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Protein synthesis elongation factor EF-1α is essential for ubiquitin-dependent degradation of certain N$^{\alpha}$-acetylated proteins and may be substituted for by the bacterial elongation factor EF-Tu

(proteolysis/N-terminally blocked proteins)

HEDVA GONEN*, CHRISTINE E. SMITH†, NED R. SIEGEL†, CHAIM KAHANA‡, WILLIAM C. MERRICK§, KALPANA CHAKRABURTTY¶, ALAN L. SCHWARTZ‖, AND AARON CIECHANOVER* **

*Department of Biochemistry and the Rappaport Institute for Research in the Medical Sciences, Faculty of Medicine, Technion-Israel Institute of Technology, P.O. Box 9649, Haifa 31096, Israel; †Monsanto, Inc., 700 Chesterfield Parkway North, St. Louis, MO 63198; ‡Department of Molecular Virology and Genetics, The Weizmann Institute for Science, Rehovot 71000, Israel; §Department of Biochemistry, Case Western Reserve University School of Medicine, 10900 Euclid Avenue, Cleveland, OH 44106; ¶Department of Biochemistry, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226; and ‖The Edward Mallincrodt Departments of Pediatrics and Pharmacology, Division of Hematology-Oncology, Children's Hospital and Washington University School of Medicine, #1 Children's Place, St. Louis, MO 63110

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ABSTRACT **Targeting of different cellular proteins for conjugation and subsequent degradation via the ubiquitin pathway involves diverse recognition signals and distinct enzymatic factors. A few proteins are recognized via their N-terminal amino acid residue and conjugated by a ubiquitin-protein ligase that recognizes this residue. Most substrates, including the N$^{\alpha}$-acetylated proteins that constitute the vast majority of cellular proteins, are targeted by different signals and are recognized by yet unknown ligases. We have previously shown that degradation of N-terminally blocked proteins requires a specific factor, designated FH, and that the factor acts along with the 26S protease complex to degrade ubiquitin-conjugated proteins. Here, we demonstrate that FH is the protein synthesis elongation factor EF-1α. (*a*) Partial sequence analysis reveals 100% identity to EF-1α. (*b*) Like EF-1α, FH binds to immobilized GTP (or GDP) and can be purified in one step using the corresponding nucleotide for elution. (*c*) Guanine nucleotides that bind to EF-1α protect the ubiquitin system-related activity of FH from heat inactivation, and nucleotides that do not bind do not exert this effect. (*d*) EF-Tu, the homologous bacterial elongation factor, can substitute for FH/EF-1α in the proteolytic system. This last finding is of particular interest since the ubiquitin system has not been identified in prokaryotes. The activities of both EF-1α and EF-Tu are strongly and specifically inhibited by ubiquitin-aldehyde, a specific inhibitor of ubiquitin isopeptidases. It appears, therefore, that EF-1α may be involved in releasing ubiquitin from multiubiquitin chains, thus rendering the conjugates susceptible to the action of the 26S protease complex.**

Degradation of cellular proteins via the ubiquitin pathway is initiated by the covalent conjugation of the protein substrate with multiple molecules of ubiquitin. The targeted protein is then degraded by a 26S protease complex. Little is known about the structural signals that target proteins for conjugation and subsequent degradation. A few proteins appear to be targeted via their N-terminal amino acid residues (1–6). However, a compelling body of evidence indicates that most cellular proteins are recognized via different signals that reside, most probably, in the "body" of the protein downstream from the N-terminal residue. (*i*) Approximately 80% of the cellular proteins are N$^{\alpha}$-acetylated (7). As for the remaining free-N-termini proteins, the rules that govern removal of the initiator methionine residue by methionine aminopeptidase suggest that in most cases this residue is cleaved only when the penultimate residue is a "stabilizing" amino acid (8). Thus, proteins with exposed destabilizing N-termini appear to be sparse. (*ii*) The ubiquitin system degrades N$^{\alpha}$-acetylated proteins in a process that does not require removal of the modifying group and exposure of a free N-terminal residue (9). (*iii*) The recognition of certain proteins with free N-termini (either destabilizing or stabilizing) is not dependent on the identity of their N-terminal residue (type IV substrates; ref. 3). (*iv*) Most convincing, mutational inactivation of the N-terminus-recognizing ubiquitin–protein ligase (E3α) in yeast is not lethal and does not result in a characteristic phenotype (1, 10). Neither the nature of the targeting signals nor the identity of the ligating enzymes has been elucidated.

We have previously shown that the degradation of certain N$^{\alpha}$-acetylated proteins requires a specific factor that is not required for the breakdown of free N-termini proteins. The factor, designated factor Hedva (FH), is required for the proteolysis of the core nucleosomal histone H2A, the cytoskeletal protein actin, and the lens protein α-crystallin (11). FH is a homodimer with a subunit molecular mass of 46 kDa. Initial analysis of the mechanism of action of FH revealed that it is not involved in the conjugation process. Rather, it acts along with the 26S protease complex and stimulates degradation of conjugated H2A. The effect appears to be specific to this group of proteins, as the factor is not required for the degradation of conjugates of several proteins with free N-termini, such as oxidized RNase A and lysozyme (11). Further analysis demonstrated that FH probably interacts with the conjugates prior to their degradation: incubation of conjugates in the presence of purified FH and the protease revealed a short, but significant, time lag that preceded initiation of degradation. The lag was completely abolished when FH was preincubated with the conjugates prior to the addition of the protease. These findings demonstrate that recognition of certain proteins and their targeting for degradation involves both conjugation of ubiquitin and degradation of the adducts by the 26S protease complex.

MATERIALS AND METHODS

Preparation of Ubiquitin-Conjugated Histone H2A. Multiply ubiquitinated histone H2A was prepared using ^{125}I-H2A, ubiquitin, ATP, and purified E1 (ubiquitin-activating enzyme), E2 (14-kDa ubiquitin-carrier protein or ubiquitin-conjugating enzyme), and E3α (ubiquitin–protein ligase) as described (11).

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**To whom reprint requests should be addressed.

Purification of FH, EF-1α, EF-Tu, and the 26S Protease Complex. FH and the 26S protease complex was purified as described (11). For sequence analysis, FH was further purified by using a salt gradient over CM-Sephadex (12). EF-1α from rabbit reticulocytes and from the yeast *Saccharomyces cerevisiae* were purified as described (12, 13). EF-Tu was partially purified as follows: 350 mg of protein of a high-speed supernatant of a sonicated culture of *E. coli* DH5α was loaded onto a 70-ml DEAE-cellulose (Whatman DE-52) column. Following washing of unadsorbed material, high salt eluate was prepared as described for the preparation of fraction II (11). Two hundred milligrams of the high-salt eluate was precipitated by $(NH_4)_2SO_4$ at 90% saturation, dialyzed, and resolved by gel filtration chromatography over Sephadex G-100-120 (11). Fractions containing EF-Tu (as detected by Coomassie blue staining and GTP binding; refs. 13 and 14) were pooled, and 15 mg of protein was resolved by a salt gradient using a 5/5 Mono Q FPLC anion-exchange column (Pharmacia). Fractions containing EF-Tu (4.5 mg) were pooled, concentrated, dialyzed, and loaded onto a 16/60 Superdex 200 FPLC gel filtration column. Elution was carried out in 20 mM Tris·HCl, pH 7.2/2 mM dithiothreitol/150 mM NaCl. Salt was rapidly removed by repeated concentration–dilution cycles using 20 mM Tris·HCl, pH 7.2/2 mM dithiothreitol and Centricon 30 ultrafiltration microconcentrators (Amicon).

Sequence Analysis of FH. Purified FH (20 μg, ≈400 pmol) was blotted from an SDS/10–20% polyacrylamide gel onto an Immobilon (Millipore) poly(vinylidene difluoride) membrane by the protocol of Matsudaira (15), and N-terminal sequence analysis was attempted. Failure to derive sequence information suggested N-terminal blockage. To obtain internal sequence information, peptide fragments were obtained from ≈40 μg of FH blotted onto nitrocellulose membrane for *in situ* trypsinolysis (16). Tryptic peptides released from the nitrocellulose were separated by reversed-phase HPLC using a Brownlee RP-300 Aquapore C_8 column developed by a gradient of 0–70% acetonitrile in 0.1–0.085% trifluoroacetic acid over 120 min. The tryptic peptides were collected by hand and selected peptides were subjected to N-terminal sequence analysis by automated Edman degradation chemistry on an Applied Biosystems model 470A gas-phase sequencer (17) using the standard sequencer cycle 03RPTH. The phenylthiohydantoin (PTH) amino acid derivatives were identified by reversed-phase HPLC analysis with an on-line Applied Biosystems model 120A PTH Analyzer fitted with a Brownlee 2.1-mm (internal diameter) PTH C_{18} column. Similarities between the obtained sequences and known protein sequences were investigated through computer-based searches of NBRF (National Biomedical Research Foundation) protein sequence databases, Genpept, SwissProt, PIR, SPUpdate, tfd, polu, GPUpdate protein databases, and NCBI (National Center for Biotechnology Information), using the BLAST network service (May 1993).

Effect of FH and EF-1α on the Degradation of Ubiquitin-Conjugated ^{125}I-H2A. Degradation of labeled H2A conjugate (≈1.5 × 10^4 cpm) was monitored as described (11) in 50-μl reaction mixtures containing 10 units of 26S protease complex. The mixture contained purified FH, EF-1α from yeast and rabbit reticulocytes, and EF-Tu as indicated.

GDP and GTP Affinity Purification of EF-1α/FH. One milligram of partially purified FH (Sephadex G-100; ref. 11) was applied to 0.5 ml of GDP-agarose as described (13) except that the loading buffer was 50 mM Tris·HCl, pH 8.5/2 mM dithiothreitol/10 mM $MgCl_2$/1 mM EDTA/5% (vol/vol) glycerol (buffer A). Following washing of the column and prior to elution with GDP, the unbound material was applied to the column two additional times. The column was then washed with buffer A/100 mM NH_4Cl (buffer B), and elution was carried out with buffer A/500 mM NH_4Cl/0.25 mM GDP (buffer C). The eluted fractions were concentrated and the buffer was rapidly changed to 20 mM Tris·HCl, pH 7.2/2 mM dithiothreitol/10% glycerol by using Centricon 30 microconcentrators. In a parallel purification procedure we used immobilized GTP and GTP-containing buffer C.

RESULTS

Sequence Analysis Reveals Identity Between FH and Protein Synthesis Elongation Factor EF-1α. Failure to derive sequence information from intact FH suggested that the protein had a modified N-terminal residue. To obtain internal sequence information, the protein was partially digested with trypsin and peptide fragments were obtained by HPLC. Sequence analysis of four internal peptides demonstrated 100% identity with rabbit reticulocyte EF-1α (Fig. 1). In retrospect, we realized that the two proteins have identical subunit molecular masses of 47 kDa. As EF-1α is encoded by a member of a gene family, we found that the internal sequences bear a varying degree of homology to other members of the family—the yeast factor, for example (data not shown). However, 100% identity was found only with the rabbit factor.

Yeast and Rabbit Reticulocyte EF-1α Stimulate Degradation of Ubiquitinated Histone H2A. To further corroborate that FH is indeed EF-1α, we tested the activity of purified elongation factors from reticulocytes and yeast. The two elongation factors stimulated the degradation of conjugated H2A, and their specific activity was similar to that of purified FH (Fig. 2).

FH Is a GTP-Binding Protein. Since the remote possibility still existed that FH constituted a minor contamination that copurified with EF-1α in all preparations and comigrated with it electrophoretically, it was necessary to demonstrate that the two proteins shared common biochemical properties. Mostly, we made use of the GTP-binding property of EF-1α.

As EF-1α is a GTP-binding protein, we wished to determine whether, following affinity binding and specific elution from immobilized GDP, EF-1α would demonstrate the same ubiquitin system-related activity as the enzyme purified by traditional methods. Indeed, the material eluted from immobilized GDP migrated in SDS/polyacrylamide gel identically

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Peptide 1:

                  ↓
EF-1α:       KK-171-IGYNPDTVAFVPISG-185
FH    :            -XXYNPDXVAFVPIXG-

Peptide 2:

                  ↓
EF-1α:       GR-257-VETGVLKPGMVVTFA-271
FH    :            -VETGVLKPGMVVTFA-

Peptide 3:

                  ↓
EF-1α:       VK-281-SVEMHHEALSQALPGDNV-298
FH    :            -SVEMHHEALSQALPGDNV-

Peptide 4:

                  ↓
EF-1α:       LK-383-SGDAAIVDMVPGKP-396
FH    :            -XGXAAIVDMVPGKP-
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FIG. 1. Sequence analysis of four internal tryptic peptides of FH displaying identity to EF-1α. Arrows denote trypsin cleavage sites and numbers denote amino acid residues in EF-1α. Underlined letters X denote ambiguous residues.

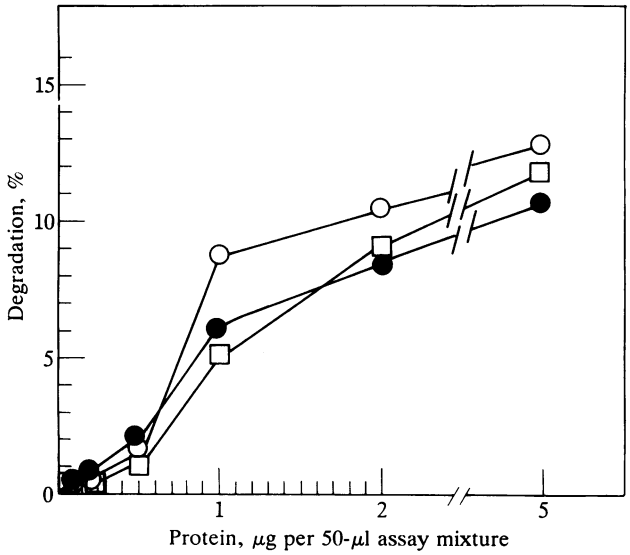

FIG. 2. Stimulation of degradation of ubiquitinated histone H2A by FH (○) and purified EF-1α from yeast (□) and rabbit reticulocytes (●).

to EF-1α that was purified by conventional methods (Fig. 3). Furthermore, the purified eluted protein stimulated degradation of ubiquitinated H2A in the presence of the 26S protease (Table 1), and its specific activity was similar (in the linear range of the reaction) to the activities of conventionally purified FH and EF-1α. Analogous results were obtained with GTP-eluted EF-1α (data not shown).

To further demonstrate that FH has a GTP-binding site, we preincubated the purified factor at 48°C for various times in the presence of various nucleotides. Only GTP, GDP, and the nonhydrolyzable analog guanosine 5′-[β,γ-imido]triphosphate, the three nucleotides that can bind to the GTP-binding site of EF-1α, protected FH from heat inactivation (Fig. 4). GMP, ATP, CTP, and UTP, which do not bind, did not protect.

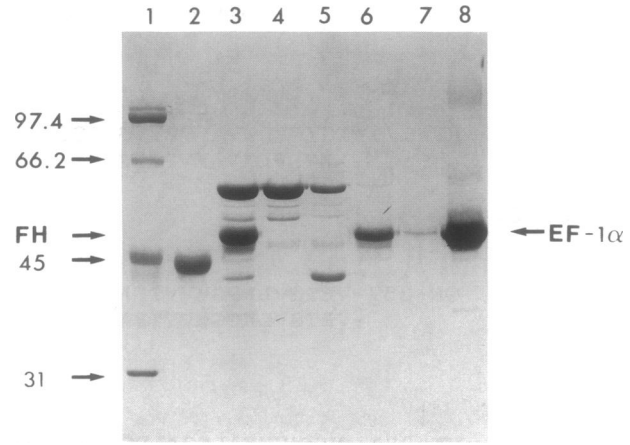

FIG. 3. Affinity chromatography-purified FH migrates identically to "conventionally" purified EF-1α in SDS/polyacrylamide gel. A crude preparation of FH was loaded onto immobilized GDP, and the specifically bound protein was eluted with a buffer containing GDP (*Materials and Methods*). Shown are comparable amounts of the different fractions. Lane 1, molecular size (kDa) markers (97.4, phosphorylase *b;* 66.2, bovine serum albumin; 45, ovalbumin; 31, carbonic anhydrase); lane 2, ovalbumin; lane 3, FH preparation prior to loading on the GDP column; lane 4, unadsorbed, low-salt (buffer A-washed) eluate; lane 5, high-salt (buffer B) eluate; lane 6, GDP (buffer C)-eluted fraction; lane 7, second elution with buffer C; lane 8, "conventionally" purified EF-1α from rabbit reticulocytes.

Table 1. Activity of conventionally purified FH and EF-1α and GDP-affinity-purified EF-1α in stimulating degradation of ubiquitin-conjugated ^{125}I-labeled histone H2A

Factor added	% degradation
FH	6.1
EF-1α	7.6
GDP-eluted EF-1α	5.7

Degradation of ubiquitin-conjugated ^{125}I-labeled H2A was carried out as described in *Materials and Methods*. The amount of FH and EF-1α used (0.75 μg per 50-μl reaction mixture) was kinetically in the linear region of the reaction. In the presence of the protease alone, degradation was 0.9%. This value was subtracted from all results.

The Bacterial Elongation Factor EF-Tu Can Substitute for EF-1α in the Proteolytic System. To finally confirm that FH is indeed EF-1α, we expressed the EF-1α human cDNA (18) in bacteria, using the pET-3a vector under the control of the T7 promoter (19). As eubacteria do not have any known active component of the ubiquitin system (see *Discussion*, however), demonstration of FH activity in bacterial extracts following expression of EF-1α cDNA would provide a strong support for the hypothesis that the two proteins are indeed identical. The expressed protein was partially soluble and was active in the proteolytic system. Surprisingly, similar activity, albeit reduced, was also detected in extracts of bacteria that were not transformed. When this activity was purified, it comigrated throughout the purification process with the bacterial elongation factor EF-Tu. In the last step in the partial purification of EF-Tu, gel filtration over a Superdex 200 FPLC column, the EF-Tu protein comigrated along with two activities: GTP-binding activity and stimulation of degradation of ubiquitinated H2A in the presence of the 26S protease complex (Fig. 5). Furthermore, homogeneous purified EF-Tu (14) also stimulated degradation of conjugated H2A in the presence of purified 26S protease complex,

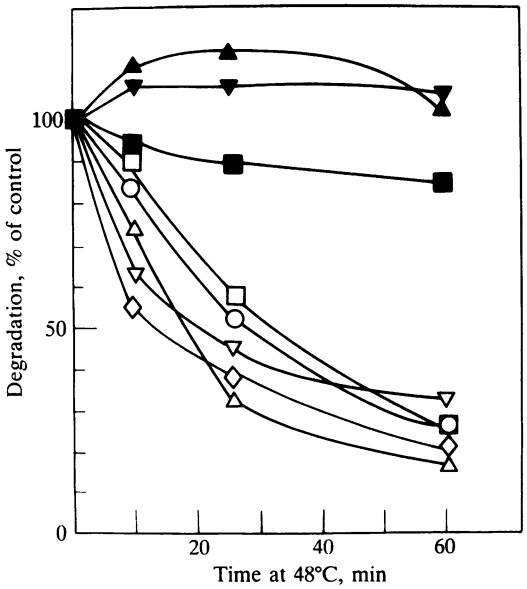

FIG. 4. FH is protected against heat inactivation by GTP, GDP, and guanosine 5′-[β,γ-imido]triphosphate. Purified FH (2 μg) was preincubated at 48°C for the indicated times in a mixture containing all the components of the final reaction mixture except for the labeled conjugates and the 26S protease complex. In addition, the preincubation mixture contained 0.2 mM GTP (▲), GDP (▼), guanosine 5′-[β,γ-imido]triphosphate (■), GMP (□), ATP (○), CTP (▽), UTP (◇), or no nucleotide (△). After preincubation, the substrate (≈2 × 10^4 cpm) and the 26S protease complex (10 units) were added. The complete reaction mixtures were incubated for 2 hr at 37°C, and degradation was monitored.

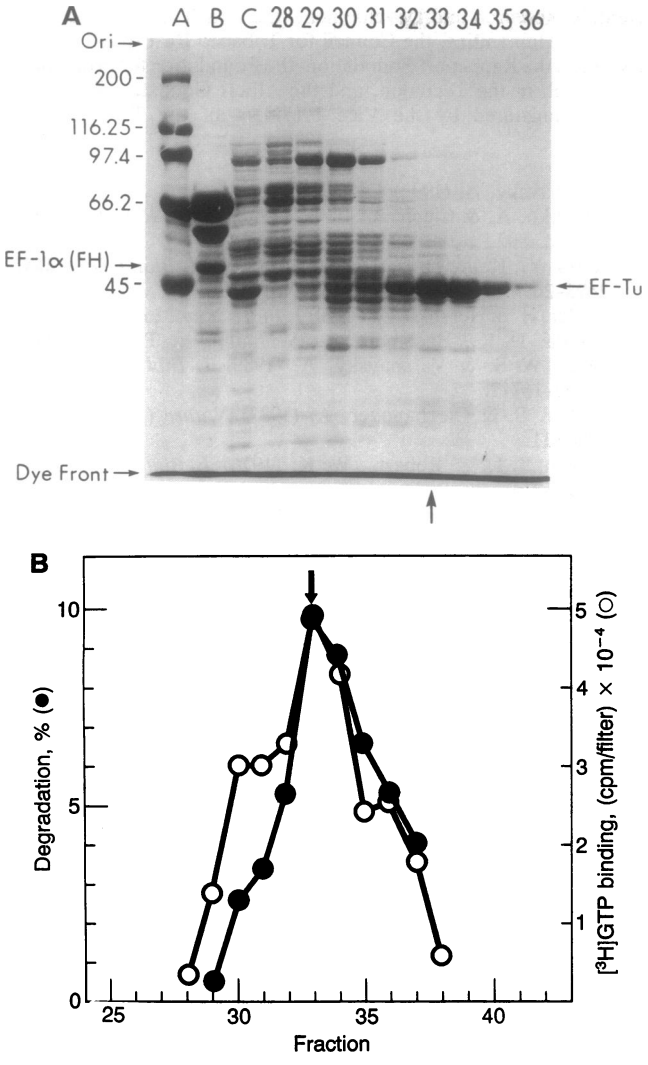

FIG. 5. Bacterial elongation factor EF-Tu copurifies with GTP-binding activity and FH-like conjugate-degradation-stimulating activity. (*A*) Gel filtration chromatography of EF-Tu: SDS/PAGE analysis. EF-Tu was purified as described under *Materials and Methods*. Mono Q-resolved protein was loaded onto a Superdex 200 FPLC column. Fractions of 2.4 ml were collected and 100-μl aliquots were resolved by SDS/10% PAGE and stained by Coomassie blue. Arrow denotes peak fraction (no. 33). Numbers above lanes denote fraction numbers. Lane A, molecular size (kDa) markers (200, myosin heavy chain; 116.25, β-galactosidase; 97.4, phosphorylase *b;* 66.2, bovine serum albumin; 45, ovalbumin); lane B, 20 μg of partially purified FH [$(NH_4)_2SO_4$ precipitation of crude reticulocyte fraction I; ref. 11]; lane C, Mono Q-resolved pooled material prior to resolution on Superdex 200. Ori, origin. (*B*) GTP-binding activity (○) and conjugate-degradation-stimulating activity (●) of EF-Tu resolved by Superdex 200 gel filtration. Ovalbumin (100 μg/ml) was added to all fractions, and the fractions were dialyzed against 20 mM Tris·HCl, pH 7.2/2 mM dithiothreitol (*Materials and Methods*). Conjugate degradation was monitored in a 25-μl reaction mixture containing 20-μl aliquots from the indicated fractions, as described (11). GTP-binding activity was determined by a filter assay with 25-μl reaction mixtures that contained 10 μl of the appropriate fractions and [³H]GTP (13).

although the preparation was only 40% as active as the mammalian factor (Table 2).

Ubiquitin-Aldehyde, a Specific Inhibitor of C-Terminal Ubiquitin Hydrolases, Inhibits FH/EF-1α-Dependent Degradation of Ubiquitinated H2A. Initial analysis of the mechanism(s) involved in the function of EF-1α in the degradation of ubiquitinated H2A revealed that an activity of a specific C-terminal ubiquitin hydrolase (isopeptidase) plays a role in the process. Ubiquitin-aldehyde, a specific inhibitor of certain isopeptidases (20), blocks almost completely the activity of both the mammalian and the bacterial factors (Table 2). Whereas FH/EF-1α can be a specific isopeptidase, it can also serve as a factor that is necessary for the activity of isopeptidase(s) contained in the 26S protease complex (see *Discussion*).

Table 2. FH, EF-1α, and EF-Tu stimulate the degradation of ubiquitinated H2A, and their activity is blocked by ubiquitin-aldehyde

Factor added	Ubiquitin-aldehyde	% degradation (% inhibition)
EF-1α	−	10.9
	+	2.9 (73)
FH	−	9.1
	+	1.7 (81)
EF-Tu	−	4.1
	+	1.1 (73)

Degradation of ubiquitin-conjugated ^{125}I-labeled H2A was carried out as for Table 1, except that 0.4 μg of the indicated factor was added per 25-μl reaction mixture. When ubiquitin-aldehyde (0.2 μg) was added, the entire reaction mixture was incubated with the inhibitor prior to the addition of the labeled conjugate.

DISCUSSION

Using several rigorous criteria, we have demonstrated that FH, a protein factor that is required for the degradation of several N^{α}-acetylated proteins, is identical to the protein synthesis elongation factor EF-1α. (*i*) Partial sequence analysis of FH reveals 100% identity to EF-1α. (*ii*) Like EF-1α, FH binds to immobilized GTP (or GDP) and can be purified in one step using the corresponding nucleotide for elution. (*iii*) Guanosine nucleotides that bind to EF-1α protect the ubiquitin system-related activity of FH from heat inactivation. In contrast, nucleotides that do not bind do not exert this effect; (*iv*) EF-Tu, the homologous bacterial elongation factor, can substitute for FH/EF-1α in the proteolytic system.

Upon analyzing the function of FH/EF-1α in the ubiquitin pathway, one faces two equally interesting and related questions: What is the mechanism of action of the protein in the proteolytic process, and why does a single protein function in two apparently opposing processes? The factor may function as an enzyme. For example, it could be a ubiquitin C-terminal hydrolase that cleaves ubiquitin moieties from certain polyubiquitin chains, thus rendering the remaining adduct more susceptible to the action of the 26S protease. Alternatively, it may serve as a chaperone that binds to the conjugate and folds it in a manner that renders it accessible to the active sites of the 26S protease complex. As for the second question, it is possible that by utilizing a single shared component, the cell can regulate protein synthesis and degradation in a rather tight manner. It is known that during rapid cell growth and division, when protein synthesis proceeds at increased rates, protein degradation is slowed down. In contrast, during conditions of stress such as starvation, protein degradation accelerates whereas proteolysis slows down. Such a regulation of the two processes could result from the predominant utilization in one process of a factor that is common to the two pathways. Mechanistically, the activity in one pathway or another could be mediated, for example, by the level of saturation of EF-1α with aminoacyl-tRNA. During phases of rapid protein synthesis, the factor is predominantly associated with charged tRNA and GTP, and the complex may be inactive in degradation. As the level of aminoacyl-tRNA decreases under stress, a large proportion of EF-1α exists in its apo form, which may be active in protein degradation but inactive in synthesis. This hypothesis can be now tested

experimentally. Indeed, initial experiments indicate that this may well be the case. (*a*) Addition of GTP slightly inhibits the activity of the EF-1α (by $\approx$30%; data not shown). The inhibition appears to be specific, as non-guanosine-based nucleotides do not affect the activity of the protein (data not shown). (*b*) Addition of RNase A or micrococcal nuclease to the reconstituted system stimulates EF-1α activity 2- to 3-fold, indicating that an RNA component may be involved in regulating the activity of this factor (data not shown). These two findings may fit well into the model that only the apoprotein is active in degradation, whereas the ternary complex of EF-1α/FH, aminoacyl-tRNA, and GTP is active during protein synthesis.

What is the physiological significance of the activity of EF-Tu? Using rigorous biochemical and molecular approaches, investigators have *not* been able to identify components or activity of the ubiquitin system in eubacteria. However, three lines of evidence indicate the existence of a "ubiquitin-like" system in prokaryotes. Tobias *et al.* (21) have demonstrated that a subset of genetically engineered species of β-galactosidase expressed in *E. coli* are targeted for degradation by the ATP-dependent Clp (Ti) protease following recognition of their N-terminal amino acid residue. Dahlmann *et al.* (22) have demonstrated a 20S proteasome complex-like structure in the archaebacterium *Thermoplasma acidophilum* (the 20S protease is the core proteolytic subunit of the 26S protease complex; ref. 2). Recently, Wolf *et al.* (23) have identified ubiquitin in *Thermoplasma*. Thus, it may well be that EF-Tu is an integral part of such a system. As bacterial proteins are not modified at their N-terminal residues, the elongation factor in these organisms may be involved in recognition of other motifs. Alternatively, it is possible that bacteria do not have a "ubiquitin-like" system and the structural component of EF-Tu that functions in the eukaryotic ubiquitin system is not functional in bacteria. It evolved late in evolution to become part of the ubiquitin system. It is clear, however, that the involvement of EF-1α and EF-Tu in the ubiquitin system cannot be attributed to some general properties of the two proteins, their pI for example. The two proteins are different from one another structurally and functionally. EF-Tu cannot substitute for the mammalian factor in cell-free translation systems. In addition, EF-1α is a basic protein and does not adsorb to anion-exchange resins even at relatively high pH. In contrast, EF-Tu has a pI of $\approx$5 and adsorbs to anion-exchange resins at neutral pH.

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