Vol. 162, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 14, 1989 Pages 89-94

Inhibition of Ubiquitin-Dependent Proteolysis by des-Gly-Gly-Ubiquitin: Implications for the Mechanism of Polyubiquitin Synthesis

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Received May 1, 1989

Cleavage of the two carboxyl-terminal glycine residues from native ubiquitin yields the proteolysis-incompetent derivative des-Gly-Gly-ubiquitin. We report here that this derivative inhibits the ATP-dependent degradation of casein and is multi-ubiquitinated but not degraded by reticulocyte lysates. Inhibition of proteolysis diminished with increasing concentration of native ubiquitin, but was not reduced by increased casein concentration. Cleavage of the last four residues from ubiquitin yielded a derivative that was a weaker inhibitor of proteolysis and a poorer substrate for ubiquitination. These results suggest that the conjugation of ubiquitin to ubiquitin during polyubiquitin synthesis involves a specific conjugation system that recognizes ubiquitin and some of its derivatives, but not general proteolysis substrates, as ubiquitin acceptors.

Conjugation of the alpha-carboxyl group of ubiquitin (Ub) to protein amino groups serves as a tag that appears to permit recognition of the conjugated protein by ubiquitin-dependent proteases (1). Proteins to which multiple ubiquitins are attached in the form of polyubiquitin are the most rapidly degraded (2,3). Polyubiquitin represents the self-conjugate of ubiquitin; i.e., covalent ubiquitin oligomers formed by the successive conjugation of the alpha-carboxyl of one ubiquitin to a lysine amino group of another (4). Recent evidence identifies the involved lysine as Lys-48 (2); however, the mechanism by which proteins are polyubiquitinated is not well understood.

Activation of the alpha-carboxyl group of ubiquitin for conjugation to proteins requires that its terminal Gly-Gly sequence be intact (5,6). The des-Gly-Gly protein has been reported to be inactive in facilitating proteolysis and non-inhibitory (6). However, in the present paper, we report that, at relatively high concentrations, des-Gly-Gly-ubiquitin inhibits ATP- (and ubiquitin-) dependent proteolysis in reticulocyte lysates. The mechanism by which it acts provides insights into pathways of protein polyubiquitination.

Materials and Methods

Native ubiquitin was purchased from Sigma and its purity confirmed by amino acid analysis and electrophoresis in both native and denaturing gel systems. Cleavage of the last two residues (-Gly-Gly) and of the last four residues (-Leu-Arg-Gly-Gly) was accomplished by digestion with 0.5% by weight trypsin (Worthington, pre-treated with L-[1-Tosyl-amido-2-phenyl] ethyl chloromethyl ketone) for 1 hr at room temperature at pH 8.7 in 0.2M $NaHCO_3$, 0.18M NaCl.

Abbreviations: Ub, ubiquitin; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; CD, circular dichroism.

0006-291X/89 $1.50

Under these conditions, cleavage of the terminal -Gly-Gly sequence (representing cleavage after Arg-74) is essentially complete and partial cleavage at Arg-72 also occurs. The modified ubiquitins were separated from the other reactants on Sephadex G-50 in 0.2M acetic acid and lyophilized. Fractionation into the des-Gly-Gly and des-Leu-Arg-Gly-Gly products was achieved by cation-exchange HPLC and the purity and identity of each product further established by amino acid analysis, mass spectrometry and electrophoresis. Iodination of ubiquitin and modified ubiquitins with Na ^{125}I was performed as previously described (7).

Rabbit reticulocyte lysates were prepared as previously described (7) with the exception that the reticulocytes were purchased from Pel-Freez Biologicals. Proteolysis assays, monitoring the rate of degradation of ^{125}I-labelled casein (or iodinated ubiquitin derivatives) and autoradiography in the presence of ^{125}I-labelled ubiquitin derivatives were also performed as described earlier (7). In view of the low molecular weights of the ubiquitin derivatives, controls were run for proteolysis studies to ensure that the undegraded proteins were precipitated by TCA.

Results

Table I reports the effects of addition of native and modified ubiquitin on the rate of ATP-dependent proteolysis of ^{125}I-labelled casein in reticulocyte lysates. Native ubiquitin is not significantly inhibitory at concentrations as high as 5 mg/ml (in excess of the endogenous lysate concentration). However, at concentrations of 2.5 and 5 mg/ml des-Gly-Gly-ubiquitin, ATP-dependent proteolysis is inhibited by 70% and 85% respectively. Inhibition by des-Gly-Gly-Ub is overcome by the addition of native ubiquitin, but is not diminished by a six-fold increase in casein concentration (Table I) .Ubiquitin from which the last four residues have been excised (des-Leu-Arg-Gly-Gly-Ub) is a significantly weaker inhibitor than the des-Gly-Gly-protein (Table I).

Table I. Effects of native and modified ubiquitins on the ATP-dependent proteolysis of casein

Casein concentration	Addition	% Inhibition
0.2mg/ml	None	0
"	Native Ub, 5 mg/ml	10
"	Des-Gly-Gly-Ub, 2.5 mg/ml	70
"	Des-Gly-Gly-Ub, 5 mg/ml	85
"	Des-Leu-Arg-Gly-Gly-Ub, 2.5 mg/ml	30
0.2 mg/ml	Des-Gly-Gly-Ub, 2.5 mg/ml	70
"	" " + Native Ub, 1mg/ml	33
"	" " + Native Ub, 2.5 mg/ml	10
0.3 mg/ml	Des-Gly-Gly-Ub, 1 mg/ml	50
1.3 mg/ml	" "	66
0.2 mg/ml	Des-Gly-Gly-Ub, 2.5 mg/ml	70
1.3 mg/ml	" "	71

Reticulocyte lysate samples containing ^{125}I-labelled casein and other additions at the concentrations listed below were incubated for 60 minutes at pH 8 (see legend, Fig. 1 for conditions) in the absence and in the presence of 5 mM ATP. The rate of ATP-dependent casein degradation was calculated from the effect of ATP on the time-dependent increase in TCA-soluble radioactivity (7). The percentage inhibition of proteolysis is calculated from the relative rates of the ATP-dependent component of casein degradation in the absence and presence of the additions noted. None of the additions had significant effects on the ATP-independent component of casein degradation.

To probe the mechanism of inhibition by des-Gly-Gly-ubiquitin, we examined its effects on ubiquitin conjugation in reticulocyte lysates. In the presence of ATP, ^{125}I-ubiquitin is conjugated to endogenous proteins to give higher molecular weight protein conjugates, as monitored on SDS gels by autoradiography (Fig. 1). In the presence of des-Gly-Gly-ubiquitin, several protein bands, which are otherwise very weak, are significantly intensified relative to other components. Examination of these bands in 10% gels (Fig. 1A) and in 12.5 or 15% gels (not shown) indicates M_r values for the principal intensified components of approximately 16,000, 24,000 and 32,000 respectively, the 24,000 M_r component occasionally being resolvable into two components. The 16,000 M_r component is clearly resolved only in the less porous gels or in 10% gels using short exposure periods or low levels of radio-labelled ubiquitin (Fig. 1A, lanes 5 and 6). These bands are separated from each other by approximately the molecular weight of ubiquitin suggesting that they represent different degrees of ubiquitination of the same protein, specifically des-Gly-Gly-ubiquitin. Intensification of these bands relative to other components occurs to a lesser extent in the presence of des-Leu-Arg-Gly-Gly-ubiquitin (Fig. 1B).

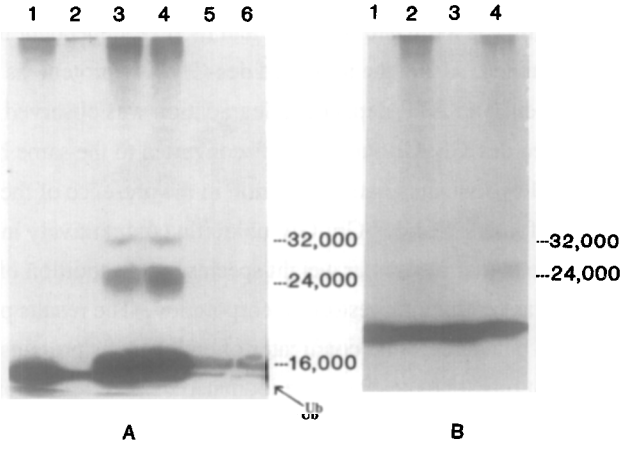

Figure 1. Autoradiographs of the effects of des-Gly-Gly-ubiquitin and des-Leu-Arg-Gly-Gly-ubiquitin on the conjugation of ^{125}I-labelled native ubiquitin in reticulocyte lysates. Samples were heated with SDS and 2-mercaptoethanol after incubation and electrophoresed on 10% polyacrylamide-SDS gels at pH 8.3. Apparent M_r values of relevant species are indicated on the right and were calculated from the migration of unlabelled Coomassie Blue-stained molecular weight standards run on the same gels. The position of unconjugated Ub is shown at the bottom of the gel, while the highest molecular weight conjugates of endogenous proteins appear as radioactivity at the top of the gels. Gel A: Each sample contained 0.0625 ml lysate in a total volume of 0.125 ml adjusted to final concentrations of 0.125 M Tris buffer, pH 8, 4 mM KCl, 4 mM $MgCl_2$, 5 mM dithiothreitol, 5 mM ATP and the additions noted. Samples were incubated for 60 min at 37° C. Lane 1, plus 0.2 mg/ml ^{125}I-Ub; lane 2, plus 0.02 mg/ml ^{125}I-Ub; lane 3, plus 0.2 mg/ml ^{125}I-Ub and 0.5 mg/ml unlabelled des-Gly-Gly-Ub; lane 4, plus 0.2 mg/ml ^{125}I-Ub and 2.5 mg/ml unlabelled des-Gly-Gly-Ub; lane 5, plus 0.02 mg/ml ^{125}I-Ub and 0.5 mg/ml unlabelled des-Gly-Gly-Ub; lane 6, plus 0.02 mg/ml ^{125}I-Ub and 2.5 mg/ml unlabelled des-Gly-Gly-Ub. Gel B: Samples were analogous to those in Gel A with the exception that ATP was only present where indicated. Samples were incubated for 90 min. Lane 1, plus 0.04 mg/ml ^{125}I-Ub, no ATP; lane 2, plus 0.04 mg/ml ^{125}I-Ub and 5 mM ATP; lane 3, plus 0.04 mg/ml ^{125}I-Ub and 2mg/ml des-Leu-Arg-Gly-Gly-Ub, no ATP; lane 4, plus 0.04 mg/ml ^{125}I-Ub, 2 mg/ml des-Leu-Arg-Gly-Gly-Ub and 5 mM ATP. (Note: To take into account the different concentrations of ^{125}I-ubiquitin on the two gels, the intensity of individual bands should be considered relative to that of other labelled components.)

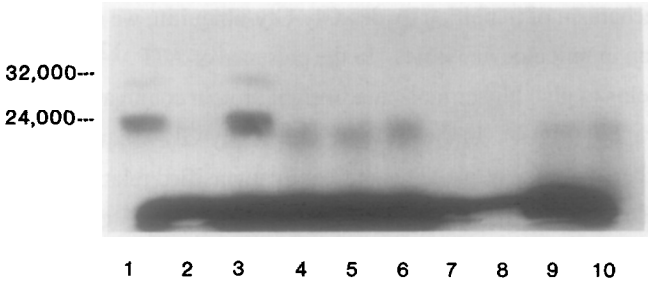

Figure 2. Autoradiographs of the incorporation of ^{125}I-labelled des-Gly-Gly-ubiquitin and - des-Leu-Arg-Gly-Gly-ubiquitin into higher molecular weight species in the presence of reticulocyte lysates. Incubation conditions were analogous to those in Figure 1, Gel A, with 5 mM ATP in all samples. SDS gel conditions were also the same as in Figure 1. Lane 1, plus 0.2 mg/ml ^{125}I-des-Gly-Gly-Ub, 90 min incubation; lane 2, same as lane 1 plus 1 mg/ml unlabelled des-Gly-Gly-Ub; lane 3, same as lane 2 plus 0.2 mg/ml unlabelled native Ub; lane 4, plus ^{125}I-des-Leu-Arg-Gly-Gly-Ub, 90 min incubation; lane 5, same as lane 4 plus 1 mg/ml unlabelled des-Leu-Arg-Gly-Gly-Ub; lane 6, same as lane 5 plus 0.2 mg/ml unlabelled native Ub; lane 7, same as lane 2, 0 min incubation; lane 8, same as lane 3, 0 min incubation; lane 9, same as lane 5, 0 min incubation; lane 10, same as lane 6, 0 min incubation. Only the lower half of the gel is shown; the upper half contains no radioactivity. Apparent molecular weights are calculated as in Figure 1.

To test the possibility that the ubiquitinated protein was des-Gly-Gly-ubiquitin , HPLC-purified des-Gly-Gly-ubiquitin was iodinated with ^{125}I and its ATP-dependent behavior in reticulocyte lysates was examined. Using the iodinated des-Gly-Gly protein as a proteolysis substrate (as above with casein), no ATP-dependent degradation was observed. Figure 2, however, shows that iodinated des-Gly-Gly-ubiquitin is converted to the same higher molecular weight species as that formed by iodinated native ubiquitin in the presence of the unlabelled des-Gly-Gly-protein. Addition of unlabelled des-Gly-Gly-ubiquitin competitively inhibits incorporation of label into the higher molecular weight species, while addition of a low concentration of unlabelled native ubiquitin restores incorporation. The results preclude the possibility that the higher M_r bands represent conjugates of endogenous proteins with traces of iodinated native ubiquitin that might be present in the iodinated des-Gly-Gly- ubiquitin preparation. They confirm instead that iodinated des-Gly-Gly-ubiquitin itself forms higher molecular weight species. Since the alpha-carboxyl group of the des-Gly-Gly-protein is not anticipated to be activatable (5), conjugation is proposed to occur by the attachment of native ubiquitin to one or more amino groups of the des-Gly-Gly-protein and the potential attachment of additional native ubiquitins to the first conjugated native ubiquitin. The enhancement of incorporation of labelled des-Gly-Gly-protein into higher molecular weight species by the addition of a low concentration of native ubiquitin (Fig. 2, lane 3) suggests that the concentration of native ubiquitin is limiting for this reaction.

Identical conjugation studies were performed with iodinated des-Leu-Arg-Gly-Gly-ubiquitin (Fig. 2). These studies were complicated by the fact that iodination of this derivative was associated with formation of a low but demonstrable level of oligomeric species, presumably reflecting oxidative covalent tyrosine-tyrosine crosslinking (8) during the iodination process. Nevertheless, the results demonstrate very little time-dependent incorporation of label from des-Leu-Arg-Gly-Gly-Ub into higher molecular weight species under the conditions used. Additionally, no ATP-dependent degradation of this derivative was observed.

Discussion

The mechanism by which des-Gly-Gly ubiquitin inhibits ATP-dependent proteolysis involves a step subsequent to ubiquitin activation. Studies elsewhere indicate that des-Gly-Gly-ubiquitin does not effectively compete with native ubiquitin for binding to activating enzyme (5). Moreover, the present data indicate that the ability to conjugate ubiquitin, which is critically dependent on ubiquitin activation, is not impaired in the presence of des-Gly-Gly -Ub.

We consider it relevant that des-Gly-Gly -ubiquitin is both a more effective inhibitor than des-Leu-Arg-Gly-Gly-ubiquitin and is also more readily ubiquitinated. The results suggest a linkage between the ubiquitination and the inhibition of protein degradation. Most significantly, the discrimination between the two ubiquitin derivatives by ubiquitin conjugating systems argues that there is a conjugation system specific for the attachment of ubiquitin to ubiquitin and that this system recognizes des-Gly-Gly -Ub more efficiently than it recognizes des-Leu-Arg-Gly-Gly -Ub. The normal conjugating systems that couple ubiquitin to proteolysis substrates must recognize a broad range of substrates, which appear to bind to conjugation factor E3 (9). While there are important factors that potentially permit discrimination among substrates at the conjugation step such as the identity of the amino-terminus (10), certain internal sequences (11) and overall conformation (12), the differences in conjugation between the two modified ubiquitin derivatives are not explained by these rules and neither appears to be degraded. Even the average conformation of the two proteins is the same as determined by CD (data not shown). Additionally, increasing casein concentration did not diminish inhibition by the des-Gly-Gly-protein, indicating that casein and des-Gly-Gly-ubiquitin do not compete for conjugation factor E3. Accordingly, we propose that the self-conjugation of ubiquitin to give polyubiquitin partially utilizes a system separate from that involved in the attachment of ubiquitin to proteolysis substrates.. In view of the fact that polyubiquitin formation appears to involve conjugation of a specific ubiquitin lysine side-chain (2), the existence of a separate and specific self-conjugating system seems reasonable if not essential. Whether this system involves a subset of the E2 conjugation factors that are recognized to carry ubiquitin (13) or yet another factor remains to be investigated.

There are three general mechanisms by which des-Gly-Gly ubiquitin might interfere with ATP-dependent proteolysis. First,the synthesis of polyubiquitinated des-Gly-Gly-ubiquitin could interfere with the synthesis of competent polyubiquitin by competing for the available ubiquitin . Second, des-Gly-Gly -Ub could compete with native Ub for ubiquitin conjugation factors via recognition by these factors of non-carboxyl-terminal regions of the ubiquitin molecule. Third, ubiquitinated des-Gly-Gly-ubiquitin might bind to the site on ubiquitin-dependent proteases that normally recognizes polyubiquitinated proteolysis substrates, if polyubiquitin is the principal recognition signal for these proteases as suggested by recent studies (2). These mechanisms are not mutually exclusive. However, the fact that there is no evidence of competition between the proteolysis substrate casein and des-Gly-Gly-ubiquitin, as should occur if their ubiquitinated forms were competing for recognition by the protease, suggests that binding of ubiquitinated des-Gly-Gly-ubiquitin to the protease is not the principal mechanism of inhibition in this case. Accordingly, it is likely that des-Gly-Gly-ubiquitin interferes with the synthesis of competent

polyubiquitin by reducing the available ubiquitin pool of activated ubiquitin and/or by binding to conjugation factors involved in the synthesis of competent polyubiquitin.

The ubiquitination of free des-Gly-Gly ubiquitin raises the possibility that competent polyubiquitin might be synthesized not solely by the growth of a polyubiquitin tail on a ubiquitin-protein conjugate, but in part by the attachment of presynthesized polyubiquitin to the protein. However, we have not yet obtained evidence that a significant steady state concentration of unconjugated oligomers of native ubiquitin can be attained in whole lysates. Therefore, either unconjugated native ubiquitin, in contrast to its des-Gly-Gly derivative, is not ubiquitinated or competent polyubiquitin is utilized quickly for conjugation to proteolysis substrates.

Acknowledgments: Dr. Bamezai is the recipient of a Charles H. Revson Foundation Fellowship in Biomedical Research. The authors are grateful to the Rockefeller Mass Spectrometry Biotechnology Research Resource, supported by the Division of Research Resources, NIH, for mass spectrometric measurements on ubiquitin derivatives.

References

1. Hershko, A. and Ciechanover, A. (1982) Ann. Rev. Biochem. 51, 335-364
2. Chau,V., Tobias, J. W., Bachmair, A., Marriott, D., Ecker, D. J., Gonda, D. K. and Varshavsky, A. (1989) Science 242, 1576-1584
3. Hershko, A., Leshinsky, E., Ganoth, D., and Heller, H. (1984) Proc. Nat'l. Acad. Sci. U.S.A. 81, 1619-1623
4. Hershko, A. and Heller, H. (1985) Biochem. Biophys. Res. Commun. 128, 1079-1086
5. Haas, A., Murphy, K. E. and Bright, P. M. (1985) J. Biol. Chem. 260, 4694-4703
6. Wilkinson, K.D. and Audhya, T.K. (1981) J. Biol. Chem. 256, 9235-9241
7. Breslow, E., Daniel, R., Ohba, R., and Tate, S. (1986) J. Biol. Chem. 261, 6530-6535
8. Sardana, V., Carlson, J. D., Breslow, E., and Peyton, D. (1987) Biochemistry 26, 995-1003
9. Reiss, Y., Kaim, D. and Hershko, A. (1988) J. Biol. Chem. 263, 2693-2698
10. Bachmair, A., Finley, D. and Varshavsky, A. (1986) Science 234, 179-186
11. Rogers, S., Wells, R. and Rechsteiner, M. (1986) Science 234, 364-368
12. Hershko, A., Heller, H., Eytan, E. and Reiss, Y. (1986) J. Biol. Chem. 261, 11992-11999
13. Pickart, C. M. and Rose, I. A. (1985) J. Biol. Chem. 260, 1573-1581