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Effect of the multicatalytic proteinase (prosome) on translational activity in rabbit reticulocyte lysates

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In a message-dependent reticulocyte lysate translation system, incorporation of [^{3}H]leucine into acid-insoluble protein is increased following selective removal of the multicatalytic proteinase (MCP) with a monospecific antibody. Re-addition of active proteinase to previously depleted lysates reverses this effect in that the same low levels of translational product are measured as in untreated lysates. Addition of histone-stimulated MCP further depresses the level of protein product. Conversely, lysates supplemented with inactivated MCP retain the higher level of translational activity which is measured after precipitation of the enzyme with antibody. In these lysates, the effect of the antibody on translational activity is inversely correlated with that on hydrolytic activity towards [^{14}C]methylcasein or N-succinyl-Leu-Leu-Val-Tyr-4-methyl-7-coumarylamide, two substrates of the MCP. These results showing that the MCP is capable of modulating translational activity in vitro, suggest an important role of this molecule in the in vivo translational process.

Multicatalytic proteinase; In vitro translation; Reticulocyte lysate; (Rabbit)

1. INTRODUCTION

The MCP is a non-lysosomal, high M_r proteinase. The enzyme, previously thought to be unique to eukaryotic cells [1], has recently been demonstrated in prokaryotic archaebacteria as well [2]. The cylinder-shaped particle [3,4] is by immunological, morphological and biochemical criteria identical with the 19–20 S RNP (also termed prosome or proteasome) [5–7]. Different functions have been ascribed to this molecule, such as mRNA-repression [8], aminoacyl-tRNA synthetase activity [9] or pre-t-RNA 5′-processing nuclease activity [10]. However, the physiological function of this molecule has remained elusive.

We [11] and others [12] have shown that the MCP is an intrinsic enzyme of rabbit reticulocytes. Furthermore, we have found that in situations of reduced cellular protein synthesis rates, the specific proteolytic activity of the MCP is significantly reduced [13,14]. Using homologous, anti-rabbit MCP antibodies, we have tested whether selective removal of the enzyme from reticulocyte lysates affects the translation of exogenous messenger RNA. Different from the findings of Horsch et al. [15], showing that in a rabbit reticulocyte translation assay, prosomes or prosomal RNA inhibit translation of a given mRNA but are without effect on another species of mRNA, our results show that the MCP (prosome) interferes with the translational activity of all mRNAs tested, and that it is the proteolytic activity of the MCP which is implicated, possibly at the level of post-translational protein processing.

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Abbreviations: BMV, brome mosaic virus; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; IgG, immunoglobulin G; MCP, multicatalytic proteinase; RNP, ribonucleoprotein; SLLVT-MCA, *N*-succinyl-Leu-Leu-Val-Tyr-4-methyl-7-coumarylamide; SDS, sodium dodecyl sulphate; TMV, tobacco mosaic virus

2. MATERIALS AND METHODS

2.1. *Materials*

Male Wistar rats (150–200 g body weight) were purchased from Ivanovas, Kisslegg, FRG. Message-dependent (nuclease-treated) rabbit reticulocyte lysate, BMV mRNA, globin mRNA and RNasin ribonuclease inhibitor were from Promega, Madison, WI, USA. TMV mRNA was from Boehringer, Mannheim, FRG. L-[^{3}H]leucine (>140 mCi/mmol) was from NEN, Boston, MA, USA. Donkey anti-sheep IgG, histone type VI-S and [^{14}C]methylcasein were from Sigma, Deisenhofen, FRG. Non-immune sheep IgG was from Nordic, Tilburg, The Netherlands. Silica gel-impregnated glass fiber sheets were from Gilman Science, Ann Arbor, MI, USA. SLLVT-MCA was from Bachem, Bubendorf, Switzerland. All other chemicals were of the highest purity available.

2.2. *Methods*

2.2.1. Preparation of MCP and anti-MCP antibodies

Purification of MCP from rabbit skeletal muscle and generation of monospecific antibodies to the enzyme in sheep was as described elsewhere [11]. Isolation of total IgG and purification of MCP-specific IgGs on MCP-affinity gel was exactly as described for the antibody to rat MCP [16]. Monospecific, polyclonal antibody to rat MCP was raised in rabbits as described previously [17].

2.2.2. SDS-PAGE

Analysis of SDS-gel electrophoretically separated proteins by immunoblotting was performed as in [18].

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Silver-staining of SDS-polyacrylamide gels was by the method of Merril et al. [19].

2.2.3. Preparation of rat liver mRNA and rat liver polysomes

Purification of total RNA from rat liver and isolation of poly(A)$^+$ RNA from this fraction, using oligo(dT)-cellulose was by the method of Okayama et al. [20].

Isolation and purification of rat liver polysomes was by the method of Ramsey and Steele [21], except that cell sap (which contains the MCP) was omitted from the isolation media.

2.2.4. Measurement of translational activity

Incorporation of L-[^{3}H]leucine into polypeptides was carried out using the Promega reticulocyte (nuclease-treated) translation kit. Unless specified otherwise, treatment of lysate with antibody, and measurement of translational activity were as follows.

The preincubation mixture contained: 12 μl of reticulocyte lysate, 1 μl of amino acid mixture minus leucine (1 mM), 1 μl of potassium acetate (1 M), 1 μl of magnesium acetate (10 mM), 80 U of RNasin (80000 U/ml) and 2.5 μl of affinity-purified IgG of a sheep anti-rabbit MCP antibody (1 mg/ml, in 20 mM Hepes/1 mM EGTA, pH 7.2). To control incubations 2.5 μl of non-immune sheep IgG instead of anti-rabbit MCP IgG was added, but at the same concentration and in the same buffer. After 30 min at 37°C, 2.5 μl of a second, donkey anti-sheep IgG (1 mg/ml in 20 mM Hepes/1 mM EGTA, pH 7.2) was added to each tube and incubation was continued for further 30 and 60 min at 37°C and 4°C, respectively. Thereafter, samples were centrifuged (Beckman SW-60 rotor, 10 min at 20000 rpm).

9.5 μl of the supernatant was carefully removed, transferred to a fresh tube, and, after adding 2.5 μl of neutralized L-[^{3}H]leucine (1 mCi/ml), translation was started by adding 1 μl of mRNA (100–150 μg/ml in 10 mM Tris-HCl/1 mM EGTA, pH 7.2) and differently treated MCP or buffer to final volume of 15 μl. Blank samples contained 1 μl of buffer instead of mRNA. The final concentration of lysate was about 40%. After 60 min at 30°C, reactions were terminated by addition of 15 μl of Tris-HCl (1 M, pH 10.7) and evaluation of counts was by spotting 2-μl aliquots on silica gel-impregnated glass fiber strips (10 × 60 mm). After developing the strips by ascending chromatography in a solvent of 30% (v/v) methanol, 10% (w/v) TCA, and 10% (v/v) acetic acid for 2 min, the lower portion of the strip containing the labeled protein was cut off and, to solubilize proteins, was reacted with 500 μl of Protosol for 30 min at 60°C. After cooling, 50 μl of glacial acetic acid was added and protein-incorporated radioactivity was determined by liquid scintillation counting at 20–25% efficiency. In some experiments, TCA-insoluble radioactivity was determined in parallel and was found to give qualitatively the same results as those obtained with chromatography on glass fiber strips. All data shown here are from the more rapid chromatographic method.

Assays were done in triplicate and variation between values was less than 5%.

To assay the effect of the anti-MCP antibody on proteolytic activity in reticulocyte lysates, preincubation conditions were identical to those for studying the incorporation of labeled amino acids into protein, except that the volume of all components was increased by a factor of 5. After preincubation and centrifugation, 50 μl of the supernatant was removed and assayed for proteolytic activity towards SLLVT-MCA and [^{14}C]methylcasein as described previously [5], except that 5 mM EDTA (final concentration) was included in the incubations.

All other experimental details are given in figure and table legends.

3. RESULTS AND DISCUSSION

3.1. *Interaction of anti-MCP antibodies with reticulocyte MCP*

In a previous study on proteolytic activities in rabbit reticulocyte lysates, we have demonstrated an almost quantitative immunoprecipitation of the MCP by an MCP-specific antibody [11]. Using a similar approach in the present study, we found that under the more stringent conditions of the reticulocyte translation assay, neither an anti-MCP specific IgG-fraction nor affinity-purified anti-MCP IgGs by themselves were capable of precipitating detectable amounts of MCP, although the antibody concentration was varied over a wide range (data not shown). Alternatively, attempts to achieve formation of precipitable antigen–antibody complexes by prolonging the preincubation period were precluded by the fact that the efficiency of subsequent translation assays was very low. However, these problems could be resolved by using affinity-purified anti-MCP IgG together with a secondary, anti-sheep IgG. Under these conditions, a precipitable complex formed. As illustrated in fig.1, SDS-gels of precipitates from control lysates, treated with non-immune sheep IgG and anti-sheep IgG exclusively reveal bands due to the IgG heavy and light chain proteins. The MCP-specific antibody additionally precipitates 8–10 peptides in the range of M_r 25–32 kDa, characteristic of the MCP [17]. That no lysate proteins other than the MCP are precipitated contrasts with findings in [22], showing that in the fly, *Drosophila melanogaster*, a fraction of the MCP is found in tight association with polysomes. If such an association existed in the reticulocyte system

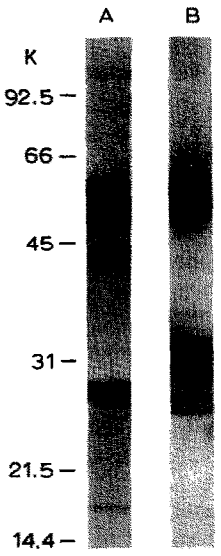

Fig.1. SDS-gel electrophoretic protein pattern of precipitates from reticulocyte lysate treated with non-immune IgG (A) or with anti-MCP IgG (B). Reticulocyte lysate was incubated with non-immune IgG plus secondary antibody or with anti-MCP IgG plus secondary antibody as described in section 2.2. After centrifugation and careful removal of supernatants, precipitates were washed 6 times with 100-μl aliquots of ice-cold 20 mM Hepes buffer, pH 7.2. Pellets were then dissolved in 50 μl of SDS sample buffer and were electrophoresed under denaturing conditions in SDS-containing slab gels with a 10–18% (w/v) polyacrylamide continuous gradient as in [11]. Protein was visualized with the silver stain method [19]. The relative molecular mass is indicated (K).

Table 1

Effect of anti-MCP antibody on proteolytic activity in reticulocyte lysate

Treatment of lysate with:	Hydrolysis of:	
	[^{14}C]Methylcasein (μg/h per ml lysate)	SLLVT-MCA (pmol/min per ml lysate)
Non-immune IgG	9 ± 0.4	630 ± 30
Anti-MCP IgG	4 ± 0.3	320 ± 10

Lysates were preincubated with either non-immune IgG or anti-MCP IgG, followed by addition of secondary, anti-sheep IgG and were centrifuged. Supernatants were supplemented with 5 mM EDTA (final concentration) and 50-μl aliquots were assayed for hydrolytic activity towards [^{14}C]methylcasein (spec. act. 2700 dpm/μg, 14 μg) or SLLVT-MCA (20 μM). Incubations lasted for 60 min at 37°C. For full experimental details, see section 2.2. Values are means ± SE of 3 different experiments

as well, bands due to co-precipitated ribosomal proteins should have been visualized with the sensitive staining method used. To test whether this lack of association is unique to the rabbit reticulocyte, we purified bound and free polyribosomes from rat liver. In electron micrographs, we only rarely detected the typical, cylinder-shaped structure of the MCP, and apparently not in association with polysomes. Furthermore, in Western blots of these fractions developed with an antibody specific for rat MCP, we could not detect any immunoreactive material (data not shown). From these results we conclude that, different from insects, the MCP in mammals is not tightly associated with polysomes. This conclusion is corroborated by data of Lockard [23] showing that, subsequent to photo-induced cross-linking of cellular proteins, the polysomal and free mRNP fractions lacked the typical low-molecular-weight 'prosomal' proteins, a finding which argues against these molecules to be in direct contact.

The above findings show that specific, precipitable antigen–antibody complex formation does occur in lysates, but they do not indicate whether precipitation is quantitative. As we have shown in previous studies on proteolytic activity in reticulocyte lysates, casein degradation is largely, and in the presence of 5 mM EDTA, SLLVT-hydrolysis is totally due to the MCP activity [11,14]. When assayed under these conditions, supernatants of lysates treated with anti-MCP were found to have lost about 50% of both peptide and protein degradative capacity (table 1). This incomplete elimination of MCP from the lysate may simply be due to the fact that the amount of antibody added was suboptimal. Indeed, in preliminary experiments where lysates were titrated with increasing amounts of anti-MCP IgG, the MCP–antibody complex precipitated, and the residual level of MCP in the supernatant was determined by quantitative immunoelectrophoresis, we found that at all IgG concentrations tested, some immunoreactive material remained in these supernatants. It is possible that the incubation time with antibody was too short for quantitative antigen–antibody complex formation, but as mentioned above, the limitations of the translation system precluded longer preincubations. In addition, due to an apparent structural heterogeneity of the MCP population in eukaryotes [2,24], it is conceivable that epitopes on some MCP molecules are not readily accessible to the antibody and that those MCP molecules 'escape' complex formation. Nevertheless, these results showing the specific immunoprecipitation and elimination of an essential MCP activity allowed us to directly address the question whether the MCP as a proteolytic enzyme is involved in the translational process.

3.2. *Effect of MCP and anti-MCP antibody on translational activity*

Table 2 shows that after treatment of lysates with anti-MCP antibodies, message-directed incorporation

Table 2

Effect on translational product formation in reticulocyte lysates treated with anti-MCP antibody

Treatment of lysate with:	[^{3}H]Leucine incorporated into acid-insoluble radioactivity (cpm/μl lysate)							
	BMV-mRNA	% Change	TMV-mRNA	% Change	Globin-mRNA	% Change	Rat liver-mRNA	% Change
n.i. IgG	14200		18500		8200		6300	
n.i. IgG + MCP	13900		18000		8000		6200	
i. IgG	17800	+125	25100	+136	11500	+140	9800	+156
i. IgG + MCP	13000		17500		7600		6000	
	(1000)		(1100)		(800)		(900)	

Lysates were preincubated with non-immune IgG (n.i. IgG) or anti-MCP IgG (i. IgG) followed by secondary antibody as detailed in section 2.2. After precipitation, lysates were charged with mRNA alone or in the presence of 1 μg purified MCP. Incubations lasted for 60 min at 30°C and radioactivity incorporated into acid-insoluble protein was determined as described in section 2.2. The values shown have been rounded to the nearest hundred. Values in brackets indicate blanks (no mRNA added). Percent change is defined as cpm measured in the acid-insoluble fraction of lysates treated with immune IgG divided by cpm measured in that of lysates treated with non-immune IgG × 100:

$$\% \text{ change} = \frac{\text{cpm (i. IgG)}}{\text{cpm (n.i. IgG)}} \times 100$$

of [^{3}H]leucine into acid-insoluble protein is significantly increased for the 4 different mRNAs tested, with BMV-mRNA and rat liver-mRNA translation products showing a 25% and 55% increase, respectively, with increases between these two values for TMV-mRNA and globin mRNA. Importantly, supplementation of previously depleted lysates with purified, active MCP reverses this effect and the levels of product formation are reduced to those of untreated lysates or even below.

Interestingly, in untreated lysates, added MCP is without effect on translational activity, probably because endogeneous MCP is at saturating levels. To test whether the proteolytic activity of the MCP is a causative factor for inhibition of translational activity, a further series of experiments was performed where the activity of the MCP was modulated prior to addition to lysates. In accordance with findings on MCP from other sources [25,26], the MCP from rabbit reticulocytes has been found to be inhibited by thiol-reactive reagents [12] and the proteinase can be activated by compounds such as SDS and fatty acids [12]. With regard to the stimulating effect of basic polyamino acids on MCP activity [27], we have found that naturally occurring, basic proteins like histones, are also activators of the MCP. Histone VI S (slightly lysine-rich) was found to be particularly effective and stimulated the MCP activity towards protein and peptide substrates about 7-fold (Kuehn, L., unpublished).

Table 3

Effect of supplementation with inactivated or activated MCP on rat liver mRNA translational product formation in reticulocyte lysates

Supplementation of lysate with:	[^{3}H]Leucine incorporated into acid-insoluble radioactivity (cpm/μl lysate)	
	n.i. IgG	i. IgG
None	7100	10700
Untreated, active MCP	6900	6800
Hg-inactivated MCP	7000	10500
Histone	5000	5300
Histone-activated MCP	3900	3100
	(1000)	

Experimental conditions were as described in table 2, with the following changes: to inactivate the MCP, enzyme was incubated with 10 mM $HgCl_2$ for 15 min at room temperature, followed by exhaustive dialysis against 20 mM Hepes buffer, pH 7.2. In another dialysis bag, active MCP was dialysed against the same buffer. Following dialysis, active enzyme was found to have retained over 95% of its original activity, whereas $HgCl_2$-treated enzyme showed no activity towards SLLVT-MCA as substrate. Activation of MCP was by incubating the enzyme with an approx. 10-fold molar excess of histone VIS for 15 min at room temperature prior to addition to lysates. Supplementation solutions contained: none, Hepes-buffer; untreated, active MCP or $HgCl_2$-inactivated MCP, 1 μg of enzyme protein; histone, 0.2 μg; histone-activated MCP, 0.2 μg of histone and 1 μg of MCP. Values shown have been rounded to the nearest hundred. Value in brackets is a blank (no mRNA added). Differences in incorporated radioactivity between values in table 2 and table 3 are due to two different rat liver mRNA preparations used

In the context of the present study, the use of histone as an activator of MCP appeared to be most appropriate since these molecules are physiologically important constituents of the cell. As shown in table 3 for a lysate charged with rat liver mRNA (similar results were obtained for BMV-, TMV- and globin-mRNA, not shown), and clearly different from the results shown in table 2, where active enzyme had been added, an MCP which had been inactivated prior to addition to antibody-treated lysates does not lower the level of translational product formation. Conversely, addition of histone-stimulated MCP lowers the level of product formation dramatically. Such a product-lowering effect can also be measured for lysates after addition of histone only, albeit in a less pronounced fashion. The latter result is probably due to residual endogeneous MCP in the lysates which, similar to the isolated enzyme, would be expected to be activated by added histone. The finding that lysates treated with non-immune IgG equally respond to the histone stimulus is supportive of this idea.

3.3. *Conclusions*

This is the first report demonstrating that the MCP is involved in protein translation as a proteolytic activity. Interestingly, a post-transcriptional control function of prosomes has been proposed earlier, based on the observation that prosomes are often associated with repressed mRNA [28]. Those authors, however, did not test for proteolytic activity in their prosomal preparations and therefore did not consider the possibility that intrinsic proteolytic activity of the MCP might, at least in part, have been responsible for their results. At present, the precise level of interaction of the MCP in the translational machinery is unknown. Furthermore, we cannot explain the apparent contradiction arising from the finding that in vivo, the specific activity of the MCP is decreased under conditions of reduced rates of protein synthesis [13,14], whereas in vitro, an inactivated enzyme is unable to reduce translational activity. On the other hand, it is noteworthy that the MCP, previously thought to be devoid of RNase activity [29], as also found in our studies showing that rabbit MCP does not degrade yeast RNA (Kuehn, L., unpublished), has recently been reported to degrade 18 S rRNA [30]. These contradictory results may only be apparent in that the MCP possesses a restricted specificity, with degradative activity against 18 S rRNA (and possibly other RNA species) but not against yeast RNA. In the present study, however, such an activity should have gone unnoticed, as RNase inhibitor had been included in all assays.

Taken together, the enzymic properties expressed in vitro suggest that the multicatalytic proteinase is a multifunctional complex with the various activities acting in concert, but at different levels, to participate in the modulation of intracellular protein metabolism.

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