Molecular and Cellular Biochemistry 292: 189–195, 2006.
DOI: 10.1007/s11010-006-9214-7
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Human homologue of SETA binding protein 1 interacts with cathepsin B and participates in TNF-induced apoptosis in ovarian cancer cells

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Abstract

Lysosomal cysteine protease cathepsin B has been reported to play an important role in apoptosis of many different cancer cells, but the regulation of cathepsin B in apoptosis is poorly understood. Human homologue of SETA binding protein 1 (hSB1) was identified to interact with cathepsin B by yeast-two hybrid method, and the interaction was confirmed *in vitro* GST pull-down assay and *in vivo* coimmunoprecipitation experiment. hSB1 was co-localized with cathepsin B in cellular lysosomes. Our previous study has shown that TNF can induce ovarian cancer cells OV-90 apoptosis and the apoptosis process is cathepsin B-depended. Here we provide evidence that overexpression of cathepsin B-interacting protein hSB1 could suppress TNF-triggered apoptosis in OV-90 cells, but has no effect on cellular cathepsin B activity. hSB1 may function as a regulator of cathepsin B-mediated apoptosis. (Mol Cell Biochem **292:** 189–195, 2006)

Key words: apoptosis, Cathepsin B, human homologue of SETA binding protein 1, OV-90 cells, TNF

Introduction

Apoptosis is an active form of cell death that plays an essential role in development and survival by eliminating damaged or otherwise unwanted cells [1]. Although caspases are well established as the main players in apoptosis, other proteases such as calpains, cathepsins may account for alternative types of apoptosis [2]. Lysosomal proteases cathepsins, in particular cathepsin B (CTSB), have been reported to be involved in the apoptosis triggered by a variety of stimuli, i.e. lysosomotropic detergents treatment, growth factor starvation and oxidative stress [3].

As a pleiotropic cytokine, tumor necrosis factor-*a* (TNF) is able to elicit complex and diverse cellular events, including apoptosis, cell growth and proinflammatory genes expression [4]. Recently increasing evidence suggests that lysosomal cysteine proteases cathepsins are released into cytosol when cells are treated with TNF, and activate the caspase-dependent or caspase-independent pathways of apoptosis, leading to cell death [3, 5–7]. In previous report, we demonstrated that ovarian cancer cells OV-90 was sensitive to TNF cytotoxicity, and cellular lysosomes underwent a permeabilization process during apoptosis, and either CTSB or caspases were involved in OV-90 cells apoptosis triggered by TNF [8].

Human homologue of SETA binding protein 1 (hSB1) gene is a recently cloned novel human gene (Genbank accession no. AF258553). It encodes a protein with 707 amino acids. Analysis of the amino acid sequence of hSB1 reveals the

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presence of one BTB domain at the N terminus followed by three WD40 domains. BTB domain, also designated as POZ domain, is involved in protein interaction and mediates presumably homo- or hetero-dimerization [9]. WD40-containing proteins are a large family found in all eukaryotes and are implicated in a variety of functions ranging from signal transduction and transcription regulation to cell cycle control and apoptosis. The underlying common function of all WD40-repeat proteins is coordinating multi-protein complex assemblies, where the repeating units serve as a rigid scaffold for protein interactions [10]. Accordingly, hSB1 appears to have at least four modalities for binding to other proteins.

As a first step toward understanding the function of hSB1, we took the approach of yeast two-hybrid library screen to identify the interacting proteins of hSB1. One binding partner we isolated was a lysosomal cysteine protease CTSB. *In vitro* pull-down assay and coimmunoprecipitation from transiently transfected cells as well as subcellular co-localization demonstrated the interaction between hSB1 and CTSB. As CTSB is a mediator of TNF-induced apoptosis in OV-90 cells [8], the effect of CTSB-binding partner hSB1 on apoptosis was investigated. Overexpression of hSB1 could suppress apoptosis triggered by TNF in OV-90 cells, while had no influence on cellular CTSB enzyme activity.

Materials and methods

Chemicals

Recombinant human TNF was kindly provided by Professor Shouyuan Zhao (Fudan University). Transcription inhibitor Actinomycin D (AcD) was purchased from ALEXIS CORPORATION. When cells were treated with TNF, AcD (0.2 μg/ml) supplemented to the medium can inhibit the transcription activation of survival genes and block the activation of NF-κB pathway mediated by TNF. LysoTracker Red DND-99, a fluorescent dye that loads predominantly into lysosomes, was from Molecular Probes, CTSB specific inhibitor CA074Me and substrate z-RR-AMC for CTSB were from BIOMOL, and propidium iodide (PI) was from Sigma.

Plasmid construction and cell transfection

Coding sequence of CTSB was in-frame constructed into the fluorescent protein expression vector pDsRed-N1 and hSB1 into pEGFP-N3 for co-localization assay. hSB1 was cloned into vector pcDNA3.1-myc-HisA(-) for analyzing effect of hSB1 overexpression on OV-90 cells apoptosis. Cells were transfected with above plasmids by means of LipofectAMINE 2000 (Invitrogen), following the manufacturer's instructions.

Multiple tissue cDNA PCR analysis

Multiple tissue single-stranded cDNAs isolated from 16 different human tissues were purchased from Clontech (MTC panel I and II) and used as templates. hSB1-specific sense primer 5′- GGCTCCACCCCACTCGCTTCCTTT -3′ and antisense primer 5′- GGCCCACTCCGCACCAGTTCCTG -3′) were used to amplify hSB1. Glyceraldehydes- 3-phosphate dehydrogenase (GAPDH) primers provided by Clontech were used in parallel as the control. All PCR reactions were performed by using KOD-Plus hot-start DNA polymerase (TOYOBO Biotech) and employing the following two-step protocol: one cycle at 94 °C for 30 sec, 40 cycles at 94 °C for 30 sec and at 68 °C for 1 min, followed by a final extension at 68 °C for 5 min. Analysis by agarose gel electrophoresis was performed with aliquot removed at cycle 30, 35 and 40 to compare amplification products from different tissues.

Yeast two-hybrid screening

Here used method was described in detail in Ref. [11]. The open reading frame of hSB1 fused to Gal4-BD domain on the pGBKT7 vector (Clontech) was used as the bait to screen human fetal liver cDNA library. The positive preys were rescued from yeast cells and then sequenced. A mating test was performed to pluck the specific protein-protein interaction in yeast.

Glutathione S-transferase (GST) pull-down assay in vitro

CTSB was cloned into the pGEX-5X-1 vector and expressed in *E. coli* strain BL21. hSB1 was cloned into pGBKT7 vector and the Myc-hSB1 fusion protein was generated by the TNT T7 Quick Coupled Transcription/Translation System (Promega). GST pull-down assay was performed as described in Ref. [11].

Coimmunoprecipitation assay in vivo

hSB1 was constructed into expression vector pCMV-HA and CTSB into pCMV-Myc. HeLa cells were cultivated in 6-well plates to 80% confluence and then co-transfected with pCMV-HA-hSB1 and pCMV-Myc-CTSB. Coimmunoprecipitation assay was performed as described previously [11]. Anti-Myc antibody was used for immunoprecipitation

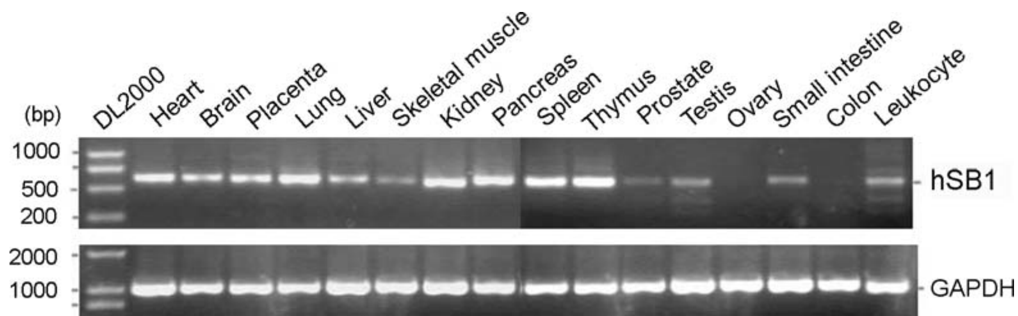

Fig. 1. Expression pattern of hSB1 in different human normal tissues. Partial cDNA sequence of hSB1 (610 bp) was amplified by PCR from multiple tissues cDNAs (Clontech MTC panel I and II). PCR products after 35 cycles were analyzed by electrophoresis on agarose gel. As a control, house-keeping gene GAPDH was amplified by PCR from the same cDNA panels.

and anti-HA antibody for analysis of immunoprecipitated proteins.

hSB1-green fluorescent protein (hSB1-GFP), CTSB-red fluorescent protein (CTSB-RFP) transfection, LysoTracker Red cell loading and confocal microscopy

After OV-90 cells were transfected with hSB1-GFP expression vector for 48 h, LysoTracker Red was loaded into cells by incubating the cells in probe-containing medium at a final concentration of 50 nM for 1 h at 37 °C. Then, cells were washed with PBS and viewed with LEICA TCSNT fluorescent confocal microscope at 480 nm and 568 nm emission wavelengths, respectively. After 48 h of co-transfection with hSB1-GFP and CTSB-RFP expression vectors, OV-90 cells were observed with above laser scanning confocal microscopy.

PI-FACS analysis

Apoptosis was quantified on the basis of the amount of sub-G1-DNA by a flow cytometry as described in Ref. [12].

CTSB enzymatic assay

Cells were first washed twice with PBS and then lysed. CTSB activity was estimated by adding 10 μl cell lysate and 50 μM z-RR-AMC to cathepsin B reaction buffer (50 mM sodium acetate, 4 mM EDTA, 8 mM DTT, 1 mM pefablock, pH 6.0). The release of 7-amido-4-methylcoumarin (AMC) (excitation 365 nm, emission 440 nm) was measured with a fluorometer (Cary Eclipse Fluorescence Spectrophotometer, VARIAN), and then normalized for the amount of protein [7]. Protein content was determined by Bradford assay (BioRad).

Results

Tissue expression pattern of hSB1

The tissue expression pattern of novel human gene hSB1 was investigated in 16 human normal tissues by PCR analysis using multi-tissue cDNA panel I and II (Clontech) as templates. As shown in Fig. 1, the expression of hSB1 was not detected in ovary and colon, weak in skeletal muscle, prostate, testis and small intestine, but abundant in the other 10 tissues examined.

Identification of hSB1-interacting proteins

To identify proteins binding to hSB1, we screened a human fetal liver cDNA library in a yeast two-hybrid assay using hSB1 as the bait. After screening of approximately 2×10^6 yeast transformants, 12 putative positive clones were obtained and sequenced. The sequence analysis in Genbank database revealed that these clones encode 10 proteins (data not shown), one of candidates is cathepsin B, a lysosomal cysteine protease. The interaction between hSB1 with CTSB was confirmed by the mating test in yeast (Fig. 2). The diploid yeast cells containing pGBKT7-hSB1 and pGADT7-CTSB can express the reporter genes and grow normally on the selective medium. The mating results indicate that hSB1 can interact with CTSB in yeast cells.

hSB1 specifically binds to CTSB

A GST pull-down assay *in vitro* was carried out to verify the interaction observed in yeast. CTSB was fused to GST and expressed in *E. coli* BL21 with the molecular weight of about 57 KDa, and the fusion partner GST was also expressed in *E. coli* BL21 as a control (Fig. 3A). Myc-tagged hSB1 was generated *in vitro* by the TNT T7 Quick

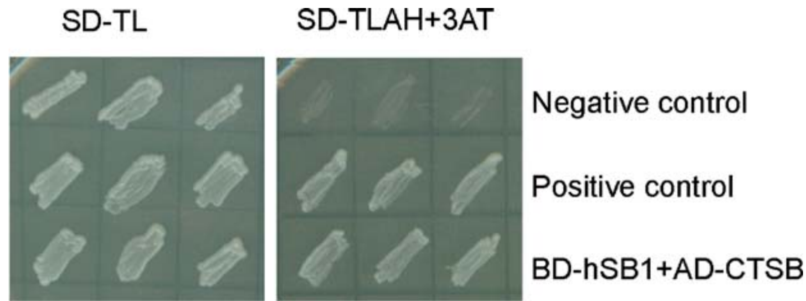

Fig. 2. Interaction of hSB1 with CTSB in yeast. CTSB was identified to interact with hSB1 by yeast two-hybrid method and the interaction was confirmed by yeast mating test. BD-hSB1 + AD-CTSB, diploid yeast cells produced by mating pGBKT7-hSB1-transformed Y187 cells with pGADT7-CTSB-transformed AH109 cells; Negative control, diploid yeast cells with pGBKT7-Lam and pGADT7-T vectors; Positive control, diploid yeast cells with pGBKT7-53 and pGADT7-T vectors. pGADT7-T, pGBKT7-Lam and pGBKT7-53 are control vectors provided in MATCHMAKER Two-Hybrid System 3. SD-TL, SD/-Trp/-Leu medium; SD-TLAH+3AT, SD/-Trp/-Leu/-Ade/-His/5 mM 3-AT medium.

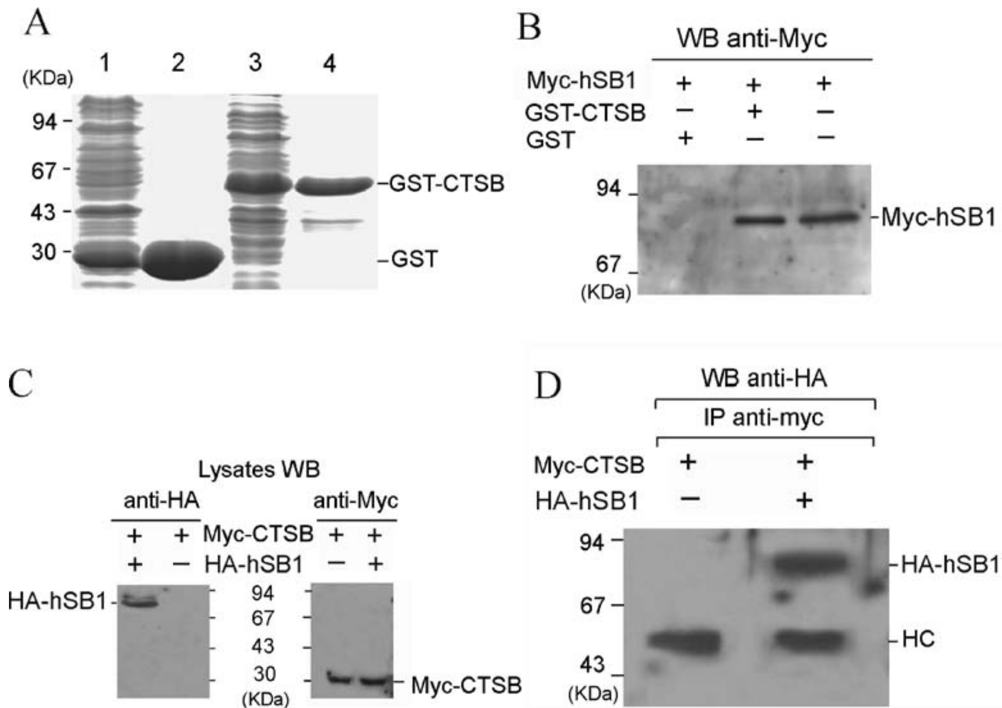

Fig. 3. Validation of the interaction between hSB1 and CTSB. (A) Expressed and purified GST and GST-CTSB proteins were analyzed by SDS-PAGE. Lane 1 and 3 represent respectively total soluble proteins of *E. coli* BL21 containing GST and GST-CTSB; lane 2 and 4 represent respectively purified GST and GST-CTSB. (B) Interaction of hSB1 with CTSB was analyzed by GST pull-down assay *in vitro*. GST-CTSB fusion protein immobilized on glutathione-Sepharose 4B beads was incubated with Myc-hSB1 translated *in vitro*. Binding proteins were immunoblotted with anti-Myc primary antibody. hSB1 binds specifically to GST-CTSB, but not to GST alone. (C) Analysis of overexpressed HA-hSB1 and Myc-CTSB in co-transfected HeLa cells by western blot. (D) Coimmunoprecipitation assay *in vivo*. The lysates from co-transfected HeLa cells were immunoprecipitated using mouse anti-Myc antibody. The precipitated proteins were eluted from the protein A/G PLUS agarose and analyzed by Western blotting using anti-HA primary antibody. Hela cells co-transfected with pCMV-Myc-CTSB and pCMV-HA was used as negative control. HC represents the heavy chain of mouse IgG.

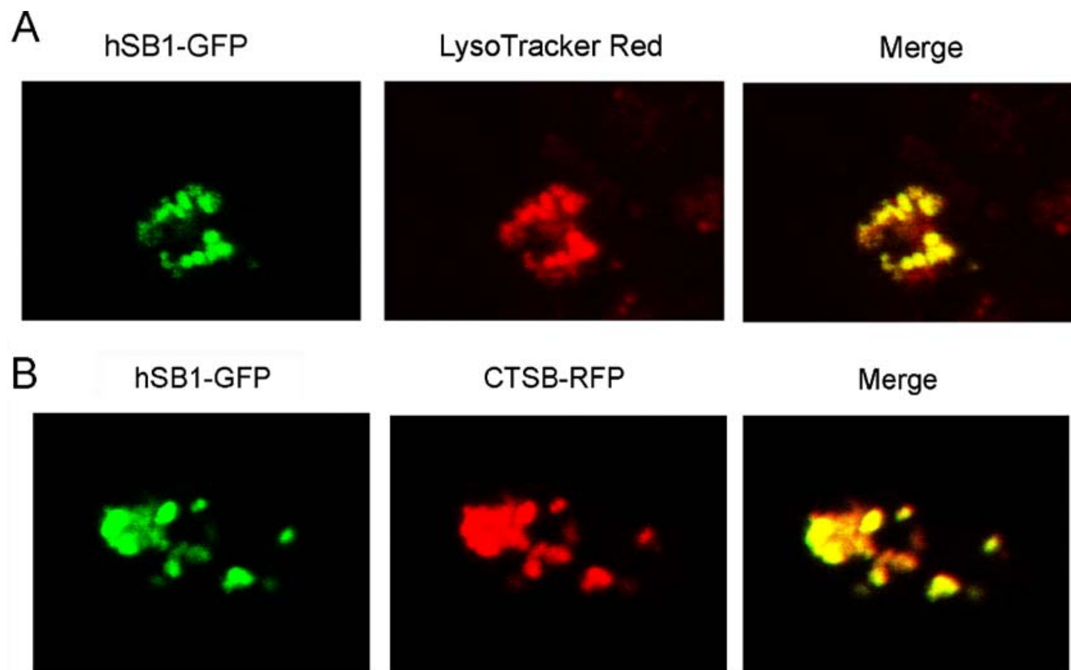

Fig. 4. Colocalization of hSB1 with CTSB in lysosomes. (A) hSB1-GFP localizes within lysosomes in OV-90 cells. After 48 h of transfection with pEGFP-N3-hSB1, OV-90 cells were loaded with LysoTracker Red for 1 h to selectively stain the lysosomal compartment and imagined by laser scanning confocal microscopy as described in details in Materials and methods. (B) hSB1-GFP colocalizes with CTSB-RFP in OV-90 cells. After 48 h of co-transfection with pDsRed-N1-CTSB and pEGFP-N3-hSB1, OV-90 cells were imagined. Overlay image demonstrates that hSB1 is colocalized with CTSB in cellular lysosomes. Magnification, 630×.

Coupled Transcription/Translation system (Promega) (Fig. 3B, lane 3). The purified GST-CTSB and GST conjugated to glutathione-Sepharose beads were incubated separately with Myc-hSB1. After stringently washing, the bound proteins were eluted and analyzed by Western blotting. It was experimentally showed that GST-CTSB conjugated on glutathione-Sepharose 4B beads specifically pulled down Myc-hSB1, while GST alone did not (Fig. 3B).

In coimmunoprecipitation assay, HeLa cells were co-transfected with pCMV-Myc-CTSB and pCMV-HA-hSB1. Western blotting analysis showed the efficient expression of CTSB and hSB1 in co-transfected HeLa cells (Fig. 3C). Monoclonal anti-Myc antibody and protein A/G-agarose were added into the cell lysates so as to precipitate Myc-CTSB. HA-hSB1 could be specifically detected in the precipitate by western blot using anti-HA antibody as primary antibody (Fig. 3D). Both the results of the GST pull-down assay *in vitro* and the coimmunoprecipitation assay confirm that CTSB interacts with hSB1.

hSB1 is co-localized with CTSB in lysosomes

To determine the subcellular localization of hSB1 protein, hSB1-GFP-transfected OV-90 cells were loaded with LysoTracker Red, a marker for the lysosomal compartment, and viewed by confocal microscopy. hSB1-GFP displayed a punctate fluorescent appearance, and colocalized with LysoTracker Red to the same vesicular compartment (Fig. 4A). CTSB-GFP, like its native protein, was reported to target to lysosomes and could be considered as a construct for indicating lysosomes [6]. When hSB1-GFP and CTSB-RFP were coexpressed in OV-90 cells, the two fluorescent proteins were completely colocalized with spots-like fluorescent appearance (Fig. 4B). This result indicates that the interaction between hSB1 and CTSB occurs in lysosomes.

Overexpression of hSB1 suppresses TNF-induced apoptosis in OV-90 cells

To investigate the function of CTSB interacting protein hSB1, hSB1 was cloned into expression vector pcDNA3.1/myc-HisA(-) and overexpressed in OV-90 cells (Fig. 5A). Cells were treated with TNF/AcD for 16 h before apoptosis analysis by PI-FACS. As shown in Fig. 5B, apoptotic level of OV-90 cells transfected with vain vector was 31.8%, while it decreased to 20.5% when hSB1 was overexpressed in OV-90 cells. If cells was preincubated with CTSB specific inhibitor

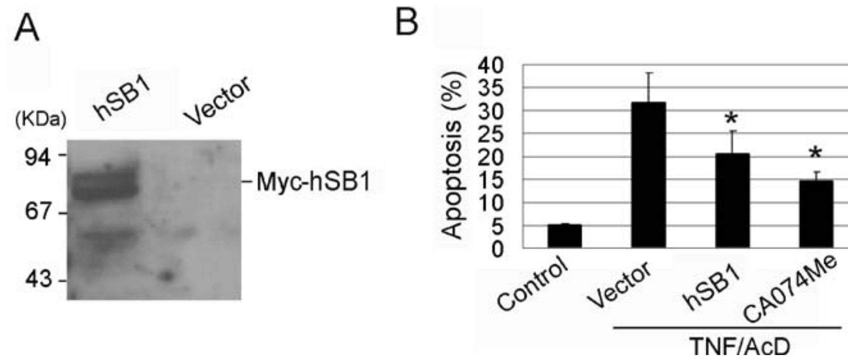

Fig. 5. Effect on TNF-induced apoptosis of hSB1 overexpressed in OV-90 cells. (A) Overexpression of hSB1 in the transfected OV-90 cells was analyzed by western blot. (B) OV-90 cells transfected with pCDNA3.1-Myc-His-hSB1 or pCDNA3.1-Myc-His were treated with 1000 U/ml TNF + 0.2 μg/ml AcD for 16 h and then harvested for apoptosis analysis. Untransfected cells were incubated with CTSB specific inhibitor CA074Me (25 μM) for 2 h before the addition of TNF/AcD. Cells transfected with vain vectors untreated with TNF served as a control. PI-FACS assay was carried out to analyze apoptotic cells in triplicate with symbol error bars indicating standard deviation. $^{*}p$-value < 0.05, as compared with the cells transfected with vain vector.

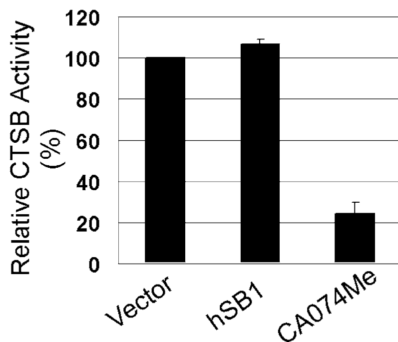

Fig. 6. Effect on cellular CTSB activity of hSB1 overexpressed in OV-90 cells. OV-90 cells transfected with vain vector (control) or pCDNA3.1-Myc-His-hSB1 or treated with CA074Me for 2 h were lysed and analyzed for CTSB activities. The activity of CTSB in the control was supposed as 100%. The results represent means of triplicate determination ± SD.

CA074Me, apoptotic cells was only 14.4%. Based on the experimental data, overexpression of hSB1 suppresses apoptosis of OV-90 cells triggered by TNF.

The effect of hSB1 overexpressed in OV-90 cells on cellular CTSB activity was investigated. The cellular CTSB activity in OV-90 cells transfected with vain vector was used as control and supposed as 100%. As shown in Fig. 6, the cellular CTSB activity was 106.3% of the control when hSB1 was overexpressed in OV-90 cells. Overexpression of hSB1 almost had no influence on cellular CTSB activity. If OV-90 cells were pretreated with CA074Me for 2 h, the cellular CTSB activity was reduced to just 24% of the control.

Discussion

CTSB, the most abundant cysteine protease in lysosomes, has long been regarded as a housekeeper enzyme engaged in intracellular protein catabolism, but recently much evidence suggests that CTSB participates in apoptotic pathway triggered by different stimuli, and may function upstream or downstream of caspases [3, 6, 7]. When CTSB is released from lysosomes to cytosol, it could induce cells apoptotic process, leading to cells death. We previously reported that lysosomal rupture happened during apoptosis of ovarian cancer cells OV-90 triggered by TNF, and either pan-caspases inhibitor zVAD-fmk or specific CTSB inhibitor CA074Me could protect OV-90 cells from TNF-induced death, which indicating the involvement of CTSB and caspases in OV-90 cells apoptosis [8].

Although it has been testified that CTSB is involved in cell death pathways, and this enzyme can not only act in concert with caspases, but also independently execute apoptotic function [3, 6, 7], the regulation of CTSB in cellular apoptosis pathways is poorly understood. In this report, we demonstrated that CTSB could interact with hSB1. hSB1 protein has 707 amino acids, featured with one BTB domain at the N terminus and following three WD40 domains. Both BTB domain and WD40 domain are reported to be involved in protein-protein interaction [9, 10]. In yeast cells the preliminary researches reveal that hSB1 interacts with CTSB through the BTB domain, while the fragment containing three-WD40 domains could not bind to CTSB (data not shown). SETA binding protein 1 (SB1) with 704 amino acids is identified in mouse and shares 92% identity with hSB1 [14]. SETA is a type of protein with multi-SH3 domains, expressed in tumorigenic astrocytes and associated with malignant transformation of astrocytes [13]. It was reported that SB1 bound to SETA's SH3 domains [14].

SETA, also known as CIN85 (Cbl-interacting protein of 85 KDa) or Ruk (regulator of ubiquitous kinase), is a multifunctional adaptor molecule and involved in both cell growth and apoptosis signaling through the interaction with

many signaling proteins, including phosphoinositol-3 kinase (PI3K), the E3 ubiqitin ligase Cbl, Grb2, p130Cas, Crk and apoptosis-linked gene 2 (ALG-2) interacting protein 1 (AIP1) [14–16]. SETA associated with Cbl has been implicated in internalization of activated EGF receptors for lysosomal degradation [17]. In addition, SETA has been shown to regulate cytoskeletal assembly [18] and modulate apoptosis in astrocytes and cultured primary neurons [15, 16]. Very recently, it was reported that SETA associates with TNF receptor 1 via Src tyrosine kinase, and overexpression of SETA can sensitize human T leukemia cells CEM A301 to TNF-triggered apoptosis [19]. But the action of SB1 in conjunction with SETA has no report. Here we show that human homologue of SB1 protein, interacts with apoptosis mediator CTSB, and overexpression of hSB1 protects ovarian cancer cells OV-90 from TNF-induced apoptotic death with no influence on cellular CTSB enzyme activity. hSB1 has many protein binding modules and may act as an adaptor in apoptotic pathway. Whether hSB1 mediates apoptosis signaling by the interaction with CTSB or with SETA protein needs to be elucidated.

It is well known that the expression level of CTSB is upregulated in many human tumors and its secreted form contributes to cancer metastasis by degrading the extracellular matrix [20]. CTSB is regarded as tumor markers correlated with unfavorable clinical prognosis [21]. Thus, CTSB has two opposing roles in tumor progression: its proapoptotic features can reduce malignancy, and its protease action in promoting invasion and metastasis can enhance malignancy. Therefore, it is important for successful treatment of cancers to profoundly understand the regulation of CTSB. Our presenting results provide a clue for the possible regulator of CTSB-mediated apoptosis.

Acknowledgements

This study was supported by grants from National Nature Science Foundation of China (30570914) and CNHLPP (2004BA711A19).

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