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Interaction of Plasminogen Activator Inhibitor-2 and Proteasome Subunit, Beta Type 1

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Abstract The apoptosis protection by plasminogen activator inhibitor-2(PAI-2) is dependent on a 33 amino acid fragment between helix C and D of PAI-2 which is probably due to the interaction of PAI-2 with unknown intracellular proteins. In this study, we used the fragment between helix C and D of PAI-2 as bait to screen a HeLa cell cDNA library constructed during apoptosis in a yeast two-hybrid system and retrieved a clone encoding 241 amino acids of proteasome (prosome, macropain) subunit, beta type 1(PSMβ1) which plays important roles in NF-κB activation. GST-pulldown experiments confirmed the interaction between PAI-2 and PSMβ1 *in vitro*. These data suggest that the antiapoptosis activity of PAI-2 is probably related to its interation with PSMβ1.

Key words plasminogen activator inhibitor type-2; proteasome (prosome, macropain) subunit, beta type 1 (PSMβ1); yeast two-hybrid system; ubiquitin

In 1993, Remokd-O'Donell reported a subset of serine protease inhibitor (serpin) superfamily with high sequence identity to chicken ovalbumin which are called ov-serpin. The ov-serpin subfamily contains a variable length of loop between helices C and D which functions in many biological processes, such as nuclear localization [1] or transglutamination [2]. Plasminogen activator inhibitor-2 (PAI-2), an important member of ov-serpin subfamily, is a multifunctional protein involved in the regulation of fibrinolysis, invasion and metastasis of cancer cells, and in regulation of apoptosis [3]. HeLa cells transfected with PAI-2 cDNA were protected from TNF-α induced apoptosis, and the antiapoptotic activity of PAI-2 probably depends on its interation with some unknown intracellular proteins via the 33 amino acid fragment [4, 5].

To explore the proteins which could interact with PAI-2, the fragment between helix C and D of PAI-2 is used as bait to screen a HeLa cells cDNA library which was constructed during apoptosis in a yeast two-hybrid system. We retrieved a clone that encodes 241 amino acids of proteasome (prosome, macropain) subunit, beta type 1 (PSMβ1). Using RT-PCR, we got the full-length cDNA of PSMβ1. GST-pulldown experiment confirmed the interaction between PAI-2 and PSMβ1 *in vitro*.

The proteasome is a large multi-subunit proteinase complex found in the cytoplasm and nucleus of all eukaryotic cells examined so far. As part of the ubiquitin-mediated protein degradation machinery, it is responsible for not only the elimination of misfolded proteins, including those derived from the lumen of the endoplasmic reticulum [6], but also many regulatory processes by which to remove unnecessary or even harmful metabolic enzymes and regulate the levels of many regulatory proteins [7,8]. The pathway is implicated in controlling cell homeostasis, growth, and development [9–11], including cell cycle progression, maintenance of chromatin structure, DNA repair, enzymatic regulation, transcription, signal transduction, and apoptosis, etc.. PSMβ1 probably plays an important role in apoptosis regulation.

Material and Methods

DNA and Plasmid Constructs

The BD vector pAS2-1NE used for the yeast two-hybrid system was a gift from Dr. Tian Yu (Harvard University). The interhelical region of C and D of PAI-2 was obtained by PCR. The primers were A1(*Nhe*Ⅰ):

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5′-AAAGCTAGCATGGCCAAGGTGCTTCAG-3′ and A2 (*Eag*I), 5′-AAACGGCCGGGATGAATGGATTTTATC-3′ and the PCR condition was as follows: 80 s at 94 ℃, 60 s at 58 ℃, 40 s at 72 ℃, 25 cycles. After *Nhe*I and *Eag*I digestion, the fragment was inserted in frame into pAS2-1NE. The plasmid pGEX-4T-1 encoding a GST protein at the N-terminus was obtained from Pharmacia. The full-length human PSMβ1 cDNA was inserted in frame into pGEX-4T-1 generating the recombinant vector pGEX-4T-1/PSMβ1. The mutant of PAI-2, without the interhelical region of C and D, was constructed as previously described [12].

Cell culture and transfection

HeLa cells were maintained in RPMI 1640 containing 10% bovine calf serum, 2 mmol/L glutamine, 50 u/ml of penicillin, and 50 μg/ml of streptomycin. Transfections were performed using LipofectAmine (Invitrogene).

Yeast two-hybrid screening and colony-lift filter assay

Two-hybrid system (Clontech) was used in this study. The interhelical region of C and D of PAI-2 was used as bait to screen a HeLa cells cDNA library as described above. The screen was performed in *Saccharomyces cerevisiae* strain AH109 (*MATa, trp1–901, leu2–3, ura3–52, his3–200*) in two-hybrid system purchased from Clontech, which expresses reporter genes conferring selective phenotype and β-galactosidase activity. The screen was carried out in Ade/His/Leu/Trp-deficient medium at 30 ℃. 10 d after co-transformation, 40 clones were screened and transferred onto a filter. It was rapidly lysed by dipping twice into liquid nitrogen and thawing at room temperature. Carefully place the filter with colonies side up, on another filter presoaked with Z buffer (60 mmol/L $Na_2HPO_4 \cdot 7H_2O$, 40 mmol/L $NaH_2PO_4 \cdot H_2O$, 10 mmol/L KCl, 0.1 mmol/L $MgSO_4 \cdot 7H_2O$, pH 7.0) containing 1 g/L X-gal and 0.27% β-mercaptoethanol. Filters were incubated at 37 ℃ until the blue colonies appeared .

Isolation of the plasmids

The plasmids from positive clones were isolated as described by Hoffam *et al*. [13]. In brief, a large positive colony was inoculated into 5 ml SD/-Trp and incubated at 30 ℃ overnight with shaking at 250 rpm. After centrifugation, the pellets were resuspended in 200 μl lysis buffer [2% Trinton X-100, 1% SDS, 100 mmol/L NaCl, 10 mmol/L Tris-HCl (pH 8.0), 1mmol/L EDTA]. 0.2 μg glass beads (Sigma) and 200 μl phenol/chloroform (1:1) were added into the lysate and the mixture was vortexed thoroughly for 10 min. The mixture was dipped into liquid nitrogen for 10 min, thawed at room temperature, and vortexed for 10 min. The supernatant was collected by spinning at 12,000 rpm for 10 min. 400 μl ice-cold ethanol was added and the pellet was spinned down by centrifugation.

Analysis of sequence homology of positive clones

The plasmids isolated from the positive clones were introduced into *E. coli* strain KC8 cells by electroporation. The sequence of the inserted DNA was analyzed by Ji Kang Inc. using BLAST program.

Amplification of the full-length human PSMβ1 cDNA by RT-PCR

The full-length human PSMβ1 cDNA was generated by RT-PCR. Briefly, total cellular RNA from HeLa cells was prepared by Trizol. RNA was analyzed by agarose gel electrophoresis and spectrophotometry. dT_{15}-primers and AMV reverse transcriptase were used to synthesize the first strand. Primers for PSMβ1 cDNA were: B1(*Eco*RI), 5′-GGC GAA TTC ATG TTG TCC TCT ACA-3′; and B2 (*Xho*I), 5′-GGC CTC GAG TCA GTC CTT CCT TAA-3′. 1 μl total cDNA product was mixed with *Taq* DNA polymerase, 50 pmol/L of each appropriate primer, 200 μmol/L of each dNTP in 100 μl buffer containing 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 0.01% BSA, 2 mmol/L $MgCl_2$. The sample was amplified for 28 cycles and the PCR condition was as follow: 40 s at 94 ℃, 40 s at 60 ℃, 80 s at 72 ℃.

GST-PSMβ1 fusion protein expression and purification

The bacterial strain Bl21 was used for the expression of GST or GST-PSMβ1 protein. The bacterial culture was induced with isopropyl-1-thio-*D*-galactopyranoside (IPTG, 0.1 mmol/L) and cultured at about 25 ℃ for 3 h. The bacterial pellet was collected by centrifugation and lysed by sonication in 10 mmol/L phosphate buffered saline (PBS), pH 7.4 (137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na_2HPO_4, 2 mmol/L KH_2PO_4) and 1 mmol/L PMSF. After centrifugation at 13,000 rpm for 10 min, the supernatant was incubated with glutathione Sepharose 4B beads (Pharmacia) for 2 h at 4 ℃ on a rotating platform. GST-PSMβ1 was expressed in an insoluble form. After denaturalization and renaturalization process, GST-PSMβ1 was purified with glutathione Sepharose 4B .

GST-pulldown experiment and Western blot analysis

HeLa cells were grown as a monolayer in 10-cm-diameter dishes and transfected with 4 μg pcDNA3-PAI-2 or pcDNA3-PAI-2ΔCD (encoding PAI-2 protein without the interhelical region of C and D) using LipofectAmine. 48 hours after transfection, cells were scraped from the dish, washed with ice-cold PBS, and lysed with ice-cold lysis buffer (137 mmol/L NaCl, 20 mmol/L Tris-HCl, pH 8.0, 0.1 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 1% NP40, 10% glycerol, 1 mmol/L PMSF, 1 g/L aprotinin) for 15 min at 4

℃ on a rotating platform. Samples were centrifugated at 13,000 rpm for 10 min, and incubated the supernatant with glutathione Sepharose 4B beads prepared in the above step "GST-PSMβ1 fusion protein expression and purification" overnight at 4 ℃ on a rotating platform. The beads were washed three times with ice-cold lysis buffer, resuspended in 100 μl loading buffer and boiled for 5 min. After centrifugation, proteins of the samples were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (Amersham Pharmacia). The membranes were blocked with 5% skimmed milk and sequentially incubated with monoclonal PAI-2 antibody (Santa Cruz) and horseradish peroxidase-conjugated secondary antibodies (Santa Cruz). Results were analyzed by ECL (Amersham Pharmacia) with X-ray film (Kodak).

Results

Identification of the positive clones and analysis of homology

Our previous study indicated that the interhelical region of C and D of PAI-2 could interact with some proteins, and IRF3 interacted with PAI-2 was identified [14]. In this study 10 d after cotransformation with recombinant vector pAS2-1NE-PAI-2CD and blank vector pACT2, 40 positive clones were screened and 36 clones showed β-galactosidase activities (data not shown).

The 36 plasmids DNA isolated from candidate clones were cloned with blank vector pAS2-1NE into AH109. Those with transcriptional activities, were chosen for further analysis. 24 clones were considered to be the candidates of PSMβ1 partners. The cDNAs of these clones were amplified by PCR and the sequences were analyzed using BLAST program of GenBank at NCBI. Our result showed that one clone was 100% homologous to the proteasome (prosome, macropain) subunit, beta type 1 (PSMβ1) (Fig.1).

Amplification of human full-length PSMβ1 cDNA and construction of pGEX-4T-1-PSMβ1

The full-length human PSMβ1 cDNA was obtained by RT-PCR (Fig.2). The agarose gel electrophoresis showed the fragment was about 1.3 kb. DNA sequencing indicated that the RT-PCR product was PSMβ1 gene. The plasmid pGEX-4T-1 encoding GST protein was inserted with full-length human PSMβ1 cDNA to generate the mammol/Lalian recombinant vector pGEX-4T-1-PSMβ1.

GST-PSMβ1 fusion protein expression and purification

The bacterial strain Bl21 was used for the expression of GST or GST-PSMβ1 protein. After induced with IPTG, each bacterial culture expressed a certain amout of corresponding protein in an insoluble form. GST-PSMβ1 was denatured with 8 mol/L urea and renatured by diluting the solution to 0.5 mol/L urea and incubated with glutathione beads (Fig. 3).

PAI-2 can interact with PSMβ1 *in vitro*

In order to further investigate the interaction of PAI-2 and PSMβ1, whole lysates of HeLa cells transfected with

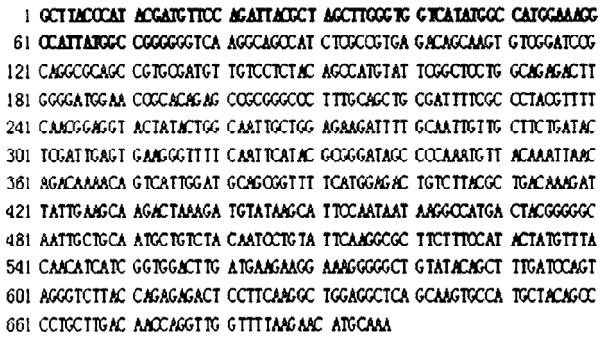

Fig. 1 Nucleotide sequence of the positive clone cDNA encoding the PSMβ1 (nucleotide sequence of pAS2-1NE in bold)

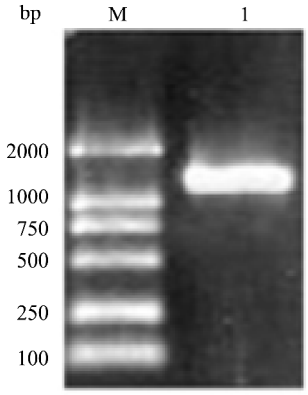

Fig. 2 RT-PCR amplification of PSMβ1 cDNA
M, marker; 1, RT-PCR product of PSMβ1.

pcDNA3-PAI-2 or pcDNA3-PAI-2ΔCD were incubated with Sepharose 4B beads bound with GST or GST-PSMβ1 fusion protein. The Sepharose 4B beads were collected centrifugation. The samples were analyzed by SDS-PAGE and immunoblotting with anti-PAI-2 antibody. As shown in Fig. 4, PAI-2 can interact with PSMβ1, however PAI-2 mutant without the interhelical region of C and D (ΔCD) can not interact with PSMβ1, indicating that PAI-2 interacts with PSMβ1 via its interhelical regions C and D.

Discussion

In this study, we found that PAI-2 could interact with

PSMβ1 via its interhelical region of C and D. As a part of the ubiquitin-mediated protein degradation machinery, proteasome is responsible for the elimination of misfolded proteins and the removal unnecessary or harmful metabolic enzymes. In the ubiquitin-mediated protein degradation pathway, proteins are first enzymatically tagged for breakdown by the covalent attachment of one or more chains of ubiquitin monomers. Attachment is via an isopeptide bond between the C-terminal glycine of ubiqutin and free lysines either in the target or in the preceding ubiqutin in the chain [15–17]. Once assembled, the multi-ubiquitin chain functions as a recognition signal for substrate degradation by the 26 S proteasome. There are 27 lysines in PAI-2 including one Lys^{87} in the interhelical region of C and D, it is probably degraded by the ubiquitin/proteasome system after ubiquitination. In our study, the protein bands of PAI-2 and PAI-2ΔCD only presente at the site of 46 kD and 42 kD respectively, suggesting that PAI-2 did not attach to ubiquitin chain and the interaction between PSMβ1 and PAI-2 was not involved in ubiquitin-mediated protein degradation pathway.

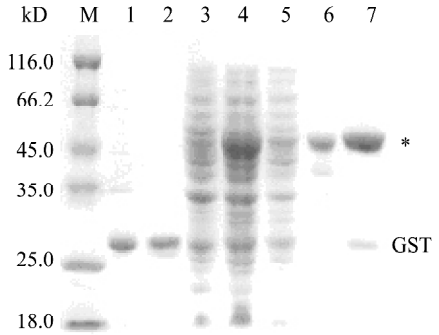

Fig. 3 ST-PSMβ1 fusion protein expression and purification

M, marker; 1, lysed bacterial pellet transformed with pGEX-4T-1 after induced with IPTG; 2, glutathione Sepharose 4B beads after incubated with GST supernatant; 3, lysed bacterial pellet transformed with pGEX-4T-1/PSMβ1 before induced with IPTG; 4, lysed bacterial pellet transformd with pGEX-4T-1/PSMβ1 after induced with IPTG; 5, supernatant of lane 4 after centrifugation; 6, deposition of lane 4 after centrifugation; 7, glutathione Sepharose 4B beads after incubated with GST-PSMβ1 fusion protein after the denaturalization and renaturalization. *GST-PSMβ1 fusion protein.

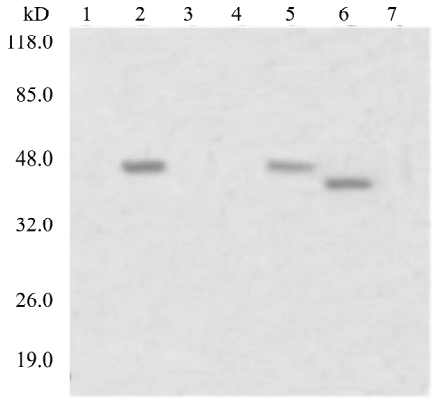

Fig. 4 Interaction between PAI-2 and PSMβ1 *in vitro*

Western blot analysis with PAI-2 antibody. 1, GST incubated with HeLa cell which transfectected with pcDNA3-PAI-2 total protein; 2, GST-PSMβ1 fusion protein incubated with HeLa cells tracfected with pcDNA3-PAI-2 total protein; 3, GST-TIAR fusion protein incubated with HeLa cells tracfected with pcDNA3-PAI-2ΔCD total protein; 4, GST-PSMβ1 fusion protein incubated with HeLa cells total protein; 5–7, the supernatant of 2–4, respectively.

Although tumor necrosis factor alpha (TNF-α) can trigger cellular apoptosis through binding to members of TNF-α receptor (TNFR) superfamily, there is evidence that TNF-α itself has little effect on the apoptosis of some kinds of cells [18]. In addition to apoptosis, treatment of cells with TNF-α can result in activation of transcription factors AP-1 and NF-κB which can induce gene expression [19,20]. The activation of NF-κB requires the degradation of its inhibitor protein IκB. In response to TNF-α, the IκBs are phosphorylated by the IκB kinase complex, resulting in the ubiquitination degradation by proteasome, and nuclear translocation of free NF-κB [19]. Some genes induced by NF-κB act to suppress TNF-α-induced apoptosis. In brief , TNF-α initiate two reverse pathways in the cells, one is to induce apoptosis, the other is to suppress apoptosis, and whether TNF-α can induce cellular apoptosis or not is dependent on the balance between the two pathways. Proteasome is an important member in the latter pathway. It can activate NF-κB indirectly by decreacing the level of IκB in cells. In this study, we found that PAI-2 can interact with one subuit of proteasome. Based on the discovery, we suggest that the antiapoptosis activity of PAI-2 is probably associated with proteasome system, and PAI-2 may activate proteasome to degrade IκB and/or protect it from the inhibition by some inhibiting factors in the cells.

In this study,we found that PAI-2 interacted with one kind of β subunit. Is it the only kind of proteasome subunits interacting with PAI-2? We compared the sequence identity of the 12 β subunit known so far (β1, β2, β3, β4, β5, β6, β7, variant 1 of β8, variant 2 of β8, variant 1of β9, variant 2 of β9, β10) by insightII, the sequence identity is 41.3%. Secondary structure simulation showed that the 12 β subunits were all constructed by several α-helices and β-sheets conjugated by some β or γ turner, and there were five conserved regions (data not shown). If PSMβ1 interacts with PAI-2 through one of the conserved region, it is possible that PAI-2 can interact with other β subunits.

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