Methylated Ubiquitin Inhibits Cyclin Degradation in Clam Embryo Extracts*

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A derivative of ubiquitin in which amino groups were blocked by reductive methylation was used to study the possible role of the ubiquitin pathway in the cell cycle-programed degradation of cyclin. It was shown previously that methylated ubiquitin can be efficiently ligated to protein substrates, but cannot form polyubiquitin chains. In the well-characterized ubiquitin-dependent proteolytic system from reticulocytes, it was found that rates of protein breakdown obtained with methylated ubiquitin are generally slower than those with ubiquitin; and thus, this derivative can be used, in some cases, as an inhibitor of ubiquitin-dependent protein degradation. The addition of methylated ubiquitin to a cell-free system from fertilized clam oocytes inhibited the degradation of both cyclins A and B. That this was due to specific interference with ubiquitin function was indicated by the observation that the supplementation of excess ubiquitin completely overcame the inhibitory action of methylated ubiquitin on cyclin degradation. These findings suggest that polyubiquitin chain formation is required for cyclin degradation.

Studies on the mode of action of a cell-free ATP-dependent proteolytic system from reticulocytes have delineated a pathway in which proteins are committed to degradation by their ligation to ubiquitin (Ub)(1) (for reviews, see Refs. 1-3). The main enzymatic steps of this pathway are as follows: (a) Initially, the COOH-terminal Gly residue of Ub is activated by the Ub-activating enzyme (E1). (b) Activated Ub is transferred to Ub carrier proteins (E2). (c) Ub-protein ligases (E3) bind suitable protein substrates and promote the transfer of Ub from E3 to ϵ-NH₂ groups of Lys residues of the protein. Two types of E3 have been isolated from reticulocytes, one of which (E₃₀) recognizes basic or hydrophobic NH₂-terminal amino acid residues in proteins (4), and the other (E₃β) that is specific for proteins that have other NH₂-terminal residues (5). Both E₃ enzymes ligate multiple Ub units to different ϵ-NH₂ groups of the substrate protein. In addition, polyubiquitin chains are bound to the substrate (6), in which one Ub is linked to Lys₄⁴ of another Ub (7). (d) Ub-conjugated proteins are degraded to small peptides by a large 26 S ATP-dependent protease complex (8).

Whereas the highly active reticulocyte system is excellent for studying the enzymatic reactions of the Ub pathway, it may not be suitable for the investigation of the problem of the regulation of the breakdown of specific proteins. This is because the reticulocyte is a highly specialized and relatively simple cell that lacks many processes present in nucleated cells. Trying to approach the problem of how the degradation of a specific protein is regulated, we have turned our attention to the case of the cell cycle-regulated degradation of cyclin (for reviews, see Refs. 9-11). Cyclin was discovered in fertilized sea urchin eggs as a protein that is synthesized during the interphase and is destroyed rapidly at the end of mitosis (12). The abrupt degradation of cyclin is apparently under tight regulation since cyclin is completely stable in prior stages of the cell cycle. It now seems that cyclins exist in all eukaryotes and that they have important roles in cell cycle control. Several types of cyclins exist, including A-type, B-type, and G1 cyclins that are synthesized and degraded at different phases of the cell cycle. All cyclins appear to be associated with p34⁰⁰-protein kinase and presumably act by regulating the activity of the kinase. Progress in this field has been greatly aided by the development of cell-free systems that reproduce programed events of the cell cycle, including cyclin degradation. In a cell-free system from Xenopus eggs, it has been shown that the synthesis of cyclin B is sufficient to drive the cell cycle (13), whereas cyclin degradation is required for exit from mitosis (14). In another cell-free system from fertilized oocytes of the surf clam Spisula solidissima, it has been shown that the timing of cyclin degradation is determined by the cell cycle stage of the enzyme system, and not by the cell cycle stage of the cyclin substrate (15).

The availability of cell-free systems that reproduce cyclin degradation has made it possible to approach the molecular mechanisms of its regulation. For example, if the programed degradation of cyclin is carried out by the Ub system, it is possible that a cyclin-specific E₃ is converted to an active form at the appropriate time of the cell cycle or that cyclin is rendered available to the action of a constitutively active Ub-protein ligase. For this reason, it had to be first determined whether or not cyclin degradation is carried out by the Ub system. In this study, we have examined this problem with the aid of a derivative of Ub that is ligated to the protein substrate, but cannot form polyubiquitin chains.
Experimental Procedures

Reducitively methylated ubiquitin (MeUb) was prepared from bovine erythrocyte ubiquitin (Sigma) by a previously described procedure (5). Free thiols and the amino groups were blocked, as determined by reaction with fluorescamine (16). Chicken egg white lysozyme, bovine β-lactoglobulin, HoloAx, OxdRNase, and S-protein were obtained from Sigma and were radioiodinated by the chloramine-T procedure (17). Fraction II (a crude extract depleted of rabbit reticulocytes by stepwise chromatography on DEAE-cellulose, as described (18).

Labeling of Clam Embryos and Preparation of Extracts—Extracts from fertilized Spisula oocytes were prepared as described by Luca and Ruderman (15), with some minor modifications. Clams (S. solidissima) were obtained in the months of June to August from the Marine Biological Laboratory (Woods Hole, MA) and were kept in seawater at 13 °C. Oocytes were obtained by dissection or by spontaneous shedding in separate containers, filtered through cheesecloth, and washed three to five times with 1-2 liters of artificial seawater. The cells were resuspended to 0.2% (v/v) in artificial seawater and kept at 16 °C with paddle stirring at 60 rpm. The oocytes were fertilized with 100-fold diluted sperm and were further cultured at 16 °C. Fertilization was >95% in all experiments, as determined by germinal vesicle breakdown. Progress in the cell cycle was monitored by fluorescent microscopy with 1 μg/ml of Hoechst 33342. At 20-25 min post-fertilization, eggs were collected on a 30-μm sieve and resuspended to 2% (v/v) in calcium-free seawater (19) at 16 °C.

Following the formation of the second polar body (55-65 min post-fertilization), 10 μCi/ml of [35S]methionine (1000-1200 Ci/mmole, ICN Radiochemicals) was added, and incubation was continued at 16 °C. Cells were collected at late interphase-early prophase (80-90 min post-fertilization) and were added to tubes containing ice-cold calcium-free seawater. The cells were washed three times with calcium-free seawater and twice with ice-cold Buffer T (see Ref. 15: 300 mM glycine, 120 mM potassium gluconate, 100 mM taurine, 100 mM Hepes, 40 mM NaCl, 2.5 mM MgCl2, adjusted to pH 7.2 with KOH). To the final cell pellet was added 0.4 volume of Buffer T, and embryos were homogenized with 10-15 strokes of a tight-fitting glass homogenizer (Kontes type B) at 0 °C. Cell breakage was essentially complete, as judged by microscopic observation. Homogenates were centrifuged twice at 13,000 rpm for 5 min, and the subsequent supernatants were frozen in liquid nitrogen and stored at -70 °C. Supernatants were then redissolved in 1 ml of electrophoresis sample buffer and subjected to electrophoresis on SDS-10% polyacrylamide gels.

RESULTS AND DISCUSSION

Influence of Reducitively Methylated Ubiquitin on Protein Breakdown in Extracts of Reticulocytes—In this study, we have used a derivative of Ub in which the amino groups had been blocked by reductive methylation (MeUb) to examine the possible role of the ubiquitin system in cyclin degradation in clam oocytes. Its use as a specific inhibitor was first investigated in the well-characterized system from reticulocytes. We had shown previously that MeUb is efficiently ligated to protein substrates, but cannot form polyubiquitin chains (6). The rate of degradation of lysozyme with MeUb was almost one-half of that obtained with Ub (6), indicating that the formation of polyubiquitin chains accelerates the rate of protein breakdown, but is not absolutely required for this process. By contrast, Chau et al. (7) have reported that a mutant Ub that contains a Lys48→Arg substitution did not effectively replace native Ub in the degradation of derivatives of β-galactosidase by reticulocyte extracts. A possible reason for this difference is that the formation of polyubiquitin chains may be more essential for the degradation of some proteins than others. In the experiment shown in Table I, the effectiveness of MeUb in stimulating the degradation of various proteins in reticulocyte extracts was compared with that of Ub. In all cases, rates of protein degradation were significantly stimulated by MeUb, but the extent of stimulation was markedly different for various protein substrates. Thus, whereas with lysozyme, β-lactoglobulin, and S-protein, rates of degradation with MeUb were 30-40% of those obtained with Ub, HoloAx and OxdRNase were degraded with MeUb at <10% of the rates obtained with Ub. There is no obvious relationship between the effectiveness of MeUb and the type of ubiquitin ligase that acts on the protein substrate since S-protein is a specific substrate for E6-β, whereas all other proteins are substrates of E 6-α (4).

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down. This expectation was verified in the experiment shown in Fig. 1, in which the effects of different concentrations of MeUb on the rate of degradation of $^{125}$I-HoLA were tested in the presence of two concentrations of Ub. At the lower concentration of Ub (1 $\mu M$), increasing concentrations of MeUb strongly inhibited the breakdown of $^{125}$I-HoLA, whereas at the higher concentration of Ub (10 $\mu M$), higher levels of MeUb were required for effective inhibition. The concentrations of MeUb required for 50% inhibition were 5–8-fold higher than the corresponding concentrations of Ub (~8 $\mu M$ MeUb at 1 $\mu M$ Ub and 50 $\mu M$ MeUb at 10 $\mu M$ Ub). These data indicate a competition situation in which MeUb (or a derivative of MeUb) has a lower affinity for the affected enzyme than Ub.

It is interesting to note that with purified $E_1 + E_2 + E_3$, the rate of conjugation of MeUb to HoLA is comparable to that of Ub. On the other hand, in unfractionated extracts (such as used for the assay of protein breakdown), levels of MeUb-HoLA conjugates are relatively low; and the low rate of degradation of HoLA to acid-soluble material in the presence of MeUb is not accompanied by a corresponding accumulation of MeUb-HoLA conjugates (data not shown). It is possible that MeUb-HoLA conjugates are poor substrates for the 26 S protease, but they do not accumulate in unfractionated extracts because they are subject to the action of isopeptidases that release free protein and Ub from conjugates that are rejected from the degradation machinery.

**MeUb Inhibits Cyclin Degradation in Extracts of Spisula Oocytes**—The effect of MeUb on cyclin degradation was examined in the cell-free system from clam oocytes established by Luca and Ruderman (15). Fertilized oocytes of the clam *S. solidissima* were labeled with $[^{35}]$S-methionine, and extracts were prepared at the beginning of the first mitosis. Upon further incubation of extracts in the presence of ATP, specific degradation of cyclins A and B could be observed (Fig. 2A). Under the conditions employed, cyclin A was degraded after a lag period of 15–20 min, and cyclin B after 25–40 min. We have confirmed the main findings of Luca and Ruderman (15), which indicate that the cell-free system faithfully reproduces cyclin degradation in intact cells. (a) Only cyclins A and B are degraded specifically, whereas all other labeled proteins, such as a major labeled band that is the small subunit of ribonucleotide reductase (23), are apparently stable; (b) cyclin degradation requires MgATP; and (c) the timing of the degradation of cyclins A and B in vitro is determined by the stage of the cell cycle at which extracts were made (15).

The possible involvement of the ubiquitin system in cyclin degradation was tested in the following incubations. (a) The addition of Ub to oocyte extracts slightly stimulated cyclin degradation (Fig. 2B). For example, a more complete disappearance of cyclin B can be seen after 30–40 min of incubation with added Ub compared with the control incubation. A possible explanation is that levels of endogenous Ub in such extracts are slightly below optimal. (b) The addition of MeUb to the cell-free system markedly inhibited cyclin degradation (Fig. 2C). This was more noticeable in the case of cyclin B, whereas inhibition of the breakdown of cyclin A by MeUb was less pronounced. Inhibition of the degradation of cyclin A by MeUb could be more easily seen following the isolation of p34$^{\text{cdc2}}$-cyclin complexes with p13-Sepharose (Fig. 3). The degradation of both cyclins B and A was slowed down, but

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**Fig. 1. Competition between MeUb and Ub on protein breakdown in reticulocyte extracts.** Experimental conditions were as described for Table I. The degradation of $^{125}$I-HoLA was determined in the presence of 1 (○) or 10 (●) $\mu M$ Ub and the indicated concentrations of MeUb.

**Fig. 2. Inhibition by MeUb of cyclin degradation in clam embryo extracts and its reversal by Ub.** $[^{35}]$S-Methionine-labeled extracts of fertilized clam oocytes were incubated as described under "Experimental Procedures" with the following additions: A, none; B, 50 $\mu M$ Ub; C, 50 $\mu M$ MeUb; D, 50 $\mu M$ MeUb and 50 $\mu M$ Ub. Numbers at the top indicate the time of incubation (in minutes) at 16 °C. The positions of cyclin A (A), cyclin B (B), and ribonucleotide reductase (RR) are indicated on the left.

**Fig. 3. Isolation of cyclins A and B from incubated extracts by p13-Sepharose.** Extracts of clam oocytes labeled with $[^{35}]$S-methionine were incubated as described under "Experimental Procedures" without additions (CONTROL) or with 50 $\mu M$ MeUb. p34$^{\text{cdc2}}$-cyclin complexes were isolated with p13-Sepharose as described under "Experimental Procedures." Numbers at the top indicate the time of incubation (in minutes) at 16 °C. The positions of molecular mass marker proteins (in kilodaltons) are shown on the right. The positions of cyclins A (A) and B (B) are shown on the left. p34$^{\text{cdc2}}$ is not labeled and is not seen on the fluorogram. A prominent labeled protein associated with p34$^{\text{cdc2}}$ of somewhat higher apparent molecular size than cyclin A was previously observed by others (25).
not arrested completely, in accordance with observations in reticulocyte extracts (Fig. 1 and Table 1). To examine whether the inhibition of cyclin degradation by MeUb is indeed due to specific competition with Ub, we have tested the influence of the combined addition of Ub and MeUb and found that high levels of added Ub completely overcome the inhibitory action of MeUb on cyclin degradation (Fig. 2D, compare cyclin B at 30–60 min with Fig. 2C).

The above data indicate that the formation of polyubiquitin chains is required for cyclin degradation. It seems reasonable to assume that polyubiquitination of cyclin takes place, but we have no direct evidence for this conclusion. We searched for cyclin-Ub conjugates as higher molecular weight bands that should be formed during the period of cyclin degradation. We could not detect such bands with p34cdc2-cyclin complexes (Fig. 3) or following immunoprecipitation with an antibody specific for conjugated ubiquitin isolated with p3-Sepharose (Fig. 4). It is possible that cyclin-Ub conjugates are rapidly degraded in Spisula oocytes by a Ub-specific protease complex; and thus, their levels are below our detection limits. It is also possible, however, that the role of Ub in cyclin degradation is indirect. For example, polyubiquitin-dependent degradation of another protein, such as an inhibitor of a cyclin-specific protease, or of a protein that has to be degraded to allow progression of the cell cycle to the stage of cyclin degradation may be involved. In addition, MeUb may act indirectly, such as by competitive inhibition of a novel Ub ligase that acts on cyclin.

While this work was in progress, Kirschner and co-workers (24) reported that Ub is ligated to bacterially expressed derivatives of cyclin in extracts of Xenopus eggs arrested in mitosis. In their work, a specific inhibitor of the ubiquitin system was not used; and therefore, the authors could not rule out the possibility that cyclin-Ub conjugates are side products rather than intermediates of cyclin degradation (24). Taken together with our findings, the cumulative evidence indicates that the Ub system carries out the cell cycle-regulated degradation of cyclin. This raises experimentally approachable questions about the mechanisms of the regulation of cyclin degradation.

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REFERENCES