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Biochemical and immunological characterization of rice homologues of the human immunodeficiency virus-1 Tat binding protein and subunit 4 of human 26S proteasome subunits

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Abstract

Previously, we isolated two cDNA clones, TBPOs-1 and TBPOs-2, encoding putative ATPases that are the rice homologues of human immunodeficiency virus-1 (HIV-1) Tat binding protein-1 and subunit 4 of human 26S proteasome. In order to determine the RNA-dependent ATPase activity of these putative proteins, the subclones from these cDNA clones were expressed in *Escherichia coli* as fusion proteins with maltose-binding protein. The recombinant proteins stimulated ATP hydrolysis in the presence of poly(U) and rice total RNA. In contrast, single- and double-stranded forms of *Hin*dIII-digested λ phage DNA are less effective at stimulating ATP hydrolysis. Western blot analysis using antisera against the TBPOs proteins showed a widespread appearance of these proteins in rice tissues and cultured cells. The TBPOs proteins were also found around the region where rice proteasomes would sediment. In addition, the TBPOs-1 protein bound to tobacco TATA-binding protein *in vitro.* Thus, we suggest that the TBPOs proteins are novel RNA-dependent ATPases characteristic of DEAD-box proteins and propose that the TPBOs proteins can exist in rice proteasomes. Further, the TBPOs-1 protein is thought to play a role in transcriptional events.

Introduction

Proteasomes are large multicatalytic protease complexes that selectively degrade intracellular proteins [4, 5, 14, 17]. 26S proteasomes degrade ubiquitin conjugates in an ATP-dependent reaction and are composed of the 20S proteasome as a catalytic core with separate complexes called 19S caps. In higher plants, 20S and 26S proteasomes have been characterized both by biochemical and immunological approaches [25, 29], and their biochemical characteristics were remarkably similar to those of proteasomes found in other eukaryotes [10]. A set of 19S cap subunits has been characterized as members of a novel family of ATPases, belonging to the DEAD-box protein family of putative RNA helicases [30]. On the other hand, some of the 19S cap subunits are similar or identical to proteins involved in transcriptional regulation. For example, the human S4 subunit has strong similarities to Tat (human immunodeficiency virus (HIV) transcriptional activator protein) binding protein-1 (TBP1), which interacts with Tat *in vitro* [22].

In a previous study we isolated two rice cDNA clones, TBPOs-1 and TBPOs-2, which share impressively high identities with human TBP1 and subunit S4 of human 26S proteasome, respectively [32]. Recently, a protein deduced from tomato cDNA clones has been also reported to be highly homologous to TBP1 and a yeast TBP1-analogous protein, YTA1A [25]. We suggest that these clones are the rice homologues of

HIV TBP1 and subunit S4 of human 26S proteasome, and that they are members of a novel family of putative ATPases in plants. Further, the TBPOs-1 and TBPOs-2 cDNA probes hybridized with homologous sequences in genomic DNA from *Arabidopsis*, suggesting the wide conservation of these genes in the genomes of higher plants.

In the present study, we show the RNA-dependent ATPase activity of the rice TBPOs-1 and TBPOs-2 recombinant proteins as a fusion to the carboxy-terminal end of maltose-binding protein (MBP). We also show that the TBPOs proteins exist in extracts from rice tissues and cultured cells by western blot analysis. Furthermore, we provide evidence suggesting that the TPBOs proteins co-sediment with the region where the rice proteasomes would sediment. By co-immunoprecipitation assay, we also found that the TBPOs-1 protein interacts with tobacco TATA-binding protein that is required for transcription by all three RNA polymerases.

Materials and methods

Materials

Rice (*Oryza sativa* L. ssp. *japonica* cv. Nipponbare) seedlings and plants were grown in a growth box and in a greenhouse at 25 °C, respectively. Suspension cultures of rice cells were carried out according to Muller and Grafe [21]. Rice cells were initiated by culture of leaves of *O. sativa* L., Nipponbare. After transfer to new media, the cells were subcultured with shaking at 125 rpm and harvested after intervals of 5 days as a logarithmic phase and 7 days as a stationary phase. Rice genes homologous to human Tat-binding protein were from the plasmids pTBPOs-1 and pTBPOs-2, a pBluescript vector (Stratagene) containing rice TBP cDNAs [32]. These plasmids encode 429 and 448 amino acid polypeptides, respectively. pMAL-c2 vector, *Escherichia coli* strain TB1 and amylose resin were purchased from New England BioLabs.

Preparation of the fusion proteins

For the preparation of fusion proteins, we amplified the coding regions of TBPOs-1 and TBPOs-2 by PCR using a 5′ primer containing the *Bam*HI site (5′-AAGGATCC**ATG**TCGTCGCCGCCGCCCGC-3′; the *Bam*HI site is underlined and the first ATG is in bold face) and a 3′ primer containing the *Xba*I site (3′-CCTCTAGA**TTA**AGCGTAATAATTTAAAC-5′; the *Xba*I site is underlined and the terminator codon is in bold face) for TBPOs-1 and a 5′ primer containing the *Eco*RI site (5′-CC-GAATTC**ATG**GGGCAGGGCACCCCGGG-3′; the *Eco*RI site is underlined and the first ATG is in bold face) and a 3′ primer containing the *Xba*I site (3′-CCTCTAGA**TCA**CATGTAAAGACCCTCCG-5′; the *Xba*I site is underlined and the terminator codon is in bold face) for TBPOs-2 [31]. The PCR products were subcloned into bacterial expression vector pMal-c2 in order to obtain recombinant TBPOs proteins fused to the carboxy-terminal end of maltose binding protein (MBP). *E. coli* strain TB-1 was transformed with the ligated DNA according to the manufacturer's instruction (New England Biolabs). The nucleotide sequence between the inserts and the carboxy-terminal end of MBP was confirmed by the dideoxynucleotide chain termination method using an automated fluorescent DNA sequencer (model 370A, Applied Biosystems). After protein induction by IPTG, the cells containing MBP-TBPOs-1 and MBP-TBPOs-2 were lysed by sonication in lysis buffer and the supernatant was loaded onto an amylose-resin affinity column. The fusion proteins were eluted with a buffer containing 10 mM maltose. The proteins were stored in a 15% glycerol solution containing 20 mM Tris-HCl pH 7.4, 200 mM NaCl and 1 mM EDTA at −80 °C and tested as enzyme source for ATPase. For the preparation of polyclonal immune serum, the fusion proteins were further purified by preparative SDS-PAGE and electroeluted from the gel (Nippon Eidou).

Assay for ATPase

The reaction mixture (40 μl) contained 20 μg of the fusion proteins in 20 mM Tris-HCl pH 7.5, 70 mM KCl, 2.5 mM $MgCl_2$, 1.5 mM DTT, 0.1 mM ATP and 2 μg of poly(U); in addition, it contained 2.5 μCi of [γ-^{32}P]ATP (3000 Ci/mmol). The reactions were carried out at 37 °C for the time periods indicated. Aliquots (5 μl at each point) were removed and processed by the consecutive additions of the following reagents [1]: (1) 125 μl of 20 mM silicotungstic acid, 20 mM sulfuric acid; (2) 300 μl of 1 mM potassium phosphate pH 7.0; (3) 125 μl of 5% ammonium molybdate, 4 M sulfuric acid; (4) 75 μl of 2.5% trichloroacetic acid, 50% acetone; and (5) 500 μl of 50% isobutyl alcohol, 50% of benzene. Additions were done at 4 °C. Under these conditions inorganic phosphate (P_i) was sequestered in the upper phase. The mixture was vortexed for 30 s and

centrifuged at 500 × g. Aliquots (100 μl) of the upper phase were mixed with 2 ml of Atomlight scintillation solution (NEN Research Products) and counted.

Antiserum

Polyclonal antiserum was raised in rabbits by multiple subcutaneous injections using the recombinant MBP-TBPOs-1 and MBP-TBPOs-2 proteins according to standard protocols. Blood was allowed to clot overnight at 4 °C and then centrifuged at 3000 rpm for 15 min. The supernatant was stored at −80 °C as the antiserum and used for immunoblotting. Antisera against 20S and 26S proteasomes were raised by injecting rabbits with proteasomes purified from spinach leaves as described previously [20].

Protein extraction and immunodetection

For the preparation of total proteins from rice, 1 g of rice tissues (mature leaves and seedlings) and cultured cells was ground in a mortar at −195 °C and extracted with 5 ml of a buffer containing 50 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 2 mM ATP and 10% glycerol. The extracts were further homogenized on ice using polytron homogenizer and centrifuged for 30 min at 12 000 rpm at 4 °C. The supernatant were stored as total protein extracts at −80 °C until further use. Protein concentration was determined with a protein assay kit (BioRad). Immunodetection of proteins on western blots was carried out with the Vectastain ABC-PO kit (Vector Laboratories). The protein extracts were boiled for 5 min, separated by 10% SDS-PAGE and blotted onto a PVDF membrane (BioRad) with a current of 1 mA/cm^2 for 90 min. The membrane was probed with rabbit polyclonal antisera made to either MBP-TBPOs-1 fusion protein or MBP-TBPOs-2 fusion protein and goat anti-rabbit IgG conjugated to horseradish peroxidase and developed with 4-chloro-1-naphthol.

Glycerol density gradient sedimentation analysis

One ml of the protein extracts was loaded on 37 ml of 10–40% glycerol gradient containing 50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol and 2 mM ATP, and centrifuged at 25 000 rpm in a SW28 rotor for 22 h at 4 °C as described previously [10]. After centrifugation, about 1 ml fractions were collected from the bottom of the tube.

Assay for proteasomes

Proteasome activity was assayed by measuring the hydrolysis of synthetic peptide substrate by a fluoromeric assay [10]. The reaction mixture (50 μl) contained 50 mM Tris-HCl pH 8.0, 8 mM $MgCl_2$, 3 mM ATP, and 0.2 mM Suc-LLVY-MCA (succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin) as substrate; in addition, it contained 20 μl of fractions from the density gradient centrifugation. The mixture was incubated at 37 °C for 30 min and the hydrolysis was terminated by the addition of 2.5 ml solution containing 100 mM Tris-HCl pH 9.0 and 0.5% SDS. Then the MCA liberated was measured fluorometrically (excitation 380 nm, emission 460 nm) and plotted against the fractions collected from the bottom of the tube.

Purification of tobacco TATA-binding protein

Recombinant tobacco TATA-binding protein [16] was overproduced in *E. coli* cells that harbored plasmid pET3a (Novagen), which include the full-length cDNA for TATA-binding protein. The recombinant tobacco TATA-binding protein was purified as described previously [35].

Co-immunoprecipitation assay for binding of the fusion proteins with tobacco TATA-binding protein

All polypeptides used in this assay were dialyzed against buffer HGDKT containing 20 mM Hepes-KOH pH 7.5, 20% glycerol, 50 mM KCl, 0.3% Triton X-100 and 1 mM DTT. Each TBPOs-1, TBPOs-2 or β-galactosidase (4 μg each) fused to maltose-binding protein (MBP) was incubated at 20 °C for 30 min in the presence or absence of tobacco TATA-binding protein (4 μg) to allow protein-protein interaction in a mixture (20 μl) containing 20 mM Hepes-KOH pH 7.5, 50 mM KCl, 2 mM $MgCl_2$, 20% glycerol, 2% polyvinyl alcohol, 0.3% Triton X-100, 1 mM DTT and 0.25 mg/ml BSA. The incubation was further continued by the addition of amylose resin (5 μl; New England Biolabs) in 15 μl of washing buffer (20 mM Hepes-KOH pH 8.0, 50 mM KCl, 2 mM $MgCl_2$, 20% glycerol, 1 mM DTT) at 20 °C for 30 min with gentle agitation to allow binding of MBP-tagged polypeptides to the resin. After the incubation, the resin was peletted by centrifugation (at 10 000 × g for 20 s) and washed twice with 250 μl of washing buffer.

The MBP-tagged polypeptide bound to tobacco TATA-binding protein was eluted from the resin with 10 μl of an elution buffer containing 20 mM maltose, 20 mM Hepes-KOH pH 8.0, 50 mM KCl, 2 mM $MgCl_2$, 20% glycerol and 1 mM DTT. The polypeptide eluted was separated on a 15% polyacrylamide gel containing 1% SDS and stained with Coomassie Brilliant Blue.

Results

RNA-dependent ATPase activity of recombinant TBPOs proteins

Previously, we have proposed that two putative rice proteins isolated from rice cDNA library, TBPOs-1 and TBPOs-2, are members of the 'DEAD box' family of putative RNA-dependent ATPases or RNA helicases, so called because they share the highly conserved region of 244 amino acids (DEAD box, 4XDEAD; ATPase B box, X = hydrophobic residues), together with other conserved elements (G4XGKT (ATPase A box, X = any amino acids) and SAT) [32]. It is of interest to determine whether TBPOs proteins can release γ-P_i from ATP in an RNA-dependent fashion. We subcloned the relevant genes from the rice TBPOs-1 and TBPOs-2 cDNA into an *E. coli* expression vector pMal-c2 and over-expressed them as fusion proteins with MBP. The release of [γ-^{32}P] from ATP by the TBPOs-1 and TBPOs-2 fusion proteins in the presence of poly(U) proceeded linearly with time for 60 min, but was not stimulated when poly(U) was absent from the reaction mixture (Figure 1). No appreciable ATPase activity was observed when the MBP-galactose fusion protein was used as the enzyme source (see Figure 1C). The amount of P_i released in 30 min of incubation with the MBP-TBPOs-1 protein increased rapidly with increasing poly(U) up to 10 ng (0.5 μg/ml, data not shown). We further investigated the effect of RNA and DNA on the ATPase activity of the fusion proteins (Table 1). The release of P_i by the TBPOs-1 protein was stimulated to a lesser extent by rice RNA than by poly(U), but rice RNA stimulated ATP hydrolysis by the TBPOs-2 protein to a level equivalent to that with poly(U). In contrast, single- and double-stranded forms of *Hin*dIII-digested λ phage DNA were less effective at stimulating ATP hydrolysis by these enzymes. Therefore, we conclude that the RNA-dependent ATPase activity is an intrinsic property of the TBPOs proteins.

Table 1. Effect of RNA and DNA on the ATPase activity of MBP-TBPOs fusion proteins

Fusion proteins	RNA or DNA added	ATPase activity (μmol)	Fold
MBP-TBPOs-1	–	2.7	1
	Poly(U)	53.8	19.1
	Rice RNA	23.3	8.6
	dsDNA	16.9	6.3
	ssDNA	18.3	6.8
MBP-TBPOs-2	–	5.3	1
	Poly(U)	78.5	14.8
	Rice RNA	82.9	15.6
	dsDNA	52.1	9.8
	ssDNA	46.8	8.8

Reaction mixture (20 μl) contained 10 μg of the fusion proteins in 20 mM Tris-HCl pH 7.5, 70 mM KCl, 2.5 mM $MgCl_2$, 1.5 mM DTT, 0.1 mM ATP, 0.5 μg of poly(U) or RNA or DNA, and 1.25 μCi of [γ-^{32}P]ATP (3000 Ci/mmol). The reactions were carried out at 37 °C for 30 min. Aliquots (5 μl) were removed and assayed as described in Materials and methods. Holds indicate as values when the amounts of released Pi with RNA or DNA is divided by that of released Pi without RNA and DNA. Each value represents the average of released phosphate in 100 μl of the upper phase from 3 independent extractions. Rice RNA, total RNA isolated from rice seedlings; dsDNA, λ phage *Hin*dIII digest; ssDNA, 2M NaOH-treated λ phage *Hin*dIII digest.

Immunological detection of native TBPOs-1 and TBPOs-2 proteins

To identify the TBPOs-1 and TBPOs-2 proteins in rice tissues and cultured cells, we prepared the immune serum against the recombinant MBP-TBPOs-1 and MBP-TBPOs-2 fusion proteins and carried out western blot analysis. The antiserum raised against the MBP-tagged TBPOs-1 protein detected a band of about 48 kDa in total protein extracts from roots, young and old seedlings, mature leaves and cultured cells of rice (Figure 2A). The TBPOs-1 protein was most abundant in mature leaves (about 80-fold more concentrated than 7-day seedlings). The mobility of the 48 kDa band closely matched that predicted from the open reading frame contained in the TBPOs-1 clone, confirming the presence of the TBPOs-1 protein. However, bands with lower molecular mass were found in the extracts of 7-day seedlings and mature leaves (Figure 2A and B). These bands are smaller than the size predicted from the open reading frame of the TBPOs-1 cDNA clone. This size difference might have resulted from proteolytic cleavage during preparation of the extracts.

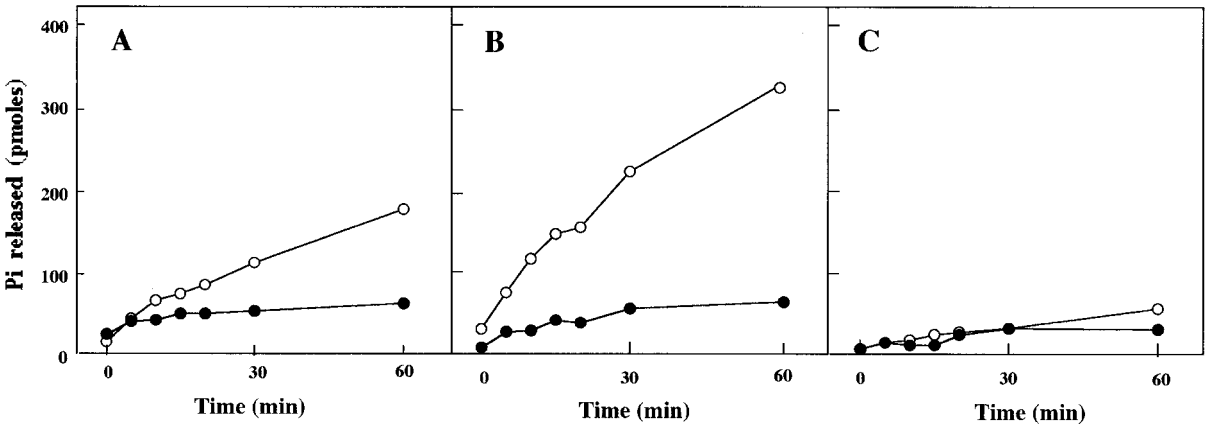

Figure 1. Time-course of ATPase activity of MBP-TBPOs fusion proteins. Reaction mixture (40 μl) contained 20 μg of the fusion proteins in 20 mM Tris-HCl (pH 7.5), 70 mM KCl, 2.5 mM $MgCl_2$, 1.5 mM DTT, 0.1 mM ATP, 2 μg of poly(U), and 2.5 μCi of [γ-^{32}P]ATP (3000 Ci/mmol). The reactions were carried out at 37 °C for the indicated time periods. Aliquots (5 μl at each point) were removed and the released phosphate was measured as described in Materials and methods. A. MBP-TBPOs-1 fusion protein. B. MBP-TBPOs-2 fusion protein. C. MBP-galactose fusion protein. ○, with poly(U); ●, without poly(U).

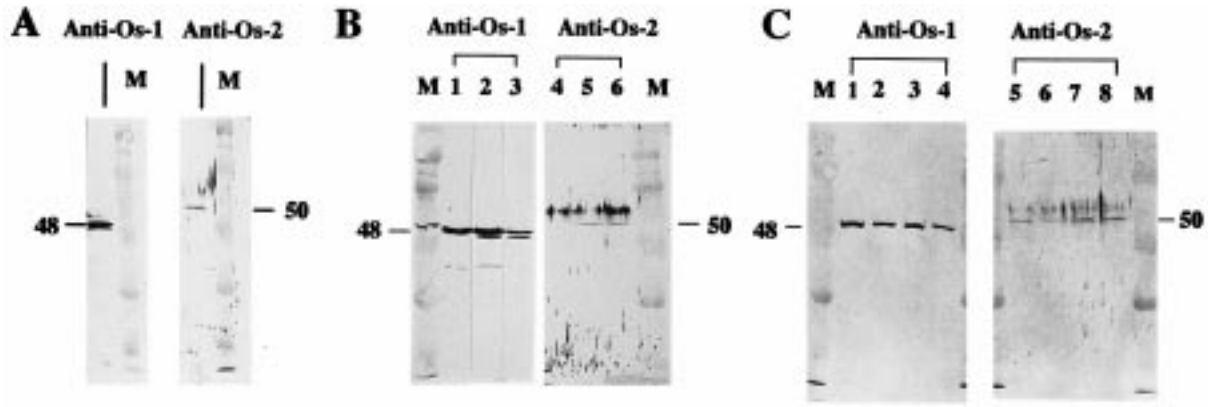

Figure 2. Identification of native TBPOs-1 and TBPOs-2 proteins in rice plant. Western blot analysis was carried out as described in Materials and methods. The blots were probed with antisera (1:500 dilution) directed against each fusion protein. Anti-Os-1 and anti-Os-2 above each panel represent the antiserum against the recombinant MBP-TBPOs-1 and MBP-TBPOs-2 proteins, respectively. The lines with 48 and 50 indicate the apparent molecular in kDa of TBPOs-1 and TBPOs-2 proteins, respectively. A. Roots of 7-day-seedlings. Each lane contained 20 μg of total protein. B. Lanes: 1 and 4, mature leaves; 2 and 5, 7-day-old seedlings in the light; 3 and 6, 7-day-old seedlings in the dark. Lane 1 contained 1.25 μg of total protein. Lanes 2, 3, 4, 5, and 6 contained 20 μg of total protein. C. Lanes 1, 2, 5 and 6, rice cultured cells at logarithmic phase; 3, 4, 7 and 8, at stationary phase. Lanes 1, 3, 5 and 7 contained 10 μg of total proteins and lanes; 2, 4, 6 and 8 contained 5 μg of total proteins. M, rainbow colored protein molecular size marker (Amersham).

Immunoblot analysis (Figure 2B) also demonstrates that the antiserum against the TBPOs-2 fusion proteins reacted with a single protein of about 50 kDa in the protein extracts except for the extract from mature leaves. The size of this band corresponded to the size of the protein deduced from the open reading frame contained in the TBPOs-2 cDNA clone, confirming the presence of the TBPOs-2 proteins. However, the signals of TBPOs-2 protein was weak in these protein extracts. In a previous report, TBPOs-2 mRNA has been also less abundant than TBPOs-1 mRNA [32]. Further, a smear band moving more slowly than the 50 kDa band appears always in cross-reaction with the antiserum raised against the TBPOs-2 protein. Although the anti-TBPOs-2 antiserum was repeatedly subjected to purification to remove the smear band on western blot, this band cannot be removed from the blot. The TBPOs-1 and TBPOs-2 proteins were present even in protein extracts obtained from dark-grown seedlings (see Figure 2B). Preimmune serum and immune ser-

um against MBP-galactose fusion protein showed no cross-reaction with proteins in any tissue when tested by western blot (data not shown).

Identification of the TBPOs-1 and TBPOs-2 proteins by glycerol density gradient centrifugation of rice extracts

The glycerol density gradient centrifugation for separation of 20S particles from 26S particles is an established method [3, 19, 24]. We repeatedly performed separation of the 20S from the 26S under conditions in which we used a ATP-generating system in addition to ATP and different gradients of glycerol density. Nevertheless, good separation was not obtained. In the present study, however, we confirmed the presence of large protease complexes such as 20S and 26S proteasomes in a crude extract of rice cells by glycerol gradient sedimentation (Figure 3). The biochemical and immunogical characteristics of the rice proteasomes resembled those of the proteasomes isolated from various other sources including human, *Drosophila* and spinach [2, 6, 10]. The critical evidence for this conclusion is: (1) that the fractions obtained from the rice extract exhibits both the SDS-insensitive protease activity for 26S proteasomes (in about fraction 25 of Figure 3A) and 0.02% SDS-activating protease activity for 20S proteasomes [36] (in about fraction 27 of the faster-sedimenting fractions as shown in Figure 3A): (2) that the former is stabilized by ATP when the extraction and the fractionation are performed (data not shown); (3) that the fractions displayed the degradation activity reacted with spinach anti-26S and -20S antibodies (the anti-20S and -26S antisera recognized multiple polypetides bands with molecular masses of 27–33 kDa and 46–120 kDa around fractions 24–29, respectively, as shown in Figure 3B and C). The slower sedimenting fractions (centred on fraction 32 of Figure 3A) also showed the Suc-LLVY-MC hydrolysis in the absence of SDS. However, no an appreciable hydrolysis was observed by treatment with 0.02 SDS. These fractions were excluded from the present studies.

Recently, it has been reported that DEAD box proteins such as Sug1, TBP1 and MSS1 are integral components of the 19S regulatory complex of the 26S proteasome [7, 8, 27]. In this connection, we wished to know whether the particular TBPOs proteins cosedimented with the rice proteasomes. To address this question, the fractions obtained by the glycerol density gradient sedimentation of the extract were probed in western blots for the TBPOs-1 and TBPOs-2 proteins. In the gradient of the extracts with ATP, the TBPOs-1 protein was detected as the main band strong in intensity, with a molecular mass of about 48 kDa, around fractions 25–27 for which SDS-insensitive degradation activity was demonstrated (Figure 4). The anti-TBPOs-2 serum also recognized a protein with a molecular mass of about 50 kDa in the same fractions of the glycerol gradient analysis, although the reaction with the anti-TBPOs-2 serum was much weaker in intensity than that with the anti-TBPOs-1 serum (data not shown). It is therefore likely that the TBPOs proteins exist in the rice proteasomes. However, attempts to further identify the TBPOs proteins in rice immunoprepicitates derived with spinach anti-26S proteasome antiserum were unsuccessful. We deferred this investigation since a reason may be an intrinsically weak association between the rice TBPOs proteins and the spinach antibody.

In vitro *binding of TBPOs proteins with tobacco TATA-binding protein*

It has been proposed that yeast Sug 1 protein, a member of a highly conserved family of ATPases that possess a DEAD-box motif, is a candidate for a transcriptional mediator [22, 28, 33]. Recently, however, Sug 1 and other related proteins such as TBP1 and MSS1 have been shown to be integral components of the 19S regulatory complex of the 26S proteasome [7, 8, 27]. It is therefore interest to examine the interaction of the TBPOs proteins with tobacco TATA-binding protein. The fusion proteins were assayed based on their ability to associate directly with tobacco TATA-binding protein. It can be seen (Figure 5) that the MBP-tagged TBPOs-1 binds to tobacco TATA-binding protein although its signal is weak in intensity, whereas no binding of the TBPOs-2 protein or β-galactosidase take place. The binding of the TBPOs-1 resembles the finding that Sug 1 protein interacts with yeast GST-tagged TATA-binding protein *in vitro* [34]. In a preliminary experiment, however, the TBPOs-1 protein did not enhance basal transcription in an *in vitro* system derived from tobacco cultured cells.

Discussion

In a previous report [32], we identified the rice homologues (TBPOs-1 and TBPOs-2) of human HIV Tat-binding protein (TBP1) and subunit 4 of human 26S proteasome from a rice cDNA library. These homo-

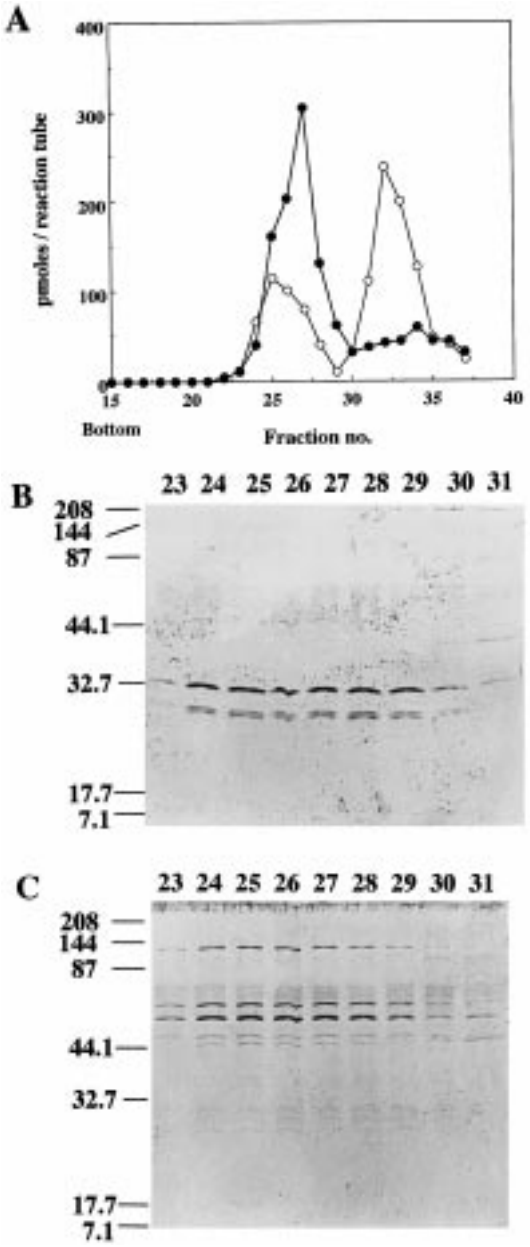

Figure 3. Sedimentation profile of rice large proteases in glycerol gradient. The protein extracts from rice 5-day-old cultured cells were subjected to glycerol gradient sedimentation. A. Protease activity was assayed by measuring the hydrolysis of a synthetic substrate (Suc-LLVY-MCA) using a fluorometric assay. The liberated MCA is plotted against fractions fractionated from the bottom of the tube. ○, without SDS; ●, with 0.02% SDS. B and C. Fractions (100 μl) of the glycerol gradient described in A were concentrated and separated on a 12.5% polyacrylamide/SDS gel. Western blot analysis was carried out as described in Materials and methods. The blots were probed with antisera (1: 500 dilution for 20S and 1: 100 for 26S) directed against purified 20S (B) and 26S (C) proteasomes. Fraction numbers are indicated at the top. Numbers in panel A correspond to fraction numbers in panels B and C. Positions of molecular mass marker proteins (kDa) are indicated at the left.

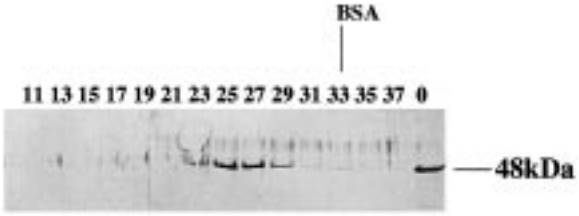

Figure 4. Identification of TBPOs-1 protein in glycerol gradient. The protein extracts were prepared from 5-day-old cultured cells. The protein extraction, glycerol gradient analysis and western blot analysis were performed as described in Materials and methods. After centrifugation, 10 μl of portions from odd numbered fractions were probed with anti-MBP-TBPOs-1 antiserum. Lane 0 indicates the original protein extract obtained 5-day-old cultured cells.

logues and their human counterparts have highly conserved primary structures because all of these proteins share a domain for putative ATPase and RNA helicases (the DEAD box and related motifs such as G4XGKT and SAT). For this reason, we have proposed that the rice homologues are members of a novel family of putative ATPases in plants. Recently, a protein deduced from tomato cDNA clones has been reported to be homologous to human TBP-1 [25]. These findings have led to the suggestion that the TBPOs proteins may be implicated in basal functions, such as transcriptional regulation and intracellular proteolysis, despite the profound differences that exist between plants and animals. However, no direct biochemical and immunological evidence to support this suggestion has been reported.

In this study, we have shown that the recombinant rice TBPOs-1 and TBPOs-2 proteins can stimulate P_i release from ATP in the presence of poly(U) or rice total RNA and the P_i release is less efficient in the presence of single- and double-stranded *Hin*dIII-digested λ DNA. These characteristics are similar to those of other RNA-stimulated ATPase which are stimulated by the addition of poly(A) or poly(U) [16, 18, 31]. Together with the results from our previous report [32], we confirm that the TBPOs proteins are members of the RNA-dependent ATPase family belonging to the DEAD box protein group [13, 18, 30]. The role of RNA stimulation on ATP hydrolysis remains still an enigma. Presumably, rice TBPOs have two kinds of ATPase activity. If TBPOs function in combination with other general transcription factors to facilitate transactivation, the RNA-depended stimulation might be involved in unwinding of RNA/DNA complexes to facilitate elongation, namely as a co-factor of elongation factor (TFIIF). On the other hand, the ATPase

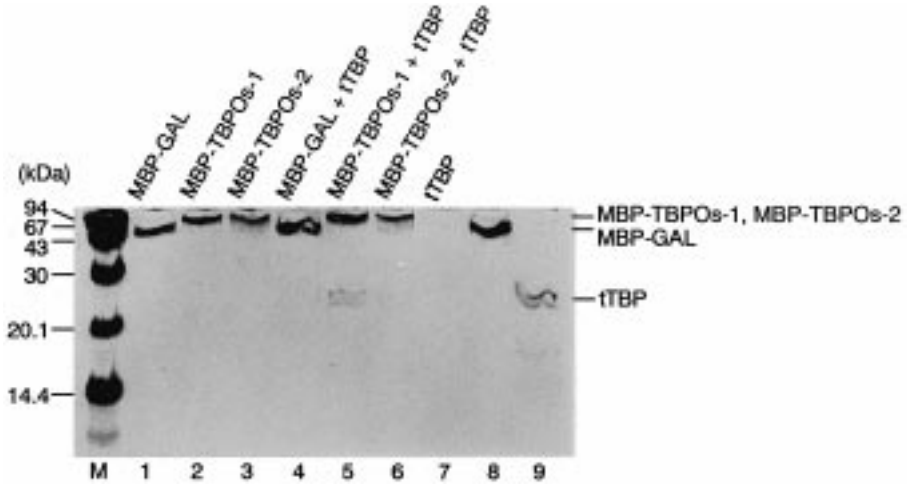

Figure 5. Analysis by co-immunoprecipitation assay of the binding between TBPOs-1 or TBPOs-2 and tobacco TATA-binding protein. tTBP in the figure represents tobacco TATA-binding protein. MBP-GAL (lanes 1 and 4), MBP-TBPOs-1 (lanes 2 and 5), or MBP-TBPOs-2 (lanes 3 and 6) was incubated in the presence (lanes 4–6) or absence (lanes 1–3) of purified recombinant tobacco TATA-binding protein as described in Materials and methods. tTBP alone was incubated as above (lane 7). Lane M, protein size markers (Pharmacia Biotech). MBP-GAL (2 μg, lane 8) or tTBP (1.5 μg, lane 9) was directly loaded onto a gel.

activity of TBPOs, in the absence of RNA, is thought to be elicited by their entry into 26S proteasomes, but the activity of unassembled TBPOs, in their free form, might be very low. It has been found that some components of the 26S proteasome do not have significant ATPase activity but such activity is exhibited after the assembly to form the 26S structure [3].

Western blot analysis using the antisera against the MBP-TBPOs-1 and MBP-TBPOs-2 fusion proteins reveal that the predicted proteins exist with sizes corresponding to those deduced from the open reading frame contained in their cDNA clones and are widespread in rice tissues and cells. Further, although we have been unable to detected the TBPOs proteins in rice immunoprecipitates which are elicited with spinach 20S and 26S antibodies, these proteins co-sediment with the region where the rice proteasomes would sediment. Thus, we propose that the TBPOs proteins are possible candidates for the regulatory components of 26S proteasome.

Previously, we have shown that rice TBPOs-1 mRNA is detected at a high level in developing seedlings [32]. Proteasome α and β subunit mRNAs from *Arabidopsis* have also co-expressed in a similar fashion [12]. It is possible that 26S proteasome play an important role in the progression of the cell cycle in plants, as in animal cells. However, in this study we found that the TBPOs-1 protein was most abundant in mature leaves, whereas we have previously shown that the TBPOs-1 mRNA is not detectable in leaves of 14-day old seedlings [32]. Furthermore, there is no signal of TBPOs-2 protein in mature leaves, but such protein is present in 7-day seedlings. At present, we have no clear-cut explanation for this contradictory finding. Recently, an mRNA of a tomato TBP1 homologue has shown to be expressed in mature leaves and flowers [25]. In this context, we cannot conclude that the plant TBP homologues are implicated only in cell cycle progression. 26S proteasomes are already known to be instrumental in the regulation of wealth of diverse cellular functions, such as transcription [26], cell death [6] and antigen presentation [13].

It has been suggested that some of 19S cap proteins are similar or identical to proteins involved in transcriptional activation [7, 8, 22]. For example, human TBP1 is a strong transcriptional activator when brought into proximity of several promoter elements [23]. Further, it has been reported that Sug-1 protein is not only a component of the 26S proteasome [27], but also a modulator of yeast transcription activation by Cdc 68 [37]. In this study we provide evidence that the TBPOs-1 protein can bind to tobacco TATA-binding protein. This binding may imply a role of ATP hydrolysis in transcriptional events. Together with the RNA dependence on ATPase activity, the binding to tobacco TATA-binding protein may be provide clues relating to the functions of the TBPOs-1 protein. This hypothesis, which needs to be rigorously addressed, would

potentially expand the known function of TBPOs gene family. However, it is unclear why the binding of TBPOs-2 with TATA-binding protein did not take place. Since we used a TATA-binding protein prepared from tobacco, a possible explanation is that the binding ability of TBPOs might be different depending on the source of TATA-binding protein. In addition, TBPOs-2 shares high identity to human 26S proteasome subunit S4 (76% identity) originally cloned as a component of human 26S proteosome, whereas TBPOs-1 shares high identity to human TBP1 (75% identity) [32]. At first, it was reported that human TBP1 suppresses Tat-mediated transactivation [22], whereas human TBP7 does not influence transcription (although 77% similar to TBP1) [23]. Human TBPs have been also shown to be components of 26S proteasomes [2, 8]. Although no clear-cut understanding of the difference between the two rice homologues on the binding with the TATA-binding protein emerged from the present studies, we think that there is a selective usage of these homologues in diverse cellular functions.

One approach to investigate the functions of the TBPOs-1 and TBPOs-2 proteins is to study the effect of inhibition of the expression of the TBPOs-1 and TBPOs-2 genes on diverse cellular processes. Efforts to use antisense DNA methodology in transgenic rice plants and to develop gene knockout rice plants to delete the functions of the TBPOs proteins are underway.

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