The N-end Rule Pathway Catalyzes a Major Fraction of the Protein Degradation in Skeletal Muscle*

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In skeletal muscle, overall protein degradation involves the ubiquitin-proteasome system. One property of a protein that leads to rapid ubiquitin-dependent degradation is the presence of a basic, acidic, or bulky hydrophobic residue at its N terminus. However, in normal cells, substrates for this N-end rule pathway, which involves ubiquitin carrier protein (E2) E2, ubiquitin-protein ligase (E3) E3, have remained unclear. Surprisingly, in soluble extracts of rabbit muscle, we found that competitive inhibitors of E3 markedly inhibited the 125I-ubiquitin conjugation and ATP-dependent degradation of endogenous proteins. These inhibitors appear to selectively inhibit E3, since they blocked degradation of 125I-Iyszyme, a model N-end rule substrate, but did not affect the degradation of proteins whose ubiquitination involved other E3s. The addition of several E2s or E3s to the muscle extracts stimulated overall proteolysis and ubiquitination, but only the stimulation by E3s or E2s was sensitive to these inhibitors. A similar general inhibition of ubiquitin conjugation to endogenous proteins was observed with a dominant negative inhibitor of E24k. Certain substrates of the N-end rule pathway are degraded after their tRNA-dependent arginylation. We found that adding RNase A to muscle extracts reduced the ATP-dependent proteolysis of endogenous proteins, and supplying tRNA partially restored this process. Finally, although in muscle extracts the N-end rule pathway catalyzes most ubiquitin conjugation, it makes only a minor contribution to overall protein ubiquitination in HeLa cell extracts.

The proteolytic system responsible for most of the protein degradation in mammalian cells is the ATP-requiring system that involves ubiquitin (Ub) and the proteasome complex (1–4). It has been generally believed that the primary role of this system is the rapid degradation of highly abnormal proteins and short lived proteins that are critical in regulating cell growth and metabolism (1–4). However, several recent studies have indicated that this nonsomalous proteolytic system is also responsible for the degradation of the bulk of cellular proteins, many of which are long lived components, in both cultured mammalian cells (5) and incubated muscles (6, 7). Moreover, in skeletal muscle extracts, the Ub-proteasome system catalyzes the complete breakdown of most soluble proteins as well as of individual myofibrillar proteins that are very stable in vivo (8). Skeletal muscle comprises most of the body proteins in mammals. Consequently, overall rates of protein degradation in muscle are tightly regulated, and mobilization of muscle protein reserves is of particular importance in energy homeostasis. For example, in fasting this degradative process is accelerated to provide amino acids for hepatic gluconeogenesis (6, 9). Muscle proteolysis also rises in a number of disease states, including cancer cachexia, sepsis, and denervation atrophy and leads to rapid muscle atrophy, primarily through activation of the Ub-proteasome system (6, 10–14).

Proteins to be hydrolyzed by this system are first modified by covalent conjugation to the protein cofactor, Ub (1, 2, 4). The linkage of a protein to a chain of Ub moieties marks it for rapid ATP-dependent degradation by the 28 S proteasome (15, 16). In this system, the Ub is first activated by the Ub-activating enzyme (E1), which forms a Ub-thioester. The activated Ub is then transferred to one of the cell's many Ub carrier proteins (E2s) and subsequently to a polypeptide substrate in a reaction catalyzed by a specific Ub-protein ligase (E3), several of which have been identified. The specificity in this proteolytic pathway appears to depend on the nature of the E2s and E3s involved, many of which have been shown to catalyze the conjugation of Ub to specific short lived proteins (1–4, 17–21). However, for most of these ubiquitination enzymes, the basis of substrate selection is not clear, nor is it known which E2(s) or E3(s) ubiquitinate the bulk of cell proteins.

One structural feature of a polypeptide that leads to its ubiquitination and degradation is the nature of its N-terminal residue (22, 23). Varshavsky and co-workers (22, 23) have shown genetically in yeast that proteins with basic, acidic, or large hydrophobic N termini are ubiquitinated and degraded very rapidly, while the same proteins bearing other N-terminal residues, e.g. methionine, are stable (22, 23). The relationship between the identity of a protein's N-terminal residue and its half-life has been termed the N-end rule pathway and was also shown to function in mammalian cells (22–25). Biochemical studies in reticulocytes have demonstrated that this selectivity is due to the Ub-protein ligase, E3 (Ubr1 and its cognate Ub carrier protein, E214k (26). E3 contains two binding sites, one specific for proteins or peptides with basic N termini and the other for substrates with bulky hydrophobic N termini (23–25). Dipeptides and amino acid methyl esters with such N termini can bind to E3 and inhibit the ubiquitination and subsequent degradation of the corresponding protein substrates both in intact yeast cells and in extracts (22–24). In addition, proteins with acidic N-terminal residues are also degraded by this pathway after undergoing a specific arginyl-tRNA-dependent N-terminal arginylation, which triggers their ubiquitination by
E3α (22, 27–32).

Despite these extensive findings, the physiological significance of the N-end rule pathway in vivo has long been unclear, and its importance has even been questioned (33). Yeast mutants that lack this pathway (i.e. mutants in Ubc2, which encodes the yeast homologue of E2\textsubscript{4k}, or in Ubr1, which encodes the yeast homologue of E3\textsubscript{α}) have only minor phenotypes (34), and efforts to identify substrates with destabilizing N termini or ones that undergo tRNA-dependent proteolysis have not succeeded thus far. We report here the unexpected finding that this ubiquitination system, involving E2\textsubscript{4k} and E3\textsubscript{α}, as well as tRNA-dependent substrate modification, is responsible for up to 60% of the ATP-dependent degradation of soluble proteins in extracts of normal skeletal muscles.

**EXPERIMENTAL PROCEDURES**

**Protein Reagents—**Ubiquitin, actin, myosin, bovine serum albumin, and human α-lactalbumin were from Sigma. Lysozyme was from Boehringer Mannheim. Radiodiodination of protein substrates was performed by the chloramine T method as described previously (8). The dominant negative inhibitor mutants of the E2\textsubscript{s} were kindly provided by Jackie Pierce, Margaret Read, and Vincent Chau (Proscript, Inc.). *Escherichia coli* strains engineered to express E2\textsubscript{4k}, E2-F1 (Ubc17), and UbcH5b were kindly provided by S. Wing, M. Scheffner, and A. Weissman, respectively.

**Muscle Extract Preparation—**Male New Zealand White rabbits (3–4 kg) were killed by lethal injection of sodium pentobarbital, and extracts from psoas muscles were prepared as described earlier (8). Homogenates were centrifuged at 30,000 × g for 30 min to remove myofibrils. “Crude extracts” were prepared by centrifuging the supernatants at 100,000 × g for 1 h and were either studied directly or fractionated on DEAE-cellulose (8) into fraction I, the resin-bound material, which contains the proteasomes and most of the enzymes required for Ub conjugation, and fraction II, the flow-through, which contains Ub and 70% of cell proteins. Both crude extracts and fraction II were then dialyzed against buffer containing 20 mM Tris-HCl, pH 7.6, 2 mM DTT, 5 mM MgCl\textsubscript{2} and 10% glycerol and stored at −70 °C until use.

**Purification of E1, E2\textsubscript{s}, and E3α—**E1 was prepared from rabbit muscle fraction II extract using Ub-Sepharose affinity chromatography by elution with AMP and PP\textsubscript{i}, as described previously (35). The E1 was further purified by MonoQ FPLC (Amersham Pharmacia Biotech). E2\textsubscript{4k} was purified from *E. coli* fraction II by MonoQ FPLC and size exclusion chromatography on Sephacryl S-100 HiPrep FPLC (Amersham Pharmacia Biotech). UbcH8b and E2-F1 were prepared directly from *E. coli* lysates using HiTrap S FPLC (Amersham Pharmacia Biotech) followed by Sephacryl S-100 HiPrep FPLC.

Partially purified E3α was prepared from crude muscle extract using a protocol kindly provided by A. Haas and co-workers.\(^2\) Crude muscle extract (35 mg) was twice passed over a 1.0-ml E2\textsubscript{4k}-Affi-Gel 10 (Bio-Rad) affinity column (prepared according to the manufacturer’s instructions using 10 μl E2\textsubscript{4k} and 5.0 ml of resin). The column was washed exhaustively with 20 ml Tris-HCl, pH 7.6, 50 mM NaCl, 1 mM DTT, and then E3α was eluted with 20 ml Tris-HCl, pH 7.6, 1 mM NaCl, 0.5 mM DTT into tubes containing bovine serum albumin to a final concentration of 0.1 mg/ml. The material was dialyzed against 20 ml Tris-HCl, pH 7.6, 5 mM MgCl\textsubscript{2}, 0.5 mM DTT, 10% glycerol and stored at −70 °C.

**Protein Degradation Assays in Skeletal Muscle Extracts—**Degradation of endogenous proteins in crude extracts and fraction II was measured by assaying the generation of free tyrosine in the trichloroacetic acid-soluble supernatant (8). Reaction mixtures contained the following in a volume of 100 μl: 20 mM Tris-HCl, pH 7.6, 5 mM MgCl\textsubscript{2}, 2 mM DTT, ATP regenerating system (10 μg of creatine phosphokinase and 10 μM creatine phosphate), 1 mM ATP, 25 μg of Ub, and approximately 1 mg of dialyzed crude extract or fraction II. Following incubation at 37 °C for 2 h, the reactions were terminated by the addition of an equal volume of 20% trichloroacetic acid. The reaction mixtures were centrifuged, and the amount of tyrosine generated in the supernatant was measured by fluorosence spectroscopy as described previously (8). When degradation of \(^{125}\text{I}\)-lysozyme, \(^{125}\text{I}\)-actin, or \(^{125}\text{I}\)-lysozyme in the crude extracts was studied, the reaction mixture contained 3 μg of labeled proteins, and their degradation was measured by following the appearance of trichloroacetic acid-soluble radioactivity using a γ-counter. The results shown are typical of those obtained in three independent experiments. The absolute amount of protein degraded in the absence or presence of ATP varied from extract to extract.

**Measurement of \(^{125}\text{I}\)-Ub Conjugation to Muscle Proteins—**To measure Ub conjugation in the crude muscle extracts, the dialyzed extracts (50 μl) were mixed with 30 μl of a buffer containing 20 mM Tris-HCl, pH 7.6, 20 mM KCl, 5 mM MgCl\textsubscript{2}, 1 mM DTT, 10% glycerol, 2 mM AMP-PNP, \(^{125}\text{I}\)-Ub (150,000 cpm, 5–10 μM), 20 μg/ml bestatin (to block aminopeptidases), 20 μg/ml GM132 (to block proteasomal activities), and 2 μg ubiquitin aldehyde (to inhibit the hydrolysis of ubiquitin conjugates by deubiquitinating isopeptidases (36)).

For ubiquitination assays in fraction II, which lack endogenous ubiquitin, preparations were centrifuged for 6–8 h at 100,000 × g to remove most of the proteasomes (37). The supernatant (60 μl in a volume of 25 μl) was then incubated with \(^{125}\text{I}\)-Ub (150,000 cpm, 5–10 μM) at 37 °C in a buffer containing 20 mM Tris-HCl, pH 7.4, 1 mM DTT, 5 mM MgCl\textsubscript{2}, 2 mM ATP-S, 20 μg/ml bestatin, and 5% glycerol in the absence or presence of the various inhibitors. All dipeptides and methyl esters were added at a concentration of 2 mM. All reactions were carried out in parallel under identical conditions. The reactions were incubated at 37 °C for 60 min and terminated by the addition of Laemmli sample buffer. Finally, equal amounts of \(^{125}\text{I}\)-labeled proteins were loaded onto the gel, and SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (38) on 12% acrylamide gels. The gels were then dried and autoradiographed.

**RESULTS**

**Inhibitors of E3α Reduce Breakdown of Endogenous Proteins in Muscle Extracts—**We reported previously that crude extracts of rabbit psoas muscle degrade endogenous proteins all the way to amino acids via the Ub-proteasome system as shown by the ATP-dependent generation of free tyrosine (8). Because this amino acid cannot be synthesized or degraded by muscle, its appearance indicates the degradation of cell proteins (8). This process occurred at a linear rate for 2 h at 37 °C, was stimulated 3–6-fold by ATP, and was blocked by removal or inhibition of proteasomes (8). To test whether the N-end rule pathway contributes to this degradation of endogenous proteins, various competitive inhibitors of E3α were added to these muscle extracts. The addition of arginine methyl ester reduced the ATP-dependent degradation of soluble muscle proteins by about 50% (Table I). The inhibitor of the hydroptic site of E3α, leucine methyl ester, also reduced proteolysis but to a lesser extent (30%). In contrast, alanine methyl ester, which does not inhibit E3α (24), caused little reduction (<10%) in the breakdown of muscle proteins. Similarly, the dipeptide inhibitor of E3α, Phe-Ala, also reduced ATP-stimulated degradation of endogenous proteins by 30%, but its isomer, Ala-Phe, had little effect on this process (<5% inhibition). There was also a low amount of ATP-independent proteolysis in these preparations, but it was not affected by these dipeptides or amino acid esters (data not shown). These observations suggest that a large fraction (perhaps 60–80%) of the ATP-dependent degradation of soluble proteins in crude muscle extracts involves E3α, the Ub-protein ligase of the N-end rule pathway.

A very similar effect of these inhibitors on proteolysis was seen after the extracts were fractionated by DEAE chromatography. Most cell proteins, including Ub, flow through the column (fraction I), but about 20% of cell proteins eluting with salt (fraction II) were bound to DEAE-Sepharose and eluted with high salt. This material (fraction II) contains E1, E2\textsubscript{4k}, E3α, and proteasomes (26, 39, 40). When ATP and Ub were added to fraction II, they stimulated the breakdown of endogenous proteins 3–5-fold (8), and as shown in Table I, most of the degradation of proteins in fraction II (like those in crude extracts) appears to involve E3α. This ATP- and Ub-stimulated degradation was inhibited when arginine methyl ester or Phe-Ala was added, while alanine methyl ester or Ala-Phe, which do not inhibit E3α, had very little effect (<5%) on proteolysis. In fact, the pattern of inhibition in fraction II with the dipeptide and methyl ester inhibitors of E3α was almost identical to that

\(^2\) R. Crinelli, O. V. Baboshina, and A. L. Haas, personal communication.
Evidence for a tRNA Requirement in Muscle Proteolysis—
Another type of substrate for the N-end rule pathway is proteins with acidic N-terminal residues. Genetic studies by Varshavsky (27) and biochemical studies by Ferber and Ciechanover (28) have shown that such proteins are modified before degradation by covalent linkage of an arginine residue to their N terminus. This modification requires arginyl-tRNA and their N terminus. This modification requires arginyl-tRNA and arginyl-tRNA synthetase. Typically, crude extracts produced 150 pmol of tyrosine in the absence of ATP, 550 pmol in its presence, and for fraction II, 125 pmol of tyrosine was generated in the absence of ATP and 700 pmol in the presence of ATP and Ub in 2 h. The ATP-independent proteolysis was subtracted from all samples, and the results were normalized to 100%. The data on this ATP-independent process are not shown, since the addition of inhibitors had no effect on degradation in the absence of ATP and/or Ub. For the RNase A inhibition assay, the reaction mixtures were preincubated for 30 min with 0.02 units of RNase A (from bovine pancreas) prior to the addition of ATP and Ub. The concentration of bovine pancreas RNase A used was 5 units. The data shown in this and subsequent tables were obtained in a single experiment and are the averages of triplicate determinations, which agreed within 10%. All experiments were repeated at least three times with similar results.

Table I
Inhibitors of the N-end rule pathway reduce the ATP-stimulated degradation of endogenous proteins in rabbit muscle extracts

| Inhibitors | Crude Fraction II | Inhibition of degradation |
|------------|------------------|--------------------------|
| None       | 0                 | 0                        |
| Arginine methyl ester | 50 | 45 |
| Leucine methyl ester | 30 | 25 |
| Alanine methyl ester | 10 | 5 |
| Phe-Ala     | 30                | 25                       |
| Ala-Phe     | 5                 | 5                        |
| RNase A     | 25                | NA                       |
| RNase A pretreated with RNase inhibitor | 5 | NA |
| RNase A + RNase inhibitor + tRNA | 15 | NA |
| DNase I     | 0                 | NA                       |

NA, not assayed.

As indicated, the reaction mixtures included various L-amino acid dipeptide or methyl ester inhibitors of E3α at 2 mM. To prevent hydrolysis of the added dipeptide, bestatin (20 μg/ml), an inhibitor of aminopeptidases, was added to all reaction mixtures, including controls. Bestatin alone reduced tyrosine production slightly (less than 20%). 100% proteolytic activity is the value measured in the absence of any inhibitors. Typically, crude extracts produced 150 pmol of tyrosine in the absence of ATP, 550 pmol in its presence, and for fraction II, 125 pmol of tyrosine was generated in the absence of ATP and 700 pmol in the presence of ATP and Ub in 2 h. The ATP-independent proteolysis was subtracted from all samples, and the results were normalized to 100%. The data on this ATP-independent process are not shown, since the addition of inhibitors had no effect on degradation in the absence of ATP and/or Ub. For the RNase A inhibition assay, the reaction mixtures were preincubated for 30 min with 0.02 units of RNase A (from bovine pancreas) prior to the addition of ATP and Ub. The concentration of bovine pancreas RNase A used was 5 units. The data shown in this and subsequent tables were obtained in a single experiment and are the averages of triplicate determinations, which agreed within 10%. All experiments were repeated at least three times with similar results.

Table II
Inhibitors of the N-end rule pathway reduce the ATP-stimulated degradation of endogenous proteins and 125I-lysozyme but not of 125I-lysozyme, 125I-myosin, and actin

| Inhibitors | 125I-Lysozyme | 125I-Myosin | 125I-Actin |
|------------|---------------|-------------|------------|
| None       | 0             | 0           | 0          |
| Arginine methyl ester | 50 | 60 | 10 |
| Leucine methyl ester | 30 | 15 | 7 |
| Ala-Phe     | 5             | 10          | 5          |
| RNase A     | 25            | NA          | 0          |

% inhibition of degradation.

Extracts were preincubated for 5 min at 37 °C with the various inhibitors. 125I-Labeled substrates, 1 mM ATP, and the ATP regenerating system were then added, and the incubation was continued for 2 h. Degradation of endogenous proteins and 125I-labeled myosin and actin was measured in the crude lysate as discussed in Table I. Also shown for comparison is the degradation of 125I-lysozyme measured in fraction II. Degradation of the 125I-labeled substrates was then measured with and without inhibitors. Since the various inhibitors had negligible effect on the ATP-independent degradation, these data are not shown. Without ATP, 0.5% of 125I-lysozyme, 1% of 125I-actin, and 1.5% of 125I-myosin were degraded, and with ATP, 4% of 125I-lysozyme, 4% of 125I-actin, and 4.5% of 125I-myosin were degraded.

Specificity of Dipeptide and Amino Acid Ester Inhibitors of E3α—Since the above inhibitors of the N-end rule pathway are weak substrate analogs and must therefore be utilized at relative high concentrations (24), it was important to establish that the reduction in ATP-dependent proteolysis shown in Tables I and II was actually due to inhibition of E3α and that these agents do not nonspecifically inhibit other ubiquitinating enzymes or the proteasome. We therefore compared in rabbit muscle extracts the effects of these inhibitors on the breakdown of exogenously added 125I-labeled lysozyme (a classic substrate of the N-end rule pathway that contains an N-terminal lysine) as well as 125I-myosin and actin that have N-acetylated N termini and are, therefore, not N-end rule substrates. The dipeptide and amino acid ester with basic N termini, which reduced breakdown of endogenous muscle proteins by 60%, inhibited similarly the rapid degradation of 125I-lysozyme. In muscle fraction II (Table II), as found previously in reticulocyte extracts (24), Lys-Ala and arginine methyl ester selectively inhibited the ATP-dependent degradation of 125I-lysozyme (60–70%), while Phe-Ala, alanine methyl ester, leucine methyl ester, or RNase A caused very little (0–15%) inhibition of 125I-lysozyme breakdown (see Table II). The Ub-mediated degradation of proteins with N-acetylated termini involves a different E3 and is not sensitive to inhibitors of E3α (41, 42).

Exogenous E2s and E3α Stimulate Proteolysis in Rabbit Muscle Extracts—To further evaluate the selectivity of the
These experiments utilized soluble crude extracts of rabbit skeletal muscles. Shown in the column "ATP-dependent proteolysis" are values for ATP-stimulated degradation of endogenous soluble proteins after subtracting the ATP-independent activity from total proteolysis. Typically, crude extracts in this specific experiment produced 230 pmol of tyrosine in the absence of ATP, 680 pmol in its presence (total proteolysis). "Sensitive to dipeptide inhibitors" is the amount of tyrosine generated due to protein degradation in the presence of dipeptide inhibitors (1 mM Lys-Ala and 1 mM Phe-Ala). "Not sensitive to dipeptide inhibitors" is what remains after subtracting "Not sensitive to dipeptide inhibitors" from total "ATP-dependent proteolysis." The addition of 1 mM Ala-Lys and 1 mM Ala-Phe had no effect on degradation. Also, the addition of various ubiquitinating enzymes or any inhibitors of E3α had no effect on ATP-independent degradation; therefore, these data are not shown. Concentration of various E2s was 1 μM, 2.5 μM of E2α preparation (described under "Experimental Procedures") was added to a total assay volume of 100 μl in appropriate reactions. For all other conditions, see the legend to Table I.

| Additions | ATP-dependent proteolysis | Sensitive to dipeptide inhibitors | Not sensitive to dipeptide inhibitors |
|-----------|---------------------------|----------------------------------|-------------------------------------|
| None      | 350                       | 230                              | 120                                 |
| Increase upon addition | pmol tyrosine |
| E1        | NSa                       | 100                              | 30                                  |
| E214k     | 130                       | 100                              | 30                                  |
| E2-F1     | 180                       | 30                               | 150                                 |
| UbcH5b    | 160                       | 0                                | 160                                 |
| E3α       | 310                       | 260                              | 50                                  |

a No significant stimulation of degradation was observed upon addition of E1.

dipeptide inhibitors and the involvement of E3α and E214k in the degradation of muscle proteins generally, we studied the effects of the addition of both of these enzymes as well as of two other E2s, E2-F1 and UbcH5b, on ATP-dependent breakdown of endogenous proteins. These experiments also allowed us to investigate whether the components of the N-end rule pathway might actually be rate-limiting for proteolysis in these muscle extracts. The addition of all of these Ub-conjugating enzymes stimulated the ATP-dependent degradation of endogenous proteins in crude muscle extracts without affecting ATP-independent proteolysis (Table III). When recombinant E214k (a major E2 in fraction II), E2-F1, or UbcH5b (both in fraction I) were added to crude muscle extracts at 1 μM, total ATP-dependent proteolysis was increased by more than 50% (Table III). These E2s had approximately equal activity/mol when assayed by thioester bond formation (data not shown). These findings suggest that the supply of E2s limits overall proteolysis under these conditions. Furthermore, the addition of E3α (partially purified by affinity chromatography on E214k-Affi-Gel 10) also stimulated the ATP-dependent degradation of endogenous muscle proteins by about 90% but had no effect on the ATP-independent degradation of muscle proteins. To determine to what extent E214k and E3α may be functioning together according to the N-end rule and whether UbcH5b and E2-F1 act through a distinct Ub-protein ligase, these agents were added in the presence or absence of dipeptide inhibitors of E3α (Lys-Ala and Phe-Ala). As expected, most of the E214k and E3α-stimulated proteolysis (70–80%) was sensitive to these dipeptides. By contrast, the E2-F1- and UbcH5b-stimulated protein degradation was unchanged upon the addition of these inhibitors. These experiments argue strongly that the dipeptide inhibitors reduce muscle protein breakdown by selectively blocking the N-end rule pathway and do not nonspecifically reduce Ub-dependent proteolysis in general.
TABLE IV
Effect of various inhibitors of the N-end rule pathway on the 125I-Ub conjugation to soluble proteins in rabbit and rat muscle extracts

| Additions                        | Inhibition of ubiquitination |
|---------------------------------|-----------------------------|
| None                            | 0%                          |
| Arginine methyl ester           | 40%                         |
| Leucine methyl ester            | 30%                         |
| Alanine methyl ester            | 5%                          |
| Phe-Ala                         | 25%                         |
| Ala-Phe                         | 10%                         |
| RNase A                         | 20%                         |
| RNase A pretreated with RNase inhibitor | 0% |
| DNase I                         | 5%                          |

Values shown are relative amounts of ubiquitination of endogenous proteins in the absence or presence of various inhibitors of E3a. 100% is the amount of 125I-radioactivity incorporated, in the absence of any inhibitor, into higher molecular weight forms (defined arbitrarily as 125I-Ub migration with molecular mass greater than 20 kDa). For all other conditions see the legend to Fig. 1.

In accord with our findings on protein breakdown, these experiments indicated that the various inhibitors that reduced overall ATP-dependent proteolysis in muscle fraction II did so by reducing Ub conjugation. Upon the addition of arginine methyl ester, Ub conjugation was inhibited by up to 50% and to a lesser extent by leucine methyl ester or Phe-Ala (Table IV). In contrast, alanine methyl ester or Ala-Phe, which do not inhibit E3a, reduced the ubiquitination of muscle proteins only slightly (<10%). In addition, preincubation of fraction II with RNase A markedly reduced conjugation formation, but not if the RNase inhibitor was also present (Table IV). Thus, these findings are in full agreement with the measurements of overall protein degradation (Table I) and indicate that most of the Ub conjugation to proteins in rabbit and rat muscle extracts involves E3a. Related experiments by us and others (42) in a reconstituted ubiquitination system with the model N-end rule substrate, 125I-α-lactalbumin, or with a substrate, 125I-troponin, that is ubiquitinated by a distinct Ub-protein ligase, E3L (42), also indicated that these inhibitors are highly selective in their effects. When the purified Ub carrier protein, E214k, E2-F1, or UbcH5b was incubated in the presence of Ub, E1, partially purified E3a, and 125I-α-lactalbumin, only E214k was able to support Ub conjugation, and this process was inhibited almost completely by Lys-Ala but not by Ala-Lys (data not shown). By contrast, only the addition of E2-F1 (and not E214k) could support the ubiquitination of 125I-troponin in fraction II, and this E2-F1-dependent ubiquitination of troponin was not sensitive to the dipeptide inhibitors of E3a (data not shown).

A Dominant Negative Form of E214k Inhibits Ub Conjugation in Muscle Extracts—The addition of recombinant E214k, at concentrations that stimulated overall proteolysis (Table III), enhanced Ub conjugation to endogenous proteins in the crude muscle extracts (Fig. 1, lane 3 versus lane 2). These findings suggest that E214k is rate-limiting in overall Ub conjugation in these extracts. By contrast, when a similar concentration of a mutated form of E214k, in which the active site cysteine at residue 88 (C88S) was mutated to serine (44), was added, it inhibited Ub conjugate formation (Fig. 1, lane 4). This Cys → Ser mutant forms a stable ester linkage with Ub, which cannot be transferred by E3a to a substrate (44). The effect of the dominant negative E214k was even more pronounced in rat muscle fraction II, where it inhibited Ub conjugation by over 75% (data not shown). As a control, a dominant negative form of another E2, UbcH10 (active site Cys314 mutated to Ser), which is involved in cyclin B ubiquitination at the end of mitosis (21, 45), had no effect on Ub conjugation in these extracts (Fig. 1, lane 5). At this concentration of the dominant negative E214k, Ub-thioester formation to another exogenously added E2 was not reduced; therefore, the dominant negative E214k did not deplete the system of E1 or ubiquitin. This inhibition by the dominant negative E214k but not by the dominant negative UbcH10, further demonstrates that the E214k/E3a pathway is a major contributor to the total amount of Ub conjugation in the muscle extracts.

The N-end Rule Pathway Is More Active in Skeletal Muscle than in HeLa Cells—Because these findings indicated a major role for the N-end rule pathway in protein breakdown in skeletal muscle, we tested whether this system is of equal importance in all mammalian cells. The conjugation of 125I-Ub to endogenous proteins could be readily measured in crude extracts from HeLa cells, where ubiquitination has been frequently studied (Fig. 2). Although overall Ub conjugation was

![Graph showing ubiquitination in muscle extracts](image)
markedly inhibited by Lys-Ala in the rabbit muscle extract, this dipeptide had little or no effect on Ub conjugation in HeLa cell lysate (compare lanes 3 and 4, Fig. 2, upper panel). However, HeLa cell lysates do contain the N-end rule pathway. Specifically, human $^{125}$I-$\alpha$-lactalbumin was ubiquitinated in the HeLa cell lysate, and this process was highly sensitive to the inhibitor Lys-Ala (Fig. 2, lower panel). Thus, the N-end rule pathway appears to play a much more important role in overall proteolysis in skeletal muscle than in rapidly dividing HeLa cells. Moreover, since Lys-Ala markedly affected $\alpha$-lactalbumin ubiquitination but not ubiquitination of endogenous proteins in the HeLa cells (even at 2 mM), these data are further evidence that this dipeptide selectively inhibited E3a.

**DISCUSSION**

The various observations presented here demonstrate that the ubiquitination system involving E3a and E2$^{14k}$ catalyzes the degradation of a large fraction of soluble proteins in skeletal muscle. Studies utilizing a variety of inhibitors of the N-end rule pathway (the dipeptides, amino acid esters, and the dominant negative inhibitor of E2$^{14k}$) as well as experiments in which we supplemented extracts with purified ubiquitination enzymes all clearly implicate E3a in protein degradation generally. The latter experiments also indicate that levels of E3a and E2$^{14k}$ enzymes are rate-limiting for overall protein breakdown in extracts of normal muscles. Therefore, protein substrates bearing destabilizing N-terminal residues appear to be present in these muscles, since a significant inhibition of protein degradation and ubiquitination was seen with competitive inhibitors that block selectively the binding of either type of substrate to E3a (24, 25). In fact, when two types of inhibitors, e.g. arginine methyl ester and Phe-Ala, were added together, they had additive effects on $^{125}$I-Ub conjugation and ATP-dependent proteolysis (data not shown). However, in these experiments, dipeptides or amino acid esters with basic N-terminal residues were consistently more effective inhibitors of overall proteolysis and ubiquitination than those with hydrophobic N termini, presumably because proteins with basic N termini are more abundant in muscle. However, some substrates of the N-end rule pathway (e.g. bovine $\alpha$-lactalbumin and soybean trypsin inhibitor) actually begin with acidic or amide-containing N-terminal residues and undergo tRNA-dependent modification that attaches N-terminal arginine residues (27, 31). The partial inhibition of overall proteolysis and Ub conjugation by RNase A and restoration of proteolysis by tRNA addition further suggests that some of the endogenous substrates in muscles have acidic N termini and undergo a tRNA-mediated arginylation reaction to enter this degradative pathway.

Although these dipeptide and amino acid esters are weak inhibitors that had to be utilized in the millimolar range, a variety of findings have confirmed that the large inhibition seen in these experiments is due to specific inhibition of E3a. For example, 1) the dipeptide and amino acid ester inhibitors of E3a suppressed Ub conjugation and the ATP-dependent, but not ATP-independent, degradation of endogenous proteins in these muscle extracts. 2) The isomers of these inhibitors Ala-Lys, Ala-Phe, or the analogous alanine methyl ester at similar concentrations had no significant effect on these processes. 3) These agents specifically inhibited Ub conjugation and ATP-dependent degradation of those model substrates, lysozyme and $\alpha$-lactalbumin, known to require E2$^{14k}$ and E3a but did not affect Ub-dependent breakdown of $^{125}$I-myosin or actin. 4) These inhibitors reduced the stimulation of proteolysis upon the addition of E2$^{14k}$ and E3a but not the stimulation induced by addition of other E2s (E2-F1 and UbcH5b). 5) Although overall Ub conjugation was markedly inhibited by Lys-Ala in the muscle extract, this dipeptide had no general effect on Ub conjugation to protein in HeLa cell lysate, where it did block ubiquitination of exogenously added $\alpha$-lactalbumin. 6) Furthermore, Howley and co-workers (46) have previously shown that these inhibitors of E3a do not affect p53 ubiquitination in other cell extracts, and Ciechanover et al. (42) have noted that they have no effect on E3L, another major E3. 7) Perhaps the strongest independent evidence for the importance of the N-end rule pathway in muscles comes from the experiments with the dominant negative form of E2$^{14k}$. This mutant E2, which can form an ester with ubiquitin but cannot transfer the Ub to a substrate, inhibited overall ubiquitination of muscle proteins. By contrast, the addition of an analogous dominant negative form of another E2, UbcH10, had no effect on Ub conjugation in these extracts. Thus these experiments, as well as those involving supplementation with E2$^{14k}$ and E3a, completely support the conclusion obtained with the low molecular weight inhibitors.

The presence in normal tissues of substrates for the N-end rule pathway was certainly not expected, since all cell proteins when synthesized begin with methionine, and the N termini of most cellular proteins are blocked by N-acetylation in muscles as in other eukaryotic cells. One possible trivial explanation for our findings could have been that in the course of muscle homogenization or extract preparation, there was some proteolytic cleavage of cell proteins leading to the generation of polypeptides with unnatural hydrophobic or basic N termini. To minimize this possibility, a variety of special procedures were employed: 1) extracts were prepared at 4 °C in the presence of 1% glycerol, immediately after dissection, or after quick freezing of the muscles in liquid nitrogen; 2) several different homogenization procedures were tried; 3) a number of protease inhibitors, including chymostatin and E-64 (20 $\mu$g/ml), EDTA (1 mM), and EGTA (1 mM) were included in the extraction buffer to block the activity of lysosomal, Ca$^{2+}$-activated and mast cell (chymase and tryptase) proteases. None of these procedures significantly altered the results.

The existence of large quantities of protein substrates for the N-end rule pathway in skeletal muscle (but not in HeLa cells) raises the possibility that a rate-limiting step in the degradation of the long lived muscle proteins is a slow exo- or endoproteolytic cleavage that exposes a destabilizing N-terminal residue. Such a modification in vivo could even be a regulated mechanism that triggers the acceleration of muscle proteolysis in fasting or other catabolic states (6). Our studies involving readaptation of different ubiquitination enzymes strongly argue that substrates for E2$^{14k}$ and E3a (as well as for the other ubiquitinating enzymes E2-F1 and UbcH5b) are present in excess. Therefore, their degradation may be regulated simply by changes in the levels of these various ubiquitinating enzymes. It is noteworthy that in muscle, E2$^{14k}$ is among the most abundant Ub carrier proteins. Moreover, its expression appears to rise together with expression of poly-Ub and proteasome subunits in atrophying muscles, when proteolysis (6) and Ub conjugation are enhanced. In fact, in related studies, we have found that the activity of the N-end rule pathway increases in physiological and pathological states where muscle protein breakdown rises. Thus, this ubiquitination system not only accounts for most of the breakdown of soluble proteins in extracts of normal muscle, but its activity is precisely regulated, and changes in its activity appear to account for much of the enhancement in Ub-dependent proteolysis in atrophying muscles.
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