A novel complete reconstitution system for membrane integration of the simplest membrane protein

Ken-ichi Nishiyama [a,d*], Masahide Maeda [b], Masato Abe [c], Takashi Kanamori [c], Keiko Shimamoto [b], Shoichi Kusumoto [b], Takuya Ueda [c], and Hajime Tokuda [a]

a Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan

b Suntory Institute for Bioorganic Research, 1-1-1 Wakayamadai, Shimamoto, Mishima, Osaka 618-8503, Japan

c Department of Medical Genome Sciences, Graduate School of Frontier Sciences, The University of Tokyo, Kashiwa, Chiba 277-8562, Japan

d Present address; Cryobiofrontier Research Center, Fuculty of Agriculture, Iwate University, 3-18-8 Ueda, Morioka, Iwate 020-8550, Japan.

*Corresponding author. Address: Cryobiofrontier Research Center, Fuculty of Agriculture, Iwate University, 3-18-8 Ueda, Morioka, Iwate 020-8550, Japan. Tel: +81 19 621 6471; Fax: +81 19 621 6240; E-mail address: nishiyam@iwate-u.ac.jp

Abstract

A complete reconstitution system for membrane integration of the simplest protein was developed by means of defined factors. A mutant version of Pf3 coat protein, 3L-Pf3 coat, requires neither signal recognition particle/Sec factors nor a membrane potential for its integration into the cytoplasmic membrane of *E. coli*. Although 3L-Pf3 coat is spontaneously integrated into liposomes composed of phospholipids, diacylglycerol completely blocks such spontaneous integrations at a physiological level. Under the conditions where spontaneous integration does not occur, 3L-Pf3 coat integration was absolutely dependent on a novel integration-stimulating factor. Combination of the PURE system, an *in vitro* translation system composed of the purified factors involved in translation in *E. coli*, with liposomes containing the highly purified integration-stimulating factor revealed multiple cycles of 3L-Pf3 coat integration, achieving the complete reconstitution of membrane integration. Based on the function of the factor, we propose that the factor is named MPIase (*M*embrane *P*rotein *I*ntegr*ase*).

Key words

diacylglycerol/integration-stimulating factor/liposomes/membrane protein integration/ MPIase/ PURE system

Introduction

Integral membrane proteins are integrated into biological membranes in a cotranslational manner with functions of integration factors. In *E. coli*, several pathways are utilized for integration (for review, see [1, 2]). Nascent chains carrying hydrophobic membrane spanning stretches are recognized by a signal recognition particle (SRP) composed of Ffh and 4.5 S RNA, followed by targeting to membrane by means of an SRP receptor, FtsY. Integration into membrane occurs at SecYEG, a protein conducting channel. On the other hands, several small membrane proteins are unable to utilize SRP system. Thus, these proteins are independent of both the SRP system and SecYEG. Moreover, these proteins are integrated into liposomes composed of phospholipids. Based on these observations, they have long been thought to integrate spontaneously. However, we found that diacylglycerol (DAG), which is contained in native membranes, completely blocks such spontaneous integrations at a concentration of physiological level, and that integration strictly depends on an integration-stimulating factor [3, 4]. This factor is involved not only in integration of SRP/Sec-dependent proteins but also in translocation of presecretory proteins [3]. Hereafter, we call the factor as MPIase (*M*embrane *P*rotein *I*nteg*rase*), based on its function.

The PURE system is an *in vitro* translation system composed of the defined purified factors involved in translation in *E. coli* [5]. The usage of PURE system enables us not only to obtain large amounts of translation products reproducibly, but also to critically examine the involvement of cytosolic factors. Therefore, development of the *in vitro* synthesis of membrane proteins and integration into liposomes only containing the necessary factors for integration is preferable for the functional and structural analysis of membrane proteins. In these analyses the integration system that reflects *in*

vivo reactions would be better, especially when membrane proteins with unknown functions are examined. In this paper, we developed further purification scheme to obtain the highly purified MPIase, followed by the establishment of the complete reconstitution system for integration of 3L-Pf3 coat by the combination of PURE system with MPIase-containing liposomes.

Materials and methods

Materials

Inverted inner membrane vesicles (INVs) were prepared from MC4100 (F^- Δ*[argF-lac]*U169 *araD rpsL150 relA1 thi deoC7 ptsF25 flbB5301*, [6]), as described [7]. Plasmid T7-7-3L-Pf3 encoding 3L-Pf3 coat [8] was kindly provided by Prof Andreas Kuhn. *Escherichia coli* polar phospholipids and 1,2-dioleoyl-sn-glycerol were purchased from Avanti polarlipids. Tran^{35}S label, a mixture of [^{35}S] methionine and [^{35}S] cycteine (>37.0 TBq/mmol), was from MP Biochemicals. Proteinase K and TLC plates were from Merck. Anisaldehyde was from Sigma. Silver staining kit was obtained from Kanto Chemicals.

Purification of MPIase

INVs purified from MC4100 (~1.5 g from protein determination) were washed with 5 M urea and then extracted with 6% sodium cholate, as described [3].

Acetone-precipitated materials were solubilized in 1.5% octyl glucoside, 50 mM triethanolamine-acetate (pH 7.5), 10% glycerol, followed by trichloroacetic acid (10%) precipitation. The resultant trichloroacetic acid-soluble materials were precipitated by 80% acetone. The precipitates were washed by acetone and diethyl ether successively.

After the dried precipitates were solubilized with 1.5% octyl glucoside, 20 mM Bistris-HCl (pH 5.8), 10% glycerol, they were applied on MonoQ column (GE Healthcare). Active fractions as to integration eluted with ~200 mM NaCl were determined and collected as described [3], followed by precipitation with acetone. The precipitates were dissolved in an equivolume mixture of the upper and lower phases of solvent A (1-butanol/tetrahydrofuran/ethanol/water : 8/7/1/20) and subjected to partition chromatography on a sephadex LH-20 column (10 x 300 mm), which had been equilibrated with the water phase of solvent A. The column was developed with the organic phase of solvent A at the flow rate of 1 ml/min. An aliquot of each fraction was subjected to TLC analysis using solvent B (chloroform/ethanol/water : 3/7/4), followed by visualization with an anisaldehyde-H_2SO_4 reagent (an ethanol solution of anisaldehyde/conc. H_2SO_4/acetic acid : 3/4/1 (v/v/v)). Fractions containing only a particular brown spot (Rf 0.35) were collected and dried.

Preparation of liposomes

Liposomes containing MPIase were prepared as follows. Phospholipids solubilized in solvent B were mixed with DAG (dioleoylglycerol; 5% of phospholipids) and MPIase solubilized in solvent B, as specified. The solvent was evaporated under stream of nitrogen gas and then under vacuum. The dried residues were suspended by sonication in 50 mM Hepes-KOH (pH 7.6), 1 mM dithiothreitol at 10 mg phospholipids/ml. Before use, the suspension was frozen, thawed and sonicated several times.

In vitro synthesis and integration of 3L-Pf3 coat

3L-Pf3 coat was synthesized by means of PURE system, which was prepared as

described previously [9] except that magnesium acetate was reduced to 9 mM to avoid aggregation of liposomes. Liposomes were added at 400 μg phospholipids/ml at the start of synthesis. The reaction mixture contained [^{35}S] methionine (~3 MBq/ml). Where specified, cold methionine (0.3 mM) was also added. Translation was allowed to proceed at 37°C for 30 min, and then divided into two parts. One (15 μl) was treated with trichloroacetic acid (5%). The other (30 μl) was incubated with proteinase K at 0.5 mg/ml at 25°C for 20 min, followed by trichloroacetic acid precipitation. After the precipitates were washed with acetone, the integrated materials were analyzed by SDS-PAGE and autoradiography, as described [3, 4].

Results

Purification of MPIase

MPIase was further purified by partition chromatography because TLC analysis revealed that previously purified preparation was contaminated by lipopolysaccharide (LPS). On the TLC plate visualized by the anisaldehyde reagent, the active preparation after the MonoQ step gave two spots, brown one with mobility of ~0.35 of Rf value and gray one with slightly lower mobility (Fig. 1A, lane 1). Since Ra-LPS prepared from MC4100 gave only a gray spot of the same mobility as the gray one (data not shown), it is suggested that the brown spot corresponds to MPIase. By means of repeated partition chromatography, the brown spot (lane 3) was completely separated from the gray one corresponding to LPS (lane 2). This preparation free from the gray one exhibited a high activity toward integration (see Figs. 2 and 3). The separated materials were also analyzed by SDS-PAGE and silver staining (Fig. 1B). The same amounts (6 μg) of the materials giving the gray (Fig. 1B, lane 1) and brown (lane 2) spots were applied to

separate lanes on an SDS-gel. Only a band of ~7 kDa was detected for each material, both exhibiting indistinguishable mobilities on the gel. However, the staining efficiency for MPIase was much lower than that of LPS. Thus, MPIase was clearly differentiated from LPS, and highly pure preparation of MPIase was obtained. The amount of MPIase purified from ~1 g INVs (from protein determination) was typically ~5 mg.

Complete reconstitution of 3L-Pf3 coat integration

We previously demonstrated that 3L-Pf3 coat, a membrane potential-independent version of Pf3 coat, is integrated in an MPIase-dependent manner [4]. Here, in order to achieve the complete reconstitution, we examined whether the PURE system can be used as a translation device for the integration assay. Also, we examined whether or not MPIase-containing liposomes can be prepared by solvent evaporation, since MPIase, DAG and phospholipids were efficiently solubilized in solvent B. When liposomes were formed without adding DAG, spontaneous integration of 3L-Pf3 coat was observed (Fig. 2). On the other hand, when DAG was included in liposomes, such spontaneous integration was effectively blocked as expected (Fig. 2). In the marked contrast, when MPIase was included in liposomes together with DAG, 3L-Pf3 coat integration was efficiently observed (Fig. 2), indicative of the complete reconstitution of 3L-Pf3 coat integration. This MPIase-dependent integration was as active as that into MPIase-containing liposomes conventionally prepared by detergent dialysis method (data not shown, [4]). These results indicate that 3L-Pf3 coat integration was reproduced by means of PURE system. Thus, it is demonstrated that no cytosolic factors but only MPIase are necessary for 3L-Pf3 coat integration.

Next, the MPIase-dependent integration was examined in detail with various

amounts of MPIase in the presence and absence of cold methionine (Fig. 3). Spontaneous integration and its blockage by DAG were again reproduced both in the presence and absence of cold methionine. As the amounts of MPIase in liposomes increased in the presence of DAG, the activities for 3L-Pf3 coat integration increased similarly in both cases. When the integration activities were plotted against the MPIase amount double-reciprocally, apparent Km and Vmax values were obtained (Fig. 3C). Essentially, both Km and Vmax values were similar in both cases. These results also indicate that the activity is already saturated when 5% of MPIase was included.

Under the conditions depicted in Fig. 3B, the amounts of synthesized 3L-Pf3 coat was determined to be 2~4 μg/ml (400~800 pmol/ml), by counting the radioactivities of the excised gel corresponding to 3L-Pf3 coat. MS analysis revealed that the molecular weight of MPIase is ~10 kDa (data not shown). Assuming that half of MPIase was reconstituted in liposomes in a correct orientation, the amount of MPIase when added at 0.05% in the integration assay was estimated to be ~10 pmol/ml. Therefore, the amount of the integrated material of 8.4% (34~67 pmol/ml) was higher than that of MPIase, suggesting that the multiple cycles of integration occur under the conditions, even if MPIase functions as a monomer.

Discussion

In this paper, we demonstrated that the complete reconstitution of integration of the simplest membrane protein was successfully developed, which reflects the *in vivo* reaction correctly. In the system, we found that no cytosolic factors other than factors involved in translation are necessary and that as a membrane component only MPIase is necessary under the conditions where no spontaneous integrations occur. We have

chosen 3L-Pf3 coat as a substrate protein, which is the simplest membrane protein in that it requires neither SRP-Sec systems nor membrane potential for integration [4, 8], because the most fundamental process of membrane integration can be analyzed with it. Therefore, our study would be the basis to further analyze the mechanisms for more complex integrations. We have previously demonstrated that some SRP/Sec-dependent membrane proteins, synthesized by PURE system, correctly integrated into urea-washed INVs [10]. Also, PURE system has been applied for the membrane protein synthesis and integration into liposomes in a spontaneous manner [11, 12]. We now found that PURE system can be applied for the MPIase-dependent integrations, indicating that our system is useful to develop the system for the *in vitro* synthesis of functional membrane proteins.

The expression level of MPIase in inner membrane could be estimated to be at least 0.5% in weight to inner membrane proteins, assuming that MPIase was quantitatively extracted from INVs. Considering that the level of SecY is ~0.1% [13], MPIase level might be higher than that of SecYEG, although the oligomeric nature of MPIase is totally unclear. This is consistent with the fact that MPIase is involved in both Sec-dependent and -independent reactions [3]. Nonetheless, when added at ~0.5% in liposomes, the obtained activity was comparable with that from wild type INVs [4], supporting that the integration reaction was correctly reconstituted.

Although LPS is a major component of outer membrane, the physicochemical properties of MPIase are quite similar to those of LPS, for example, the solubility in trichloroacetic acid and mobility on an SDS-gel, both of which have puzzled to identify MPIase. Now, we could differentiate MPIase from LPS clearly, and therefore LPS-free preparation of MPIase could be obtained in large amount. The structural analysis on

MPIase is now on progress, indicating that MPIase is not a proteinaceous molecule.

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Figure legends

Fig. 1. Detection of the highly purified MPIase. (A) The MonoQ fraction (lane 1), and gray (lane 2) and brown (lane 3) fractions separated by partition chromatography were spotted on the TLC plates and developed by solvent B, followed by visualization with anisaldehyde. The positions corresponding to origin and front were indicated by arrows. (B) The gray (lane 1) and grown (lane 2) fractions (6 μg each) analyzed in (A) were applied on SDS-gel and stained with silver. Molecular weight marker (lane M) was also applied.

Fig.2. Spontaneous and MPIase-dependent integration of 3L-Pf3 coat synthesized by PURE system. 3L-Pf3 coat protein was synthesized using PURE system in the presence of liposomes, which contain DAG and MPIase, as specified. After 30-min synthesis at 37°C, the reaction mixture (50 μl) was divided into two parts. One part (15 μl) directly received 5% trichloroacetic acid, while the other part (30 μl) was mixed with an equal volume of 1 mg/ml proteinase K (PK) as indicated. After 20-min digestion at 25°C, the membrane-protected fragment (MPF) was recovered by trichloroacetic acid (5%) precipitation. The integration efficiency was indicated at the bottom of the autoradiogram as a percentage of the synthesized protein.

Fig. 3. Effect of the amount of MPIase on 3L-Pf3 coat integration. 3L-Pf3 coat was synthesized as the legend to Fig. 2, in the absence (A) and presence (B) of 0.3 mM cold methionine. The integration efficiencies were also indicated. (C) The integration

activities obtained in A and B were double-reciprocally plotted against the MPIase content in the liposomes. Apparent Km and Vmax values were calculated and indicated in both cases.

Figure 1

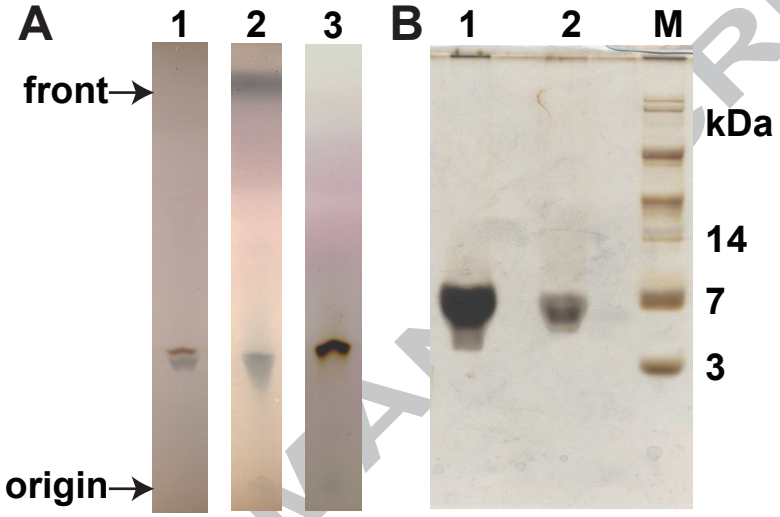


Fig. 1 Nishiyama et al.

Figure 2

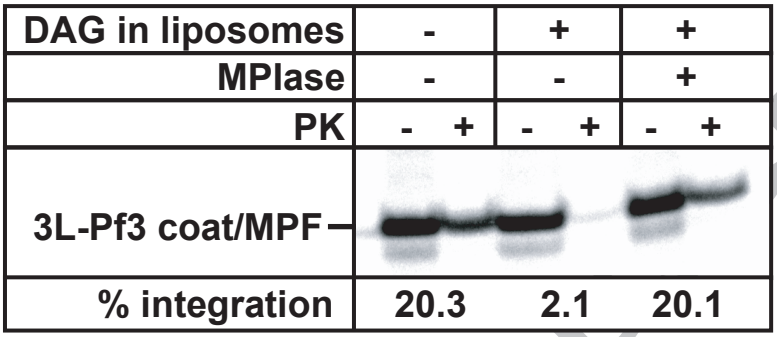
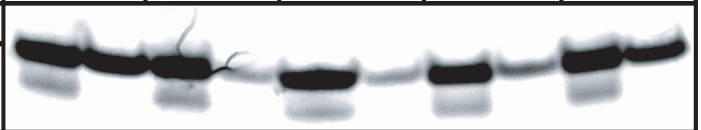


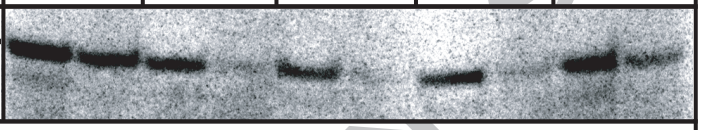
Fig. 2 Nishiyama et al.

Figure 3

A -cold Met

DAG in liposomes	-	+	+	+	+
MPlase	-	-	0.05%	0.5%	5%
PK	- +	- +	- +	- +	- +
3L-Pf3 coat/MPF					
% integration	38.9	3.9	5.4	12.3	24.3

B +cold Met

DAG in liposomes	-	+	+	+	+
MPlase	-	-	0.05%	0.5%	5%
PK	- +	- +	- +	- +	- +
3L-Pf3 coat/MPF					
% integration	32.6	5.4	8.4	16.8	24.6

C

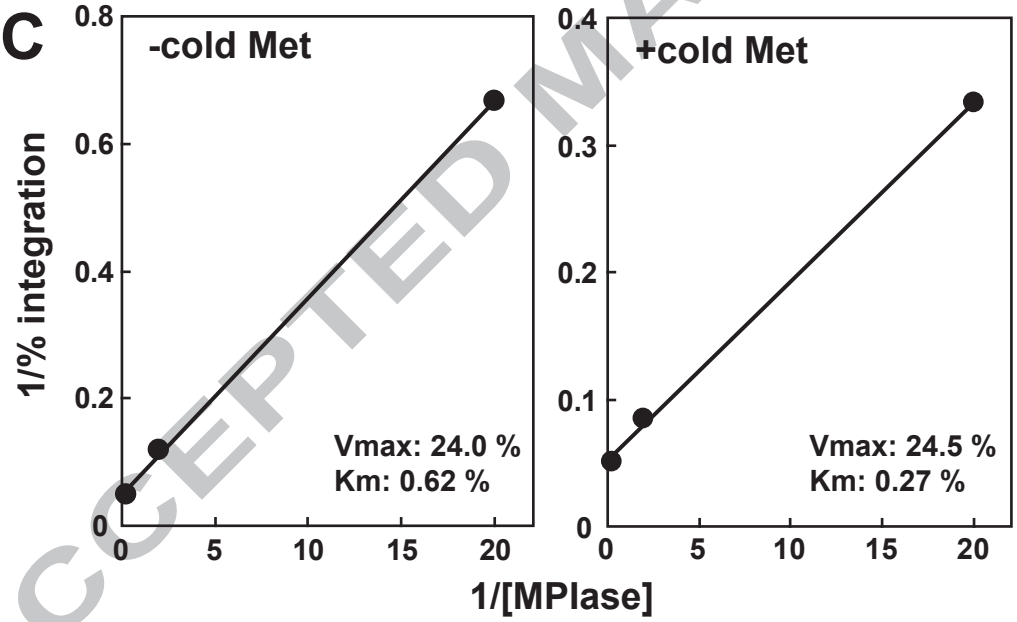


Fig. 3 Nishiyama et al.