN through MADD can act as a key regulator of TRAIL-induced apoptosis. Thus, modulation of the PTEN–MADD signaling pathway might represent an effective and novel therapeutic approach to treat cancer.

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