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Ubiquitinated Proteasome Inhibitor Is a Component of the 26 S Proteasome Complex†

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ABSTRACT: Western blot analysis, using a polyclonal antibody to the 240-kDa endogenous inhibitor of the 20 S proteasome, revealed that the inhibitor is a component of the 26 S complex. Although isolated inhibitor displayed a single 40-kDa band on SDS–PAGE, the antibody detected a 55-kDa component in the 26 S proteasome complex. Ubiquitin polyclonal antibody recognized the same 55-kDa component but did not react with free 40-kDa inhibitor subunit. Addition of purified 40-kDa inhibitor to a ubiquitin ligating system also generated the 55-kDa species. In crude erythrocyte extracts, most of the inhibitor migrated at 55 kDa in the presence of ATP but shifted to 40 kDa in the absence of ATP, consistent with removal of ubiquitin. It is suggested that ubiquitination of the inhibitor may be involved in regulating assembly and/or activity of the 26 S proteasome complex.

Proteasomes are high molecular weight multicatalytic proteinases found in the cytosol and nucleus of many cells (Orlowski, 1990; Tanaka & Ichihara, 1990; Rivett, 1989; Hough et al., 1988). These multisubunit complexes have been implicated in the degradation of normal and modified proteins (Matthews et al., 1989), cell proliferation (Fujiwara et al., 1990), cancer (Kumatori et al., 1990), development (Klein, 1990), and antigen processing (Ortiz-Navarrette et al., 1991; Brown et al., 1991). 20 S proteasomes have a molecular mass of approximately 700 kDa and consist of a family of subunits (25–35 kDa) arranged in cylinders (Tanaka et al., 1988; Baumeister et al., 1988; Puhler et al., 1992). Several lines of evidence suggest that 20 S proteasomes can assemble with two other factors in the presence of ATP to form a larger (1500 kDa) 26 S proteasome complex (Peters et al., 1991; Ganoth et al., 1988; Eytan et al., 1989; Orino et al., 1991; Driscoll & Goldberg, 1990). However, it has also been claimed that certain 26 S preparations have little or no detectable proteasome subunits in the 25–35-kDa range (Kuehn et al., 1992). Although 20 S proteasomes are able to degrade or process certain proteins, the 26 S complex appears to be involved in ATP/ubiquitin-dependent proteolysis (Peters et al., 1991; Ganoth et al., 1988; Eytan et al., 1989; Orino et al., 1991; Driscoll & Goldberg, 1990; Hough et al., 1987; Waxman et al., 1987; Okada et al., 1991). Ubiquitin, a 76 amino acid polypeptide, can be conjugated to other proteins by an ATP-dependent pathway which leads to the formation of isopeptide bonds (Hershko & Ciechanover, 1992). It is generally thought that ubiquitination serves to "tag" proteins for recognition by the 26 S proteasome complex (Hershko & Ciechanover, 1992). However, ubiquitination may also serve other functions since a number of proteins which are ligated to ubiquitin are stable (Rechsteiner, 1987).

We now demonstrate that 40-kDa subunits of the 240-kDa proteasome inhibitor (Murakami & Etlinger, 1986) are associated with the 26 S proteasome complex from human erythrocytes. In addition, Western blot analysis, using antibodies to the inhibitor and ubiquitin, indicates that inhibitor contained within the 26 S complex is ubiquitinated, suggesting a role of this modification in regulating assembly and/or activity.

EXPERIMENTAL PROCEDURES

Preparation of 26 S Proteasome Complex and Inhibitor. 26 S proteasome complex was isolated by a procedure which was essentially as previously described (Hough et al., 1987). Human erythrocyte extract was adsorbed on DEAE-cellulose

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(DE52) and eluted with 0.5 M KCl to obtain "fraction II", which was then supplemented with 2 mM ATP, 5 mM $MgCl_2$, 1 mM DTT, 10 mM phosphocreatine, and 10 μg/mL of creatine kinase, followed by incubation at 37 °C for 1 h. The complex was precipitated twice with ammonium sulfate at 38% saturation and then chromatographed on a Sephacryl S300 column. Fractions which hydrolyzed Suc-Leu-Leu-Val-Tyr-AMC[1] in the presence of ATP were pooled and rechromatographed on a Bio-Gel HT column. The active fractions were pooled, concentrated, and subjected to glycerol density gradient centrifugation (10–40% v/v) at 100000*g* using a SW 28 rotor. The active 26 S fraction from a glycerol gradient was further purified by 4.5% nondenaturing PAGE as described previously (Li et al., 1991). After electrophoresis, the gel was incubated with a buffer containing 50 mM Tris-HCl, (pH 8.0), 2 mM ATP, 50 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, and 10% glycerol at 37 °C for 10 min. Suc-Leu-Leu-Val-Tyr-AMC (0.1 mM) in the same buffer was added and incubated at 37 °C for 1 h. Free AMC released by hydrolysis was visualized under ultraviolet irradiation. Parallel native gel slices containing peptidase activity were extracted in the above buffer and subjected to Western blot analysis.

Proteasome inhibitor, containing a single 40-kDa band after SDS–PAGE, was prepared as described previously (Murakami & Etlinger, 1986).

Western Blot Analysis. 26 S proteasome complex isolated by nondenaturing PAGE, and 40-kDa inhibitor was subjected to 12.5% SDS–PAGE and transferred to a nitrocellulose membrane as described (Weitman & Etlinger, 1992). Blots were incubated in blocking buffer (5% nonfat dry milk/0.02% Tween-20/PBS) for 2 h at room temperature. The blots were placed in a multichannel miniblotter (Immunetics) and incubated for 15 h at 4 °C with blocking buffer containing 40-kDa inhibitor-specific polyclonal antibody to be described in detail elsewhere (Guo, Li, and Etlinger, manuscript in preparation) or ubiquitin conjugate-specific polyclonal antibody (a gift provided by Dr. Victor Fried). Blots were washed with blocking buffer for 1 h with several changes and incubated with ^{125}I-labeled goat anti-rabbit Ig (ICN) diluted to 200000 cpm/mL for 1 h at room temperature. Following a wash as above, blots were dried and exposed to Kodak X-Omat film at –70 °C.

Ubiquitin Ligase System. The ubiquitin–protein ligase system (E1, E2, E3) was prepared as previously described (Hershko et al., 1989). A ubiquitin affinity column was eluted with 1 M KCl containing 50 mM Tris-HCl pH 7.2 (KCl eluate), 2 mM AMP, and 0.04 mM sodium pyrophosphate in the above Tris buffer (AMP-PPi eluate); 10 mM dithiothreitol in the same Tris buffer (DTT eluate); and 50 mM Tris-HCl, pH 9.0, containing 2 mM DTT (pH 9 eluate). The eluates were combined as indicated and mixed with purified 40-kDa inhibitor in a total reaction volume of 50 μL containing 2 mM ATP, 2 μg of ubiquitin (Sigma), 50 mM Tris-HCl (pH 7.2), 5 mM $MgCl_2$, and 2 mM DTT. Following incubation at 37 °C for 30 min, the reaction was terminated by the addition of SDS–PAGE sample buffer at 100 °C for 5 min and subjected to 12.5% SDS–PAGE.

RESULTS

26 S proteasome complex was isolated in the presence of ATP from human erythrocytes using a glycerol gradient as previously described (Hough et al., 1987). The preparation was subjected to native PAGE and visualized by detection of hydrolysis of a fluorogenic peptide (Figure 1). The active band was eluted from a parallel gel and analyzed by SDS–PAGE, which revealed many protein bands in the 25–120-kDa range after silver staining (Figure 2), similar to previous reports (Hough et al., 1987; Waxman et al., 1987). Western blot analysis was carried out using a specific antibody generated against the 240-kDa inhibitor. Inhibitor antibody recognized a single subunit within the 26 S complex migrating at approximately 55 kDa (Figure 2, lane 2), whereas isolated inhibitor contained only a single protein migrating at 40 kDa (Figure 2, lane 3).

To determine whether the apparent increased size of the protein in the 26 S complex resulted from conjugation of

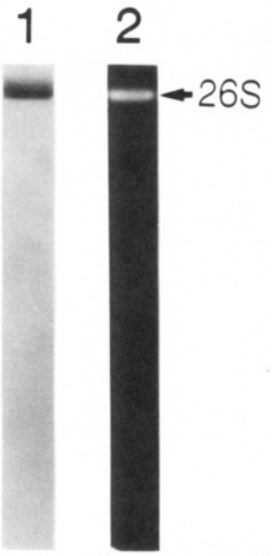

FIGURE 1: Nondenaturing PAGE analysis of active 26 S proteasome complex. Lane 1 was stained with Coomassie blue and lane 2 incubated with Suc-Leu-Leu-Val-Tyr-AMC and visualized with UV irradiation.

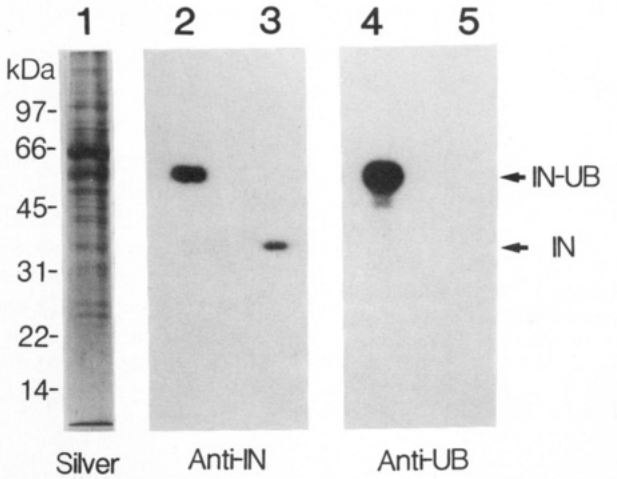

FIGURE 2: Western Blot analysis of 40-kDa inhibitor and ubiquitin within the 26 S proteasome complex purified by nondenaturing PAGE and subjected to 12.5% SDS–PAGE. Protein eluted from an active (unstained) 26 S complex band (Figure 1) was analyzed by 12.5% SDS–PAGE with silver staining (lane 1). Also shown are binding of inhibitor antibody (lane 2) and ubiquitin antibody (lane 4) to purified 26 S proteasome complex and binding of inhibitor antibody (lane 3) and ubiquitin antibody (lane 5) to isolated 40-kDa inhibitor. Molecular mass markers are indicated at the left: phosphorylase (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (22 kDa), and lysozyme (14 kDa). IN-UB indicates the putative inhibitor–ubiquitin conjugate (55 kDa), whereas IN indicates unmodified inhibitor (40 kDa).

[1] Abbreviations: DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; AMC, 7-amino-4-methylcoumarin.

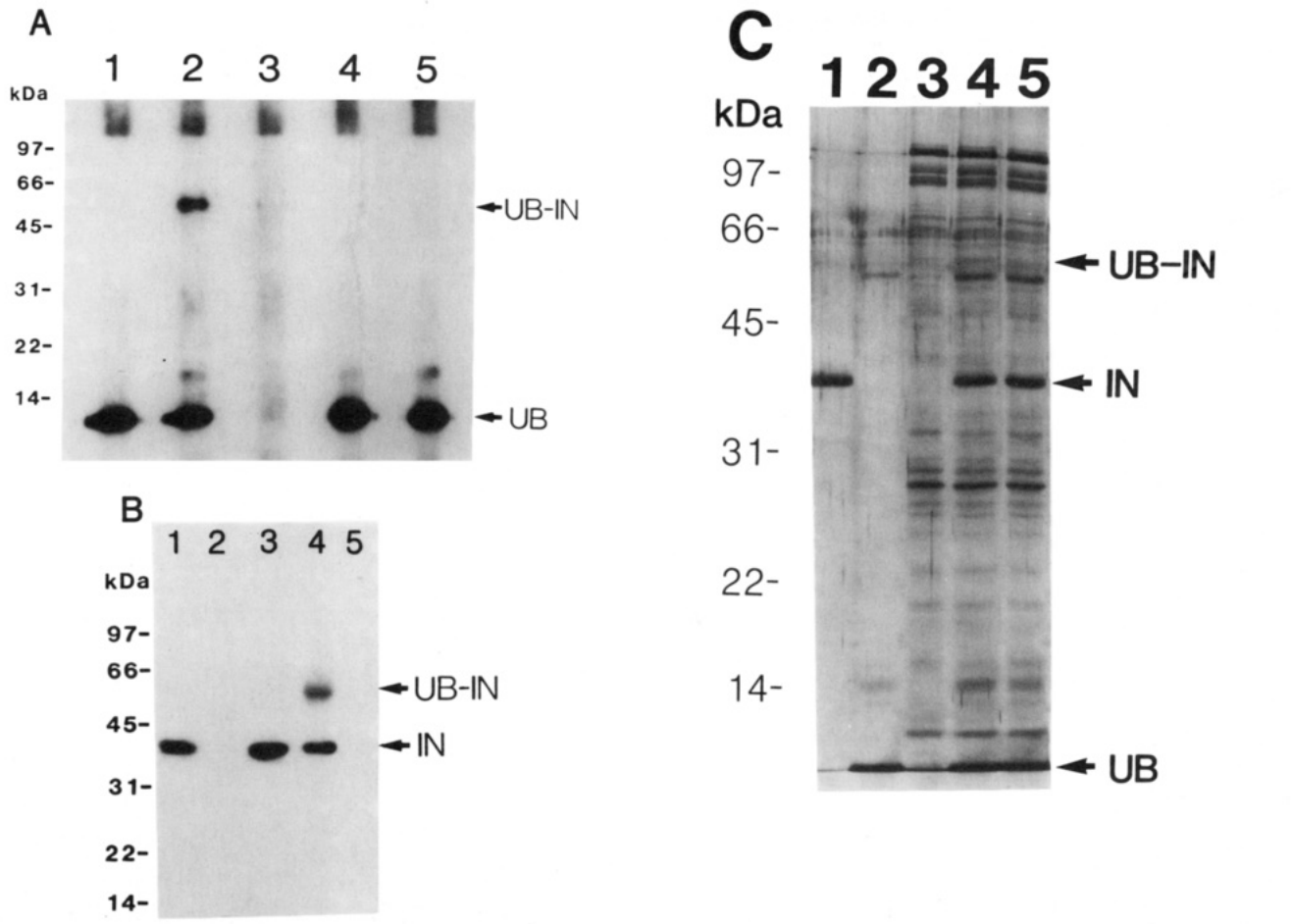

FIGURE 3: Western Blot analysis (A, B) and silver staining (C) of ubiquitin–40-kDa inhibitor conjugate generated in the presence of ubiquitin–protein ligase system (E_1, E_2, E_3). (A) Immunostaining with antiubiquitin: lane 1, ubiquitin; lane 2, ubiquitin, inhibitor, and E_1, E_2, and E_3 in the presence of ATP; lane 3, E_1, E_2, and E_3; lane 4, inhibitor, ubiquitin, and E_1, E_2, and E_3 in the absence of ATP; lane 5, ubiquitin and E_1, E_2, and E_3 in the presence of ATP. (B) Immunostaining with antiinhibitor: lane 1, inhibitor; lane 2, ubiquitin; lane 3, inhibitor, ubiquitin, and E_1, E_2, and E_3 in the absence of ATP; lane 4, inhibitor, ubiquitin, and E_1, E_2, and E_3 in the presence of ATP; lane 5, E_1, E_2, and E_3. (C) lane 1, inhibitor; lane 2, ubiquitin; lane 3, E_1, E_2, and E_3; lane 4, inhibitor, ubiquitin, and E_1, E_2, and E_3 ligase system in the presence of ATP; lane 5, same as lane 4 in the absence of ATP. Reaction mixtures contained the following amounts of ubiquitin affinity column eluates: KCl eluate (8.2 μg), AMP eluate (2.9 μg), DTT eluate (3.0 μg), and pH 9 eluate (3.2 μg). 2.5 μg of 40-kDa inhibitor was added as indicated. After incubation at 37 °C for 30 min, samples were subjected to 12.5% SDS–PAGE electrophoresis, followed by Western blot analysis or silver staining. Ubiquitin–40-kDa inhibitor conjugate is indicated by UB-IN. Free 40-kDa inhibitor is indicated as IN and free ubiquitin as UB.

ubiquitin to 40-kDa inhibitor, Western blot analysis was also performed with an antibody able to recognize ubiquitin which is free as well as conjugated to proteins (Figure 2). This antibody detected a protein migrating at precisely the same position (55 kDa) as that seen using the inhibitor antibody (Figure 2, lane 4). Despite the multitude of components visualized in the 26 S complex by silver staining, no other component was detected by this ubiquitin antibody (Figure 2). Evidence of a specific association with this complex is suggested since another proteasome inhibitor composed of 50-kDa subunits is not present in the 26 S complex and neither 40- nor 50-kDa components (unmodified or ubiquitinated) are found in the smaller 20 S proteasome (Li et al., 1991; Weitman & Etlinger, 1992; Li and Etlinger, unpublished observations).

To obtain additional evidence that the 40-kDa inhibitor is ubiquitinated, isolated 40-kDa inhibitor was incubated in the presence of ubiquitin and a ligase system containing E_1, E_2, and E_3 (Figure 3). These components have been shown previously to catalyze a pathway of reactions which result in the ATP-dependent activation of ubiquitin and subsequent conjugation of this polypeptide to proteins (Hershko et al., 1989). In the presence of inhibitor, a major ubiquitin conjugate was generated in an ATP-dependent manner as detected by Western blotting. This protein migrated with an apparent molecular mass of 55 kDa (Figure 3A, lane 2), indistinguishable from the ubiquitinated inhibitor detected within the 26 S proteasome (Figure 2). Although components within the ligase preparation may also form conjugates (e.g., the band migrating at 20 kDa in Figure 3A, lanes 2 and 5, and Li and Etlinger, unpublished observations), the prominent 55-kDa component was only seen when 40-kDa inhibitor was added to the complete ligase system (Figure 3A). Furthermore, a single band in the corresponding 55-kDa position was also detected using antibody to the inhibitor (Figure 3B, lane 4). Accumulation of a band at 55 kDa was also visualized directly with silver staining although it is not well resolved (Figure 3C, lane 4). In the absence of ATP, the ubiquitin antibody recognized only free ubiquitin (Figure 3A, lane 4).

To examine whether ubiquitinated inhibitor is stable in crude cell extracts, Western blot analysis was carried out on erythrocyte lysates incubated in the presence of ATP (Figure 4). In these preparations, binding of antibody to inhibitor indicated that most of the inhibitor migrates at 55 kDa, corresponding to the ubiquitinated species, whereas a smaller fraction (approximately 15%) comigrated with unmodified inhibitor at 40 kDa (Figure 4). When erythrocyte extract was incubated with hexokinase and glucose to deplete ATP,

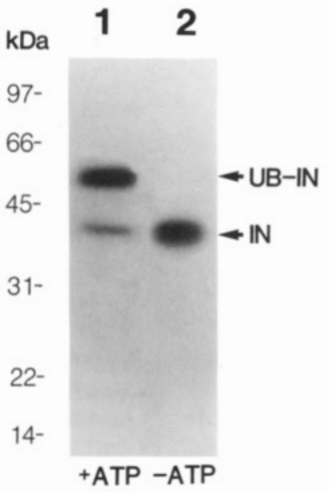

FIGURE 4: Western blot analysis of free 40-kDa inhibitor released from a conjugate in erythrocyte extract. Lane 1 displays inhibitor antibody binding in extracts incubated in the presence of ATP. Lane 2 shows inhibitor antibody binding in extracts incubated in the absence of ATP. Ub-IN indicates the ubiquitin–inhibitor conjugate, and IN indicates free inhibitor. Crude human erythrocyte extract was prepared as described previously (Speiser & Etlinger, 1983), and 40 μL was mixed with 50 mM Tris-HCl (pH 7.2), 5 mM $MgCl_2$, and 2 mM DTT in the presence of 2 mM ATP or an ATP trap consisting of 10 mM glucose and 0.44 unit of hexokinase in a total volume of 50 μL. Following incubation at 37 °C for 60 min, the reaction was stopped by the addition of SDS–PAGE sample buffer and heating at 100 °C for 5 min. An aliquot was separated by 12.5% SDS–PAGE. After electrophoresis, Western blot analysis was carried out using inhibitor polyclonal antibody as described in the legend to Figure 2.

all the detectable inhibitor appeared at 40 kDa. This shift was consistent with the net release of ubiquitin from inhibitor in the absence of ATP since the conjugation pathway, but not most of the ubiquitin isopeptidase activity, is ATP requiring (Hershko & Ciechanover, 1992).

DISCUSSION

The present studies have shown that the 40-kDa subunit of the 240-kDa proteasome inhibitor is a component of the 26 S proteasome complex. After completion of the present work, a study concluded that the inhibitor corresponds to the CF-2 component of the 26 S complex, consistent with the results presented here (Driscoll et al., 1992). In addition, we have now demonstrated that the inhibitor is present as a 55-kDa ubiquitin conjugate within the 26 S complex. Ubiquitination of a component of the proteolytic machinery suggests a novel function for this covalent modification distinct from the generally accepted role of ubiquitin as a substrate tagging signal. Our conclusion that the ubiquitinated 40-kDa inhibitor subunit is a component of the 26 S proteasome complex is supported by the identification of a single polypeptide within the 26 S proteasome, purified by native PAGE, which is recognized by specific antibodies both to purified 40-kDa inhibitor and to ubiquitin. Secondly, production of the corresponding component was carried out using a ubiquitin ligase system in a manner that required addition of 40-kDa inhibitor; conjugates which formed from endogenous proteins in the ligase system were present at much lower levels and with different mobility than the 55-kDa protein. Thirdly, conversion, in crude erythrocyte lysates, of the 55-kDa component to a 40-kDa protein is accompanied by loss of ubiquitin. Finally, it should be emphasized that only a single ubiquitinated species was detected in our 26 S preparations, making the comparison to the corresponding 55-kDa band in the crude lysate and ligase preparations unambiguous. Thus, we conclude that the 55-kDa protein is a ubiquitinated 40-kDa inhibitor within the 26 S complex.

The subunit structure of the 26 S proteasome complex is not well established. Although numerous components (25–120 kDa) are displayed after SDS–PAGE, there is considerable variability, particularly in the region of the 25–35-kDa bands which constitute the subunits of the small 20 S proteasome (see references cited above). Criteria for purity are based upon homogeneity by native PAGE or by electron microscopy which in both cases may not readily distinguish copurifying contaminants from components of the complex. Thus, unambiguous definition of the large 26 S proteasome complex must await future investigations. However, recent studies identifying the 240-kDa inhibitor as CF-2 argue that this factor is necessary for ATP-dependent proteolysis of ubiquitinated proteins (Driscoll et al., 1992). We have referred to the inhibitor by its ability to block activity of the small 20 S proteasome. However, the conclusions of the present study indicating the presence of this unique ubiquitinated protein in the 26 S preparation are valid irrespective of the presence or absence of specific subunits, e.g., 20 S proteasome components, in the 26 S complex.

As mentioned above, attachment of ubiquitin to proteins is thought to serve a "tagging" function leading to recognition and proteolysis of the conjugated proteins by the 26 S proteasome complex (Hough et al., 1988; Matthews et al., 1989; Hough et al., 1987; Waxman et al., 1987; Hershko & Ciechanover, 1992). However, in such cases, addition of branched polyubiquitin moieties appears necessary, often producing a family of large adducts (Chau et al., 1989). On the other hand, attachment of single ubiquitins to a protein is usually not associated with rapid degradation (Rechsteiner, 1987; Goldknopf & Busch, 1977; Siegelman et al., 1986). Our results are consistent with the latter possibility since attachment of one or two ubiquitins would best explain a single conjugate species with an apparent size of 55 kDa and because the inhibitor–ubiquitin conjugate is stable in cell lysates (Figure 4). Thus, we speculate that ubiquitination may affect assembly or activity of the 26 S complex.

Although all of the 40-kDa subunit is ubiquitinated in the 26 S complex, prior assembly into the complex is not required for ubiquitination since it was possible to generate these conjugates using a ligase system in the absence of proteasomes (Figure 3). On the other hand, some of the inhibitor in cell lysates exists in the free form (Figure 4). Possibly, ubiquitination influences the equilibrium between free and assembled components favoring the 26 S complex, in turn, determining the level of ATP/ubiquitin-dependent proteolytic activity.

In order for the 26 S complex to be capable of degrading an appropriate substrate molecule, inhibitor must be modulated so that proteasome activity is derepressed at the appropriate instant. This process could involve interaction of ubiquitin, ubiquitinated substrates, and/or ATP with inhibitor, proteasomes, or other undefined components of the complex which may be coupled to the protease (Speiser & Etlinger, 1983; Murakami & Etlinger, 1986; Etlinger et al., 1989; Driscoll et al., 1992). Ubiquitination of the 40-kDa component described in the present study might participate in such a process.

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