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# Multi-Ubiquitination of a Nascent Membrane Protein Produced in a Rabbit Reticulocyte Lysate[1]

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**During a large-scale *in vitro* translation analysis of a human full-length cDNA bank, we found many clones producing *in vitro* translation products showing ladder bands on a fluorogram with the equidistance of about 9 kDa at the position larger than the molecular mass expected from the open reading frame. We have analyzed a clone showing a typical pattern of the ladder bands. This clone encoded a 188-amino acid polypeptide containing a putative transmembrane domain. A green fluorescent protein-tagged polypeptide expressed in COS7 cells was localized in the endoplasmic reticulum and the Golgi apparatus. The ladder bands were observed in a rabbit reticulocyte lysate system, but not in a wheat germ extract system. Addition of the glutathione S-transferase-fused ubiquitin into the lysate caused upward shifts of the ladder bands. Addition of microsomal membranes prevented the formation of the ladder bands. Time course experiments demonstrated that the *in vitro* translation products increased in the presence of microsomal membranes, but were gradually degraded in their absence. These results suggest that the ladder formation resulted from the ubiquitination of misfolded polypeptide that failed to translocate to its proper position, and that an exclusion mechanism of misfolded membrane protein works in the rabbit reticulocyte lysate system.**

**Key words: full-length cDNA, *in vitro* translation, membrane protein, post-translational modification, ubiquitin.**

A cell-free protein synthesis system using a rabbit reticulocyte lysate is useful tool to estimate the molecular weight of protein encoded by mRNA (*1, 2*). Furthermore, because the reticulocyte lysate contains enzymes required for modifying the translated product, this system has been used to investigate various post-translational modifications of proteins: cleavage of a signal peptide from a nascent presecretory protein (*3*), translocation of a nuclear-encoded mitochondrial protein into mitochondria (*4*), the cleavage of the initiator methionine and $N^{\alpha}$-acetylation (*5*), myristylation (*6*), polyisoprenylation (*7*), phosphorylation (*8*), *O*-linked glycosylation (*9*), and so on.

We have used the rabbit reticulocyte lysate system to perform a large-scale *in vitro* translation analysis of human full-length cDNA clones to determine their molecular masses and to investigate post-translational modifications of their translated products (*10*). To date, hundreds of cDNA clones have been translated in the reticulocyte lysate. Most clones gave products of a reasonable size calculated from the deduced amino acid sequence, but some clones gave products showing unexpected band patterns on a fluorogram after SDS-polyacrylamide gel electrophoresis (*e.g.*, much larger or smaller bands than expected, and plural or smeared bands). Recently, we reported two cDNA clones showing such abnormal bands: polyubiquitin cDNA producing processed ubiquitin molecules (*11*) and a ubiquitin-like NEDD8 cDNA producing a high molecular weight conjugate (*12*).

In the present study, we analyzed a cDNA clone encoding a putative endoplasmic reticulum membrane protein, whose *in vitro* translation product showed, in addition to the main band, a ladder of bands with higher molecular weight. We demonstrate that the high molecular weight ladder-like bands are produced by the multiple conjugation of ubiquitin (Ub) to the nascent polypeptide. The appearance of ladder bands was prevented by addition of microsomal membranes in the translation reaction. These results suggest that, in the absence of its target for translocation, a nascent membrane protein would fail to fold into its active conformation, and in consequence, it would be ubiquitinated and degraded as an aberrant protein by the ubiquitin-proteasome pathway in the lysate.

## MATERIALS AND METHODS

*Materials*—A $T_NT$ coupled rabbit reticulocyte lysate system, $T_NT$ coupled wheat germ extract system and canine pancreatic microsomal membranes were obtained from

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Abbreviation: β-ME, β-mercaptoethanol; GFP, green fluorescent protein; GST, glutathione S-transferase; PBS, phosphate-buffered saline; TCA, trichloroacetic acid; Ub, ubiquitin.

Promega. L-[$^{35}$S]methionine ($^{35}$S-Met) (37 TBq/mmol), [$\alpha$-$^{32}$P]dCTP (110 TBq/mmol), and Rainbow [$^{14}$C]methylated protein molecular markers (148 kBq/ml) were purchased from Amersham Pharmacia. Synthetic oligonucleotides were obtained from Nihon Bioservice. The GST Gene Fusion System was purchased from Amersham Pharmacia. GST-fused ubiquitin (GST-Ub) was generously provided by Dr. Osaka (ERATO).

*cDNA Cloning and Sequencing*—Human fibroblast cell line HT-1080 cDNA library was described in the previous paper (*10*). The sequencing reaction was performed by the dideoxy method using a dye primer cycle sequencing kit (Applied Biosystems). The reaction mixture was electrophoresed on a model 377 automated DNA sequencer (Applied Biosystems).

*In Vitro Transcription and Translation*—*In vitro* transcription and translation were carried out using a $T_NT$ coupled rabbit reticulocyte lysate system or a $T_NT$ coupled wheat germ extract system in the presence of $^{35}$S-Met according to the manufacturer's instructions. The reaction products were separated by SDS-polyacrylamide gel electrophoresis using 10-20% polyacrylamide gradient gel (Daiichi Kagaku). The gel was fixed in 10% acetic acid-20% methanol for 1 h with gentle agitation, soaked in Amplify (Amersham-Pharmacia) for 30 min, and dried on a gel dryer. It was then exposed to X-OMAT AR Kodak film at −80°C or at room temperature for the appropriate time.

*Quantification of Radiolabeled Proteins*—Each band on a dried gel was identified by merging the developed X-ray film and the gel, and then the band was cut from the gel. An aliquot of the reaction mix was spotted on a piece of Whatman 3MM filter paper, and the total synthetic proteins were precipitated with 10% trichloroacetic acid (TCA). The radiolabeled proteins in the gel pieces or filters were determined by counting radioactivity in a TRI-CARB 2300TR liquid scintillation analyzer (Packard, USA) with Aquasol 2 universal LSC cocktail (Dupont).

*Northern Blot Analysis*—Human multiple tissue Northern blots I and II (Clontech) were hybridized with a $^{32}$P-labeled *Eco*RI-*Hin*dIII fragment of HP10122 cDNA. The fragment was labeled with [$^{32}$P]dCTP using a Ready-To-Go DNA labeling kit (Amersham Pharmacia). Pre-hybridization and hybridization were carried out using the Express Hyb Hybridization system (Clontech) at 65°C for 1 h according to the manufacturer's protocol. The filter was washed with 0.1 × SSC containing 0.1% SDS at 50°C for 1 h.

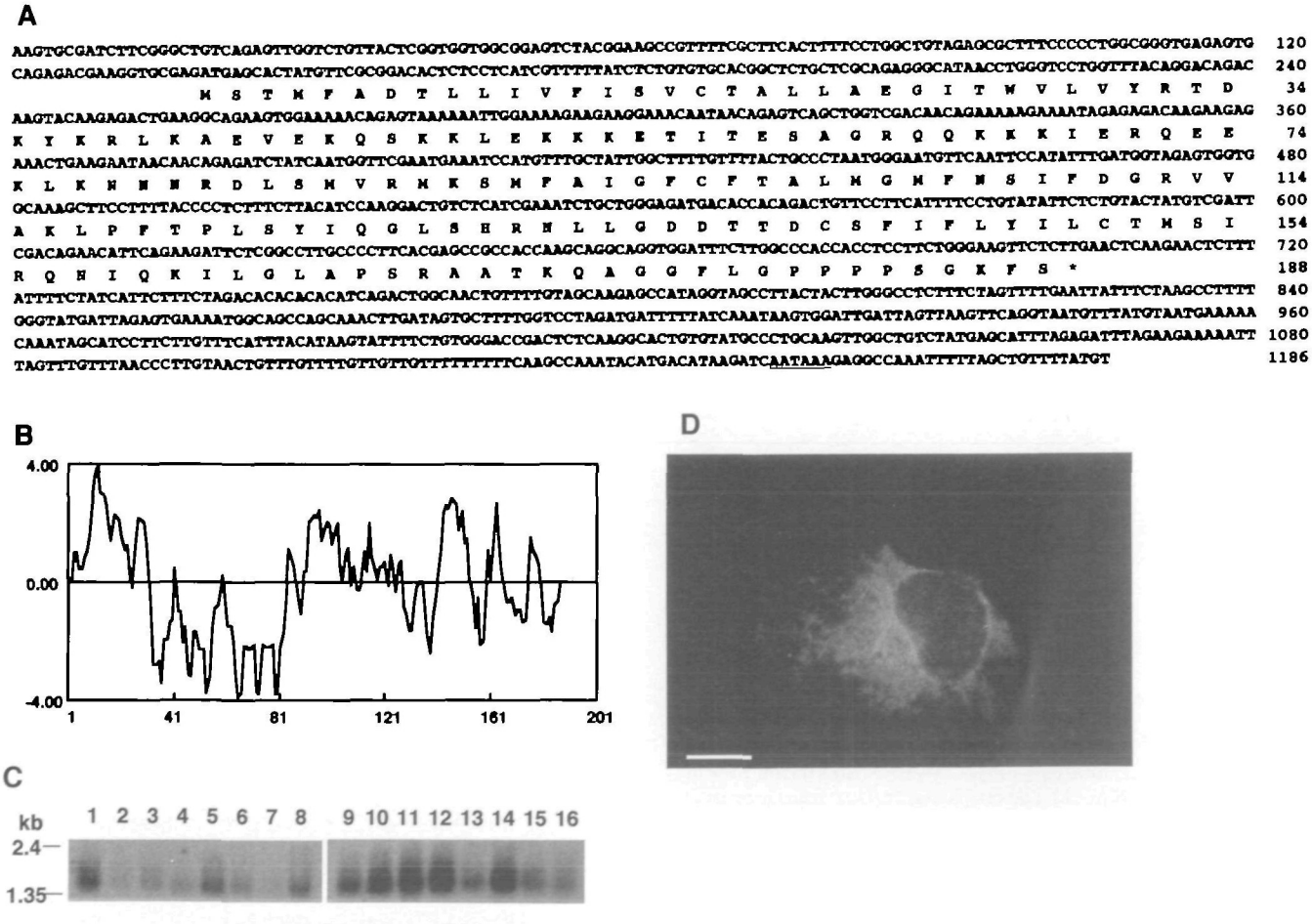


Fig. 1. **Characterization of the clone HP10122.** (A) The nucleotide sequence of the cDNA and the deduced amino acid sequence. The putative poly (A) addition signal is underlined. (B) Hydropathy plot calculated by the Kyte and Doolittle method (*18*). (C) Expression profile in adult human tissues. 1, heart; 2, brain; 3, placenta; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, pancreas; 9, spleen; 10, thymus; 11, prostate; 12, testis; 13, ovary; 14, small intestine; 15, colon; 16, peripheral blood leukocytes. (D) Localization of GFP-tagged HP10122 expressed in the COS7 cell. Scale bar = 10 $\mu$m.

The signal was detected by autoradiography.

*Construction of GFP-Tagged Polypeptides*—To produce a GFP-tagged fusion protein, the coding region of cDNA was amplified by polymerase chain reaction using a sense primer with an *Eco*RI site and an antisense primer with a *Sal*I site: 5′-ccgaattgagagaacttcccagaaggagg-3′ and 5′-ccctcgagatgagcactatgttcgcggaca-3′. The fragment digested with *Eco*RI and *Sal*I was inserted into pEGFP-$N_1$ (Clontech).

*Transfection and Microscopic Analysis*—COS7 cells were cultured in Dulbecco's modified Eagle medium (Gibco-BRL) supplemented with 10% fetal calf serum and antibiotics (50 U/ml penicillin and 50 $\mu$g/ml streptomycin, Gibco-BRL) under 5% $CO_2$ at 37°C. Cells ($3\times10^4$) plated onto a chamber of a Falcon 4-chamber culture slide (Becton Dickinson) were grown overnight and transiently transfected with 2.5 $\mu$g of the expression vector using a lipofection reagent, Transfectam™ (BIOSEPRA), according to the manufacturer's protocol. After 24 h, the cells were washed three times with ice-cold phosphate-buffered saline (PBS), then fixed in 4% paraformaldehyde in PBS for 15 min. The fixed cells were washed and mounted with Perma Fluor aqueous mounting medium (Shandon/Lipshaw). The cells were imaged with an Eclipse E800 microscope (Nikon) equipped with an MRC1000 confocal laser system (Bio-Rad).

## RESULTS

*Characterization of cDNA and Its Encoding Protein*—A full-length cDNA clone HP10122 was isolated from a human HT-1080 cDNA library. The cDNA contains a 5′-untranslated region of 138 bp, an open reading frame of 567 bp, and a 3′-untranslated region of 481 bp (Fig. 1A). The open reading frame encodes a putative polypeptide of 188 amino acids with a calculated molecular mass of 21.2 kDa. The hydropathy plot shows the existence of the hydrophobic region at positions 1-30, 90-119, and 141-154 (Fig. 1B). The N-terminal hydrophobic region was predicted to be a transmembrane helix according to SOSUI program (*13*, http://www.tuat.ac.jp/adv_sosui). The amino acid sequence showed high similarity to the African malaria mosquito hypothetical protein TU37B2 (GenBank accession number AF042732) with 74.9% identity and the *Caenorhabditis elegans* hypothetical protein F22B5.10. (EMBL accession number Z50044) with 62.0% identity. Recently, an almost identical cDNA that was shorter than our clone by 11 bp of the 5′-terminal sequence was deposited as clone 24483 unknown mRNA in the GenBank (accession number AF070626).

Northern blot analysis was performed to discover the expression pattern of mRNA in various human tissues. The HP10122 mRNA was detected as a single band of 1.5 kb in all tissues but with different expression levels: abundant in the thymus, prostate, testis and small intestine, while scarce in the brain, placenta, lung and kidney (Fig. 1C).

To study the subcellular localization of the protein encoded by the HP10122 cDNA, a GFP-tagged protein expression vector was constructed and transfected into COS7 cells. After 24 h of incubation, the COS7 cells expressing the fusion protein were fixed and their fluorescence was imaged by use of a fluorescent microscope equipped with a confocal laser system. The protein was detected in the ER and the Golgi apparatus surrounding the nucleus (Fig. 1D).

*Formation of a Ladder-Band Pattern by In Vitro Translation*—The cDNA clone was transcribed and trans-



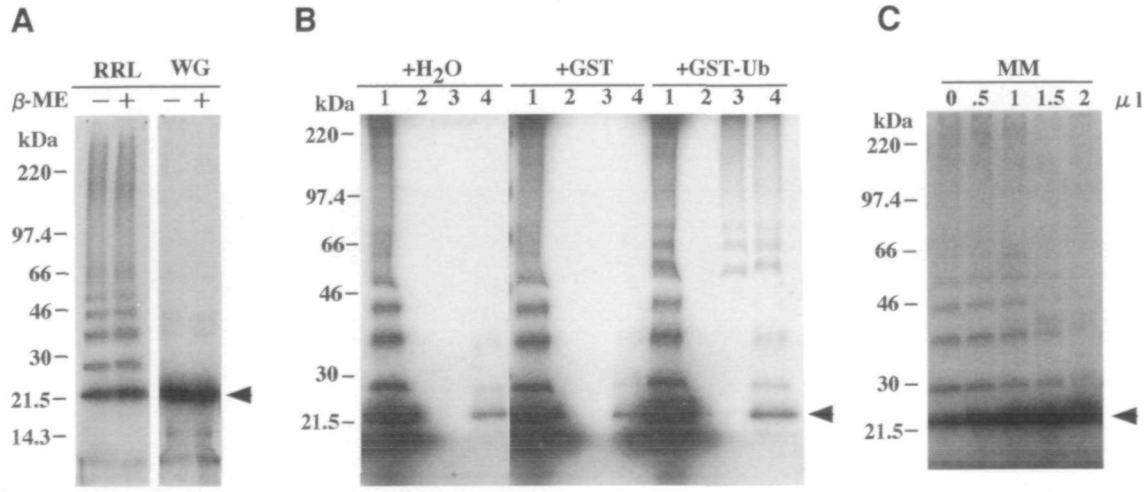


Fig. 2. **SDS-PAGE analysis of the *in vitro* translation product of the HP10122 cDNA.** (A) The cDNA was *in vitro* translated in the T7T coupled rabbit reticulocyte lysate (RRL) or wheat germ extract (WG) system for 90 min at 30 or 25°C, respectively. Ten microliters of the reaction solution contained 0.5 $\mu$g of cDNA, 0.7 $\mu$l of $^{35}$S-Met, 4 $\mu$l of the RRL or the WG, 20 nM essential amino acids (Met free), and 3.3 U of T7 RNA polymerase. The translation product was separated by 10-20% SDS-PAGE with (+) or without (−) $\beta$-ME. (B) The band-shift induced by addition of GST-Ub. One microgram of GST or GST-Ub was added to 10 $\mu$l of *in vitro* translation solution prior to the reaction. $H_2O$ was used as negative control. All other translation conditions were the same as in (A). Half of the total product was incubated with anti-GST antibody or glutathione-Sepharose 4B at 4°C for 1 h to affinity-purify the GST-ubiquitinated proteins. The immunoprecipitate with anti-GST was purified by use of Protein A-beads. The entire matrix was washed and the adsorbed proteins were eluted with Laemli's SDS-PAGE sample loading buffer. 1, original translation product; 2, pre-immune rabbit IgG-binding proteins; 3, anti-GST IgG-binding proteins; 4, glutathione-Sepharose 4B-binding proteins. (C) Inhibition of the Ub-conjugation by adding canine pancreatic microsomal membrane (MM) into the RRL. The amount of MM indicated at the top was added to the translation solution prior to the reaction. All other translation conditions were the same as in (A).

lated *in vitro* using a $T_NT$ coupled rabbit reticulocyte lysate system or a $T_NT$ coupled wheat germ extract system. The *in vitro* translation products were separated on 10-20% polyacrylamide gel in the presence or absence of $\beta$-mercaptoethanol ($\beta$-ME) (Fig. 2A). In the wheat germ system, the translation products of the HP10122 cDNA appeared on a fluorogram as a single main band with a molecular mass of 23 kDa, which is similar to that predicted from the deduced amino acid sequence. The main band of the product in the reticulocyte system was observed at the same size as that in the wheat germ system, but several extra ladder bands and smear bands with higher molecular masses than the main band emerged (Fig. 2A). The molecular masses of the ladder bands were 28, 35, 44, 53, and 64 kDa. The difference in molecular mass between the ladder bands was 5-11 kDa. The addition of $\beta$-ME or DNase or RNase treatment had no obvious effects on the ladder pattern, suggesting that a peptide of about 9 kDa in the lysate was covalently bound successively to the nascent *in vitro* translation product during the reaction. These results are suggestive of ubiquitination, because the size of the band-shift is similar to the molecular mass of the Ub molecule, and multi-ubiquitination of exogenous proteins has been reported to occur in the rabbit reticulocyte (*14*).

*Band-Shift by Addition of GST-Ub*—To examine whether the ladder bands were caused by conjugation of Ub to the *in vitro* translated protein, GST-Ub was added to the *in vitro* translation reaction mixture. The addition of GST-Ub but not GST caused the appearance of several new bands with high molecular masses. These additional bands were shown to contain GST-Ub by the fact that they could be purified by immunoprecipitation using anti-GST antibody or by use of glutathione-Sepharose 4B beads (Fig. 2B). The molecular masses of the new bands were 60, 67, and 76 kDa. The smallest of them corresponded in size to the conjugate between the main translation product (23 kDa) and GST-Ub (approximately 36 kDa), suggesting that GST-Ub bound to the translated protein instead of the endogenous Ub to induce a 36-kDa upward shift of the main band. The remaining ladder bands correspond in size to conjugates between multi-ubiquitinated proteins and Ub-GST.

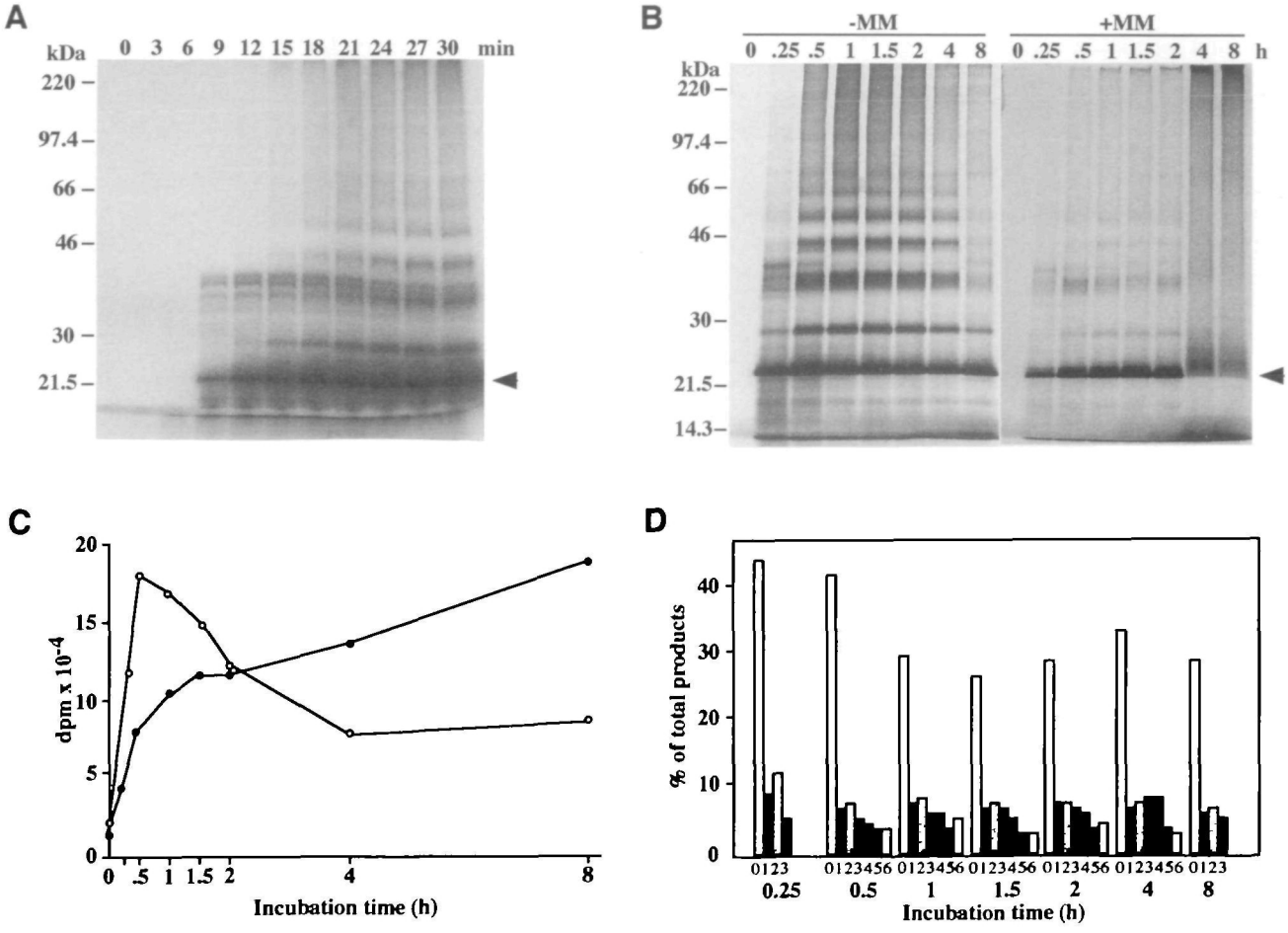


Fig. 3. **The time courses of the ladder pattern and the total amount of the translation product of clone HP10122.** The *in vitro* translation was carried out at 30°C in 50 $\mu$l of reaction mixture in the absence of microsomal membranes (A) or in 25 $\mu$l of reaction mixture with or without addition of 2 $\mu$l of microsomal membranes (B). A 2-$\mu$l aliquot was taken from the reaction mixture at the time indicated at the top, and the reaction was stopped by the addition of Laemli's sample buffer. The products were separated by 10-20% SDS-PAGE under reducing conditions. (C) A 2-$\mu$l aliquot of each sample in (B) was spotted on a piece of filter paper. After fixing in boiling 10% TCA and washing with acetone and ethanol, its radioactivity was measured in a scintillation counter. (D) The proportion of each band to the total amount of *in vitro* translation product at various incubation times is shown by a bar, below which is indicated the number of Ub molecules conjugated to the product.

*Inhibition of the Ladder-Band Formation by Microsomal Membranes*—Localization analysis indicated that the protein encoded by HP10122 is transported into the ER or the Golgi from the cytosol after translation and stays there as a resident protein. Addition of canine pancreatic microsomal membranes has been used to examine this kind of translocation in an *in vitro* translation system (*15*). Addition of microsomal membranes to the *in vitro* translation mixture inhibited the ubiquitination of the protein dose-dependently (Fig. 2C). These results suggest that the *in vitro* translated product is transported into microsomes before undergoing ubiquitination. Alternatively, the disappearance of the ladder bands may be explained by degradation by a proteasome included in the microsomal membrane fraction or by inhibition of multi-ubiquitination by another factor included in the fraction. These possibilities, however, were excluded by the fact that addition of the microsomal membranes did not affect the ladder- and smear-band pattern observed in the *in vitro* transcription/translation system of polyubiquitin cDNA (data not shown), which might result from multi-ubiquitination and polyubiquitination of reticulocyte lysate-derived proteins as described in the previous paper (*11*).

*Time-Course of Multiple-Band Formation*—Figure 3A shows band formation at an early stage of *in vitro* translation of the HP10122 cDNA. Several bands of 33-40 kDa appeared after 9 min, and thereafter the production of mono-ubiquitinated products gradually increased. Figure 3B shows long-range time courses of the ladder-band formation in the presence or absence of microsomal membranes. In the absence of the membranes, the ladder bands increased and high molecular weight smear bands appeared, but after 4 h, both types of bands weakened. In the presence of the membranes, the formation of ladder bands was inhibited, and after 4 h, high molecular weight products incapable of penetrating into the gel were observed. It is noted that some bands around 35 kDa observed at an early stage were still present after 30 min.

Figure 3C shows the time course of the total amount of synthesized proteins measured by a TCA precipitation method. In the absence of microsomal membranes, the products sharply increased during the initial 30 min, then gradually decreased. This result is consistent with the disappearance of the ladder and high molecular weight bands shown in Fig. 3B, suggesting that polyubiquitinated proteins were degraded by proteasomes contained in the lysate. In the presence of the membranes, the products increased with incubation time, but the initial rate of increase was slow compared with that in the absence of the membranes.

Figure 3D shows the molecular ratio of multiply ubiquitinated products at various incubation times. The main band accounted for more than 40% of the total products translated during the initial 30 min and gradually decreased to about 30%. The proportion of multiply ubiquitinated products with fewer than 7 Ub molecules remained almost constant (less than 10%) throughout the incubation time. This result suggests that the polyubiquitinated products were preferentially recognized as a target for degradation by proteasome.

## DISCUSSION

The ladder-band formation of the *in vitro* translated product was shown to result from the multi-ubiquitination of the nascent translation product that failed to translocate to the membrane, on the basis of the following results: (i) the difference in molecular mass between the ladder bands is similar to the molecular mass of Ub; (ii) addition of GST-Ub fusion protein caused upward shifts of the ladder bands corresponding to the molecular mass of GST-Ub; (iii) addition of microsomal membranes prevented the ladder formation; (iv) the ladder and smear bands gradually decreased by degradation, in the same way that multi-ubiquitinated proteins are degraded by proteasome in the lysate. It has been reported that exogenous substrates such as lysozyme, globin, and lactalbumin are multi-ubiquitinated in the reticulocyte lysate (*14*) and degraded by proteasomes (*16*), but there has been no report of the multi-ubiquitination of the nascent protein produced in the *in vitro* translation system.

Our results suggest that the multi-ubiquitination occurs cotranslationally and that several proteins might strongly interact with translated products prior to mono-ubiquitination. These interacting proteins may act as sensor proteins to detect the abnormal protein and mediate the ubiquitination. Usually the nascent protein is bound with chaperons to form a proper folding structure (*17*). It will be interesting to investigate the difference between the chaperon for folding and the protein for sensing the misfolded protein. Analysis of the interacting proteins at the early stage of *in vitro* translation may provide useful information to elucidate the recognition mechanism of abnormal proteins. The multi-ubiquitination occurred only slightly in the wheat germ extract. This may be due to the lack of interacting proteins such as chaperons and/or ubiquitin-conjugating enzymes in the wheat germ extract.

We found many other clones in our full-length cDNA bank whose translation products showed the ladder pattern. A common feature of the proteins encoded by these cDNAs is the presence of at least one putative transmembrane domain, like the protein described in this study. Membrane proteins, however, did not always exhibit the ladder pattern. Most membrane proteins show high molecular weight smear bands rather than a clear discrete band in the absence of microsomal membranes. The pattern may depend on the molecular size, the hydrophobicity of the product, and/or the presence of interacting substances.

The question remains whether different sites of the *in vitro* translation product were mono-ubiquitinated or one site had a single multi-ubiquitin chain. Site-directed mutagenesis of the target clone in combination with the present system may allow determination of the ubiquitination site. In this way, the ubiquitination system using the *in vitro* translation system offers a potentially useful tool to investigate the mechanism of Ub-conjugation.

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