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Identification and Initial Characterization of a Specific Proteasome (Prosome) Associated RNase Activity*

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We have identified and characterized a specific nuclease activity to be tightly associated with proteasomes. Using tobacco mosaic virus RNA (TMV-RNA) as substrate to analyze and quantify the cleavage reaction, we supply several lines of evidence that this nuclease activity is an integral part of proteasomes. Thus, RNase activity was coincident with the elution profiles of proteasomes at each stage of purification. Proteasomal nuclease activity was resistant to strong dissociation conditions using 480 mM KCl, 0.5% sodium lauroylsarcosinate, and 6 M urea. This nuclease activity remained associated with an urea-resistant subcomplex of the proteasome comprising a specific set of proteins. Finally the digestion of TMV-RNA led to a well defined pattern of RNA fragments while 5 S ribosomal RNA and globin mRNA were not degraded. These results provide further evidence that proteasomes are able to discriminate between different RNAs, and the possible involvement of proteasomes in translation control is discussed.

Subribosomal fractions of archaebacteria (Grziwa *et al.*, 1991) and eukaryotic cells contain a large multiprotein complex with a molecular mass of about 700 kDa (mammalian cells and cells of avians, Schmid *et al.* (1984); human cells, Martins de Sa *et al.* (1986); plant tissue cells, Schliephacke *et al.* (1991); yeast cells, Heinemeyer *et al.* (1991); for review, see Tanaka *et al.* (1992) and Rivett (1993)). This particle is very stable, resistant *in vitro* to high ionic strength and to 1% sodium lauroylsarcosinate, a strong detergent (Schmid *et al.*, 1984). Studies by electron microscopy show that this particle has a cylinder shaped structure (Kleinschmidt *et al.*, 1983). The cylinder consists of a stack of four discs, each disc or ring consists of 7 subunits (Pühler *et al.*, 1992) which migrate in Laemmli PAGE[1] in the range of 19,000–35,000 daltons. Two-dimensional protein gel electrophoresis revealed up to 20 individual proteins, the number varies between species (duck, mouse, and HeLa cells, Martins de Sa *et al.* (1986); for review, see Rivett (1993)). In addition, purified preparations of the complex contain low molecular weight RNAs as we published about 10 years ago (Schmid *et al.*, 1984). Controversy still exists concerning the amount of RNA per particle and the diversity of these RNAs. Several different types of RNA, one 120 nucleotides long and a more heterogenous population of molecules with 80–90 nucleotides, were reported by different groups (Arrigo *et al.*, 1985; Martins de Sa *et al.*, 1986; Pühler *et al.*, 1992). RNA is an intrinsic part of the complex as shown by nuclease digestion assays, where a strand of about 80 nucleotides was found to be protected (Dineva *et al.*, 1989). However, this RNA is not considered to be a structural component of the particle (Pühler *et al.*, 1992).

The best characterized properties of the proteasome are its multiple endopeptidase activities (Arrigo *et al.*, 1988; Wilk and Orlowski, 1983). The 20 S proteasome is considered to be the enzymatic core of a larger complex, with a molecular mass of about 1500 kDa, which digests ubiquitin-conjugated proteins in an ATP-dependent fashion (Hough *et al.*, 1987; Eytan *et al.*, 1989; Driscoll and Goldberg, 1990; Peters *et al.*, 1993). Furthermore, there is evidence for the involvement of the 20 S proteasome in the generation of the major histocompatibility complex class I binding peptides (Ortiz-Navarrete *et al.*, 1991; Martinez and Monaco, 1991).

We and others have demonstrated that proteasomes can interfere with protein synthesis *in vitro* (Horsch *et al.*, 1989; Kühn *et al.*, 1990). While no inhibition was observed with globin mRNA and cellular mRNAs of HeLa cells, the translation of TMV-RNA and mRNA isolated from adenovirus-infected HeLa cells was impeded (Horsch *et al.*, 1989). Recent work of our laboratory indicates that proteasome prevents the formation of 80 S initiation complexes but not the early phase of initiation (Homma *et al.* 1994). In addition, we have shown that proteasomes associated with tobacco mosaic virus RNA and poly(A)$^+$ mRNA from adenovirus-infected cells (Horsch *et al.*, 1990). To elucidate these points in particular, we have analyzed the relations between the proteasomes and TMV-RNA more closely. Our results demonstrate that proteasomes hydrolyze TMV-RNA creating well defined fragments and we show that integrity of the proteasomal complex is not necessary for RNase activity.

EXPERIMENTAL PROCEDURES

Cell Fractionation Procedure and Isolation of Proteasomes from Calf Liver Cells—The preparation of the post-mitochondrial supernatant has been described (Tomek *et al.*, 1990).

The post-mitochondrial supernatant was layered over 10 ml of 30% (w/w) sucrose in centrifugation buffer (20 mM Tris-HCl, pH 7.4, 50 mM KCl, 5 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 0.1 mM EDTA, 200 mM saccharose) and centrifuged to sediment ribosomes and polyribosomes (Beckmann rotor Ti 45, 42,000 rpm, 2 h, 4 °C). To separate the cytoplasmic proteins, post-polyribosomal supernatants were sedimented again through 10 ml of 30% (w/w) sucrose. After centrifugation (Beckmann rotor Ti 45, 42,000 rpm, 19 h, 4 °C) the pellets of post-ribosomal particles were resuspended in TBK 300 and approximately 1,500 A_{280} units were applied to 100 ml of a Q-Sepharose fast flow anionic exchanger in a C26/40 column (Pharmacia Biotech Inc.) equilibrated with TBK buffer containing 300 mM KCl. Particles eluted by a step gradient to 600 mM KCl were concentrated by ultracentrifugation (Beckmann Ti

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[1] The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; mRNP, messenger ribonucleoprotein; TMV, tobacco mosaic virus; MOPS, 4-morpholinepropanesulfonic acid; FPLC, fast protein liquid chromatography; pCp, cytidine 3′-5′-[5′-^{32}P]biphosphate.

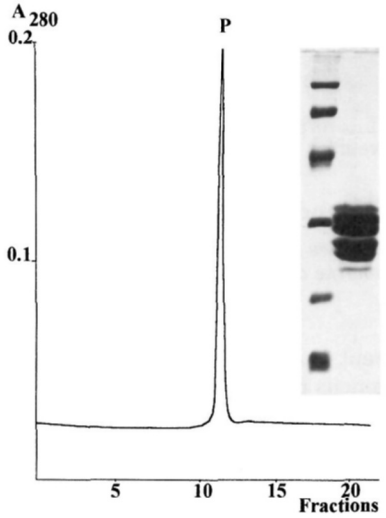

FIG. 1. **Purity of proteasomes from calf liver cells.** Proteasomes of calf liver cells were prepared as described under "Experimental Procedures." The final step of purification was a gel filtration on FPLC Superose 6 columns. Proteasomes eluted from Superose 6 columns were precipitated with 10% trichloroacetic acid (final concentration) and solubilized in sample buffer (Laemmli, 1970). The proteins were separated on a 12.5% polyacrylamide gel and stained with Coomassie Blue (Laemmli, 1970). Absorbance at 280 nm (— —). *Inset*, protein pattern of proteasomes eluted from Superose 6. The molecular mass markers are listed under "Experimental Procedures."

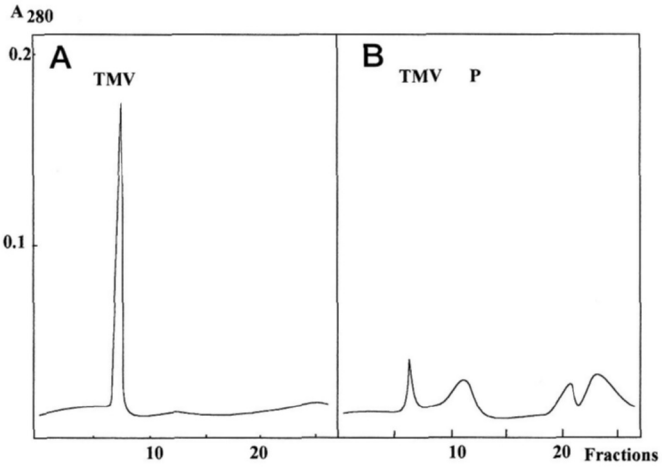

FIG. 2. **Degradation of TMV-RNA by proteasomes.** Eluted fractions of proteasomes from Superose 6 columns were diluted 1+3 with FPLC low salt buffer to obtain a final concentration of 120 mM KCl. Then the suspension was incubated with TMV-RNA for 10 min at 37 °C. Subsequently the total assay (200 μl) was immediately analyzed by chromatography on Superose 6 equilibrated in TBK 120. *A*, 7.5 μg of TMV-RNA incubated in TBK 120 without proteasomes. *B*, 7.5 μg of TMV-RNA incubated in TBK 120 with 10 μg of proteasomes.

45, 42,000 rpm, 19 h, 4 °C). Sediments were resuspended and applied to a Fast Protein Liquid Chromatography (FPLC) Mono Q column (HR 5/5 Pharmacia) equilibrated in TBK 50. A linear salt gradient up to 600 mM KCl was formed. Proteasomes were eluted at 390 mM and further purified by gel filtration on a FPLC Superose 6 column (HR 10/30 Pharmacia-LKB) equilibrated with Tris-HCl buffer, pH 7.4, containing 480 mM KCl (TBK 480) as described by Tomek *et al.* (1990).

Protein Gel Electrophoresis—Proteins and particles were precipitated with 10% trichloroacetic acid (final concentration). Electrophoresis of proteins was on one-dimensional SDS-polyacrylamide gels (PAGE according to Laemmli (1970)). Molecular mass markers were: phosphorylase *b* (94 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20 kDa), and lactalbumin (14 kDa).

Assay for TMV Digestion—Purified TMV-RNA of *Nicotiana rustica* was purchased from DSM Braunschweig (Germany). The quantity of proteasomes was determined by the Bio-Rad assay. TMV-RNA was incubated with proteasomes at 37 °C. The volume of each assay was 200 μl. Incubation buffers were adapted to the buffers which we used for the purification of proteasomes on Superose 6 and Mono Q columns. Thus proteasomes eluted in TBK 480 were diluted 1+3 with low salt buffer (20 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 7 mM 2-mercaptoethanol). Final salt concentrations were identical with TBK 120. Proteasomes eluted in detergent buffer were adjusted to 3 mM $MgCl_2$ and 120 mM KCl with 20 mM Tris-HCl, pH 7.4, 6 mM $MgCl_2$, 7 mM 2-mercaptoethanol, 240 mM KCl (dilution 1:1). Final salt concentrations were identical with TBK 120 + 0.25% Sarkosyl.

RNA Extraction and Gel Electrophoresis—TMV-RNA, rabbit globin mRNA (Life Technologies, Inc.), and 5 S rRNA (Boehringer Mannheim) were labeled *in vitro* with ^{32}P at the 3′ end. Labeling was carried out with [^{32}P]pCp in a reaction catalyzed by T4 RNA ligase according to Peattie (1979).

The digests of TMV-RNA were extracted from enzymatic assays with chloroform/phenol (Perry *et al.*, 1972) and precipitated with 2.5 volumes ethanol + 0.3 M sodium acetate. RNA was analyzed on MOPS-agarose gels containing formaldehyde according to Sambrook *et al.* (1989).

Treatment of Proteasomes with 0.5% Sarkosyl, 6 M Urea, or Micrococcus Nuclease—Fractions of proteasomes eluted from Superose 6 columns were incubated with 0.5% Sarkosyl and chromatographed again on Superose 6 equilibrated in detergent buffer (20 mM Tris-HCl, pH 7.4, 0.5% Sarkosyl, 7 mM 2-mercaptoethanol). Alternatively the fractions were incubated with crystalline urea (final concentration 6 M) and further applied to a Superose 6 column equilibrated in urea buffer (20 mM Tris-HCl, pH 7.4, 6 M urea, 60 mM KCl, 3 mM $MgCl_2$, 7 mM 2-mercaptoethanol). Eluted peak fractions were dialyzed overnight against TBK 120.

Purified proteasomes (500 ng) were incubated with 5 μg of micrococcus nuclease for 2 min at 37 °C in the presence of 3 mM $CaCl_2$. After that 5 mM EGTA was added to block the nuclease activity of this enzyme.

RESULTS

Proteasomes Degrade RNA—The objective of this work was to show that proteasomes harbor a specific RNase activity. For this approach, proteasomes were isolated by FPLC. Fig. 1 shows that proteasomes eluted from Superose 6 columns as one sharp peak of absorbance, corresponding to their characteristic molecular mass of about 700 kDa. No other particles or proteins were observed. Subsequently eluted particles were analyzed by Laemmli PAGE (Fig. 1, *inset*). All proteins visualized by Coomassie Blue banded in a range of 19–35 kDa, corresponding to the characteristic subunit pattern of purified proteasomes.

As a source of mRNA, we used tobacco mosaic virus RNA which was tested recently with proteasomes in cell free translation systems (Homma *et al.*, 1994). TMV-RNA is a polycistronic messenger with an apparent molecular mass of about 2000 kDa. It separates well from proteasomes which elute at about 700 kDa from gel filtration columns.

To demonstrate the degradation of TMV-RNA, in a first series of investigations, we checked our apparatus and solutions to ensure that they were free of RNase activity. For this approach, we incubated TMV-RNA at 37 °C in buffers we used for digestion. Then we applied the assays on Superose 6 columns we used for the purification of proteasomes and the analysis of the digests. As demonstrated in Fig. 2*A* there was no visible degradation of TMV-RNA after these procedures.

After 10 min of incubation in TBK 120 with an approximately 4-fold excess (molar ratio) of proteasomes, 88% of TMV-RNA was degraded comparing the peaks of absorbance before and after digestion (Fig. 2*B*). Interestingly, there was no visible erosion of the sharp peak of absorbance of TMV-RNA, which would be typical for a random degradation in the presence of exonucleases or nonspecific RNase activity. Other experiments showed that proteasomal RNase activity is rather sensitive. When a suspension of proteasomes was treated for 10 min at 100 °C, RNase activity was completely abolished. Repeated freezing and thawing led to the same effect. In addition, TMV-RNA was not degraded in the absence of Mg^{2+}.

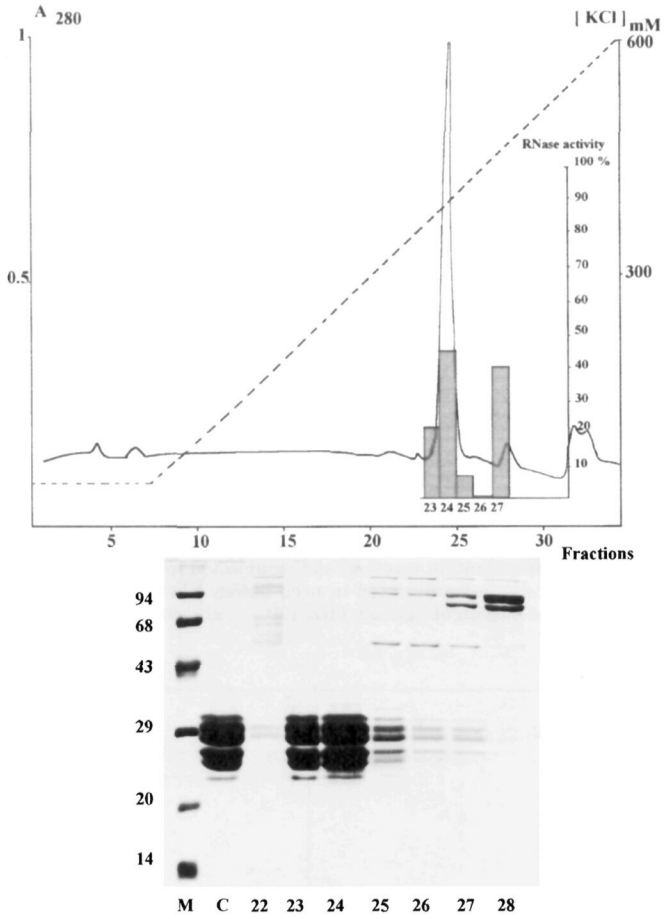

FIG. 3. **Detection of RNase activity of subribosomal particles analyzed by ionic exchange chromatography on FPLC Mono Q columns.** Subribosomal particles of calf liver cells were prepared as described under "Experimental Procedures." Approximately 1500 A_{280} units were chromatographed on a column of Q Sepharose fast flow. Fractions eluted with Tris buffer containing 600 mM KCl were concentrated by ultracentrifugation and applied to a FPLC Mono Q HR 5/5 column. Bound particles were eluted by a linear salt gradient ranging from O to 600 mM KCl. Samples (50 μl) of the 1-ml fractions (23–27) were analyzed for RNase activity using TMV-RNA as substrate as described under "Experimental Procedures." In addition 900 μl of fractions 22–28 were incubated with 10% trichloroacetic acid (final concentration) to precipitate particles and proteins. All precipitates were analyzed by Laemmli PAGE. *Top*: —, absorbance at 280 nm; - - -, linear salt gradient. *Bottom*: *C*, purified proteasomes. *M*, marker proteins.

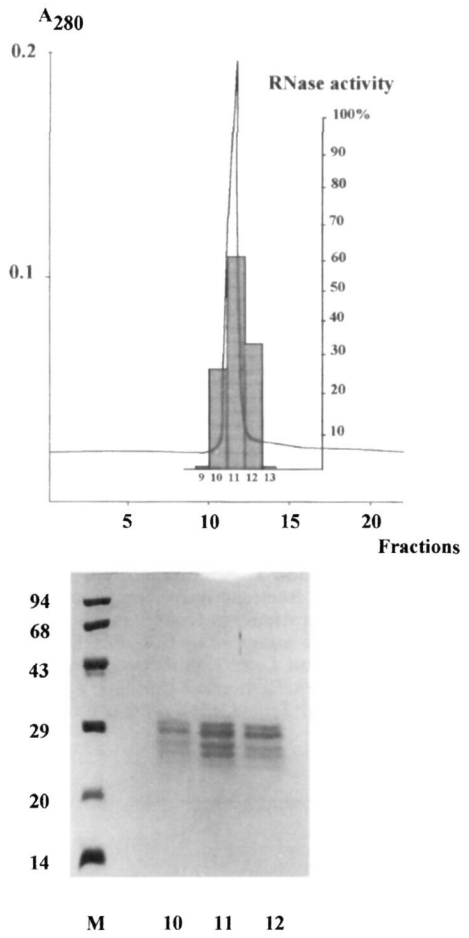

FIG. 4. **RNase activity coelutes with purified proteasomes.** Fractions 11 eluted from three to four FPLC Mono Q columns were pooled and concentrated by step elution on a Mono Q column and chromatographed on a FPLC Superose 6 column equilibrated in TBK 480. Samples (50 μl of the 1 ml fractions 9–13) were analyzed for RNase activity using TMV-RNA as substrate. In addition 900 μl of fractions 9–13 were incubated with 10% trichloroacetic acid (final concentration) to precipitate particles and proteins. Subsequently they were analyzed by Laemmli PAGE and visualized by Coomassie Blue stain. *Top*: —, absorbance at 280 nm. *Bottom*: *M*, marker proteins; fractions 10, 11, and 12 (see *top*).

RNase Activity Coelutes with Proteasomes during Purification—The question arose as to whether RNase activity was indeed an integral part of proteasomes. To address this question, we wanted to know if RNase activity follows proteasome distribution during purification. For these experiments we incubated fractions eluted from the different columns we used for purification with TMV-RNA for 10 min at 37 °C as described under "Experimental Procedures." The presence of proteasomes was determined by Laemmli PAGE (Fig. 3, *bottom*). Our experiments revealed at least two maxima of RNase activity (Fig. 3, *top*). The first eluted exactly with the most prominent peak of absorbance containing pure proteasomes (Fig. 3, *top*, fractions 23 and 24).

The second maximum of RNase activity was found in fraction 27, containing proteins in the molecular range of 50–150 kDa and proteasomal proteins as minor components. Fractions 23 and 24 were pooled and further purified by gel filtration on a Superose 6 column (Fig. 4). Proteasomes eluted with one sharp peak of absorbance in fraction 11 (Fig. 4, *top*), corresponding to a molecular mass of about 700 kDa as we have determined earlier with marker proteins; no other peaks were observed. In this case, RNase activity coeluted exactly with the elution volume of proteasomes and Laemmli PAGE confirmed the purity of proteasomes (Fig. 4, *bottom*).

RNase Activity of Proteasomes Resists to 0.5% Sodium Lauroylsarcosyl and 6 M Urea—It was possible that proteasomes might still be contaminated by proteins, with similar molecular weights as proteasomal proteins, which remain unspecifically attached to the surface of the genuine complex. Such proteins can be eliminated with buffers containing Sarkosyl. We have shown that proteasomes resisted this rather strong detergent and they sedimented in sucrose gradients according to their characteristic 19 S value (Schmid *et al.*, 1984). Under these conditions, ribosomes and mRNP complexes dissociated completely in RNA and protein components.

We incubated proteasomes purified by Superose 6 chromatography in detergent buffer containing 0.5% Sarkosyl and subjected the particles again to gel filtration. Proteasomes eluted with the same molecular weight (data not shown), and their protein composition did not change during this procedure (Fig. 5, *lane 3*). Then we tested detergent washed proteasomes for RNase activity. After 10 min of incubation in TBK 120 + 0.25% Sarkosyl with an approximately 12-fold excess (molar

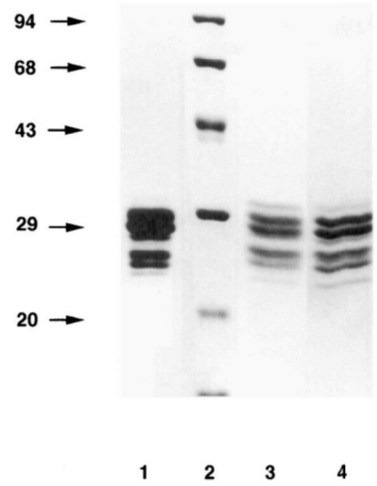

FIG. 5. **SDS-PAGE of proteasomes exposed to 0.5% Sarkosyl and 6 M urea.** Proteasomes were washed with 0.5% Sarkosyl or alternatively with 6 M urea as described under "Experimental Procedures" or in the legends of Figs. 6 and 7. After repeating chromatography on Superose 6 columns, peak fractions were incubated with 10% trichloroacetic acid (final concentrations) to precipitate the proteasomes. The concentrated particles were analyzed by SDS-PAGE and the gels were colored with Coomassie Blue. *Lane 1,* proteasomes exposed to 6 M urea (fraction 13, see Fig. 7); *lane 2,* marker proteins; *lane 3,* proteasomes washed with 0.5% Sarkosyl; *lane 4,* proteasomes eluted from Superose 6 at 480 mM.

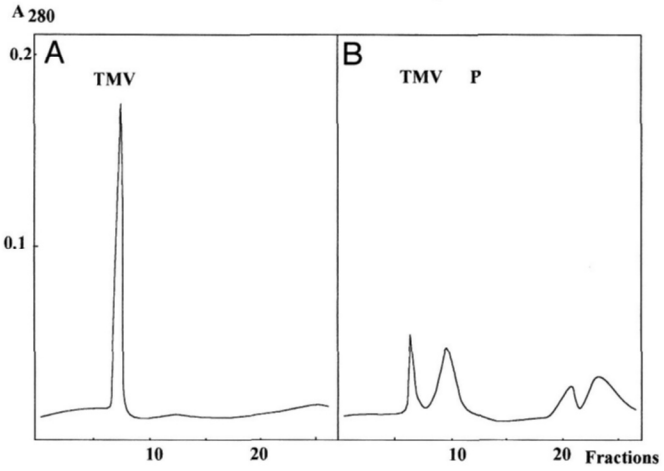

FIG. 6. **Degradation of TMV-RNA by proteasomes exposed to 0.5% Sarkosyl.** Fraction 11 (Fig. 4) from Superose 6 columns containing pure proteasomes (0.8–1 A_{280}/ml) was incubated with 0.5% Sarkosyl (final concentration) and applied again to a Superose 6 column equilibrated in detergent buffer. Eluted peak fractions were diluted 1:1 with 20 mM Tris-HCl, pH 7.4, 6 mM $MgCl_2$, 240 mM KCl, 7 mM 2-mercaptoethanol and incubated with TMV-RNA for 10 min at 37 °C. Then the total assay (200 μl) was immediately analyzed by chromatography on a Superose 6 column equilibrated in TBK 120. *A*, 7.5 μg of TMV-RNA incubated without proteasomes in TBK 120 + 0.25% Sarkosyl. *B*, 7.5 μg of TMV-RNA incubated with 30 μg of proteasomes in TBK 120 + 0.25% Sarkosyl.

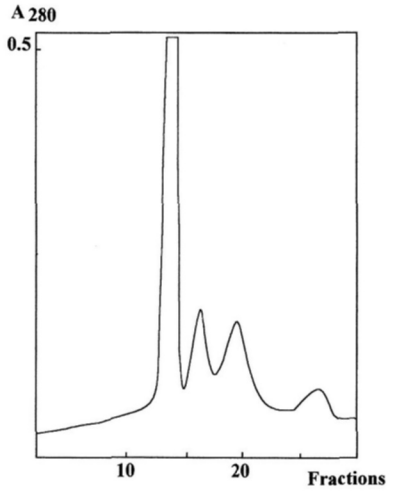

FIG. 7. **Gel filtration of proteasomes exposed to 6 M urea.** Fractions of pure proteasomes in TBK 480 were incubated with crystalline urea (final concentration was 6 M) and resubjected to gel filtration on a Superose 6 column equilibrated in urea buffer. Eluted peak fractions were dialyzed overnight against TBK 120. —, absorbance at 280 nm.

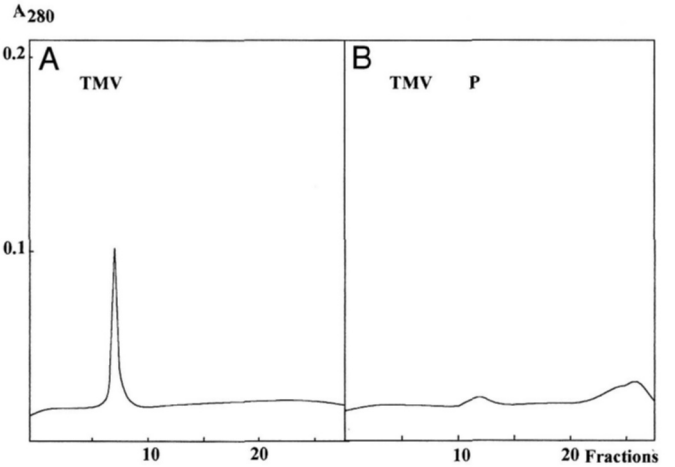

FIG. 8. **Degradation of TMV-RNA by proteasomes exposed to 6 M urea.** A suspension of proteasomes in TBK 480 (1 A_{280}/ml) was incubated with crystalline urea (final concentration 6 M) and loaded on a Superose 6 column equilibrated in urea buffer. After elution with the same buffer fraction 13 (Fig. 7) was dialyzed overnight against TBK 120. TMV-RNA (4 μg) was added to 200 μl of the dialyzed fraction and incubated at 37 °C for 10 min. Subsequently the total assay was analyzed by chromatography on Superose 6 equilibrated in TBK 120. *A*, 4 μg of TMV-RNA incubated without proteasomes in TBK 120. *B*, 4 μg of TMV-RNA incubated with 5 μg of urea-washed proteasomes in TBK 120.

ratio) of proteasomes, 76% of TMV-RNA was degraded (Fig. 6*B*). However, the cleavage reaction was slightly inhibited under these conditions.

Alternatively we used high concentrations of urea to wash purified proteasomes eluted from Superose 6 columns. For this approach, proteasomes were exposed to Tris buffer containing 6 M urea and again subjected to gel filtration on Superose 6 column in the presence of 6 M urea. In this case the molecular weight of the genuine complex changed, the most prominent peak eluted later in fraction 13, which indicated that proteasomes lost some proteins during this procedure (Fig. 7). Indeed the protein composition of urea washed proteasomes was different compared to untreated or Sarkosyl washed complexes (Fig. 5, *lane 1*). However, RNase activity remained associated with the washed subcomplex (Fig. 8). Further dissociation of the subcomplex and subsequent chromatography in the presence of urea revealed that proteasomal RNase activity is associated with at least one distinct proteasomal subunit (preliminary results not shown). In addition, we preincubated proteasomes with Micrococcus nuclease to destroy any copurifing RNA species which might modify proteasomal RNase activity. However, TMV-RNA hydrolysis was not affected by this procedure. Summarizing, all these results clearly showed that RNase activity is an integral part of proteasomes.

Specificity of Proteasomal RNase Activity—To demonstrate the specificity of the nuclease activity of proteasomes, we analyzed the digests of TMV-RNA by electrophoresis on MOPS-

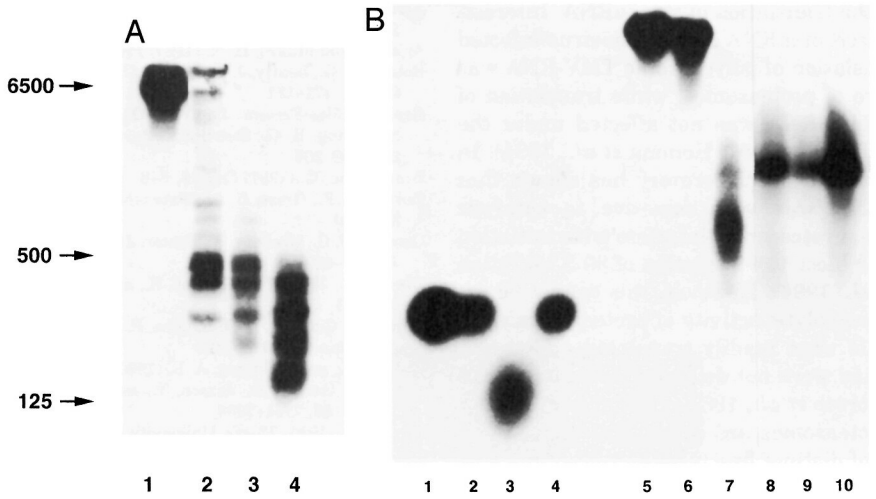

FIG. 9. **Analysis of TMV-RNA digests by electrophoresis on agarose gels.** A suspension of unwashed proteasomes (20 μl) in TBK 120 was incubated with [^{32}P]pCp-labeled RNAs. After extraction with chloroform/phenol the digests were analyzed on MOPS, 2% agarose gels containing formaldehyde. Gels were run at 60 V for 4 h. *A*: *lane 1,* 1 μg of TMV-RNA incubated for 10 min in TBK 120 (control); *lane 2,* 1 μg of TMV-RNA digested with 800 ng of proteasomes for 10 min; *lane 3,* 1 μg of TMV-RNA digested with 800 ng of proteasomes for 30 min; *lane 4,* 1 μg of TMV-RNA digested with 1.6 μg of proteasomes for 10 min; *B: lane 1,* 400 ng of 5 S rRNA incubated for 10 min in TBK 120 (control); *lane 2,* 400 ng of 5 S rRNA (control); *lane 3,* 400 ng of 5 S rRNA digested with 100 units of RNase T1 for 10 min; *lane 4,* 400 ng of 5 S rRNA incubated for 10 min with 1 μg of proteasomes; *lane 5,* 1 μg of TMV-RNA (control); *lane 6,* 1 μg of TMV-RNA incubated for 10 min in TBK 120 (control); *lane 7,* 1 μg of TMV-RNA digested with 600 ng of proteasomes for 10 min; *lane 8,* 250 ng of globin mRNA incubated with 1 μg of proteasomes for 15 min; *lane 9,* 250 ng of globin mRNA incubated for 15 min in TBK 120 (control); *lane 10,* 250 ng of globin mRNA (control)

agarose gels. For this approach, TMV-RNA was labeled at the 3′ end with [^{32}P]pCp. Starting from the 3′ end of TMV-RNA, we identified at least 5 well defined fragments which cover a length of about 500 nucleotides, they accumulated after different times of digestion or quantity of proteasomes in the assay. We could not detect fragments smaller than 120 nucleotides (Fig. 9*A*). 5 S rRNA was not digested by proteasomes (Fig. 9*B*). However, this RNA was cleaved by T1 RNase. In this case all processed fragments migrated at the front of the gel. Also globin mRNA resisted to proteasomal RNase activity (Fig. 9*B, lane 8*), which correlates well to our recently published *in vitro* translation experiments which will be discussed below.

DISCUSSION

In this report we demonstrate that proteasomes harbor specific RNase activity. We have shown that TMV-RNA is an ideal substrate to analyze and quantify the cleavage reaction and we supply several lines of evidence that nuclease activity is an integral part of proteasomes. Degradation of TMV-RNA was not random, liberating mononucleotides or very small oligonucleotides, which suggests that proteasomes have endonuclease activity. Most interestingly the cleavage reaction was rather RNA specific since 5 S rRNA and globin mRNA were not digested by proteasomes.

These results gave further evidence that proteasomes are able to discriminate between different mRNAs and we postulate that proteasomes are also involved in the control of translation. Intensive experimental efforts have led to identification and characterization of transcriptionally regulatory mechanisms; however, there exists little precise evidence for the control of gene expression at the level of translation. The best studied example is the translation regulation of ferritin mRNA. It was shown that cytoplasmic ferritin mRNA underwent a redistribution from an inactive free mRNP pool to translationally active polyribosomal mRNPs, after iron induction (White and Munro, 1988). This mechanism is controlled by a cis-acting element in the 5′ leader region of ferritin mRNA which associates strongly with a transacting element, a 90-kDa protein, in the absence of iron (Walden *et al.*, 1989; Aziz and Munro, 1987).

Several years ago, it was shown that free mRNA bound to transacting proteins (free mRNPs) are blocked for translation, while deproteinized mRNA stimulated protein synthesis in cell free systems (Schmid *et al.*, 1983a, 1983b; Imaizumi-Scherrer *et al.*, 1982; Vincent *et al.*, 1983). Several reports support the idea that there is a close relationship between proteasomes and untranslatable free mRNPs (Schmid *et al.*, 1984; Martins de Sa *et al.*, 1986). We and others have shown that free mRNPs migrate with proteasomes in subribosomal fractions of HeLa cells and erythroblasts. Interestingly, proteasomes always appear linked with the free mRNP particles when low salt conditions were used throughout purification (Schmid *et al.*, 1984; Martins de Sa *et al.*, 1986; Nothwang *et al.*, 1992a, 1992b). In addition, previously we have shown that proteasomes associated *in vitro* with TMV-RNA and mRNA from adenovirus-infected cells, and Bey *et al.* (1993) have demonstrated that the 27-kDa protein, an α-type subunit of proteasomes, has one distinct mRNA binding domain. A widely accepted argument for a possible relationship among proteasomes and RNA is the presence of small RNA molecules in pure preparations of proteasomes (Arrigo *et al.*, 1985; Martins de Sa *et al.*, 1986). The amount of RNA detected in proteasomes varies, it is not stoichiometric (Pühler *et al.*, 1992) and the RNA is very heterogenous in size and sequence (Skilton *et al.*, 1991; Nothwang *et al.*, 1992b; Martins de Sa *et al.*, 1986; Gaedik, 1988).[2] These data reflect that proteasomal RNAs could be interpreted as residual parts of larger RNAs. This correlates well with the following observations. Proteasomal RNAs were reported to have 5′-P and 3′-OH termini (Schmid *et al.*, 1984; Martins de Sa *et al.*, 1986) and we found that proteasomal RNase activity creates TMV fragments which we easily could label at the 3′ end with [^{32}P]pCp but not with [γ-^{32}P]ATP at the 5′ end. This is in good agreement with a model for mRNA decay which describes that endonucleases initiate mRNA decay by cleaving mRNAs at specific sites to provide exposed 3′-OH termini (Yajnik and Godson 1993).

If proteasomes associated with a given mRNA, they should

[2] M-N. Pouch, F. Petit, J. Buri, Y. Briand, and H-P. Schmid, unpublished results.

influence in some way the translation of this mRNA. Interestingly, the *in vitro* synthesis of mRNA from adenovirus-infected HeLa cells and the translation of polycistronic TMV-RNA was impeded in the presence of proteasomes, while translation of HeLa mRNA and globin mRNA was not affected under the same conditions (Horsch *et al.*, 1989; Homma *et al.*, 1994). In addition very recent work of our laboratory has shown that translation of TMV-RNA was very sensitive to catalytic amounts of proteasomes. Proteasomes interfere with initiation of protein synthesis, and block the formation of 80 S initiation complexes (Homma *et al.*, 1994). However, this cannot be explained simply by the proteolytic activity of proteasomes since mRNAs from HeLa cells were readily translated and the *in vitro* synthesized proteins were not degraded by proteasomes (Homma *et al.*, 1994; Horsch *et al.*, 1989). All these data favor the hypothesis that proteasomes are involved in translation control by degradation of distinct free mRNAs containing specific sequences or secondary structures.

There could be signals for degradation of RNAs which are recognized by proteasomes and we assume that the degradation process is rather selective. In eukaryotic cells there is only little evidence about the nature and function of RNases which are involved in a selective control of proteins synthesis. However, in some prokaryotic model systems it was shown that protein synthesis is regulated by a selective decay of a coding sequence within a polycistronic mRNA (Brawerman, 1987; Belasco *et al.*, 1985; Burton *et al.*, 1983). The selectivity in mRNA decay is ensured by site specific endonucleases like RNase E which is also involved in mRNA processing and the processing of 9 S rRNA into 5 S rRNA (Yajnik and Godson, 1993; Mudd and Higgins, 1993). Indeed there exists some parallels between proteasomes and such RNases: the proteasomal RNase activity we describe in this paper led to a cleavage pattern typical for endonucleases, and proteasomes are able to discriminate between RNAs (see our results and Horsch *et al.* (1989, 1990)).

Interestingly RNase activity of proteasomes has been proposed earlier, based on the observation that fractions containing proteasomes hydrolyzed 18 S rRNA and purified proteasomes were shown to be involved in the processing of pre-tRNA (Tsukahara *et al.*, 1989; Castano *et al.*, 1986). However, the later observation was challenged recently by Doria *et al.* (1991), and others have reported that they did not degrade soluble yeast RNA (Kühn *et al.*, 1990). These contradictory results demonstrate again that RNase activity of proteasomes is rather specific.

Clearly, much work is needed to map the cleavage sites on TMV-RNA. Currently we are studing the factors which modulate the RNase activity of proteasomes. Furthermore, these investigations have to be extended to other RNA species to demonstrate how proteasomes discriminate between RNAs.

Taken together, the multienzymatic properties expressed *in vitro* and *in vivo* suggest that proteasomes are multifunctional complexes, which participate in the pathways of intracellular protein breakdown and RNA metabolism.

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