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Research Article

A novel member of the Rhomboid family, RHBDD1, regulates BIK-mediated apoptosis

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Abstract. Rhomboid family members are widely conserved and found in all three kingdoms of life. They are serine proteases and serve important regulatory functions. In the present study, a novel gene highly expressed in the testis, RHBDD1, is shown to be a new member of the Rhomboid family, participating in the cleavage of BIK, a proapoptotic member of the Bcl-2 family. The RHBDD1-involved proteolytic modification is upstream of the BIK protein degradation pathway. Mutagenesis studies show that the amino acid residues glycine142 and serine144 of RHBDD1 are crucial for its activity in cleaving BIK at a site located in the transmembrane region. Overexpression or knock-down of RHBDD1 in HEK 293T cells can reduce or enhance BIK-mediated apoptosis, respectively. The present findings suggest that, by acting as a serine protease, RHBDD1 modulates BIK-mediated apoptotic activity.

Keywords. RHBDD1, BIK, cleavage, degradation, apoptosis.

Introduction

Proteolysis is a highly effective regulatory mechanism. A well known proteolytic mechanism is "ectodomain shedding" [1], by which extracellular domains of integral membrane proteins are shed and released, with ADAM family proteases as the best known example [2]. A new regulatory paradigm of proteolysis is the regulated intramembrane proteolysis (RIP) [3], where some proteases, including cysteine proteases, serine/threonine proteases and metalloproteases, cleave their substrates within the transmembrane domains, resulting in the production of segments containing cytoplasmic or extracellular/luminal domains. A subset of such fragments has been shown to serve as biological effectors at other intracellular loci [4, 5]. RIP can be mediated by two mechanisms: two-step cleavage, such as the metalloprotease for Notch cleavage [6]; and one-step cleavage, mediated by Rhomboid serine proteases. RIP is used in signaling pathways in organisms ranging from bacteria to humans [3].

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Rhomboid proteases constitute probably the most widely conserved polytopic-membrane-protein family identified to date [7] and share conserved biochemical characteristics throughout the entire kingdom. In Drosophila, they regulate the EGF receptor signaling pathway, thereby controlling growth and development [8, 9]. In yeast, they play important roles in mitochondrial membrane remodeling [10]. In parasites, proteases containing Rhomboid domain take part in invasion [11]. Despite the functions in lower eukaryotes, the function of vertebrate Rhomboid proteases remains unknown. Identification of target substrate is crucial in studying the function of vertebrate Rhomboid protease; however, only a limited number of substrate proteins have been identified. A recent exciting finding is the discovery of a mammalian Rhomboid protease, PARL (Presenilin-associated rhomboid-like) and its substrate – OPA1 (Optic Atrophy 1), whose function was shown to be associated with apoptosis[12–14]. In the present study, we focused on RHBDD1, a protein highly-expressed in human testis containing a rhomboid domain with unknown function.

Bcl-2-family proteins are known to interact with each other in promoting or inhibiting apoptosis. Bcl-2-related proteins can either be anti-apoptotic, such as BCL-XL, Mcl-1, BCL-W and A1/BFL-1; or proapoptotic, such as Bax, Bik, and Hrk (BH3-only). The multiplicity of factors and the complex regulation of these proteins allow refined control over apoptosis. Many modifications, such as proteolysis, can regulate the activation of these proteins. For example, caspase-8 can cleave full length Bid and produce the truncated form (tBid), which in turn translocates into the mitochondria [15, 16]. In the present study, BIK was identified as an interacting protein in a yeast two-hybrid screening using RHBDD1 as bait, raising the possibility that BIK is a potential substrate of RHBDD1. Therefore, the present study aimed to determine the function of RHBDD1 and establish its role in BIK processing and apoptotic regulation.

Materials and methods

Plasmid constructions. *Rhbdd1* and *bik* genes were amplified from a human testes cDNA library (CytoTrap XR custom and premade libraries purchased from Stratagene.) by PCR (the sense primer of *rhbdd1:* ATGCAACGGAGATCAAGAGGG, the antisense primer of *rhbdd1:* CTGGCTATCGAATCTGTG; the sense primer of *bik:* CCGCCAGAG GAGAAATGTCT, the antisense primer of *bik:* TGTTCCAGCACTA TCTCGGG). A pCDNA6/V5-HisB vector (Invitrogen) was inserted a HA or Flag tag sequence between Nhe I and Hind III sites in the upstream of the multiple cloning site. *rhbdd1* and *bik* PCR products were cloned into pCDNA6/V5-HisB-HA/Flag and pEGFP-N1 under the control of CMV promotor. pSilencer-1.0-U6 (Ambion) was used to construct RNAi vectors. An RNAi oligo against *rhbdd1* was cloned into pSilencer-1.0-U6 (the sense primer: TAGATGGTTTGCCTATGTTTCAAGAGAACATAGGCAAACCATCTACTTTTTG, the antisense primer: AATTCAAAAAGTAGATGGTTTGCCTATGTTCTCTTGAAACATAGGCAAACCATCTA). All point mutants were generated using Molecular Cloning Method (chapter 13, protocol 6, site-specific mutagenesis by overlap extension). The following chimeras were constructed: G142A, S144A (with glycine142, serine144 replaced by alanine) and BIK (GG/FF) (with glycine 153, 154 of BIK both replaced by phenylalanines). ΔM is a truncated RHBDD1, lacking the segment from 134 to 203 residues. All sequences of the mutations were verified by sequencing analysis. They were cloned into pCDNA6/V5-His-HA/Flag vectors.

Cell culture and transfection. HeLa and HEK 293T cells were cultured in fresh DMEM with 10% FBS. Adherent cells were passaged every two–three days with 0.5 mg/mL trypsin (1:250) and 0.53 mM EDTA. All transfections were performed using Lipofectamin 2000 reagent (Invitrogen).

Northern blot assay. In the Northern blot assay a commercial mRNA blot membrane, Human Adult Normal Tissue mRNA Northern Blot XII (Chinese National Human Genome Center, Beijing (CHGB). SinoGenoMax Co., Ltd) was used. The specific cDNA probe was labeled with (α^{32}P) dCTP by random primed labeling and purified on Sephadex G 50 resin. Hybridization was performed overnight at 68 °C; the blot was washed three times with 2×SSC buffer containing 0.5% SDS at 10-, 10- and 20-minute intervals at room temperature. Finally, the blot was exposed to a film for 2–4 days at –70 °C.

Western blot assay. Cells were lysed in a lysis buffer [20 mM HEPES (pH 7.5), 0.1% SDS, 1% Triton X-100, 120 mM NaCl, 10% glycerol, 10 mM tetrasodium pyrophosphate, 20 mM β-glycerolphosphate, 1 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL Pepstatin A, and 10 μg/mL leupeptin]. 80 μg of protein were loaded into 15% SDS-PAGE gel and transferred onto PVDF membrane. Polyclonal rabbit anti-RHBDD1 antibody was prepared in our lab. The following antibodies were purchased from commercial suppliers: anti-BIK (Cell Signaling Technology, #4592). anti-HA and anti-

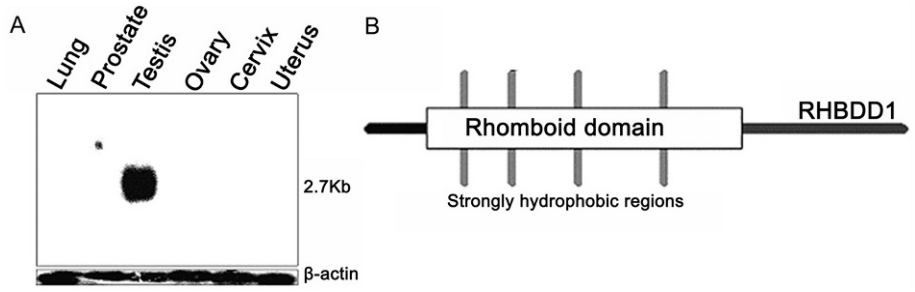

Figure 1. Structural characteristics and tissue distribution of RHBDD1. (*A*) Northern blotting assay for rhbdd1 in various human tissues. A C-terminal 300bp cDNA sequence of rhbdd1 was used as the specific probe labeled with (a-32P) ATP. A 2.7Kb transcript was detected in the testis. (*B*) The structure of RHBDD1 protein. The Rhomboid domain is located in N-terminal of RHBDD1; four strongly hydrophobic regions were found within the Rhomboid domain.

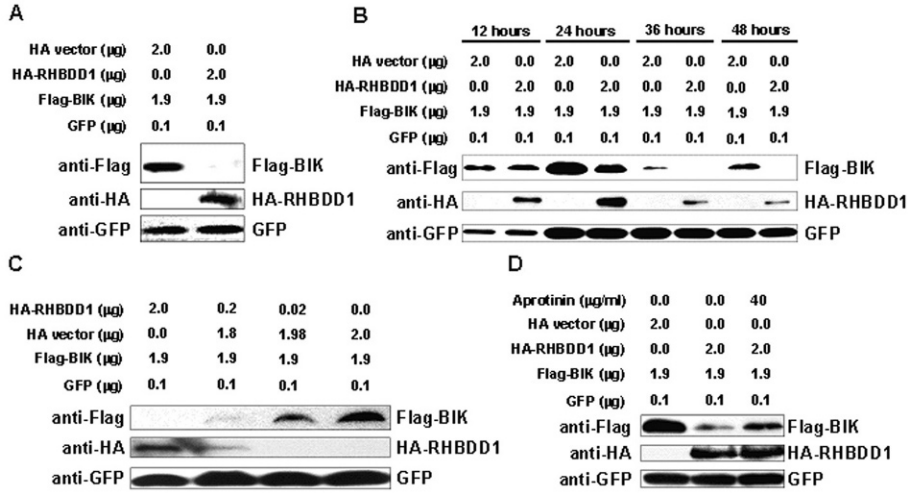

Figure 2. RHBDD1 cleaves BIK. (*A*) Co-transfection of HA-RHBDD1 and Flag-BIK in HEK 293T cells showed decreased level of full length BIK due to its cleavage. (*B*) Time-dependent cleavage of Flag-BIK (Harvest cells at different time points posttransfection). (*C*) Dose-dependent cleavage of Flag-BIK (Harvest cells after 48 hours). (*D*) Aprotinin inhibited BIK cleavage mediated by RHBDD1 (addition of fresh complete DMEM medium containing 40μg/mL Aprotinin at four hours post-transfection and incubation for 24 hours). GFP was used as a transfection and loading control.

Flag (Sigma). anti-caspase 3, anti-GFP, and anti-β-actin (Santa Cruz). Immunodetection was performed using the ECL system (Santa Cruz).

Induction of apoptosis in HEK 293T cells. HEK 293T cells were transfected with pSilencer-RHBDD1 or pSilencer-control in knock-down system and pCDAN6-HisB-HA-RHBDD1 or pCDAN6-HisB-HA in overexpression system. At four hours post transfection, fresh medium with 1μM Velcade was added to the cells for further incubation of 20 hours before harvest for detection of procaspase 3 cleavage.

Results

***Rhbdd1* gene is highly expressed in human testes.** *Rhbdd1* cDNA was isolated from a human testis library by PCR. The estimated size of *rhbdd1* transcript was about 2.7Kb. Northern blot assay showed that it was highly expressed in human testes as compared to other tissues examined (Fig. 1A). This gene was shown to be expressed in a number of human cell lines, such as HEK 293T, HepG2, HT1080 and MCF-7 (data not shown). Bioinformatics analysis of RHBDD1 indicated that it consisted of 315 amino acids, containing a conserved Rhomboid domain and four strongly hydrophobic regions (Fig. 1B).

RHBDD1 induces BIK cleavage as a serine protease. To determine the interacting protein of RHBDD1, a yeast two-hybrid screening was conducted using RHBDD1 as a bait in a human cDNA library (Shanghai Genomics, China). BIK was identified and the occurrence of interaction between the two proteins was verified by β-galactose assay.

Since Rhomboids are the only intramembrane serine proteases that release the cleaved fragments from transmembrane domains [17], single transmembrane BIK may be the candidate substrate of RHBDD1. when HA-RHBDD1 was co-transfected with Flag-BIK, the later was cleaved effectively with observed decrease in its full length form (Fig. 2A). To further establish an action of RHBDD1, the time and dose-dependency of this enzyme on the rate of cleavage of full length BIK was investigated. As shown in Figure

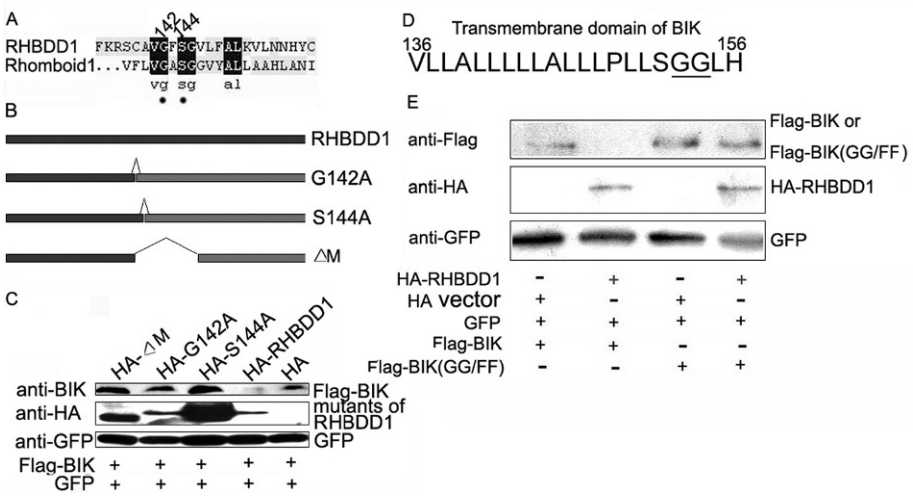

Figure 3. RHBDD1 is a serine protease with BIK as the substrate. (*A*) The alignment between conserved regions of RHBDD1 and Rhomboid1. Black dots are the conserved residues. (*B*) Representative structural models of three RHBDD1 mutants (G142A, S144A and ΔM). (*C*) The BIK cleavage analysis by the three mutants of RHBDD1. (*D*) The peptide sequence of the BIK transmembrane domain. (*E*) BIK (GG/FF), a mutant in which the GG (153,154) motif is replaced with FF sequence. Note that minimal cleavage of the full length mutant BIK (GG/FF) as compared to the prominent cleavage of the intact full length BIK. GFP was used as a transfection and loading control.

2B, the cleavage of BIK was increased gradually from 12 to 48 hours, with the increased expression of RHBDD1 as observed at 48 hours (Fig. 2C). To demonstrate RHBDD1 as a serine protease, the effect of a serine specific inhibitor, Aprotinin [18], on the RHBDD1-induced BIK cleavage was studied and the results showed that Aprotinin caused a significant decrease in RHBDD1-induced BIK cleavage (Fig. 2D), indicating that the effect of RHBDD1 on BIK cleavage may be mediated by an action similar to a serine protease. Taken together, these results suggest that BIK is a potential target substrate of this novel rhomboid protease.

Mutations of conserved rhomboid protease glycine 142 and serine 144 residues hinder RHBDD1 ability to cleave BIK. To further demonstrate that RHBDD1 is a new member of the rhomboid family capable of cleaving BIK as a serine protease, the structure of RHBDD1 was compared with that of Drosophila rhomboid1, a classical rhomboid protease [9], which contains two conserved residues, glycine142 and serine144 (Fig. 3A). To confirm if these residues were essential for the enzymatic activities, each of them was separately replaced by an alanine, or the region containing the GFSGV motif, which was similar to the conserved catalytic motif, GASGG of rhomboid1, was deleted (Fig. 3B). The point mutation of glycine or serine (G142A or S144A) and GFSGV deletion mutant (ΔM) resulted in a decrease in the ability of RHBDD1 to cleave BIK (Fig. 3C). The fact that RHBDD1 contains two conserved and essential residues for cleavage similar to that of the classical rhomboid1 protease suggests that it is a new member of the protease family.

Identification of the critical sites of BIK for RHBDD1 induced cleavage. All Rhomboid proteases cleave the transmembrane domain of their substrates, and thus, the cleavage site of BIK may be located within the transmembrane region. Since the GA/GG motif located in the transmembrane domain of a substrate has been reported to be the primary determinant for rhomboid proteases recognition [19] and the transmembrane domain of BIK also contains a GG motif (Fig. 3D), loss-of-function by point mutation experiment was conducted and the results showed that the RHBDD1-induced cleavage of the mutant BIK, designated as BIK (GG/FF) with glycine residue replaced by phenylalanine was extremely low (Fig. 3E), indicating that the GG motif of BIK is the key determinant recognized by RHBDD1 serine protease.

Primary cleavage of BIK by RHBDD1 is a necessary step prior to its degradation. In the present study, the cleaved products of BIK were hardly detectable probably due to subsequent degradation since BIK is known to be degraded by the proteasome-dependent mechanism [20–22]. Therefore, measuring the decrease of the full length BIK level was used to reflect the cleavage of this protein by RHBDD1. Endogenous BIK levels were analyzed in RHBDD1 knockdown or overexpressing HEK 293T cells, which endogenously expressed both proteins, with or without the presence of the proteasome inhibitor, Velcade.

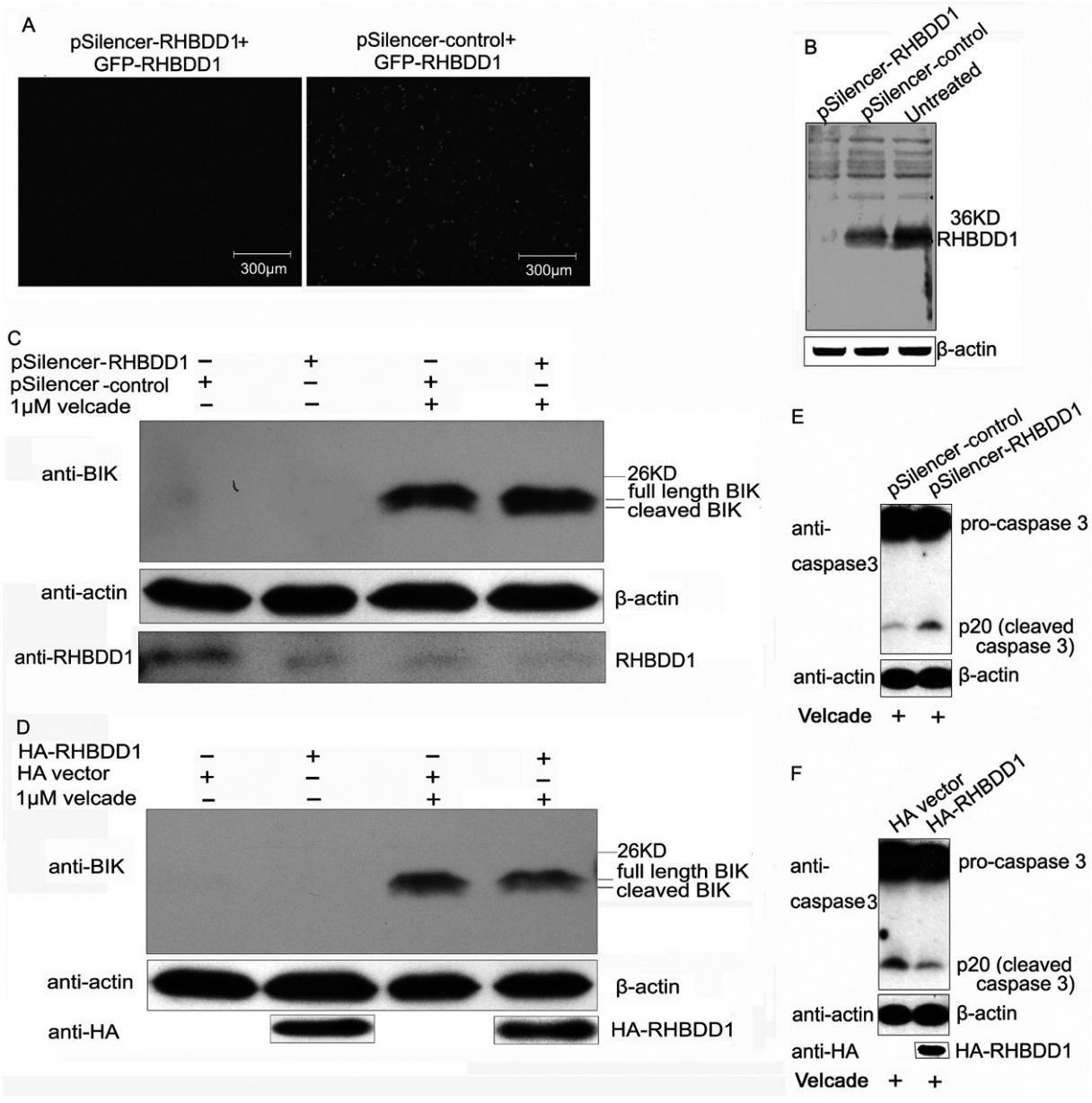

Figure 4. RHBDD1 regulates BIK-mediated apoptosis. (*A*) An effective shRNA were designed for knocking down rhbdd1. Efficient knock down of GFP-RHBDD1 fusion protein in HeLa cells, as indicated by GFP signal, by pSilencer-RHBDD1 shRNA and control. When pSilencer-RHBDD1 and GFP-RHBDD1 were cotransfected into HeLa cells for 24 hours, most of mRNA for GFP-RHBDD1 fusion gene underwent degradation, as shown by less GFP positive signal as compared to more GFP positive signal in the negative control shRNA from pSilencer-control. (*B*) Western blot showing efficient knock down of endogenous RHBDD1 (molecular weight of about 36KD) in HEK 293T cells. In the left lane, the disappearance of ~36KD band on Western blot indicated that most endogenous RHBDD1 expression was knocked down by pSilencer-RHBDD1 in these cells. (*C*), (*D*) The analysis of full length BIK and cleaved BIK in RHBDD1 knockdown or upregulating HEK 293T cells, with or without the presence of proteasome inhibitor, Velcade, showing BIK accumulation in RHBDD1 knockdown but a decrease in RHBDD1 overexpressed HEK 293T cells. BIK was undetectable in the absence of Velcade. Double bands for BIK on Western blot appeared with the treatment of proteasome inhibitor, Velcade. The lower one was the cleaved BIK. (*E*), (*F*) The assay of procaspase 3 cleavage in RHBDD1 knockdown or overexpressing HEK 293T cells in the presence of Velcade. P20 was a cleaved subunit of procaspase 3 during apoptosis. For all experiments, at four hours posttransfection, 1 μM of Velcade was added to cells and incubated for 20 hours.

A RNAi of RHBDD1 was designed and found to be effective in knocking down exogenous GFP-RHBDD1 expression in HeLa cells (Fig. 4A) and endogenous RHBDD1 (36kDa) expression in HEK 293T cells (Fig. 4B). BIK was undetectable in RHBDD1 knockdown or overexpressed cells with the absence of proteasome inhibitor (the left panel of Fig. 4C and D), indicating that the cleaved form of BIK was very unstable in physiological conditions. RHBDD1, as a serine endopeptidase, could not

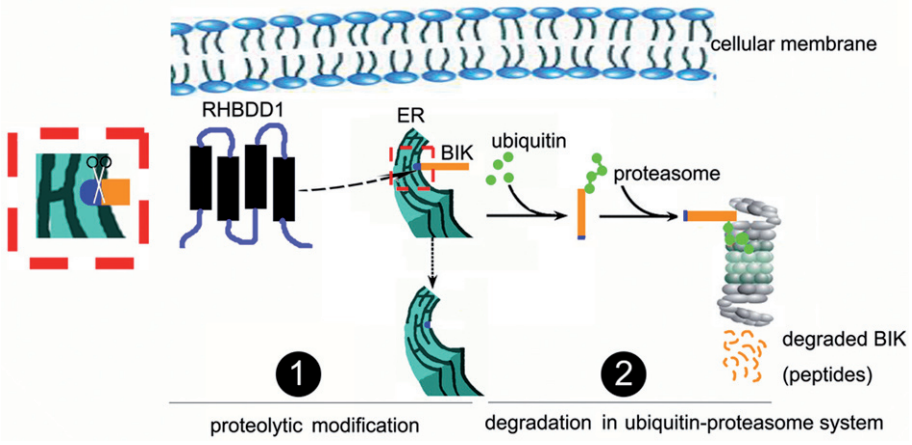

Figure 5. The sketch model depicting cleavage and degradation pathway mediated by RHBDD1.

directly degrade BIK since RHBDD1 suppression did not result in the accumulation of BIK (the left panel of Fig. 4C). BIK appeared as double bands on Western blotting in the inhibitor treatment. The smaller band on Western blotting corresponded to the RHBDD1-cleaved form of BIK, which was cleaved at the C-terminal transmembrane domain at a GG motif. This corresponded to an elimination of seven amino acids and reflected the shift of less than 1 kDa for BIK band, which was difficult to resolve clearly in the Western blot analysis (Fig. 4C and D). The amount of full length BIK in RHBDD1 knockdown cells was increased as compared to that observed in the empty vector transfected control with the treatment of the proteasome inhibitor (the right panel of Fig. 4C), suggesting cleavage of BIK by RHBDD1 prior to proteasome-dependent degradation. On the other hand, The amount of full length BIK in RHBDD1 transfected cells was decreased as compared to that in the control cells in the presence of the proteasome inhibitor (the right panel of Fig. 4D), indicating RHBDD1 cleaving modification accelerated the degradation of BIK. The amount of cleaved BIK did not obviously change in Velcade-treated cells with RHBDD1 knockdown or overexpression, as compared with control, suggesting the evanescence of these cleaved fragments. These findings suggested that RHBDD1 could cleave BIK, and that this proteolytic process was upstream of proteasome-dependent degradation.

RHBDD1 regulates apoptosis by BIK cleavage. Since BIK is a proapoptotic member of the Bcl-2 family, its cleavage induced by RHBDD1 may affect apoptotic activity in the cells. To determine the cellular function of RHBDD1, HEK 293T cells were used as a model since they endogenously express both RHBDD1 and BIK. When RHBDD1 expression was down-regulated by a RNAi, full length BIK accumulation occurred in these cells as compared with the negative control (pSilencer-control) (Fig. 4C). This was associated with a concurrent increase in procaspase 3 cleavage, which was indicated by an increase in a cleaved subunit of procaspase 3, p20, during apoptosis when the cells were challenged with Velcade (Fig. 4E). On the other hand, full length BIK level was decreased by overexpression of RHBDD1 (Fig. 4D), which was associated with a concurrent decrease in p20 subunit as compared with the HA empty vector group when the cells were under Velcade challenge (Fig. 4F). The above findings suggest that RHBDD1 can regulate apoptosis by BIK cleavage in HEK 293T cells.

In addition, MCF-7 and HepG2 cells were used. When the cells were challenged with (S)-(+) camptothecin (CPT) to induce apoptosis, RHBDD1 knockdown expression by a RNAi (pSilencer-RHBDD1)(Supp. Fig. 1A) was associated with a concurrent increase in procaspase 7 cleavage, indicated by an increase in p17, which is a cleaved subunit of procaspase 7 during apoptosis, as compared with the negative control (pSilencer-control) (Supp. Fig. 1B). Under the same condition, on the other hand, overexpression of RHBDD1 was associated with a concurrent decrease in procaspase 7 cleavage, indicated by the decrease in p17 subunit as compared with the HA empty vector group (Supp. Fig. 1C). Similarly, RHBDD1 knockdown in HepG2 cells resulted in much more apoptosis than that in the control (Supp. Fig. 2). The above findings suggested that RHBDD1 was an anti-apoptotic protein.

Discussion

The present study has demonstrated that RHBDD1, a new gene highly expressed in human testis, is a novel member of the rhomboid family of serine proteases. Apart from the bioinformatic hints of its rhomboid

serine protease-like structure [17], RHBDD1 can be classified as a novel member of the mammalian rhomboid family based on the current findings. First, RHBDD1 induces BIK cleavage in a time and dose-dependent manner. Second, its activity is sensitive to a specific serine protease inhibitor, Aprotinin. Third, it contains a GFSGV motif which is homologous to the key region at the active center of classical serine proteases [9] with glycine142 and serine144 being the essential sites for its activity to induce BIK cleavage. Fourth, GG motif in the transmembrane region of BIK is crucial for enzymatic recognition as expected for cleavage by serine proteases. Taken together, RHBDD1 appears to possess the characteristics of a rhomboid serine protease.

RHBDD1 cleaves BIK within the C-terminal transmembrane region to release an N-terminal bulk form containing the functional domain for apoptotic activity [25] from endoplasmic reticulum (ER), and the short form will be rapidly degraded in proteasome system. RHBDD1-induced cleavage of BIK may result in the cleaved BIK being destined to degradation by a proteasome-dependent mechanism, which explains why a decrease in the full length BIK was detected in our experiments, which indicates cleavage of BIK, but hardly the cleaved fragment of BIK. BIK is integrated almost exclusively in the membrane of the endoplasmic reticulum (ER) [23, 24], and it is degraded by a proteasome-dependent mechanism [20–22]. We hypothesize that BIK protein degradation may take two steps. First, RHBDD1 cleaves the transmembrane region of BIK and the major bulk of the protein is released into the cytosol from the endoplasmic reticulum (ER). Second, the cleaved BIK fragment is degraded rapidly by the proteasome (Fig. 5). This notion was supported by the observed increase of full length BIK by RHBDD1 knockdown and its decrease by RHBDD1 overexpression in the presence of the proteasome inhibitor. Also the intact, noncleaved BIK was very stable in transfected cells, indicating that RHBDD1 mediated-cleavage may be upstream of the BIK degradation pathway. In our study, RHBDD1 was not localized in the ER, but in the mitochondria. Informatics prediction shows that RHBDD1 may be localized in mitochondria (k-NN Prediction). GFP-RHBDD1 was co-localized with mitochondrial marker, Mito Tracker Red CMXRos (Molecular Probe) (Supp. Fig. 3A), and transfected RHBDD1 was present in mitochondrial fraction (Supp. Fig. 3B). Interestingly, there is also a report that Rhomboid1 is a Golgi protein whose substrate, Spitz, is normally retained in the ER. Spitz must be moved to the Golgi apparatus by a carrier molecule, Star, where it is subjected to the proteolytic action promoted by Rhomboid1 [8]. A similar scenario may be envisioned for RHBDD1 and its substrate BIK.

Since BIK is a proapoptotic member of the bcl-2 family, we believe that the RHBDD1-induced changes in BIK levels may be an important regulatory mechanism for fine-tuning the apoptotic process in mammalian cells. Indeed the present results have demonstrated that the cleavage of BIK by RHBDD1is associated with apoptosis as indicated by changes in the levels of cleaved procaspase 3, p20, a key signaling molecule in apoptosis. It would be interesting to explore further whether the signaling pathway involved in RHBDD1-regulated apoptosis is unique or not, and whether it has possible cross-talk with other signaling pathways.

In closing, the present study has provided evidence indicating that RHBDD1 is a novel Rhomboid protease that can regulate the apoptotic activity of the cells by affecting the stability of BIK. It is interesting to note that some investigators have reported that *in vivo* delivery of BIK to cancer cells could induce apoptosis, which may be used as a novel strategy of cancer therapy [26–29]. Since RHBDD1 suppression can induce BIK accumulation and increase the sensitivity of cells to apoptotic stimuli, this serine protease may also be considered as a candidate target for therapy against cancer. Further studies exploring the potential applications of this novel serine protease may prove to be beneficial for cancer therapy.

Electronic supplementary material. Supplementary material is available in the online version of this article at springerlink.com (DOI 10.1007/s00018-008-8452-0) and is accessible for authorized users.

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