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Mos is degraded by the 26S proteasome in a ubiquitin-dependent fashion

Naruhiro Ishida[a], Keiji Tanaka[b], Tomohiro Tamura[b], Mayumi Nishizawa[c], Kenji Okazaki[c], Noriyuki Sagata[c] and Akira Ichihara[b]

[a]*Central Research Laboratories, Santen Pharmaceutical Co., Ltd., Osaka 533, Japan,* [b]*Institute for Enzyme Research, The University of Tokushima, Tokushima 770, Japan* and [c]*Division of Molecular Genetics, Institute of Life Science, Kurume University, 2432-3 Aikawa, Kurume, Fukuoka 830, Japan*

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Mos, the *c-mos* proto-oncogene product, is a key regulator of cell cycle progression Recently, rapid turnover of Mos in an early stage of meiotic maturation of *Xenopus* oocytes was found to be mediated by the ubiquitin pathway, but the protease responsible for its breakdown was not identified In the present study, we found that ^{35}S-labeled Mos synthesized in an in vitro transcription/translation system was degraded ATP- and time-dependently by the 26S proteasome, but not by the 20S proteasome, in the presence of a ubiquitin-ligation system. The 26S proteasome did not degrade a mutant Mos in which Ser^3 was replaced by Asp^3 that is metabolically stable in oocytes, indicating a similarity in the proteolytic events in vivo to those observed in vitro in the present work This is the first demonstration that the proteasome catalyzes the ATP-dependent degradation of a naturally occurring, short-lived oncoprotein by the ubiquitin pathway This finding suggests that the proteasome may regulate the intracellular stability of various oncoproteins.

ATP-dependent proteolysis; Mos; Proteasome, Ubiquitin system

1. INTRODUCTION

Ubiquitin (Ub) confers a degradation signal to target proteins by covalent attachment to them through a multi-step enzymatic process catalyzed by Ub-activating enzyme E1, Ub-conjugating enzyme E2 and Ub recognition protein E3 [1]. Recently, proteins with aberrant structures and naturally occurring short-lived proteins related with cell cycle control were reported to be degraded selectively by the Ub pathway. Indeed, the degradations of a regulatory component of MPF (M-phase promoting factor), cyclin, the oncoprotein Myc and the tumor-suppressor protein p53 were reported to be ATP-, and Ub-dependent (reviewed in [2]). Based on in vitro [3] and in vivo [4] studies, the proteasome, a central enzyme catalyzing a nonlysosomal proteolytic pathway, has been proposed to be the enzyme responsible for breakdown of proteins targeted by ubiquitination. Moreover, ubiquitinated proteins have been suggested to be degraded ATP-dependently by the 26S proteasome complex [1,2,5,6]. The features of this unusually large complex containing a multicatalytic 20S proteasome have been studied using various model proteins, such as lysozyme, α-lactoalbumin, β-lactoglobulin and β-galactosidase, but it is still not clear whether the complex is actually involved in the Ub pathway of degradation of important naturally occurring proteins. Recently, the *c-mos* proto-oncogene product Mos, a key regulator of the meiotic cell cycle [7], was shown to be very unstable in the early stage of oocyte maturation in *Xenopus* and this extreme instability was found to be due to its rapid breakdown by the Ub pathway [8]. It was, therefore, of interest to examine whether the 26S proteasome is responsible for preferential degradation of ubiquitinated Mos. In this work, we found that the 26S proteasome catalyzed ATP- and Ub-dependent breakdown of Mos in a reconstituted proteolytic system, consisting of the 26S proteasome and a Ub-system.

Correspondence address K. Tanaka, Institute for Enzyme Research, The University of Tokushima, Tokushima 770, Japan

2. MATERIALS AND METHODS

2.1. *Preparation of ^{35}S-labeled Mos*

Xenopus c-mos and mutant mRNAs were transcribed using an SP6/T7 Transcription kit (Boehringer Mannheim) from the corresponding full-length clones (pSP64-MPSP encoding the native Mos and its derivative pSP64-MPDP encoding mutant Mos with Asp^3 in place of Ser^3) with SP6 RNA polymerase [8]. The mutant *c-mos* cDNA was prepared as described [8] The mRNAs were translated in the presence of [^{35}S]methionine in a rabbit reticulocyte lysate (Amersham).

2.2. *Assay of Mos degradation*

For assay of Mos degradation, 1 μl (about 5,000 cpm) of [^{35}S]Mos translated in a reticulocyte lysate was incubated at 37°C for 120 min in a total volume of 100 μl of reaction mixture containing 100 mM Tris-HCl (pH 9 0), 5 mM $MgCl_2$, 2 mM ATP, 1 mM dithiothreitol, 2 U/ml pyrophosphatase, 7.5 μg of Ub and 1 μg of purified 26S proteasomes in the presence or absence of the Ub–protein ligation enzymes E1 + E2 and E3. Reaction products precipitated with cold acetone were subjected to SDS-PAGE, and the disappearance of [^{35}S]Mos was monitored fluorographically. For measurement of the activity without Mg^{2+}, 20 mM EDTA was added to the assay mixture. Highly purified E1 + E2 and partially purified E3, which were added to an assay mixture in amounts of 12 μg and 30 μg, respectively, were

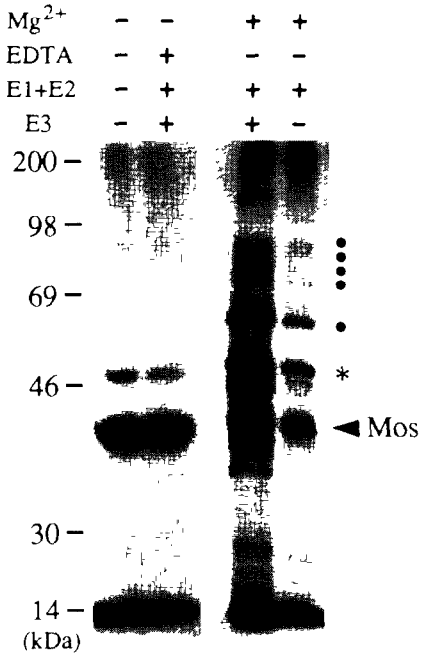

Fig. 1. In vitro ubiquitination of Mos by the Ub-system. For details of the assay mixture, see section 2. EDTA was added at a final concentration of 20 mM. The asterisk shows a band of artifact, which may be a [^{35}S]Met-tRNA Closed circles on the right show positions of ubiquitinated Mos. The arrowhead shows the position of Mos. The ^{14}C-labeled molecular weight markers (Amersham) used were myosin (200 kDa), phosphorylase-b (98 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa) and lysozyme (14 kDa).

obtained from a rabbit reticulocyte extract by Ub-Sepharose affinity chromatography as described before [9]. The 20S proteasome [10] and 26S proteasome [11] were purified as reported.

3. RESULTS

3.1. *In vitro ubiquitination of Mos*

Recently, Nishizawa et al. [8] reported that Mos is ubiquitinated before being broken down in *Xenopus* oocytes. We examined whether Mos is ubiquitinated in vitro by incubating it with Ub and purified E1 + E2 and E3. As shown in Fig. 1, ^{35}S-labeled Mos of 39 kDa was multi-ubiquitinated. The ubiquitination was Mg^{2+}-dependent and therefore ATP-cleavage-dependent. These results suggest that Mos is a target of the Ub system. The ubiquitination pattern was consistent with that obtained with a reticulocyte lysate by Nishizawa et al. [8]. Interestingly, the multi-ubiquitination of Mos was markedly stimulated by the addition of E3 in the presence of E1 and E2, suggesting that the ubiquitination of Mos is largely E3-dependent. This finding is consistent with the 'second-codon rule', which mimics the N-end rule mediated by E3 [12], for Mos degradation observed in *Xenopus* oocytes [8].

3.2. *Degradation of Mos by the Ub-system with the 26S proteasome*

The 26S proteasome containing a 20S proteasome is proposed to be an ATP-dependent protease in mammalian cells [1,6,10]. We examined whether the 26S proteasome is involved in the ATP-dependent degradation of [^{35}S]Mos and whether post-translational modification by ubiquitination is essential for Mos degradation in vitro. As shown in Fig. 2 (left panel), [^{35}S]Mos was degraded significantly, but only gradually, by the 26S proteasome in the presence of ATP only, but the further addition of a Ub system greatly stimulated its degradation by the 26S proteasome (Fig. 2, right panel). This

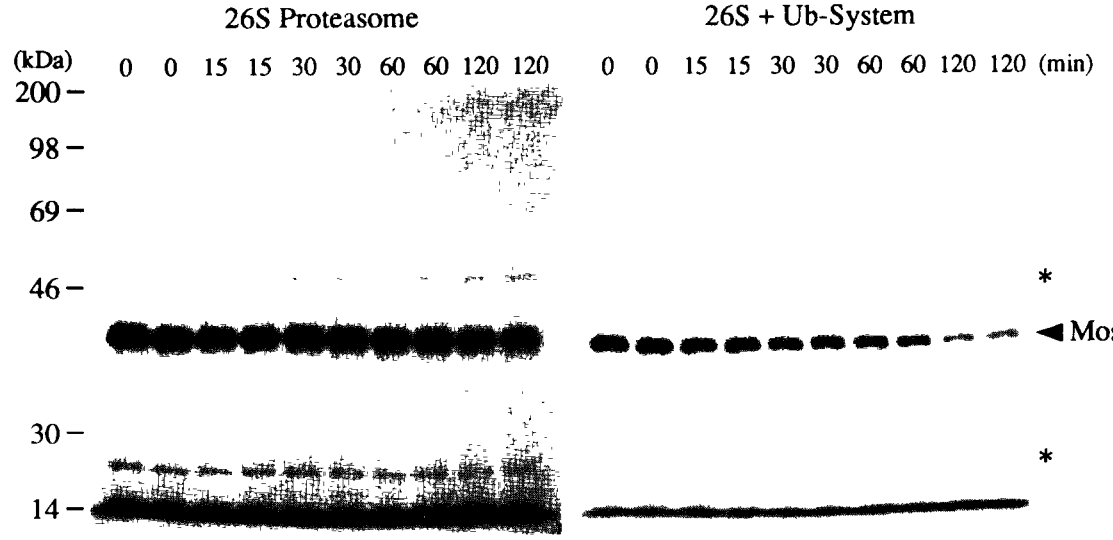

Fig. 2. Time course of Mos degradation by the 26S proteasome. [^{35}S]Mos synthesized in vitro was incubated at 37°C with the 26S proteasome (left panel), or the 26S proteasome and a Ub-ligated system, consisting of Ub, E1 + E2 and E3 (right panel) for the indicated times. Symbols are as for Fig. 1 Samples were run in duplicate. Asterisks show bands of artifacts. The upper asterisk is as for Fig. 1 The lower asterisk indicates a band thought to be a product translated from the second Met of Mos mRNA

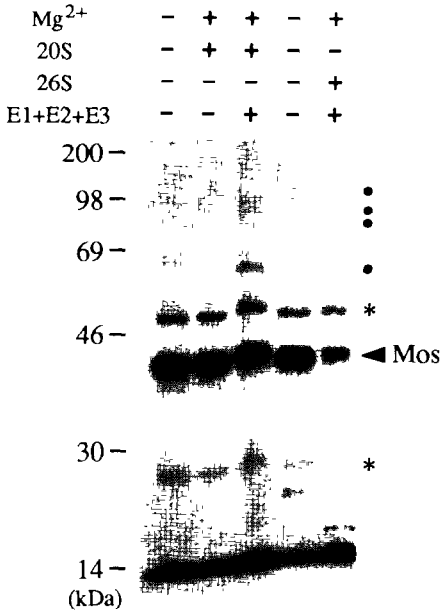

Fig. 3. Degradation of Mos by the 20S proteasome. The system was as for Fig. 2, except that the purified 20S proteasome (1 μg) was used instead of the 26S proteasome. Symbols are as for Fig. 1.

finding suggests that the 26S proteasome catalyzes Mos degradation in a ubiquitination-dependent manner. Interestingly, in this system, no band of ubiquitinated Mos was detected even on longer exposure of the fluorographic film, suggesting rapid degradation of Ub-ligated Mos by the 26S proteasome. Possibly, the ubiquitination step is rate-limiting, and the subsequent degradation is so rapid that no intermediates were detectable. The Ub-dependent degradation of Mos by the 26S proteasome seemed to be ATP-dependent, because replacement of Mg^{2+} by EDTA almost completely inhibited the degradation of Mos that had been ubiquitinated by the Ub system (data not shown). The 20S multicatalytic proteasome did not degrade [^{35}S]Mos in the presence or absence of the Ub-ligation system (Fig. 3). Moreover, significant multi-ubiquitination of Mos occurred on addition of the Ub-system in the presence of the 20S proteasome (Fig. 3), as seen in Fig. 1. This finding is consistent with the observation that the 20S proteasome also has no effect on the degradation of [^{125}I]lysozyme–Ub conjugates [11].

3.3. *Absence of degradation of mutant MPDP-Mos by the 26S proteasome*

To obtain further information on Mos degradation in the present reconstituted proteolytic system, we examined the degradation of a mutant Mos named MPDP-Mos, in which Ser^3 of native Mos (named MPSP- Mos) is replaced by Asp^3. This mutant is metabolically stable in oocytes, consistent with increase in stability observed on phosphorylation of Ser^3 in native Mos [8]. As shown in Fig. 4 (left panel), [^{35}S]MPDP-Mos, which was synthesized in vitro, was not degraded

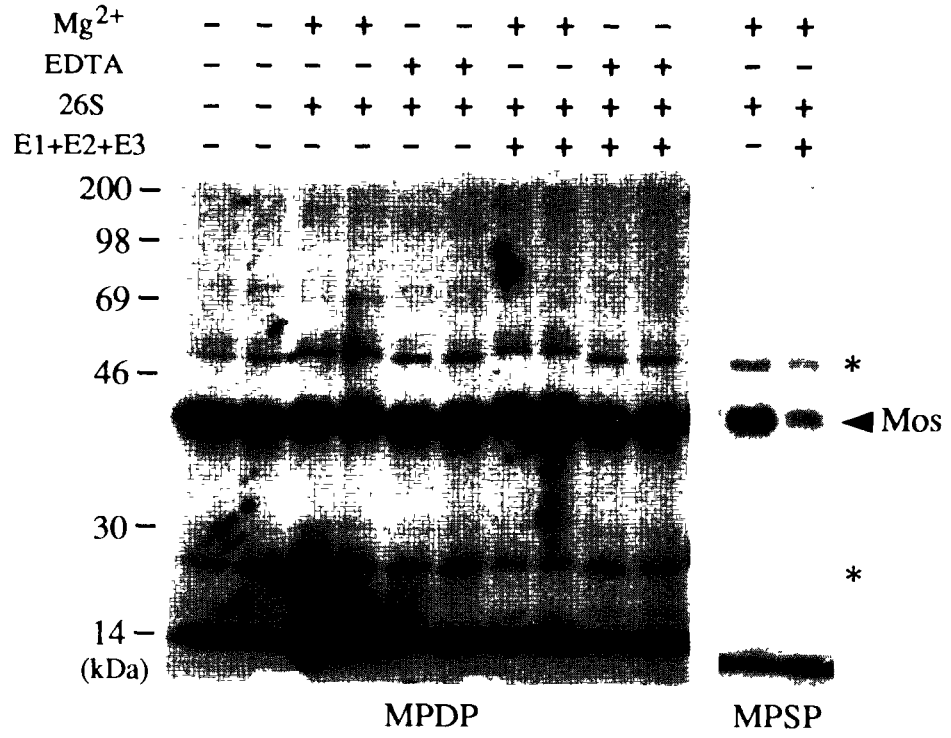

Fig. 4. Ubiquitination and degradation of mutant MPDP-Mos in which Ser^3 of native MPSP-Mos was replaced by Asp^3. The degradations of MPDP-Mos (left panel) and MPSP-Mos (right panel) were examined. The arrowhead shows the position of Mos. Note that no larger bands of the ubiquitinated Mos were detected. Symbols are as for Fig. 1.

appreciably by the 26S proteasome with or without ATP and/or the Ub-conjugation system. This was in marked contrast to the rapid degradation of the native MPSP-Mos in the presence of ATP and the Ub-conjugation system (Fig. 4, right panel and Fig. 2). On replacement of Ser^3 by Asp^3, Mos acquires a negative charge in the N-terminal region, which might result in loss of its recognition by E3 in the Ub system, as observed in a *Xenopus* oocyte system [8]. This possibility is supported by the finding that incubation of [^{35}S]MPDP-Mos with the Ub-ligation enzymes did not result in generation of ubiquitinated bands of larger size (Fig. 4, left panel), an observation that is consistent with results obtained with a reticulocyte extract [8]. These findings show that Mos is degraded ATP-dependently by the 26S proteasome in a Ub-dependent fashion.

4. DISCUSSION

Many investigators including our group have reported purification and characterization of the 26S proteasome complex containing a 20S proteasome and its catalysis of ATP-dependent degradation of proteins modified by ubiquitination [1,2,6,11]. But the natural substrates of this enzyme complex have not been identified. In the present study, we showed that Mos is a true substrate of the proteasome degraded by the Ub pathway (Fig. 2). Nishizawa et al. [8] reported that introduction of a negative charge into Ser^3 located in the N-terminal region of Mos by phosphorylation prevents its recognition by the Ub-ligated system, possibly by the E3 recognition protein. This result is consistent with our finding that a mutant MPDP-Mos with an artificially introduced negative charge was not a substrate of the Ub-dependent degradation system (Fig. 4). Thus, the unmodified Mos, which is an unstable form in *Xenopus* oocytes, is degraded by the 26S proteasome after its ubiquitination.

This degradation of [^{35}S]Mos was not much faster than the degradation of [^{125}I]lysozyme–Ub conjugates by the 26S proteasome [11]. There are several possible explanations for this finding. For example, another ATP-dependent protease distinct from the 26S proteasome may be involved in Mos degradation, or different Ub-ligation enzymes from those used in this study might be responsible for the ubiquitination of Mos. Alternatively, the [^{35}S]Mos used as a substrate might have been partly phosphorylated during its synthesis in a reticulocyte lysate. Furthermore, the characteristics of the degradation system of amphibian oocytes, such as the optimal temperature, may be somewhat different from those of the mammalian system.

There are two classes of the mammalian Ub-recognition protein E3 named E3α and E3β, which are distinguished by their selective degradations of Type I/II proteins with basic or bulky hydrophobic side chains and of Type III proteins with small uncharged residues at their N-termini, respectively [1]. The N-terminal residue of Mos is unique in being Pro, no other proteins with an N-terminal Pro residue having been reported to be ubiquitinated. It will be interesting to determine which type of E3 catalyzes the ubiquitination of Mos.

We have reported that ATP is required not only for ubiquitination, but also for subsequent degradation of ubiquitinated proteins [13]. However, recently we found that the 26S proteasome catalyzes ATP-dependent degradation of ornithine decarboxylase (ODC) without ubiquitination, when ODC is associated with an antizyme, an ODC inhibitory protein, induced by polyamines [14]. Therefore, the 26S proteasome recognizes not only the Ub moiety, as demonstrated by Mos degradation here, but also the antizyme complex and possibly other degradation signals. The 26S proteasome complex is thus an important enzyme for energy-dependent proteolysis in cells, and may be responsible for proliferation and differentiation of cells through selective removal of various regulatory proteins involved in cell-cycle progression.

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