Inhibition of Ubiquitin-mediated Proteolysis by the Arabidopsis 26 S Protease Subunit S5a*

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A variety of protease inhibitors have been used to study ubiquitin-dependent proteolysis by the 26 S protease. However, these inhibitors lack complete specificity and thus affect ubiquitin-independent pathways as well. We recently identified an Arabidopsis protein, MBP1, that is homologous to subunit 5a (S5a) of the human 26 S protease complex. MBP1 and S5a bind mult ubiquitin chains with high affinity and presumably facilitate the recognition of ubiquitin conjugates by the 26 S protease. We show here that free MBP1 can be a potent inhibitor of ubiquitin-dependent proteolysis in several cell-free systems. When added to reticulocyte lysates or to Xenopus egg extracts, the plant protein effectively blocked the degradation of mult ubiquitinated lysozyme and cyclin B, respectively. MBP1 did not enhance the removal of ubiquitin from lysozyme or affect the ability of the 26 S complex to hydrolyze fluorescent peptides. These data suggest that the plant protein specifically interferes with the recognition of ubiquitin conjugates by the 26 S protease. Thus MBP1, human S5a, and their homologs should prove to be valuable reagents for investigating cellular events mediated by ubiquitin-dependent proteolysis.

A major proteolytic pathway in eukaryotes involves covalent attachment of ubiquitin to protein substrates. Although attachment of ubiquitin monomers may reversibly affect the structure or function of a protein (eg, histone H2A), in most cases conjugation of mult ubiquitin chains to a protein promotes its rapid degradation (1-6). For example, in Xenopus egg extracts, cyclin B accumulates during interphase and is degraded within a few minutes at metaphase (7, 8). Attachment of ubiquitin chains to cyclin B is a prerequisite for its rapid destruction (9-11).

The 26 S protease is the only enzyme identified so far that is able to degrade ubiquitinated proteins in an ATP-dependent reaction (12). This large, multisubunit enzyme is composed of a regulatory complex and the multicatalytic protease or 20 S proteasome. The regulatory complex is comprised of 15 or more different subunits (13) whereas the multicatalytic protease has at least 14 unique subunits (14). We previously identified a 50-kDa subunit of the regulatory complex that binds ubiquitin-lysozyme conjugates and free mult ubiquitin chains (15). In an effort to isolate a cDNA encoding this protein we discovered that two subunits of the regulatory complex have apparent molecular masses of 50 kDa (16). Two-dimensional polyacrylamide gel electrophoresis (PAGE)† revealed that the more acidic of these subunits (S5a) binds mult ubiquitin chains whereas the function of the more basic subunit (S5b) is currently unknown (16).

In addition to binding free mult ubiquitin chains, S5a exhibits markedly increased affinity for longer chains (15). These properties are consistent with the preference of the 26 S protease for substrates modified with long mult ubiquitin chains (17). Further evidence that S5a selects ubiquitin conjugates for proteolysis comes from binding studies using chains synthesized from mutant ubiquitins. Binding of these chains to S5a correlates with their ability to support ubiquitin-mediated proteolysis.2

We recently isolated an Arabidopsis thaliana cDNA that encodes a mult ubiquitin binding protein (MBP1) homologous to subunit 5a of the 26 S protease.3 Two-dimensional PAGE of the recombinant MBP1 demonstrated that it has a molecular mass and pl virtually identical to S5a from the human 26 S protease. Like S5a, MBP1 binds free mult ubiquitin chains and has a preference for longer chains. Because MBP1 has a high affinity for mult ubiquitin chains and large quantities of recombinant protein can be expressed in Escherichia coli, we examined the effects of adding it to in vitro systems competent for ubiquitin-mediated proteolysis. Here we show that the Arabidopsis MBP1 protein can be used as a specific inhibitor of ubiquitin-mediated proteolysis.

EXPERIMENTAL PROCEDURES

Source of Proteins—MBP1 was synthesized with an N-terminal histidine tag in E. coli BL21(DE3) cells following insertion of the MBP1 cDNA into the pET 15b vector (Novagen).4 Histidine-tagged MBP1 was purified from induced cells by metal-chelate chromatography according to the manufacturer’s recommendations. The histidine-tagged and non-tagged versions of MBP1 have been shown to exhibit virtually identical properties.5 An expression vector encoding cycA90 was provided by M. Glotzer and M. Kirschner (9). The cycA90 protein was expressed and purified as described previously (9). Full-length 35S-labeled Xenopus cyclin B2 was produced by in vitro translation in nuclease-treated Xenopus egg extract (18). Briefly, electrically activated Xenopus egg extract was prepared and successively treated with RNase A and RNase inhibitor before addition of TRNA, [35S]methionine (4.3 Ci/mmol), and cyclin B2 mRNA synthesized from an expression vector (18). After 40 min the extract was divided into aliquots and frozen at −80°C until use. High molecular weight mult ubiquitinated 125I-lysozyme and rab-

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1 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; S5a, subunit 5a of the 26 S protease; MBP1, mult ubiquitin binding protein; SLVY-MCA, succinyl-l-leucyl-l-leucyl-l-valyl-l-tyrosine-4-methylcoumaryl-7-amide.

2 Beal, R., Deveraux, Q., Gang, X., Rechsteiner, M., and Pickart, C. Proc. Natl. Acad. Sci. U. S. A., in press.

3 van Nocker, S., Deveraux, Q., Rechsteiner, M., and Vierstra, R. Proc. Natl. Acad. Sci. U. S. A., in press.

4 van Nocker, S., Deveraux, Q., Rechsteiner, M., and Vierstra, R. Proc. Natl. Acad. Sci. U. S. A., in press.
RESULTS

Inhibition of Ubiquitin-Lysozyme Conjugate Degradation by MBP1—Because Arabidopsis MBP1 binds mult ubiquitin chains with high affinity, we suspected that upon addition to in vitro extracts it would associate with ubiquitin conjugates, thereby preventing their recognition by the 26 S enzyme. To test this possibility, histidine-tagged MBP1 was synthesized in E. coli, purified by metal-chelate chromatography, and added to in vitro systems capable of degrading ubiquitin conjugates. Protoplysis of ubiquitin-125I-lysozyme conjugates in reticulocyte lysate was inhibited by 85% upon addition of 5 μg of MBP1 and virtually eliminated after adding 50 μg of the protein (Fig. 1A). Equivalent amounts (5 μg) of four other histidine-tagged proteins did not affect proteolysis (Fig. 1B). Among the proteins unable to inhibit degradation was S5b, a 26 S protease subunit of unknown function that was expressed and purified in the same way as MBP1 (16).

Persistence of Multiubiquitinated Lysozyme in the Presence of MBP1—Although we hypothesized that MBP1 would block proteolysis of ubiquitin-lysozyme conjugates by competitive binding, the recombinant protein could have stimulated detachment of mult ubiquitin chains from lysozyme by isopeptidases (23). Chain removal or shortening would convert the ubiquitin-lysozyme conjugates to undermodified or free lysozyme molecules, both of which are poor substrates for the 26 S protease (20). This possible mechanism of inhibition was addressed by electrophoresis of samples taken from the degradation assays. The autoradiogram in Fig. 2A shows that the high molecular weight ubiquitin-lysozyme conjugates were not converted to unmodified or undermodified forms of lysozyme but, in fact, were stabilized in the presence of MBP1. The initial loss of high molecular weight ubiquitin-lysozyme conjugates was more than 3-fold slower in the presence of the plant protein (Fig. 2B). Stabilization of conjugates in the presence of MBP1 indicates that its association with the ubiquitin-lysozyme conjugates prevented their recognition by isopeptidases as well as the 26 S protease.

Inhibition of Cyclin Degradation by MBP1—To determine whether MBP1 could inhibit proteolysis of a natural substrate of the ubiquitin pathway, we examined its effect on the degradation of cyclin B in Xenopus egg extracts. In embryonic cells, destruction of cyclins by ubiquitin-mediated proteolysis is a prerequisite for completion of mitosis (8, 9). The cyclin degradation phase of the cell cycle is normally brief, but addition of truncated cyclin missing the N-terminal 90 amino acids (cycl90) can arrest the egg extracts with the cyclin degradation system constitutively activated (9). In these extracts full-length cyclin B2 was rapidly degraded (Fig. 3, left panel). Increasing amounts of MBP1 progressively inhibited cyclin destruction with the addition of 330 μg/ml MBP1 resulting in a 5-fold increase in the half-life of cyclin (Fig. 3, right panel). Because the egg extracts are extremely concentrated (~30 mg/ml), the added MBP1 represents only a small percentage of total protein (~1%). This amount, however, appears sufficient to associate with endogenous ubiquitin conjugates of cyclin and effectively inhibit their recognition by the 26 S protease.

MBP1 Does Not Inhibit ATP-dependent Peptidase Activity by the 26 S Protease—As described above, stabilization of high molecular weight ubiquitin-lysozyme conjugates in the presence of MBP1 suggests that the mechanism of inhibition is by competition with subunit 5a in the 26 S protease. Nonetheless, more complicated mechanisms for MBP1-directed inhibition are possible. Since MBP1 or S5a must interact with other subunits in the 26 S complex, an excess of the free subunit might associate with the 26 S protease in a non-physiological manner and inactivate it. To address this possibility, we determined the effect of MBP1 on ATP-dependent peptide hydrolysis by the 26 S protease from HeLa cells. The partially purified HeLa enzyme was incubated with ubiquitin-lysozyme conjugates or fluorogenic peptides in the presence or absence of MBP1. As with reticulocyte lysate, the plant protein inhibited ATP-dependent degradation of mult ubiquitinated lysozyme (Fig. 4, left panel). However, it did not significantly affect ATP-dependent peptide hydrolysis by the 26 S protease (Fig. 4, right panel). Since ATP-dependent stimulation of peptide hydrolysis requires an intact 26 S protease (21), we conclude that...
Inhibition of Ubiquitin-mediated Proteolysis

MBP1 inhibits conjugate degradation without disrupting the 26 S complex or affecting its peptidase activities. This is consistent with the hypothesis that free MBP1 molecules bind mult ubiquitin chains and prevent their recognition by the 26 S protease. It remains possible, however, that MBP1 interacts with the 26 S protease and blocks the degradation of ubiquitated proteins without affecting the hydrolysis of small peptides, although we find this mechanism unlikely due to the high affinity of MBP1 for mult ubiquitin chains. Moreover, if MBP1 interferes directly with 26 S protease function, then addition of MBP1 to reticulocyte lysate or partially purified 26 S protease prior to the addition of mult ubiquitinated lysozyme might be expected to enhance inhibition by MBP1. However, we found that mixing MBP1 with the labeled conjugates and then adding the mixture to reticulocyte lysate or partially purified 26 S protease was the most effective way to inhibit degradation of mult ubiquitinated lysozyme.

DISCUSSION

Mutations in ubiquitin pathway enzymes and 26 S protease subunits have been used to investigate ubiquitin-mediated proteolysis in mammals, yeasts, and plants (3, 6, 24). For example, mutations in yeast 26 S protease subunits and ubiquitin-conjugating enzymes (E2s) have provided valuable information on natural substrates of the ubiquitin pathway and its involvement in cell cycle regulation (25–32). Similar genetic approaches in higher eukaryotic cells have been less successful. Mammalian cells containing a thermolabile ubiquitin-activating enzyme (E1) have been used to provide evidence for the involvement of ubiquitin-mediated proteolysis in a variety of cellular processes including autophagy, antigen presentation, and p53 degradation (33–36). However, these temperature-sensitive mammalian cell lines continue to transfer ubiquitin, albeit slowly, to cellular proteins at the restrictive temperature (37). In addition, high molecular weight ubiquitin conjugates accumulate at elevated temperatures, a result not readily explained by the inactivation of E1 (37). Therefore, considerable caution must be used in interpreting results from mammalian cell lines containing temperature-sensitive E1s (37, 38).

Chemical inhibitors have also been used to investigate the ubiquitin pathway. Early experiments by Haas and Rose (39) demonstrated that hemin inhibits the degradation, but not the formation, of ubiquitin conjugates. This finding provided the foundation for identification of the 26 S protease (19). It was subsequently found that hemin inhibits proteolysis by the mult catalytic protease or 20 S proteasome (20), the proteolytic core of the 26 S protease, and affects other aspects of the ubiquitin pathway, including conjugation and disassembly (40). Thus, the oxidized heme derivative cannot be used to selectively inhibit ubiquitin-mediated proteolysis by the 26 S protease. The same is true for peptide aldehydes, compounds recently used to implicate the proteasome pathway in the processing of antigens (41, 42), NF-kB (43), and the cyclin-dependent kinase inhibitor p27 (44). However, peptide aldehydes inhibit a wide variety of cysteine and serine proteases (45, 46), making them unsuitable as strict diagnostic reagents for the 20 S protease or the 20 S proteasome. More recently, it has been reported that amyloid β protein inhibits ubiquitin-dependent proteolysis in vitro (47). Because the amyloid protein inhibited cleavage of fluorogenic peptides by the 20 S proteasome as well, it is doubtful that amyloid β protein will permit clear distinction between ubiquitin-dependent and ubiquitin-independent proteolysis by the 26 S enzyme.

Here, we have shown that recombinant MBP1, human S5a, and their homologs may be ideal reagents for specifically inhibiting ubiquitin-mediated proteolysis. MBP1 inhibited degradation of ubiquitin-lysozyme conjugates and cyclin (Figs. 1 and 3) but had little effect on the ATP-dependent hydrolysis of fluorogenic peptides (Fig. 4). Although substantial levels of MBP1 were required to significantly inhibit degradation of cycin B, this is expected if inhibition requires that the added MBP1 molecules bind and sequester mult ubiquitin chains attached to protein substrates. For example, the steady state concentration of mult ubiquitin chains in mammalian cells is approximately 5–10 μM as estimated from immunological assays (48) and microinjection experiments (49). Therefore, it is not surprising that 8 μM MBP1 was needed to block cyclin B degradation in Xenopus egg extract. Moreover, previous estimates of total mult ubiquitin chains in our preparation of 125I-ubiquitin-lysozyme conjugates (15) allow us to calculate that in the experiment shown in Fig. 4 conjugate degradation was eliminated by adding one MBP1 molecule per ubiquitin tetramer. This ratio also supports the idea that sequestration of mult ubiquitin chains is the mechanism by which MBP1 inhibits conjugate degradation.

The ability of MBP1 to reduce markedly cyclin degradation in Xenopus egg extracts is encouraging for those who would use it as an inhibitor. Crushed Xenopus eggs yield a very concentrated extract that supports a variety of complicated biological
processes such as mitosis and nuclear envelope reconstitution. Inhibition of cyclin degradation in Xenopus egg extract makes it likely that ectopic expression of MBP1, human S5a, and their homologs will provide a useful method for inhibiting ubiquitin-dependent proteolysis within living cells.

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