Ubiquitylation of Nascent Globin Chains in a Cell-free System*

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The ubiquitin/proteasome pathway for degradation of completed and nascent globin chains was evaluated using a cell-free in vitro coupled transcription/translation assay. No decrease in radiolabeled globin chains was observed when ubiquitin, energy regenerating source (or ATP), and E1 and E2 enzymes were added 30 min after the start of translation when globin chain synthesis had plateaued. In contrast, the addition of these components prior to the start of translation resulted in no radiolabeled globin chains after 30 min. The loss of radiolabeled globin chains was dependent on ATP concentration; the higher the concentration, the less the radiolabeled globin chains formed. Prior to the initiation of transcription/translation, cell extract was preincubated with the proteasomal inhibitor MG132 in the absence of globin chain expression vector after which ubiquitin-protein isopeptidase inhibitor, Ubal, and expression vector were added in the presence of 1.5 mM ATP. Thereafter, radiolabeled monoubiquitylated and multiubiquitylated globin chains with few unmodified globin chains were formed. Our results suggest that polyubiquitylated globin chains are localized to the polysomal fractions. These results suggest that nascent globin chains are potential targets for ubiquitylation and debubiquitylation during or soon after translation and that ATP levels play a role in the balance between polypeptide synthesis and degradation.

In general, cells have the capability of repairing errors of incorrectly folded nascent peptides or removing them during translation as well as degrading unnecessary and/or misfolded cytoplasmic proteins (1). The important factors in this process include molecular chaperones and energy-dependent proteases. Much is known regarding protein synthesis during erythropoiesis, but molecular chaperones and energy-dependent degradation including ubiquitin-mediated proteolysis of globin chains in erythroid cells are not completely understood, even though characterization of the mammalian ubiquitin-conjugating enzymes was done primarily using reticulocyte extracts (2). In fact, in human erythocyte differentiation, a combination of selective protein synthesis, degradation, and nuclear extrusion brings about extensive cellular reorganization and dramatic changes in protein composition (3). The roles of ubiquitin in protein degradation as well as some of the enzymes involved were elucidated in biochemical studies using cell-free systems (2).

A major pathway of protein breakdown involves covalent conjugation of proteolytic substrates to ubiquitin, a 76 amino acid polypeptide (4, 5). Conjugation of ubiquitin to the substrate proceeds via a three-step mechanism. Initially, ubiquitin is activated in its C-terminal Gly by the ubiquitin-activating enzyme (E1) via an ATP-dependent reaction. Following activation, one of several E2 enzymes (ubiquitin-carrier proteins or ubiquitin-conjugating enzymes) transfers ubiquitin from E1 to a member of the ubiquitin protein ligase family, E3, to which the substrate protein is bound, resulting in the formation of a polyubiquitin chain. The polyubiquitin-conjugated protein is then degraded through the 26 S ATP-dependent proteasome complex (5). The identification of specific translated proteins for degradation by the ubiquitin system appears to be based on recognition by particular E2 and E3 families of specific motifs in the targeted protein such as the N-terminal amino acid residue or certain amino acid sequences elsewhere in the protein (5, 6). Although the enzymatic mechanism by which ubiquitin is conjugated to protein has been well characterized, the process by which protein substrates, especially incompletely folded nascent polypeptide chains, are recognized co-translationally by the ubiquitin conjugation machinery is unresolved (2, 5–7). In fact, recently, Turner and Varshavsky (8) showed that the folding of nascent proteins competes with co-translational degradation via the ubiquitin conjugation pathway.

Understanding the process of degradation of excess and/or incompletely folded globin chains as well as assembly of globin chains is critical for a comprehensive analysis of formation of stable and functional hemoglobins in vivo. In addition, molecular controls of co-translational, ubiquitin-mediated degradation as well as folding/assembly of globin chains should be relevant to the etiology of the hemoglobinopathies and their therapy.

Our previous assembly studies of the formation of Hb A and Hb F using purified globin chain subunits and a cell-free coupled transcription/translation system suggested that α-chains bind to monomeric non-α-chains during or soon after translation but prior to the formation of stable homodimers (9, 10). Thus, this binding promotes the formation of functional tetrameric hemoglobin in vivo. Understanding the process of degradation of excess globin chains as well as the assembly of globin chains in vivo is critical for gene therapy for sickle cell disease, because one therapeutic scenario involves the induction of excess globin chains (11). Thus, quality control including

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degradation of free excess or misfolded single globin chains as well as proper folding and assembly of subunits to form functional hemoglobin in cells is necessary to prevent accumulation of single chains, leading to red blood cell destruction.

Previous studies showed that human α-globin was degraded by ATP-dependent proteolysis, whereas there was no ubiquitin-mediated degradation of tetrameric hemoglobin (12–14). However, in vitro energy-dependent proteolysis of purified α-globin chains was very slow (t1/2 ~ 7 h) and they were poor substrates for ubiquitylation (12). In this report, we evaluated the ubiquitin/proteasome pathway in synthesis and degradation of completed as well as nascent globin chains during translation using a coupled transcription/translation cell-free system in vitro.

EXPERIMENTAL PROCEDURES

cDNA Expression Vectors for Proteins—The plasmids pcDNA α, pcDNA β, pcDNA γ, pcDNA β-actin, pcDNA platelet factor 4, pcDNA GATA1, and pcDNA histone deacetylase contained the SP6 or T7 RNA polymerase promoter and the full-length cDNAs coding for these proteins. A commercially available wheat germ cell-free transcription/translation system obtained from Promega (Madison, WI) was used to generate radiolabeled polypeptide chains corresponding to the cDNA inserts (9). Transcription in vitro by SP6 or T7 RNA polymerase generates the mRNAs, which then are translated in the wheat germ cell-free transcription/translation system that contains 15–30 units/ml creatine kinase, 15–30 μM creatine phosphate, and ~1.5 mM ATP as an energy source as well as a concentration of 20 mM for each amino acid (with the exception of Met), 0.75 mM magnesium acetate, 90–110 mM KCl, and 5–20 mM dithiothreitol.

Expression of Proteins in a Wheat Germ Cell-free Transcription/Translation System—The expression of proteins in a cell-free coupled transcription/translation system was performed using a Tnt SP6 or T7-coupled wheat germ extract system kit (Promega) containing [35S]methionine (Amersham Biosciences). A typical 50-μl reaction containing DNA template (2 μg) in the presence of cyano-hemin (S × 10^{-6} M) (10) was incubated for 30–60 min at 30 °C. Synthesized radiolabeled proteins were analyzed by SDS-PAGE and then visualized and quantified following autoradiography using a phosphorimaging analysis system (Storm 84D) (Amersham Biosciences).

In vitro transcription/translation reactions to promote ubiquitylation were incubated in a total volume of 15.4 μl including an energy regenerating source (ERS) containing 5 mM Mg2+-ATP, creatine phosphokinase (0.5 units/ml), and creatine phosphate (10 μM) (Boston Biochem, Boston, MA). In addition, ubiquitin (1.5 mM) (Sigma), E1 and E2 enzymes (Boston Biochem, MA), ubiquitin-mediated proteolysis inhibitor MG132 (Z-Leu-Leu-Leu-CHO) (138 μM), and an inhibitor of deubiquitylation, ubiquitin aldehyde (Ub-CH) (6 μM), which inhibits some deubiquitylating enzymes (5–7), were added to some of the reactions. After incubation at 30 °C, 5 μl of electrophoresis sample buffer was added to reactions and 15 μl was used for SDS-PAGE analysis of labeled protein bands.

Antibody-mediated Isolation of Radiolabeled Bands Using Ubiquitin and β-Globin Chain Antibodies—Polyclonal antibodies for ubiquitin and β-globin chains were obtained from Calbiochem and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. For isolation of radiolabeled ubiquitylated β-globin chains using β-globin and ubiquitin antibodies, after translation, 100 μl of in vitro transcription/translation reactions was incubated for 1 h at 4 °C with 5 μg/ml rabbit polyclonal ubiquitin antibody or 10 μg/ml human β-globin antibody to form antigen-antibody complexes. Reactions were incubated with 50 μl of protein G/A-Sepharose beads (Amersham Biosciences) for 1 h at 4 °C to bind
antibody-antigen complexes. The mixture then was washed with the 500 μl of immunoprecipitating buffer (100 mM KCl, 200 mM HEPES, pH 7.5, 2 mM EDTA, 1 mM dithiothreitol, and 0.1% (v/v) Triton X-100) four times and centrifuged at 10,000 g for 30 s. The supernatants were removed, and the pellets were resuspended in 40 μl of lithium dodecyl sulfate sample buffer (Invitrogen) and then were boiled for 10 min prior to electrophoresis on 12% (w/v) SDS-PAGE gels followed by autoradiography.

**RESULTS**

**Lack of Ubiquitin-mediated Proteolytic Degradation of Radiolabeled β-Globin Chains after Synthesis in a Cell-free Transcription/Translation System**—Our previous results using a β-globin chain expression vector in the presence of hemin showed major radiolabeled bands of ~16 kDa after SDS-PAGE, which co-migrated with purified human β-globin chains, whereas the expression plasmids lacking cDNA inserts showed no bands (9). Some radiolabeled bands were observed of higher as well as lower molecular masses than ~16 kDa (Fig. 1, A and B), indicating the production of radiolabeled, ubiquitylated, and partially degraded globin chains, respectively. The degree of ubiquitin-mediated degradation of newly synthesized globin chains was assessed following the addition of ubiquitin and an ERS after radiolabeled chain synthesis had plateaued (30 min) and further translation was terminated by the addition of cycloheximide. The addition of ubiquitin, ERS, E1 and E2 enzymes resulted in the generation of radiolabeled 52-kDa bands accompanied by decreased inten-
In addition, results using an at 30°C for 5 min at 30°C were preincubated for 5 min at 30°C. Results are expressed as relative formation (%) of radiolabeled added at zero time in the cell-free coupled transcription/translation reaction. Results using 100% as the intensity value observed in the absence of Ubal (Fig. 1C). Results were the same whether or not unlabeled β-globin chains were present during translation. In addition, results using an α- or β-globin chain expression vector were the same, showing little or no ubiquitin-mediated proteolysis of previously synthesized radiolabeled globin chains. 

Ubiquitin-mediated Proteolytic Degradation of Nascent Globin Chains Made in a Cell-free System—Newly translated nascent globin chains must undergo folding, heme insertion, and assembly with partner globin chains to form functional heterodimers and tetramers of Hb (9). Proteolytic degradation of nascent globin chains was evaluated by monitoring the effects of the addition of ubiquitin, ERS, or E1 and E2 enzymes at zero time prior to the initiation of transcription/translation and following the incubation at 30°C in the cell-free system.

Radiolabeled β-globin chain levels were assessed by SDS-PAGE following 30- and 60-min incubations with 35S-labeled methionine employing the wheat germ cell-free system containing a β-globin cDNA expression vector. Autoradiographic intensities of radiolabeled β-globin chain bands following 30- and 60-min incubations were the same with or without ubiquitin addition at zero time (Fig. 2A, compare lanes 3 and 4 to lanes 1 and 2). In contrast, under the same conditions, the addition of ubiquitin and ERS at zero time resulted in no measurable ~16-kDa β-chain radiolabeled bands following 30- and 60-min incubations (Fig. 2A, lanes 5 and 6). The loss of radiolabeled bands was dependent on ERS concentration. The dilution of ERS led to higher amounts of radiolabeled globin chain bands. Furthermore, the addition of E1 and E2 enzymes to reactions containing ubiquitin and ERS showed no radiolabeled globin chain band; however, higher molecular mass bands were evident (>52 kDa) (Fig. 2A, lanes 7-10). These results of no measurable radiolabeled globin chain band during transcription/translation in the presence of ubiquitin and ERS were the same regardless of whether α-, β-, or γ-globin cDNAs were used (Fig. 2, B and C, lanes 1–6, respectively).

We next attempted to identify the component(s) in the ERS (e.g. ATP, pyrophosphatase, creatine phosphokinase, creatine phosphate, and dithiothreitol), which was responsible for inhibiting accumulation of radiolabeled bands coded by the cDNA inserts in the transcription/translation reactions. A lack of appearance of radiolabeled bands could be due to the inhibition

![Image](http://www.jbc.org/Downloaded_from.png)
of polypeptide chain synthesis or to increased degradation of newly synthesized nascent globin chains. The addition of pyrophosphatase alone did not decrease radiolabeled β-globin chain band intensity, although pyrophosphatase and ATP did decrease the band intensity. The addition of >5 mM Mg$^{2+}$-ATP (~3.5 times higher than the optimal amount required for protein synthesis) was critical for complete inhibition of radiolabeled globin chain accumulation (Fig. 3). Also noteworthy is that the addition of ERS containing 5 mM Mg$^{2+}$-ATP and ubiquitin in the presence of E1 and E2 enzymes resulted in no ~16-kDa radiolabeled band but rather in the generation of radiolabeled ~52-kDa bands (Fig. 2A, lanes 7 and 8). This finding indicates that formation of multiubiquitylated globin chains, which can become substrates for proteolysis, occurred when ERS and >5 mM Mg$^{2+}$-ATP were present. Furthermore, increasing the concentration of added ATP to 5 mM resulted in a linear decrease in the amount of radiolabeled β-globin chains. These findings suggest that the absence of radiolabeled bands when ubiquitin and ERS are added prior to the initiation of protein synthesis is attributed mainly to increased degradation of newly synthesized nascent globin chains rather than to inhibition of protein synthesis.

It is also noteworthy that translation in wheat germ extracts is known to be sensitive to ATP concentration and that either a higher or lower concentration than ~1 mM causes a significant “decrease” in radiolabeled protein synthesis (15). Such decreases in protein synthesis in the presence of high Mg$^{2+}$-ATP (e.g. 3–5 mM) may be in part the result of increased degradation as shown by our present studies. 

Effects of Proteasome and Deubiquitylation Inhibitors on Ubiquitin-mediated Proteolytic Degradation of Nascent Globin Chains in the Presence of High (5 mM) and Low (1.5 mM) ATP Levels—We attempted to clarify whether the absence of radiolabeled globin chain bands after the addition of ubiquitin and ERS prior to initiation of translation is dependent on ubiquitylation of nascent globin chains and subsequent proteolytic degradation through the 26 S proteasome. Therefore, we examined the effects of a specific proteasome inhibitor, MG132, and an inhibitor of ubiquitin-protein isopeptidase, Ubal, on radiolabeled globin chain levels. These inhibitors are known to markedly increase the accumulation of ubiquitin protein conjugates coincident with a more decreased rate of proteolytic degradation. Reactions were done in the presence of high ATP (e.g. 5 mM), which should promote degradation. Radiolabeled ~16-kDa β-globin chain bands were observed in the presence of these inhibitors after 30- and 60-min incubations in the reactions containing ERS (Fig. 2A, lanes 11 and 12). These results were the same as those when using α-cDNA expression vector (Fig. 2B, lanes 7 and 8). The addition of E1 and E2 enzymes in the presence of ubiquitin, ERS, and inhibitors prior to transcription/translation led to decreased radiolabeled intensity of the ~16-kDa β-globin chain band compared with no enzyme addition (Fig. 2A, compare lanes 9 and 10 with lanes 11 and 12). Furthermore, higher molecular mass radiolabeled bands (~52 kDa) appeared after a 30-min incubation in the presence of the enzymes, ERS, ubiquitin, and inhibitors, which may correspond to multiantibiquitylated globin chains (Fig. 2A, lane 9). In addition, preincubation of the cell extract prior to initiation of transcription/translation with MG132 in the absence of expression vector followed by the addition of Ubal and expression vector resulted in the accumulation of monoubiquitylated and multiubiquitylated globin chains. In contrast, in the absence of Ubal under the same conditions, unubiquitylated globin chains were present even after a 60-min incubation.

Ubiquitylation of synthesized globin chains was also assessed in the presence of MG132 and Ubal at low ATP (1.5 mM), which should minimize proteolytic degradation. Upon preincubation of the cell extract prior to initiation of transcription/translation with MG132 in the absence of expression vector followed by the addition of Ubal, ~1.5 mM ATP and expression vector resulted in a ladder of several high molecular mass radiolabeled bands ranging from ~22 to ~80 kDa. The ~22- and ~52-kDa bands correspond to the expected monoubiquitylated and to four ubiquitylated globin chain bands, respectively (Fig. 4, panel A, lanes 5 and 6). Decreases in the radiolabeled 16-kDa band accompanied by increases in the higher molecular mass bands were dependent on Ubal concentration with higher Ubal concentrations resulting in a decreased 16-kDa band. The intensity of the 16-kDa band decreased with increases in Ubal concentration in the presence of MG132 (Fig. 4, panel B). In contrast, without MG132 preincubation at low concentrations of Ubal, radiolabeled bands were not observed (Fig. 4, panel C, lanes 3 and 4). But at higher Ubal concentrations, a ladder of high molecular mass radiolabeled bands ranging from ~22- to ~80 kDa with no 16-kDa band was observed (Fig. 4, panel C, lanes 6 and 7). These results indicate that degradation of ubiquitylated protein is stimulated at low but inhibited at higher concentrations of Ubal as previously reported (14). Also suggested is that, under these conditions, all of the nascent globin chains are potential targets for ubiquitylation.
Identification of Radiolabeled Bands Using Ubiquitin and β-Globin Chain Antibodies—We also found endogenous “free” ubiquitin in some of the cell-free systems at −1.5 mM by immunoblot analysis using polyclonal anti-ubiquitin antibody (data not shown). Endogenous levels of ubiquitin appear sufficient for ubiquitylation of nascent globin chains without additional ubiquitin, because ERS or 5 mM ATP alone without the addition of ubiquitin gave similar SDS-PAGE results in which no radiolabeled globin chains were observed (data not shown). Documentation of ubiquitylated globin chains was shown using β-globin and ubiquitin antibody affinity purification of radiolabeled chains (Fig. 5). The β-globin antibody selectively isolated the 16-kDa non-ubiquitylated and higher molecular mass radiolabeled bands, which should include polyubiquitylated globin chain bands (lane 3), whereas ubiquitin antibody isolated higher molecular mass radiolabeled bands >16 kDa including the 22- and 52-kDa bands (lane 6). The use of non-immune serum showed no enrichment of radiolabeled bands (lanes 2 and 5).

Polysome Profiles of Reactions in the Presence and Absence of Puromycin—To determine whether ubiquitylation of nascent polypeptides could be detected on polysomes, polysome profiling was performed using sucrose density gradient analysis of the transcription/translation products after 30-min globin chain synthesis. Because puromycin releases nascent polypeptides from polysomes, we further incubated reaction products after the 30-min synthesis with or without puromycin for an additional 10 min at room temperature (Fig. 6). In the absence of puromycin, higher molecular mass radiolabeled bands were located in the polysome fractions (Fig. 6, fractions 7–11 in panels A and B), indicating the presence of polysome-bound ubiquitylated nascent globin chains. In contrast, after treatment with puromycin, almost all of the radiolabeled high molecular mass bands are no longer present on polysomes (Fig. 6, panel A’, compare fractions 7–11 in panels B and B’). These results further support our contention that polysomal-bound nascent globin chains are ubiquitylated during translation.

Proteolytic Degradation of Other Nascent Proteins in the Wheat Germ Cell-free Transcription/Translation System—To verify that proteolytic degradation via ubiquitylation of nascent polypeptides in this system was not specific for globin chains, we evaluated expression vectors coding for β-actin, which was transcribed by SP6, and GATA-1, platelet factor 4 and histone deacetylase-1, which were transcribed by T7 RNA polymerase promoters (Fig. 7). Results generated with these non-globin expression vectors in which little or no radiolabeled bands were observed in the presence of ERS and ubiquitin added prior to transcription/translation were similar to results with globin cDNA. In contrast, in the presence of ERS and MG132 and Ubal, mainly decreased levels of non-ubiquitinated full-length radiolabeled protein bands were observed, also similar to results with globin cDNA expression vectors (arrows in Fig. 6).
Ubiquitin/Proteasome Pathway of Nascent Globin Chains

Fig. 7. Proteolysis of β-actin, GATA-1, platelet factor 4, and histone deacetylase-1 in a cell-free system in the presence of ubiquitin, ERS, and proteasome inhibitors. SDS-PAGE was done followed by autoradiography of in vitro transcription/translation reactions containing β-actin (A), GATA-1 (B), platelet factor 4 (C), or histone deacetylase-1 (D) cDNA expression vectors incubated with 35S-labeled methionine for 30 min in the presence (+) or absence (−) of ubiquitin, ERS (5 mM ATP) or inhibitor (138 μM MG132 + 6 μM Ubal). Arrows indicate positions of each cDNA-coded protein.

DISCUSSION

Ubiquitylation of proteins involves covalent modification that targets proteins for degradation by the 26 S proteasome (4, 5). Substrates for degradation include potentially toxic, aggregation-prone misfolded proteins that can be synthesized de novo as a result of mutation or by errors in translation or transcription. Furthermore, nascent polypeptides emerging from the ribosome may, in the process of folding, present structural features that serve as degradation signals similar to those recognized by the ubiquitin system in misfolded or otherwise damaged proteins (8). Previous experiments bearing on co-translational degradation of nascent proteins used proteasomal and deubiquitylation inhibitors and cell-free systems targeting for degradation (8). However, our results using proteasomal and deubiquitylation inhibitors and cell-free systems suggest that nascent polypeptide chains are recognized as substrates for ubiquitylation during translation and that ATP levels could be a key determinant in the balance between protein synthesis and degradation.

Finally, our protein synthesis experiments using ubiquitin aldehyde at ATP levels required for protein synthesis (~1.5 mM) revealed that almost all of the nascent globin chains could be ubiquitylated. Even though the mechanisms of protein folding in cells are still not clear, the folding of proteins in vivo is apparently co-translational (24, 25). Furthermore, the folding of newly synthesized nascent proteins is facilitated by chaperones, which may prevent misfolding and aggregation (22). It may be difficult to identify incompletedly folded nascent polypeptides without chaperones. Because all of the newly formed globin chains in the absence of chaperones and/or partner chains may be targets for ubiquitylation during and/or soon after translation, deubiquitylation also may be critical for protein fate. This finding is consistent with our results using an inhibitor of deubiquitylation and a ubiquitin-protein isopeptide inhibitor in which the equilibrium is shifted favor the formation of the ubiquitin-conjugated form of a nascent polypeptide. Under such conditions, ubiquitylated chains might attain proper folding or chaperone interactions, which could stimulate deubiquitylation of these chains. The molecular basis for recognition of properly folded structures of nascent polypeptides in vivo is not known, but might operate at both the ubiquitylation as well as possible deubiquitylation of polypeptides during translation may be critical to protein fate.

It is known that ubiquitylation/deubiquitylation including 26 S proteasomal protein degradation is involved in multiple processes (e.g. cell cycle and division, response to stress and extracellular modulation, morphogenesis of neuronal networks, modulation of cell surface receptors, ion channels and secretory pathways, DNA repair, transcription, immune and inflammatory responses as well as biogenesis of organelles, and apoptosis) (2, 21–23). Nascent polypeptide chains might be expected to be protected from degradation in vivo, either because they are sterically shielded by chaperones or because their translation time is short compared with the time required for targeting for degradation (8). However, our results using proteasomal and deubiquitylation inhibitors and cell-free systems suggest that nascent polypeptide chains are recognized as substrates for ubiquitylation during translation and that ATP levels could be a key determinant in the balance between protein synthesis and degradation.

Fig. 7). These results suggest that co-translational protein ubiquitylation is a general finding for any protein translated in this system.
and molecular chaperones for nascent chains are needed to understand the formation of functional hemoglobin tetramers as well as the co-translational balance between synthesis and degradation for protein quality control in general.

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