Communication

Cotranslational Ubiquitination of Cystic Fibrosis Transmembrane Conductance Regulator in Vitro*

(Received for publication, December 18, 1997, and in revised form, January 27, 1998)

Sachiko Sato‡, Cristina L. Ward, and Ron R. Kopito§

From the Department of Biological Sciences, Stanford University, Stanford, California 94305-5020

Ubiquitination is a covalent protein modification that can target proteins in eukaryotic cells for degradation by the 26 S proteasome. Substrates for this degradation pathway include abnormal proteins that arise from misfolding and/or mutation. How and when the ubiquitination machinery recognizes misfolded proteins and targets them for degradation remains largely unknown. We have previously shown that cystic fibrosis transmembrane conductance regulator (CFTR), is rapidly degraded in a ubiquitin-dependent manner, whereas the nascent polypeptide chains are still elongating on ribosomes. These data establish that ubiquitination precedes the completion of full-length polypeptide chains. Moreover, ubiquitin was also found to be transferred to nascent CFTR chains while attached to ribosomes. Together, these data establish that ubiquitination, which is widely assumed to occur cotranslationally, may occur posttranslationally and suggest a role for ubiquitination early in protein biosynthesis.

Ubiquitin is a highly conserved 76-amino acid polypeptide that plays a central role in determining protein turnover (reviewed in Refs. 1–3). Cofacial attachment of ubiquitin serves as signal that targets proteins for degradation by the 26 S proteasome. Substrates for this degradation pathway include potentially toxic, aggregation-prone misfolded proteins that can be synthesized de novo as a result of mutation or of errors in translation or transcription. Although the enzymatic mechanism by which ubiquitin is conjugated to degradation substrates has been well characterized (reviewed in Ref. 4), the processes by which misfolded protein substrates are initially recognized by the Ub1 conjugation machinery remain largely unknown.

Genetic and biochemical studies suggest that some substrate specificity resides in the enzymatic machinery of the ubiquitin conjugating system, particularly the ubiquitin-conjugating enzymes (E2s) and ubiquitin-protein ligases (E3s), which function in transferring thiol-activated ubiquitin intermediates to lysine ε-amino groups on the target polypeptide (2, 3). However, attachment of a single ubiquitin is not sufficient to mark a protein for degradation (5). Efficient degradation by the 26 S proteasome appears to require the presence on the target protein of “multiubiquitin” polymers often composed of more than 20 ubiquitins (5, 6). Proteasomal degradation of a protein depends therefore not only on the presence of covalently attached ubiquitin but also on the length of the multiubiquitin chain and possibly the type of internal ubiquitin-ubiquitin linkage. An additional level of specificity is suggested by the presence in cells of multiple ubiquitin isopeptidases that appear to “edit” multiubiquitin chains and suggest that the ultimate fate of a protein may be determined by kinetic partitioning between ubiquitination and de-ubiquitination (7, 8).

Recent data implicate the ubiquitin-proteasome pathway in the degradation of misfolded integral membrane proteins (9) including CFTR (10, 11). It is well established that CFTR folding is an inefficient process in vivo (12, 13). The majority (50–75%) of newly synthesized wild type CFTR molecules are rapidly degraded by a pathway that involves both ubiquitination and proteasome activity (10, 11). The common ΔF508 mutation increases the inefficiency of CFTR folding (to greater than 99%) but does not change the kinetics or ubiquitin dependence of its degradation (12, 13). CFTR and ΔF508 degradation in vivo occur without a detectable lag following biosynthesis (12). Moreover, the apparent rate of CFTR synthesis in vivo (assessed by the incorporation of [35S]Met) is increased 2–3-fold in the presence of proteasome inhibitors. Together these data suggest that recognition of CFTR misfolding by the ubiquitin-conjugating machinery may be closely linked to translation and may not be easily reconciled with the generally held assumption that ubiquitination is a “post-translational modification.” To clarify this point, we address in this paper when nascent CFTR chains are tagged with ubiquitin by employing a cell-free system. Our data indicate that nascent CFTR polypeptides can become ubiquitinated prior to release from the translation apparatus. These data establish that ubiquitin-conjugating machinery can recognize nascent polypeptides during their translation and suggest that the destiny of nascent polypeptides may be established by ubiquitination, whereas the polypeptide chains are still elongating on ribosomes.

MATERIALS AND METHODS

In Vitro Transcription, Translation, and Immunoprecipitation—HA-CFTR and HA-ΔF508 cDNA were generated by introducing the HA epitope (YPYDVPDYA) between codons 109 and 110 of human CFTR

* This work was supported by Grant DK43994 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a Human Frontier Science Program Long-term Fellowship. Present address: Research Center for Infectious Diseases, CHUL, Laval University, Quebec G1V 4G2, Canada.

§ Established Investigator of the American Heart Association. To whom correspondence should be addressed: Dept. of Biological Sciences, Stanford University, Stanford, CA 94305-5020. Tel.: 650-723-7581; E-mail: kopito@stanford.edu.

1 The abbreviations used are: Ub, ubiquitin; CFTR, cystic fibrosis transmembrane conductance regulator; HA, influenza hemagglutinin epitope tag; RRL, rabbit reticulocyte lysate; RM, canine pancreas rough microsomes; PAGE, polyacrylamide gel electrophoresis.

2 C. L. Ward and R. R. Kopito, unpublished observations.
and ΔF508 cDNA, respectively, by polymerase chain reaction mutagenesis. RNA was transcribed from linearized HA-CFTR and HA-ΔF508 templates using T7 polymerase (Ambion Inc.). Because native CFTR contains 5’ sequences that interfere with translational initiation in vitro, the 5’ leader and the first 18 codons of CFTR were replaced with the 5’ region of a viral internal ribosomal entry sequence and an S-tag antigen (pCTE-4b; Novagen). RNA was translated in 60% RRL (Promega) supplemented with 20 μM amino acid (except Met), [35S]Met (0.4 mCi/ml), 0.75 mM magnesium acetate, 90–110 mM KCl, 2 mM dithiothreitol in the presence or the absence of canine pancreas microsomes (RM) (Promega; 2.5 A260 units) at 30 °C. Where indicated, translations were terminated by addition of RNA (following 5 min of preincubation at 30 °C) followed after 5–10 min by addition of 75 μM atracuriumbesic acid. Translation was terminated either by adding ice-cold 2× SDS-PAGE sample buffer or by dilution with ice-cold buffer A (50 mM Tris, 1 mM DTT, 2 mM puromycin (2 mM) to release the polypeptide from ribosomes together with [125I] to 4–6 × 105 cpm/g using Iodogen (Pierce) and purified by gel filtration. For in vitro ubiquitination in RRL, translation reactions (50 μl) were conducted as above (except that [35S]Met was replaced with unlabeled Met) and in the presence of [35S]Ub (2–5 × 104 cpm/μl).

Separation of Ribosome-bound Polypeptide Chains—Run-off translation of HA-CFTR RNA (truncated at codon 920) was terminated with cycloheximide (2 mM) to stabilize the ribosome nascent chain complex or puromycin (2 mM) to release the polypeptide from ribosomes by formation of an ATP depletion mixture containing hexokinase (0.4 mg/ml) and n-glucose (5 mM). Samples to which puromycin was added were further incubated at 30 °C for 5 min (for translation without RM) or 30 min (for translation with RM) as indicated. The RM fraction was sedimented as above and solubilized in 3% digitonin in buffer E (50 mM HEPES-KOH (pH 7.5), 500 mM potassium acetate, 5 mM magnesium acetate, 0.5 mM EDTA) for 30 min on ice. The solubilized fraction was pelleted by 10 min of centrifugation at 14,000 × g at 4 °C and then applied onto a 1.2-M sucrose gradient (15–50%) in 3% digitonin/buffer E and centrifuged for 90 min in TLA 100.2 at 100,000 × g. The gradient was fractionated (0.1 ml/fraction), and one-fifth of each fraction was used to monitor truncated HA-CFTR polypeptide by immunoblotting. The remainder was diluted in buffer A and immunoprecipitated with anti-HA antibody. For purification of ribosome-polypeptide in the presence of RM, translation mixture was precleared and subjected to sedimentation on a sucrose density gradient in buffer E without detergent.

RESULTS AND DISCUSSION

To monitor the fate of nascent CFTR polypeptide chains, an HA epitope tag was inserted near the N terminus of CFTR and ΔF508 (Fig. 1a). This construct, designated HA-CFTR (or HA-ΔF508) was efficiently translated in vitro in a RRL supplemented with canine pancreas microsomes (RM), giving rise to a single predominant polypeptide of molecular mass of 140 kDa (Fig. 1b) that was identified as full-length HA-CFTR because it was immunoprecipitated by antibodies both to the extreme C terminus of CFTR and to the N-terminal HA epitope. Most HA-CFTR translated in the presence of RM bound to canavalin A-Sepharose, suggesting that the glycosylated polypeptide (if not shown). Moreover, the mobility of HA-CFTR translated in the absence of RM was ~2–4 kDa lower than its mobility in the presence of RM, consistent with the presence of ~1–2 core N-linked oligosaccharide chains (Fig. 1c). Finally, HA-CFTR was not extracted from RM membranes at pH 11.5 under conditions sufficient to extract the luminal protein β-lactamase (Fig. 1d).

Together, these data demonstrate that full-length HA-CFTR is efficiently translated, glycosylated, and integrated into RM membranes in vitro and establish that this cell-free system is a suitable model for the early stages of CFTR biosynthesis.

To study ubiquitination of nascent CFTR polypeptides in vitro, HA-CFTR or HA-ΔF508 were translated in the presence of [35S]Ub, and HA-CFTR polypeptide chains were purified from the translation mixture by immunoprecipitation with anti-HA antibody. For purification of ribosome-polypeptide in the presence of RM, translation mixture was precleared and subjected to sedimentation on a sucrose density gradient in buffer E without detergent. The high molecular weight of [125I] label relative to unmodified CFTR could be due to mult ubiquitination at a single lysine or to monoubiquitination at multiple lysines, although the data do not discount the possibility that conjugation of single Ub could promote CFTR aggregation. There was no significant difference in the degree of ubiquitination between HA-CFTR and HA-ΔF508 (Fig. 2a), in agreement with previous data indicating that both ΔF508 and the ~75% of CFTR that
fails to fold are ubiquitinated in vivo (10). By contrast, significantly more label was incorporated into HA immunoprecipitates when the translation was conducted in the absence of RM, consistent with the expectation that CFTR, a hydrophilic polypeptide integral membrane protein, is likely to be severely misfolded when translated in the absence of membranes. To test if the [125I]-labeled smear associated with HA-CFTR is due to the small fraction of CFTR molecules that fail to be translocated and to become membrane integrated, we isolated RM. As expected, the majority of core-glycosylated CFTR (Fig. 2b, lower panel) together with the majority of the [125I]-labeled protein (Fig. 2b, upper panel) cosedimented with RM. When we tested for glycosylation in detergent extracts of RM from HA-CFTR-programmed lysates, we found that most of the [125I]-labeled protein was adsorbed with concanavalin A-Sepharose (data not shown). Together, these data strongly suggest that RRL can transfer Ub onto both glycosylated membrane-associated and untranslocated CFTR polypeptides.

To precisely determine the timing of ubiquitination relative to translation, we monitored the kinetics of [125I]Ub incorporation into a cohort of HA-CFTR molecules synthesized in a synchronized RRL extract. 10 min after translation was initiated by the addition of HA-CFTR mRNA, the translation reaction was synchronized with auranincarboxylic acid to prevent re-initiation (16). Full-length HA-CFTR monitored by immunoblotting (Fig. 3a, lower panel) or by [35S]Met incorporation in parallel translations (data not shown) was first detectable after 30 min, corresponding to a rate of CFTR synthesis of 50–70 amino acid residues/min, comparable with previously reported rates (17). By contrast, high molecular weight, [125I]Ubiquitinated HA-immunoreactive polypeptide was evident in the autoradiograms after 20 min (Fig. 3a, upper panel), suggesting that ubiquitination of the translating polypeptide precedes the comma translation, we monitored the kinetics of [125I]Ub incorporation (17). By contrast, high molecular weight, [125I]Ubiquitin, either in the absence (lanes 1–3) or the presence (lanes 4–6) of RM. Labeled protein was isolated from translation extracts by immunoprecipitation with anti-HA antibody, separated by 6% SDS-PAGE, and detected by autoradiography. Mobility of full-length HA-CFTR is indicated at left. b, ubiquitinated HA-CFTR is associated with microsomes. HA-CFTR was immunoprecipitated from supernatant (S) and detergent-solubilized microsome fraction (P) isolated from translation extracts conducted as described for a. Samples were analyzed for [125I]Ubiquitin incorporation by autoradiography (upper panel) or for HA-CFTR by immunoblotting (lower panel).
in fractions 7–10 were ribosome associated. Although the release of nascent chains from membrane-bound ribosomes was inefficient, when truncated HA-CFTR was translated in the presence of RM, a fraction of ubiquitinated truncated HA-CFTR chains were shifted to lighter fractions by puromycin treatment (data not shown), suggesting that nascent membrane-integrated chains that are attached to ribosomes are also modified by Ub. Taken together, these data show that ribosome-associated nascent CFTR polypeptides chains can be modified cotranslationally with ubiquitin. Protein folding is the process by which the linear information in the genetic code is converted into the three-dimensional structures of proteins. Because incorrectly folded proteins expose hydrophobic surfaces and therefore have a strong tendency to form insoluble potentially toxic aggregates, eukaryotic cells possess "quality control" mechanisms that monitor the progress of protein folding and selectively degrade misfolded proteins (reviewed in Ref. 18). Intermediates in the normal pathway of protein folding also transiently expose aggregation-prone hydrophobic surfaces; the formation of aggregated, off-pathway products in cells is minimized by sequential, transient interaction of nascent, partially folded proteins with molecular chaperones (19).

How the cellular quality control machinery discriminates between bona fide intermediates in the folding pathway and aggregation-prone products that lie off of the folding pathway is not known. Misfolded ΔF508 CFTR molecules appear to remain associated with the molecular chaperones calnexin (20) and Hsp70 (21) until they are degraded, suggesting that molecular chaperones may contribute to quality control. However, because these chaperones also lie on the folding pathway, it is unlikely that chaperone binding is the sole signal for the degradation machinery. The existence of an editing isopeptidase recently identified as a component of the 26 S proteasome (7) raises the possibility that multiquadubiquitination is reversible and that Ub-dependent proteasomal targeting is not sufficient for efficient degradation.

The data in this paper are the first evidence demonstrating that protein ubiquitination can occur while the nascent polypeptide chain is still attached to the ribosome. Cotranslational ubiquitination could occur because of early recognition of a misfolding/ubiquitination signal by the cellular quality control apparatus. Alternatively, the reversible conjugation of Ub to nascent chains might itself serve a chaperone-like role where the choice between folding and degradation would be determined by the competition between net elongation or shortening of a Ub chain. Future studies will be needed to discriminate between these two possibilities.

REFERENCES

1. Ciechanover, A. (1994) Cell 79, 13–21
2. Hochstrasser, M. (1996) Annu. Rev. Genet. 30, 405–439
3. Jentsch, S. (1992) Annu. Rev. Genet. 26, 179–207
4. Herskovic, A., and Ciechanover, A. (1992) Annu. Rev. Biochem. 61, 761–807
5. Chau, V., Tobias, J. W., Bachmair, A., Marriott, D., Ecker, D. J., Gonda, D. K., and Varshavsky, A. (1989) Science 243, 1576–1583
6. Deveraux, Q., Ustrell, V., Pickart, C., and Rechsteiner, M. (1994) J. Biol. Chem. 269, 7059–7061
7. Lam, Y. A., Xu, W., DeMartino, G. N., and Cohen, R. E. (1997) Nature 385, 737–740
8. Shaffer, J. R., and Cohen, R. E. (1996) Biochemistry 35, 10886–10893
9. Kopito, R. R. (1997) Cell 88, 427–430
10. Ward, C. L., Omura, S., and Kopito, R. R. (1995) Cell 83, 121–127
11. Jensen, T. J., Lou, M. A., Pind, S., Williams, D. B., Goldberg, A. L., and Riordan, J. R. (1995) Cell 83, 129–135
12. Ward, C. L., and Kopito, R. R. (1994) J. Biol. Chem. 269, 25710–25718
13. Lukacs, G. L., Chang, X. B., Bear, C., Kartner, N., Mohamed, A., Riordan, J. R., and Grinstein, S. (1993) J. Biol. Chem. 268, 21592–21598
14. Do, H., Falcone, D., Lin, J., Andrews, D. W., and Johnson, A. E. (1996) Cell 85, 369–378
15. Haas, A. L., and Rose, I. A. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 6845–6848
16. Stewart, M. L., Grollman, A. P., and Huang, M. T. (1971) Proc. Natl. Acad. Sci. U. S. A. 68, 97–101
17. Frydman, J., Ninmesgern, E., Ohnaka, K., and Hartl, F. U. (1994) Nature 370, 111–117
18. Hammond, C., and Helenius, A. (1995) Curr. Opin. Cell Biol. 7, 523–529
19. Hartl, F. U. (1996) Nature 381, 571–579
20. Pind, S., Riordan, J. R., and Williams, D. B. (1994) J. Biol. Chem. 269, 12784–12788
21. Yang, Y., Janich, S., Cohn, J. A., and Wilson, J. M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 9480–9484
Cotranslational Ubiquitination of Cystic Fibrosis Transmembrane Conductance Regulator *in Vitro*
Sachiko Sato, Cristina L. Ward and Ron R. Kopito

*J. Biol. Chem.* 1998, 273:7189-7192.
doi: 10.1074/jbc.273.13.7189

Access the most updated version of this article at http://www.jbc.org/content/273/13/7189

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 21 references, 8 of which can be accessed free at http://www.jbc.org/content/273/13/7189.full.html#ref-list-1