186 (1984).
16. C. Shiozawa, S. Sewada, M. Inazawa, E. J. Diener, *J. Immunol.* **132**, 1892 (1984).
17. J. P. Deans, J. F. Krowka, T. Mosmann, L. M. Pilarski, *J. Immunogenet.* **14**, 103 (1987).
18. P. C. Kwong and H.-S. Teh, *Immunology* **61**, 143 (1987).
19. R. R. Quinones, J. Lunney, R. E. Gress, *Transplantation* **46**, 143 (1988).
20. L. E. Samelson, A. M. Weissman, F. A. Robey, I. Berkower, R. D. Klausner, *J. Immunol.* **137**, 3254 (1986).
21. U. K. Laemmli, *Nature* **227**, 680 (1970).
22. Human rIL-2 was a gift from Cetus (Emeryville, CA), and mouse rIL-4 was provided by Immunex (Seattle, WA). We thank J. Ashwell and A. Singer for critical reviews and comments during preparation of this manuscript.

8 February 1989; accepted 21 April 1989

Degradation of Proteins with Acetylated Amino Termini by the Ubiquitin System

ARIE MAYER,* NED R. SIEGEL, ALAN L. SCHWARTZ, AARON CIECHANOVER†

A free NH_2-terminal group has been previously shown to be an obligatory signal for recognition and subsequent degradation of proteins in a partially fractionated and reconstituted ubiquitin proteolytic system. Naturally occurring proteins with acetylated NH_2-termini—most cellular proteins fall in this category—were not degraded by this system. Other studies have suggested that the identity of the NH_2-terminal residue is important in determining the metabolic stability of a protein in vivo (N-end rule). Whole reticulocyte lysate and antibodies directed against the ubiquitin-activating enzyme (E1) have now been used to show that such acetylated proteins are degraded in a ubiquitin-dependent mode. Although fractionation of lysate does not affect its proteolytic activity toward substrates with free NH_2-termini, it completely abolishes the activity toward the blocked substrates, indicating that an important component of the system was either removed or inactivated during fractionation. An NH_2-terminal "unblocking" activity that removes the blocking group, thus exposing a free NH_2-terminus for recognition according to the N-end rule, does not seem to participate in this pathway. Incubation of whole lysate with labeled histone H2A results in the formation of multiple ubiquitin conjugates. In contrast, the fractionated system is devoid of any significant conjugating activity. These results suggest that a novel conjugating enzyme (possibly a ubiquitin-protein ligase) may be responsible for the degradation of these acetylated proteins by recognizing structural features of the substrate that are downstream and distinct from the NH_2-terminal residue.

CONJUGATION OF UBIQUITIN TO certain proteins can trigger their degradation both in vitro and in vivo (*1*). A major question concerns the specificity of the system, that is, the identification of the structural features of a protein that render it susceptible to ubiquitin ligation. A free α-NH_2 group of some proteins is necessary for ligation of the proteins to ubiquitin and their subsequent degradation by crude reticulocyte fraction II [the high-salt eluate from reticulocyte lysate fractionated on a DEAE cellulose column (*2*)] supplemented with ubiquitin (*3*). Naturally occurring Nα-acetylated proteins are not degraded by this system (*3*). The identity of the NH_2-terminal residue affects the stability of a protein in vivo (*4*); genetically engineered derivatives of β-galactosidase had half-lives varying from a few minutes to many hours. Although it has recently been shown that the degradation signal includes a specific Lys residue that is proximal to the NH_2-terminal residue, the variation in the half-lives could be attributed entirely to the amino acid residue in the NH_2-terminal position (*5*).

We have shown that an Arg moiety must be transferred posttranslationally in a tRNA-dependent mode to acidic NH_2-termini (Asp or Glu) of proteins before their recognition by the ubiquitin system (*6*). A similar modification must also take place in proteins with an Asn, Gln, or Cys residue in this position (*7*). At least part of the selection of proteins for ubiquitin-dependent degradation is mediated by distinct recognition sites on ubiquitin-protein ligase (E3) for basic NH_2-termini (which also recognizes Arg-modified residues) and bulky hydrophobic NH_2-termini (*8*). It is also clear from these studies, however, that the NH_2-terminal residue, although an important recognition signal, is not the only one and most probably not the predominant structural feature. For example, a third site on E3 was classified that probably recognizes structural features of the proteins downstream from the NH_2-terminal residue (*8*). However, even the substrates recognized by this E3 had free NH_2-terminal residues; substrates with modified NH_2-termini were not recognized by this E3 and were not degraded by the DEAE-fractionated system (*2, 3, 8*). It is not known whether the different recognition sites reside within one E3 molecule or whether distinct E3 molecules recognize specific subsets of proteolytic substrates (*1, 8*).

Proteins with acetylated NH_2-termini constitute about 80% of the soluble cellular proteins in Ehrlich Ascites tumor cells (*9*). Apparently, most cellular proteins are degraded in an adenosine 5′-triphosphate (ATP)–dependent mode (*10*), and it is possible that the NH_2-terminal–blocked proteins could be degraded in the cell in an ATP-dependent, but ubiquitin-independent, mode (*11*). Another possibility is that these proteins can be degraded by the ubiquitin system; this possibility would imply that during the initial fractionation and separation of the system in vitro (*2*), a crucial factor necessary for their degradation is removed or inactivated. We have now tested these two hypotheses.

Table 1. Degradation of substrates with free and Nα-blocked NH_2-termini in crude reticulocyte fraction II and in reticulocyte lysate. BSA, lysozyme, α-crystallin (Sigma), histone H2A (Boehringer Mannheim), and actin (Worthington) were radioiodinated as described (*6*). In the case of α-crystallin, only the A chain was labeled, although both chains have blocked termini (*26*). Degradation was measured as described (*27*). Results represent a typical experiment. In five independent repeats with three different enzymatic preparations, the variation did not exceed 50% when degradation rates were below 10%, and 15% when degradation rates were above 10%. Each experiment was performed in duplicate. Variation among duplicates did not exceed 7%.

Substrate	Degradation (%)			
	Crude reticulocyte fraction II		Reticulocyte lysate	
	−ATP	+ATP	−ATP	+ATP
BSA	0	32	0	36
Lysozyme	7	29	5	31
Histone H2A	4	5	2	60
α-Crystallin	4	7	2	54
Actin	5	4	1	43

A. Mayer and A. Ciechanover, Unit of Biochemistry and the Rappaport Institute for Research in the Medical Sciences, Faculty of Medicine, Technion-Israel Institute of Technology, Post Office Box 9649, Haifa 31096, Israel.
N. R. Siegel, Life Sciences Division, Monsanto Inc., St. Louis, MO 63198.
A. L. Schwartz, Edward Mallinckrodt Departments of Pediatrics and Pharmacology and Division of Hematology-Oncology, Children's Hospital, Washington University School of Medicine, St. Louis, MO 63110.

*Present address: Department of Cell Biology, New York University Medical Center, New York, NY 10016.
†To whom correspondence should be addressed.

We first found that naturally occurring Nα-acetylated proteins (histone H2A, α-crystallin, and actin) are degraded in whole reticulocyte lysate in an ATP-dependent mode (Table 1). In contrast, and in agreement with previous results (*3*), these proteins were not degraded in the partially fractionated ubiquitin system [crude reticulocyte fraction II supplemented with ubiquitin (*2*)]. Substrates with free NH_2-termini [bovine serum albumin (BSA) and lysozyme] were degraded efficiently in both the fractionated system and the unfractionated lysate.

Ubiquitin-dependent degradation of a specific substrate in crude reticulocyte fraction II can be tested by the addition or omission of ubiquitin. Testing ubiquitin-dependent degradation of a protein in whole lysate is more difficult, because the lysate contains all the components of the system required for its activity, including ubiquitin. We therefore made an antibody to affinity-purified E1, the first enzyme in the cascade of ubiquitin ligation; this enzyme has been shown to be an obligatory component of the proteolytic system both in vitro and in vivo (*12*). The enzyme activates ubiquitin to a high-energy intermediate by a two-step reaction. In the first step, ubiquitin is activated to a high-energy adenylate intermediate, a reaction that can be monitored by assaying ATP–inorganic pyrophosphate (ATP-PP_i) exchange (*12*). The immunoglobulin G (IgG) fraction of the antiserum was affinity-purified on immobilized E1. Protein immunoblot analysis revealed that the purified antibody [anti-E1(IgG)] recognizes specifically E1 (*13*) and inhibits E1-dependent activation of ubiquitin and consequently ubiquitin conjugation and ubiquitin-dependent degradation (Table 2). The inhibition is reversible in that after removal of the antibody all the inhibited functions of E1 can be restored by the addition of affinity-purified E1.

We used this antibody to test whether the degradation of some Nα-acetylated proteins is E1-dependent. The degradation of actin, histone H2A, and the A chain of α-crystallin was strongly and specifically inhibited by anti-E1(IgG) (Fig. 1). After removal of the antibody, the inhibition could be alleviated by the addition of purified E1. Much less anti-E1(IgG) was needed to inhibit the degradation of histone H2A than was necessary to inhibit the degradation of actin and α-crystallin. It is possible, for example, that different species of E1 are required for the degradation of different substrates and that the affinities of the antibody for the various species of E1 are not identical [see (*14*)]. Other Nα-acetylated proteins (such as histone H4) were degraded in a similar way (*15*). The ubiquitin system can therefore degrade Nα-blocked proteins, and an essential component that is required for this activity is removed or inactivated during the partial separation of the reticulocyte lysate.

Ubiquitin-dependent degradation of substrates occurs in two main successive steps, conjugation of ubiquitin to the substrate and degradation of the protein moiety of the conjugate with the release of free and reusable ubiquitin (*1*). To determine in which of these two steps the putative component participates, we incubated ^{125}I-labeled histone H2A in reticulocyte lysate in the presence of ATP and found that multiple ubiquitin conjugates were formed (Fig. 2). In contrast, fraction II was devoid of any conjugating activity. Substrates with free NH_2-termini, such as oxidized ribonuclease A (oxRNase A), were conjugated to ubiquitin by both the lysate and fraction II (Fig. 2).

Two possible functions of the factor are: (i) the factor is a deacetylase or an acylaminohydrolase [both activities have been described (*16*); however, their physiological roles have not been delineated], or (ii) the factor is participating in the conjugation of ubiquitin to substrates with intact, blocked NH_2-termini and therefore recognizes structural features of the protein that are distinct from the free NH_2-terminal residue. If the factor removes the acetyl group (or acylamino or acylpeptide groups) and thus exposes a free NH_2-terminus, the processed substrates should then be degraded in a ubiquitin-supplemented fraction II in accordance with the rules described for degradation of proteins with exposed NH_2-termini.

Three independent lines of evidence exclude an NH_2-terminal exposing activity as a possible function of the factor. (i) The three ^{125}I-labeled Nα-acetylated substrates were

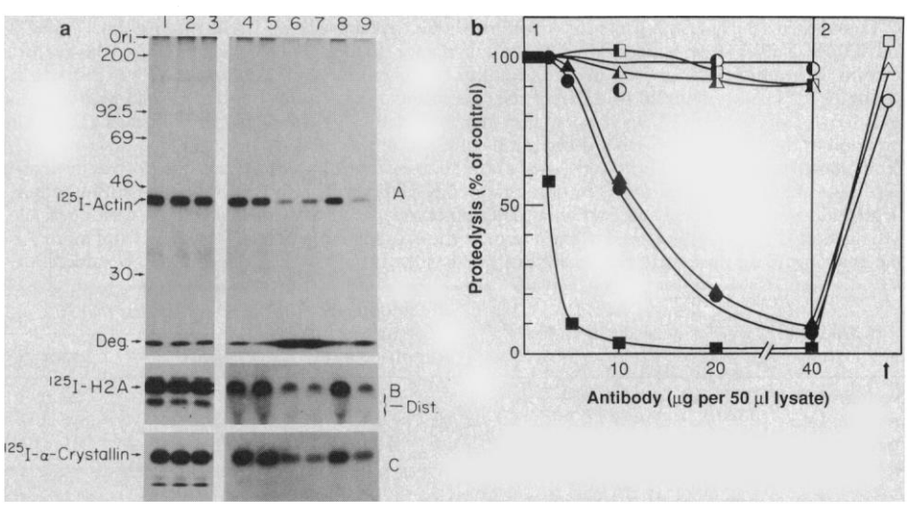

Fig. 1. Degradation of Nα-acetylated proteins in crude reticulocyte fraction II and in reticulocyte lysate; inhibition by anti-E1(IgG) and reconstitution with E1. (**a**) Reaction mixtures (50 μl) containing 220 μg of crude reticulocyte fraction II and 3 μg of ubiquitin (lanes 1 to 3) or 30 μl of reticulocyte lysate (lanes 4 to 9) were incubated for 45 min at 37°C with 50 m*M* tris HCl (*p*H 7.6), 5 m*M* $MgCl_2$, 2 m*M* dithiothreitol, and either 0.5 μg of hexokinase and 10 m*M* 2-deoxyglucose (lanes 1, 2, 4, and 5), or 0.5 m*M* ATP, 10 m*M* creatine phosphate, and 10 μg of creatine phosphokinase (lanes 3 and 6 to 9). Affinity-purified nonimmune IgG (25 μg) was present in the reaction described in lane 7, and affinity-purified anti-E1(IgG) (25 μg) was present in the reactions described in lanes 8 and 9 (*32*; see legend to Table 2). A 50% suspension of Protein G–Sepharose beads (10 μl) was then added to all the reaction mixtures (*32*; see legend to Table 2). After removal of the beads, 45 μl of the supernatant were used for continuation of the reaction in a final volume of 50 μl. Affinity-purified E1 (0.5 μg; see legend to Table 2) was added to the reaction described in lane 9. Degradation was initiated by the addition of 0.5 μg (~750,000 cpm) of each substrate (panel A, ^{125}I-labeled actin; panel B, ^{125}I-labeled histone 2A; panel C, ^{125}I-labeled α-crystallin), and reaction mixtures were either incubated for 2 hours at 37°C (lanes 2, 3, and 5 to 9) or not incubated (lanes 1 and 4). Samples (5 μl) of each reaction mixture were loaded onto 10% (actin), 12.5% (α-crystallin), and 18% (histone H2A) SDS-polyacrylamide gels (*27, 29*), separated by electrophoresis, and autoradiographed. Molecular mass markers are as follows: 200 kD, myosin; 92.5 kD, phosphorylase B; 69 kD, BSA; 46 kD, ovalbumin; 30 kD, carbonic anhydrase (^{14}C-labeled; Amersham). Deg. denotes ^{125}I-labeled low molecular mass degradation products. Dist. denotes distortion of the migration of histone due to hemoglobin, which migrates to a similar distance. (**b**) (Panel 1) Degradation of ^{125}I-labeled histone H2A (squares), ^{125}I-labeled α-crystallin (triangles), and ^{125}I-labeled actin (circles) in reticulocyte lysate was measured in reaction mixtures (50 μl) as described in (a) and in (*27*) and the legend to Table 2 (except that the incubation with the antibody was carried out with 50 μl of lysate) in the presence of the indicated amounts of anti-E1(IgG) (closed symbols) or nonimmune IgG (semiclosed symbols). (Panel 2) After removal of the antibody as described (legend to Table 2), affinity-purified E1 (0.5 μg) was added (arrow) to the reaction mixtures that had contained 40 μg of immune IgG per 50 μl of lysate. The experiment was performed three times with essentially the same results. Shown is one typical experiment.

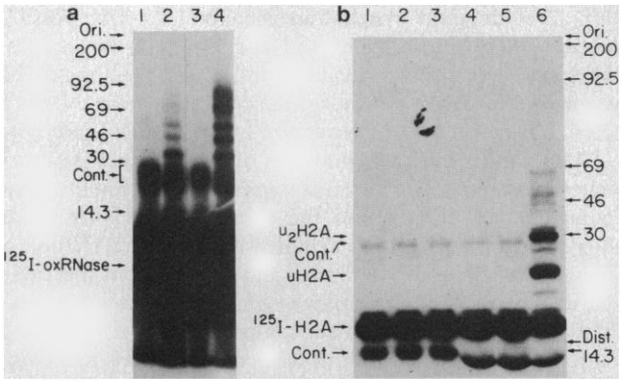

Fig. 2. Conjugation of ubiquitin to ^{125}I-labeled oxidized RNase A (**a**) and ^{125}I-labeled histone H2A (**b**) by crude reticulocyte fraction II (a, lanes 1 and 2; b, lanes 1 to 3) and reticulocyte lysate (a, lanes 3 and 4; b, lanes 4 to 6). Conjugation was performed as described (*6*) in a reaction mixture (25 μl) containing 110 μg of crude reticulocyte fraction II or 15 μl of reticulocyte lysate. ^{125}I-labeled substrates (0.5 μg; ~750,000 cpm) were used. Reaction mixtures (5 μl) were loaded onto polyacrylamide gels (a, 12.5%; b, 18%) (*27, 29*) (see legend to Fig. 1). Lanes a1, a3, b2, and b5, without ATP; lanes a2, a4, b3, and b6, with ATP; lanes b1 and b4, without incubation. uH2A and u_2H2A denote the putative mono- and diubiquitin conjugates of histone H2A; Cont. denotes radiolabeled contamination in the commercial preparation; Dist. is described in the legend to Fig. 1. Molecular mass markers are as described in the legend to Fig. 1 except that 14.3 kD denotes lysozyme.

Table 2. Functional characterization of the anti-E1 antibody. ATP-PP_i exchange was measured essentially as described (*12*). The reaction mixture (50 μl) contained 0.5 μg of affinity-purified E1 (*12*) and 3 μg of either affinity-purified anti-E1(IgG) or nonimmune IgG [IgG fraction from goat serum purified on Protein G–Sepharose (Pharmacia-LKB)] (*32*). The mixtures were incubated for 30 min at 37°C, and then 10 μl of a 50% suspension of Protein G–Sepharose beads [in 20 m*M* tris-HCl (*p*H 7.2) and 0.02% NaN_3] were added. After gentle mixing for 10 min at room temperature, the reaction mixture was centrifuged for 10 s in an Eppendorf microcentrifuge, and the reaction was initiated by adding [^{32}P]PP_i and ubiquitin to 45 μl of the supernatant. To a parallel tube incubated with immune IgG, 0.5 μg of purified E1 was added after removal of the IgG with Protein G–beads and before initiation of the reaction. ^{125}I-labeled ubiquitin conjugation was measured essentially as described (*12*). Crude reticulocyte fraction II (90 μg) (used as the source of the ubiquitin-ligase system) was incubated with 6 μg of purified anti-E1(IgG) or nonimmune IgG; 0.5 μg of E1 was used to reconstitute activity. Degradation of ^{125}I-labeled substrates was determined essentially as described (*6, 27*). Crude reticulocyte fraction II (220 μg) was used as the source of the degradative system. Where data from more than one experiment are presented, results are expressed as the mean ± the range of values obtained.

Addition	ATP-PP_i exchange (%) (n = 2)	Ubiquitin conjugation (pmol) (n = 1)	Degradation (%) (n = 3)	
			BSA	Lysozyme
Nonimmune IgG	41 ± 4	16	27 ± 4	22 ± 3
Immune IgG	0	2	1 ± 1	6 ± 2
Immune IgG + E1	38 ± 4	18	24 ± 3	19 ± 3

incubated in reticulocyte lysate in the presence of anti-E1(IgG) at concentrations that inhibit proteolysis. After the antibody was removed, the lysate was dialyzed and a small portion (2 μl per 50 μl of reaction mixture) of the dialyzed lysate was then added either to crude reticulocyte fraction II supplemented with ubiquitin or to untreated reticulocyte lysate. Although fraction II did not degrade the substrates to any measurable extent, the lysate was proteolytically active toward these proteins (*17*). (ii) Degradation of the substrates was not susceptible to inhibition by dipeptides known to inhibit the ubiquitin-dependent degradation of proteins with specific subsets of NH_2-terminal residues. The dipeptide His-Ala, which inhibits the degradation of proteins with basic NH_2-termini (His, Arg, and Lys) (*1, 8*) and proteins with NH_2-termini that are posttranslationally modified by the addition of an Arg residue before their degradation (Asn, Asp, Gln, Glu, and Cys) (*6, 7*), did not have any effect on the degradation of the Nα-acetylated substrates (*18*). The dipeptide Trp-Ala, which inhibits the degradation of proteins with bulky hydrophobic NH_2-termini (Trp, Leu, Phe, and Tyr) (*1, 8*), similarly did not have any effect (*19*). Proteins with either an Ala, Ser, Thr, Ile, Gly, Met, or Val residue in the NH_2-terminal position are stable (*4*), and it is not known whether their degradation by the ubiquitin system requires recognition of this residue (*1, 4, 7, 8*). Exposure of such termini, therefore, does not seem to render them susceptible to rapid proteolysis. It is not clear whether Pro in the NH_2-terminal position plays a role in recognition of the protein (*4, 7*). The first Pro residue is located substantially distal to the NH_2-terminal position in all three substrates (27th, 26th, and 8th residue in actin, histone H2A, and α-crystallin, respectively), and exposure at this site would have been detected by a change in the apparent molecular mass of the labeled proteins following incubation with the anti-E1(IgG), a change that could not be detected (Fig. 2). (iii) Actin has four successive acidic amino acid residues after the acetyl group (*20*). Removal of the acetyl moiety, either alone or along with any combination of the first, second, and third amino acids, should render the degradation of the remaining molecule sensitive to ribonuclease A (RNase A), because a tRNA-dependent transfer of an Arg residue to the newly exposed acidic NH_2-terminal residue would be required for its further degradation (*6*). However, RNase A had no effect on actin degradation in reticulocyte lysate (*21*). The participation of the putative factor in removal of the acetyl group together with a long polypeptide from the NH_2-terminal region (thus exposing a new nonacidic, free NH_2-terminal residue substantially downstream from the original one) was also ruled out. In the presence of anti-E1(IgG), actin (as well as histone H2A and α-crystallin) retained its authentic molecular mass on SDS-polyacrylamide gels (Fig. 1, lane 8) (*22*).

All the substrates used in our study can be found as conjugates of ubiquitin in the cell (*23*). However, it is not known whether the modification of these proteins in vivo signals them for degradation. In our study we used these proteins as model substrates only. Our results show that the ubiquitin system can conjugate and subsequently degrade Nα-acetylated proteins. Furthermore, the recognition capability does not seem to require an exposed NH_2-terminal residue and may be directed to a structural feature (or features) of the substrate that is distinct and possibly downstream from the NH_2-terminal residue. Most of the studies on the ubiquitin system and analysis of its components have been performed with the in vitro reticulocyte system. A role for the system in the degradation of cellular proteins in vivo has been shown for the general population of short-lived proteins (*12*) and for the light-induced degradation of phytochrome (*24*). Because the vast majority of cellular proteins are blocked in their NH_2-terminal position, a potential role for the ubiquitin system in their turnover in vivo is physiologically important.

The factor lost during fractionation of reticulocyte lysate on the ion-exchanger may be a novel ubiquitin-protein ligase (E3). Alternatively, it may play a role in the function of the already described E3, which interacts with substrates that have free NH_2-termini, however, at regions distinct from these residues (*8*). In reticulocyte lysate, nondegradable proteins with blocked amino groups (both α and ε) can compete with known proteolyzable substrates of the ubiquitin system for binding to ubiquitin-con-

jugating factors (*25*). It is possible that both Nα-acetylated proteins and proteins with free NH_2-termini that do not use this residue as a recognition marker share the same ubiquitin-conjugation factor or factors.

REFERENCES AND NOTES

1. A. Hershko, *J. Biol. Chem.* **263**, 15237 (1988).
2. A. Ciechanover, Y. Hod, A. Hershko, *Biochim. Biophys. Res. Commun.* **81**, 1100 (1978).
3. A. Hershko *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 7021 (1984).
4. A. Bachmair, D. Finley, A. Varshavsky, *Science* **234**, 179 (1986).
5. A. Bachmair and A. Varshavsky, *Cell* **56**, 1019 (1989).
6. S. Ferber and A. Ciechanover, *J. Biol. Chem.* **261**, 3128 (1986); *Nature* **326**, 808 (1987); A. Ciechanover *et al.*, *J. Biol. Chem.* **263**, 11155 (1988).
7. D. K. Gonda, A. Bachmair, I. Wünning, W. S. Lane, A. Varshavsky, in *The Ubiquitin System*, M. Schlesinger and A. Hershko, Eds. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988), pp. 97–105.
8. A. Hershko, H. Heller, E. Eytan, Y. Reiss, *J. Biol. Chem.* **261**, 11992 (1986); Y. Reiss, D. Kaim, A. Hershko, *ibid.* **263**, 2693 (1988).
9. J. L. Brown and W. K. Roberts, *ibid.* **251**, 1009 (1976).
10. A. L. Goldberg and A. St. John, *Annu. Rev. Biochem.* **45**, 747 (1976); A. Hershko and A. Ciechanover, *ibid.* **51**, 335 (1982).
11. K. Tanaka, L. Waxman, A. L. Goldberg, *J. Cell Biol.* **96**, 1580 (1983).
12. A. Ciechanover, S. Elias, H. Heller, A. Hershko, *J. Biol. Chem.* **257**, 2537 (1982); A. Hershko, H. Heller, S. Elias, A. Ciechanover, *ibid.* **258**, 8206 (1983); D. Finley, A. Ciechanover, A. Varshavsky, *Cell* **37**, 43 (1984); A. Ciechanover, D. Finley, A. Varshavsky, *ibid.*, p. 57.
13. A. Mayer, A. L. Schwartz, A. Ciechanover, unpublished data.
14. A. Mayer, R. Gropper, A. L. Schwartz, A. Ciechanover, *J. Biol. Chem.* **264**, 2060 (1989); P. M. Hatfield and R. D. Vierstra, *Biochemistry* **28**, 735 (1989).
15. A. Mayer *et al.*, unpublished data.
16. F. Wold, *Annu. Rev. Biochem.* **50**, 783 (1981); Y. Endo, *Biochim. Biophys. Acta* **628**, 13 (1980); W. Gade and J. L. Brown, *J. Biol. Chem.* **253**, 5012 (1978); G. Radhakrishna and F. Wold, *ibid.* **261**, 9572 (1986).
17. A. Mayer *et al.*, unpublished data.
18. ———, unpublished data.
19. ———, unpublished data.
20. J. H. Collins and M. Elzinga, *J. Biol. Chem.* **250**, 5915 (1975).
21. A. Mayer, A. L. Schwartz, A. Ciechanover, unpublished data.
22. ———, unpublished data.
23. I. L. Goldknofp and H. Busch, *Cell Nucleus* **6**, 149 (1978); B. E. Nickel and J. R. Davie, *Biochemistry* **28**, 964 (1989); L. T. Hunt and M. O. Dayhoff, *Biochim. Biophys Res. Commun.* **74**, 650 (1977); J. H. Jahngen *et al.*, *J. Biol. Chem.* **261**, 13760 (1986); E. Ball *et al.*, *Cell* **51**, 221 (1987).
24. J. Shanklin, M. Jabben, R. D. Vierstra, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 359 (1987).
25. E. Breslow, R. Daniel, R. Ohba, S. Tate, *J. Biol. Chem.* **261**, 6530 (1986).
26. A. Mayer, N. R. Siegel, A. L. Schwartz, A. Ciechanover, unpublished results.
27. Degradation was measured in reaction mixtures (50 μl) containing 220 μg of crude reticulocyte fraction II and 3 μg of ubiquitin (Sigma), or 30 μl of reticulocyte lysate [prepared as described (*2*) except that the ATP-depleting step was omitted] as described (*6*). Reaction mixtures without ATP and ATP-regenerating system were incubated in the presence of 0.5 μg of hexokinase (Boehringer Mannheim) and 10 m*M* 2-deoxyglucose. The identity of each protein and the state of its NH_2-terminal residue (free or blocked) were determined by automated Edman degradation. A gas-phase sequenator (Applied Biosystems, Inc. model 470A) was used for the degradation (*28*). The respective phenylthiohydantoin–amino acid derivatives (PTH-aa) were identified by reversed-phase high-performance liquid chromatography (RP-HPLC) in an on-line fashion with a PTH-aa analyzer (Applied Biosystems, Inc., model 120A) fitted with a Brownlee (PTH-C18 column (internal diameter, 2.1 mm). Samples of protein (20 μg) were separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE), according to the procedure of Laemmli [(*29*); the histone molecule was separated using a modification of the Laemmli system as described (*30*)]. Coomassie blue staining of samples separated independently showed that each protein was at least 90% pure. The samples were then transferred onto polyvinylidene difluoride paper (*31*) (Immobilin, Millipore), and the appropriate bands were cut and sequenced directly in an NH_2-terminal fashion. For those samples that failed to show free NH_2-termini (histone H2A, actin, and α-crystallin), solution-phase digestions were carried out with *N*-tosyl-l-phenylalanine chloromethyl ketone-treated trypsin (Worthington). RP-HPLC purified internal fragments were then sequenced in an NH_2-terminal fashion in order to substantiate the presence of the desired protein. The identity of all proteins used was confirmed.
28. M. W. Hunkapiller, R. M. Hewick, R. J. Dreyer, L. E. Hood, *Methods Enzymol.* **91**, 399 (1983).
29. U. K. Laemmli, *Nature* **227**, 680 (1970).
30. B. D. Hames, in *Gel Electrophoresis of Proteins: A Practical Approach*., B. D. Hames and D. Rickwood, Eds. (IRL Press, Oxford, 1981), pp. 66–67.
31. P. Matsudaira, *J. Biol. Chem.* **262**, 10035 (1987).
32. Affinity-purified E1 (0.5 mg) (*12*) was injected into a goat in Freund's complete adjuvant (primary immunization). Immunization was performed again every 4 weeks with 100 μg of E1 in Freund's incomplete adjuvant. After the third immunization, the animal was bled on a regular basis 7 days after each injection. Serum (20 ml) was affinity-purified on a column of 10 ml of Protein G–Sepharose as described for purification of IgG on Protein A–Sepharose (*33*). The IgG fraction was then subjected to affinity purification on affinity-purified rabbit E1 (*12*) immobilized to CH-activated Sepharose (Sigma; 12.5 mg of protein per 3 ml of matrix) as described (*33*).
33. A. Hershko, E. Eytan, A. Ciechanover, A. L. Haas, *J. Biol. Chem.* **257**, 13964 (1982).

34. We thank A. Hershko for making available unpublished results on the degradation of chemically acetylated lysozyme in reticulocyte lysate, M. Mueckler for help with the computer search of the naturally occurring Nα-acetylated proteins, S. Elias for initial work with the anti-E1 antibody, S. Adams for advice and support, and M. Schainker for typing the manuscript. Supported by grants from the U.S.-Israel Binational Science Foundation (8500059/3), Monsanto, the Foundation for Promotion of Research in the Technion, and the Research Fund of the Vice President of the Technion for Research. A.L.S. is an Established Investigator of the American Heart Association. A.C. is a Research Career Development Awardee of the Israel Cancer Research Fund and an American Cancer Society Eleanor Roosevelt Memorial Fellow.

30 January 1989; accepted 27 April 1989

Adipsin and Complement Factor D Activity: An Immune-Related Defect in Obesity

BARRY S. ROSEN, KATHLEEN S. COOK, JULIA YAGLOM, DOUGLAS L. GROVES, JOHN E. VOLANAKIS, DEBORAH DAMM, TYLER WHITE, BRUCE M. SPIEGELMAN

Adipsin is a serine protease that is secreted by adipocytes into the bloodstream; it is deficient in several animal models of obesity, representing a striking example of defective gene expression in this disorder. Recombinant mouse adipsin was purified and its biochemical and enzymatic properties were studied in order to elucidate the function of this protein. Activated adipsin has little or no proteolytic activity toward most substrates but has the same activity as human complement factor D, cleaving complement factor B when it is complexed with activated complement component C3. Like authentic factor D, adipsin can activate the alternative pathway of complement, resulting in red blood cell lysis. Decreased (58 to 80 percent) complement factor D activity, relative to lean controls, was observed as a common feature of several experimental models of obesity, including the *ob/ob*, *db/db*, and monosodium glutamate (MSG)–injected mouse and the *fa/fa* rat. These results suggest that adipsin and the alternative pathway of complement may play an unexpected but important role in the regulation of systemic energy balance in vivo.

THE ADIPOCYTE SERVES AS THE MAJOR repository of energy stores in higher organisms. The hormonal and biochemical control of many of the steps of lipogenesis and lipolysis in adipocytes in normal and diseased states remain to be elucidated. Molecular cloning has been used to identify gene products that may be important in adipocyte metabolism, including adipsin, a member of the serine protease gene family (*1*, *2*).

Adipsin messenger RNA (mRNA) is tissue-specific; it can be detected primarily in fat tissue (*2*) and sciatic nerve (*3*). The encoded protein has several features suggesting that it can function as an active serine protease, including a catalytically ac-

B. S. Rosen, K. S. Cook, J. Yaglom, D. L. Groves, B. M. Spiegelman, Dana-Farber Cancer Institute and the Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115.
J. E. Volanakis, Department of Medicine, University of Alabama at Birmingham, Birmingham, AL 35294.
D. Damm and T. White, Metabolic Biosystems, Inc., Mountain View, CA 94043.