Membrane perturbing factor in reticulocyte lysate, which is transiently activated by proteases

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Abstract Proteases have been used to examine the topology of proteins on various membranes. We reexamined the conditions of protease treatment for rough microsomal membranes and found that proteinase K degraded the lumenal proteins in the presence of reticulocyte lysate. The lysate treated with either heat or *N*-ethylmaleimide no longer promoted the degradation. The reticulocyte dependent degradation was also observed with papain, trypsin, and elastase. This activity was transiently generated by treating reticulocyte lysate short-term with trypsin. We thus concluded that a membrane perturbing factor(s) must exist in reticulocyte which is transiently activated by protease treatment.

Key words: Membrane topology; Protease treatment; Membrane degradation

1. Introduction

Protease treatment is one of the useful criteria for the sequestration of proteins in biomembranes [1]. For instance, when the precursor of secretory protein was synthesized in a cell free system including rough microsomal membranes (RM), the mature protein which was translocated into the lumenal space was resistant to proteases, while the precursor form with signal peptide was readily degraded [2]. In the case of integral membrane proteins, only the domains or loops exposed on the cytoplasmic side were accessible to the externally added proteases [3,4]. Pore forming protein which showed a β-barrel structure was also protease resistant when correctly integrated in the membrane [5,6]. Such an assay supposes that the segment of the proteins within either the membrane or the lumenal space should not be accessible to proteases if the membrane structure is kept intact.

In our ongoing study of the topogenesis of membrane proteins on the endoplasmic reticulum membrane (e.g. [7,8]), we found that the secretory proteins synthesized and translocated across the membrane in the reticulocyte lysate (RL) cell free system were readily degraded by proteinase treatment at room temperature. In this study we examined the effect of proteases on the lumenal proteins of RM and demonstrated that protein factor in RL was transiently activated by a low concentration of proteases and to make lumenal proteins accessible to the externally added proteases.

2. Materials and methods

2.1. Materials

Proteinase K (Merk), trypsin (Sigma), papain (Sigma), elastase (Sigma), and trypsin inhibitor (Sigma) were obtained from the indicated sources. Rabbit RL [9] and RM from dog pancreas [10] were prepared as previously described. RM was washed with 25 mM EDTA and treated with staphylococcal nuclease to remove endogenous mRNAs as previously described [10].

2.2. Assay for in vitro degradation of mature prolactin

cDNA encoding preprolactin (pPL) was subcloned between *Nco*I and *Xba*I of pCITE2c (Novagen). The plasmid was linearized by *Xba*I and transcribed by T7 RNA polymerase. The RNA was translated in an RL cell free system (15 μl containing 27% RL (24 mg/ml)) in the presence or absence of RM (1.4 μg/15 μl) at 30°C for 40 min as described previously [6]. The translation reaction was terminated by 2 mM puromycin and then further incubated at 30°C for 10 min. The translation mixture was directly subjected to protease treatment.

Where indicated, the membrane vesicles were recovered from the translation reaction mixture through the sucrose cushion (containing 0.5 M sucrose, 50 mM HEPES/KOH (pH 7.5), 100 mM KCl, 5 mM MgoAc, and 1 mM DTT) by ultracentrifugation at 100 500×*g* for 15 min. RM was resuspended in a small volume of buffer B (containing 0.25 M sucrose, 50 mM triethanol amine (pH 7.4), and 1 mM DTT) and frozen immediately by liquid nitrogen.

The reaction mixture (15 μl) for the degradation assay contained the resuspended RM (1.4 μg), 37.5 mM HEPES/KOH (pH 7.5), 2.5 mM DTT, 138 mM KCl, 2.1 mM MgoAc, and various amounts of RL and proteases. After incubation at 30°C for 20 min, the reaction was terminated with trichloroacetic acid and the protein precipitates were analyzed by SDS-PAGE. The radioactive bands of proteins were visualized and quantitated by an image analyzer (Fuji). Endogenous proteins in the lumenal space, BiP and protein disulfide isomerase (PDI) were detected by immunoblotting using anti-KDEL monoclonal antibody (StressGen).

3. Results

3.1. Treatment with low concentration proteinase K resulted in a loss of mature secretory protein

We have often used proteinase K to examine the membrane topology of in vitro synthesized proteins. To establish the appropriate conditions for the protease treatment of membranes in a cell free assay system, we examined the effect of the protease concentration on the integrity of in vitro synthesized proteins (Fig. 1). Bovine preprolactin (pPL) was synthesized in the absence or presence of rough microsomal membranes (RM) in a rabbit RL cell free system. When synthesized in the absence of RM, a single band corresponding to the precursor form which possessed signal peptide was observed (Fig. 1A, lane 1). Upon synthesis in the presence of RM, mPL was newly detected in addition to the precursor form (lane 2). Aliquots of the translation mixture were treated

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Abbreviations: mPL, mature prolactin; PDI, protein disulfide isomerase; pPL, preprolactin; RL, reticulocyte lysate; RM, rough microsomal membrane

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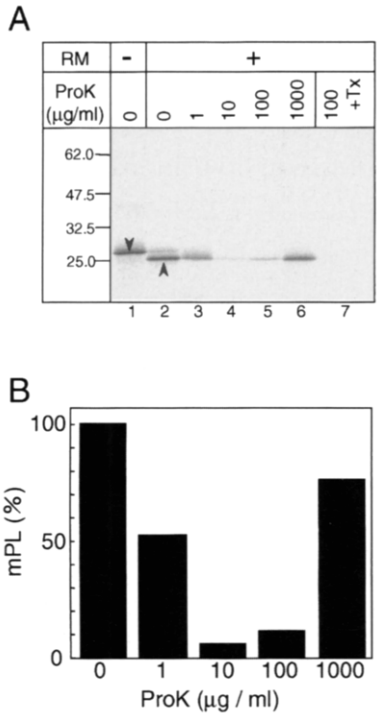

Fig. 1. Low concentration proteinase K degraded mPL in the ER lumen. A: In vitro synthesis of prolactin and proteinase K treatment. mRNA encoding bovine pPL was translated in the absence (−) or presence (+) of RM with RL system. After the translation reaction at 30°C for 40 min, aliquots (15 μl) of translation mixture were treated with proteinase K at various concentrations (0–1000 μg/ml) for 20 min at 30°C. In the experiment for lane 7, proteinase K treatment was carried out in the presence of 1% Tx-100. After the proteinase K treatment, the proteins were precipitated by trichloroacetic acid and analyzed by SDS-PAGE and a subsequent image analysis. The bands of pPL and mPL are indicated by downward and upward arrow heads, respectively. B: Quantitation of the remaining mature prolactin by an image analysis.

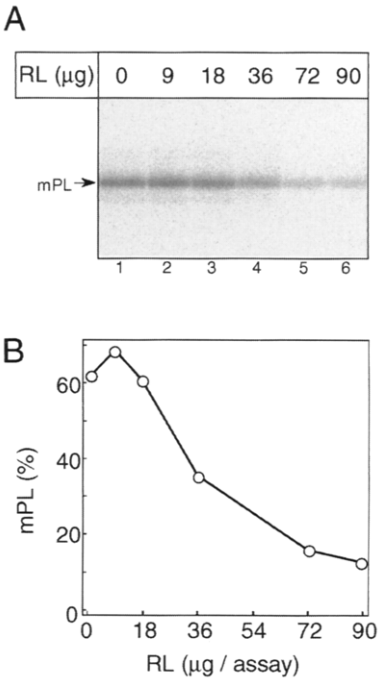

Fig. 2. Reticulocyte lysate was required for the degradation of mPL. A: After the in vitro translation reaction, RM was isolated by ultracentrifugation and then resuspended in reaction buffer (15 μl) for the degradation assay. The membranes in the reaction mixture were subjected to proteinase K (20 μg/ml) treatment in the presence of the indicated amounts of RL. B: Quantitation of the remaining mature prolactin in each reaction.

with various amounts of proteinase K at 30°C (lanes 3–6). The precursor form was readily degraded. To our surprise, the mature form was also degraded at low concentrations of proteinase K (10–100 μg/ml) (lanes 4 and 5), whereas it was rather resistant against higher concentration (1000 μg/ml) of the protease (lane 6). The mature form was completely degraded when the membrane was solubilized by non-denaturing detergent, Tx-100 (lane 7).

3.2. *The degradation of mature prolactin was dependent on reticulocyte lysate*

To examine the requirements for the degradation of mPL by proteinase K at low concentrations, the membrane was isolated by ultracentrifugation after in vitro protein synthesis. When the membrane was treated with the proteinase K (20 μg/ml) in the presence of less than 18 μg/ml RL, no RL dependent degradation was observed (Fig. 2, lanes 1–3). Efficient degradation was observed at higher doses of RL (lanes 4–6, and B), thus indicating that some factors exist in RL, which made the mPL accessible to the externally added proteinase K. As noted in panel B, the dose curve seems to be saturable, and higher dose (more than 90 μg/assay) of the lysate did no longer improve the degradation efficiency (data not shown). Such an RL dependent degradation was not observed with protease treatment at 0°C. Furthermore, the activity diminished by incubation at temperatures higher than 60°C or treatment with *N*-ethylmaleimide (data not shown), indicating that proteinaceous factor in the RL promoted the degradation of the mPL in the lumen. ATP and other nucleotide triphosphates were not required and apyrase treatment to remove nucleotide triphosphates from the reaction mixture did not have any effect either.

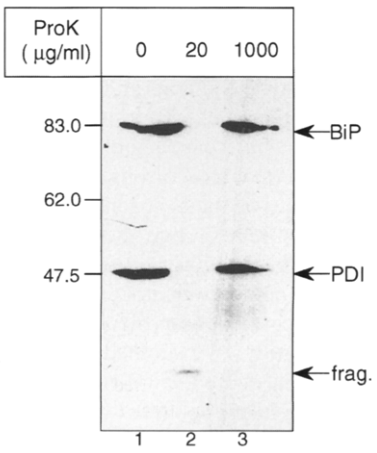

Fig. 3. RL mediated degradation of the endogenous proteins of RM. RM (7.0 μg/50 μl reaction) was subjected to a degradation assay using RL (6 mg/ml) and the indicated amount of proteinase K. After incubation at 30°C for 20 min, proteins were analyzed by SDS-PAGE and the subsequent immunoblotting with anti-KDEL antibody. Protein bands were visualized with chemiluminescence reagent (ECL, Amersham) by exposing for 2 min. Degraded fragment was indicated (frag.).

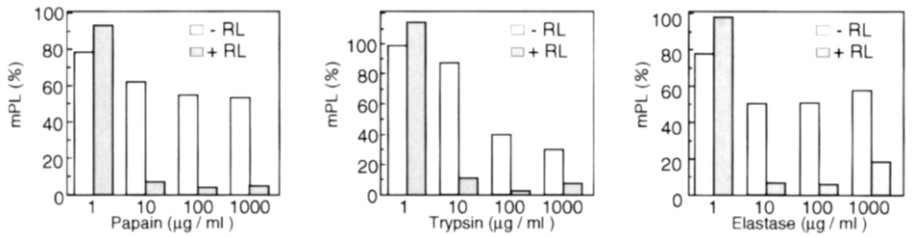

Fig. 4. RL induced degradation of lumenal proteins by other proteases. Papain, trypsin, and elastase of indicated concentrations were used instead of proteinase K in the degradation assay. The assay was carried out in either the presence (+) or the absence (−) of RL (6.0 mg/ml). Other procedures were the same as in Fig. 2. The quantity of the remaining mPL was determined by an image analysis.

The cytoplasmic fraction of the rat liver did not mediate the degradation of the lumenal proteins. When several lots of RL were tested, the degradation activity correlated with the activity of cell free protein synthesis: the RL lot with a higher translation activity showed a higher membrane perturbing effect. Hereafter, we denote the factor which mediates the degradation of the lumenal protein 'RL-factor'.

3.3. The endogenous lumenal proteins were also degraded

To examine the fate of the endogenous lumenal proteins, RM treated with proteinase K was probed by immunoblotting using anti-KDEL monoclonal antibody (Fig. 3). When RM was treated with proteinase K at the lower concentration (20 μg/ml), BiP and PDI, which are both lumenal soluble proteins of ER, were degraded (lane 2), whereas the proteinase K at the higher concentration (1000 μg/ml) did not degrade them at all (lane 3). This observation indicated that the externally added proteinase K degraded not only the newly synthesized mPL but also endogenous lumenal proteins.

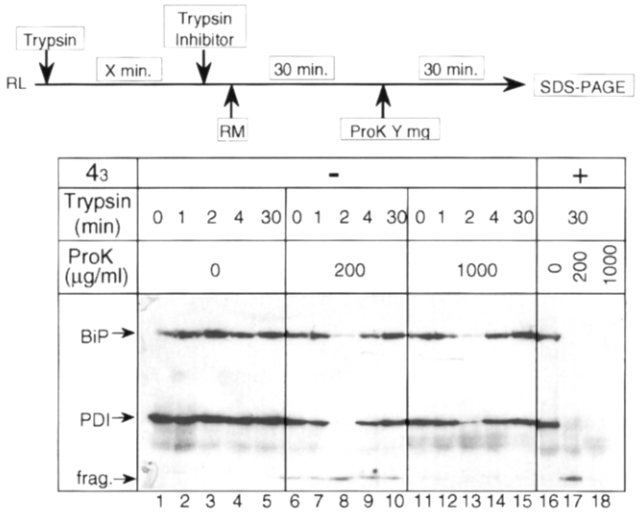

Fig. 5. RL-factor is transiently activated by short-term treatment with trypsin. RL (6.0 mg/ml) was treated by trypsin (20 μg/ml) in the reaction mixture (50 μl) of degradation assay for various periods (X = 0 to 30 min) at 30°C, and the reaction was terminated by trypsin inhibitor (40 μg/ml) on ice for 5 min. RM (7.0 μg) was incubated with the trypsin treated RL for 20 min at 30°C, and then was treated with proteinase K at various concentrations on ice. The proteins were analyzed by SDS-PAGE and subsequent immunoblotting. The bands of BiP and PDI are indicated by arrows, and peptide fragments are also shown (frag.). As a positive control, 0.1 mM pore forming peptide (4_3) was added instead of the trypsin treated RL.

3.4. RL-factor was transiently activated by trypsin treatment

We next examined whether other proteases (papain, trypsin, and elastase) degraded the lumenal protein in a similar way. RM vesicles containing mPL were treated with various concentrations of proteases in the presence or absence of RL. Three proteases similarly degraded mPL depending on the RL-factor at lower concentrations (Fig. 4). In contrast to the proteinase K, papain and elastase also degraded the prolactin even at the highest concentration (1000 μg/ml) and such degradation was directly related to the presence of RL. It was thus suggested that the RL dependent degradation of mPL by the externally added protease was not specific to proteinase K.

To examine the possibility that the pretreatment of RL with protease produces a membrane perturbing factor, RL was treated with trypsin (20 μg/ml) for various periods, and the reaction was stopped by trypsin inhibitor. Next, RM was incubated with the trypsin treated RL for 5 min at 30°C, and then was subjected to proteinase K treatment at 0°C for 20 min. When the RM was incubated with the RL pretreated by trypsin for 2 min, both PDI and BiP were degraded even with the highest dose of proteinase K (1000 μg/ml) (Fig. 5, lanes 8 and 13). In contrast, a longer pretreatment of RL with the trypsin resulted in a loss of the RL-factor activity (lanes 9, 10, 14, and 15). It is thus clear that the RL-factor was transiently activated with trypsin and then rapidly inactivated by prolonged treatment with trypsin. Such trypsin inactivation also supported the notion that the RL-factor is a proteinaceous factor. The RL-factor which had been activated with trypsin treatment for 2 min was also sensitive to both the heat treatment and NEM treatment (data not shown), thus indicating

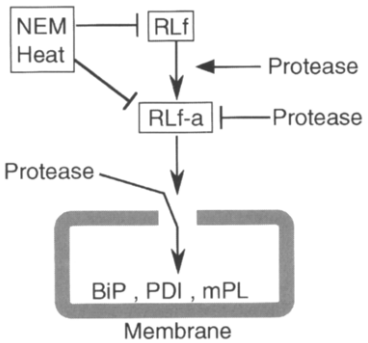

Fig. 6. Working model of RL-factor. The RL-factor (RLf) that is transiently activated by proteases permeabilizes microsomal membrane and makes the lumenal proteins accessible to externally added proteases. Both the RL-factor and its activated form (RLf-a) are sensitive to NEM, heat, and proteinase K.

that RL-factor is proteinaceous even after it was activated by trypsin. As a positive control for membrane perturbation, amphiphilic peptide, 4_3, Ac-(Leu-Ala-Arg-Leu)$_3$-NHCH$_3$ [11,12] was used. In the presence of the peptide, both proteins were completely degraded.

4. Discussion

We herein demonstrated that RL contains proteinaceous factor (RL-factor) which was transiently activated by protease treatment and thus permeabilized microsomal membranes (Fig. 6). This factor was inactivated by heat treatment and NEM treatment. The activity seemed to be specific to reticulocyte and was not found in the liver cytosol and rabbit erythrocyte lysate.

Interestingly this factor is similarly activated by various proteases (proteinase K, trypsin, papain, and elastase), and it is also readily inactivated by higher concentrations of proteinase K and prolonged incubation with trypsin. We can thus speculate two mechanisms of activation of RL-factor: first, it contains a hinge region which should be accessible to the various proteases used; second, some inhibitory factor which is sensitive to various proteases should bind to the active RL-factor. The RL-factor activated by short-term trypsin treatment perturbed the membrane as reported with amphiphilic peptide, 4_3 [11]. Once it perturbed the membrane, it was no longer inactivated with proteinase K. Various attempts to purify RL-factor are now in progress.

It is likely that at higher concentrations proteinase K immediately inactivated the active form of RL-factor. In contrast, papain and elastase did not inactivate it, probably due to their substrate specificity. Although trypsin (10 μg/ml) efficiently degraded the lumenal protein depending on the RL-factor, at higher concentration it degraded them in spite of the absence of RL-factor (Fig. 4). For some unknown reason, trypsin non-specifically degraded the membrane at higher concentrations.

In the absence of RL, 30–40% of mature form prolactin was degraded by proteinase K even in the absence of RL-factor (Fig. 2). This level of degradation was also observed at the highest concentration used in this study (Fig. 1). This RL independent degradation of mPL can be explained from two points of view: first, in vitro synthesized prolactin might be dislocated from the lumen to the cytoplasmic side as observed by Ooi and Weiss [13]; second, the membrane vesicle should not be completely sealed.

The membrane perturbing activity might be related to organelle degradation during the maturation of reticulocytes. Recently, van Lyen et al. reported that the 15-lipoxygenase permeabilizes the intracellular membrane and is also responsible for the intracellular degradation of organelles [14]. In contrast to the 15-lipoxygenase, the RL-factor reported herein requires short-term treatment with protease in order to exert its unique activity. Our preliminary results suggested that the RL-factor was not co-fractionated with 15-lipoxygenase (data not shown).

It should finally be noted that the data presented in this study clearly show pitfalls in analyzing the membrane topology of proteins with proteases. To avoid them, the membranes should not be treated with proteases at room temperature (15–30°C) and they should instead be kept on ice when proteases are added in the presence of RL.

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