Role of Ribosomes in Reinitiation of Membrane Insertion of Internal Transmembrane Segments in a Polytopic Membrane Protein†

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ABSTRACT: The topogenesis of membrane proteins with a single transmembrane (TM) segment is well understood. However, understanding the topogenesis and membrane assembly of membrane proteins with multiple TM segments (polytopic) is still incomplete. Recently, several studies on P-glycoprotein (Pgp) suggested that the topogenesis of polytopic membrane proteins is likely more complicated than anticipated. While studying the mechanism by which Pgp topogenesis is determined, we unexpectedly found that ribosomes or proteins associated with ribosomes are involved in regulating the membrane insertion and folding of Pgp during its translation. We discovered that when Pgp was translated by wheat germ ribosomes *in vitro*, TM3 could not reinitiate the insertion of the protein into microsomal membranes following the membrane insertion of TM1 and TM2. In contrast, TM3 could reinitiate membrane insertion when the protein was translated by rabbit reticulocyte ribosomes. These findings suggest that ribosomes or proteins associated with ribosomes play an important role in membrane insertion and folding of TM segments of Pgp and that rabbit reticulocyte and wheat germ ribosomes may use different mechanisms to control the membrane insertion of the same nascent peptide. We propose that ribosomes or proteins associated with ribosomes help reinitiate insertion of internal TM segments into the membrane by dissociation and reassociation with the protein-conducting channel in ER membranes.

The biogenesis of a membrane protein occurs in at least three steps: targeting, translocation, and membrane integration. This complex process involves many proteins or factors, such as the signal recognition particle (SRP), SRP receptor, Sec61p complex, and the translocating chain-associating membrane (TRAM) protein [for reviews, see Walter and Johnson (1994), Corsi and Schekman (1996), Andrews and Johnson (1996), and Rapoport et al. (1996)]. Ribosomes have also been suggested to participate in the cotranslational membrane insertion of nascent proteins by interacting with the protein-conducting channel in the ER membrane (Hegde & Lingappa, 1996).

The biogenesis of most polytopic membrane proteins (proteins with two or more transmembrane segments) in eukaryotic cells is thought to be similar to that of proteins with a single transmembrane (TM) segment. The polytopic membrane proteins likely acquire their final membrane orientation(s) during or immediately after synthesis on the rough ER (Goldman & Blobel, 1981; Braell & Lodish, 1982; Brown & Simoni, 1984; Wessels & Spiess, 1988). Topogenic sequences involved in signal-anchorage and stop-transfer activities have been identified in polytopic membrane proteins of eukaryotes (Friedlander & Blobel, 1985; Audigier et al., 1987; Lipp et al., 1989; Chavez & Hall, 1991; Silve et al., 1991). A polytopic topology is probably generated by the sequential translocation and membrane integration of independent topogenic sequences (Blobel, 1980; Wessels & Spiess, 1988; Lipp et al., 1989; Hartmann et al., 1989; Skach & Lingappa, 1993).

Recent studies on a polytopic membrane protein, P-glycoprotein (Pgp), suggest that the folding and topogenesis of polytopic membrane proteins are more complicated than previously thought (Zhang & Ling, 1991; Skach et al., 1993; Bibi & Béjà, 1994; Borel & Simon, 1996; Zhang, 1996). Using expression systems derived from rabbit reticulocytes, frog oocytes, bacteria, and mammalian multidrug-resistant cells, several putative TM segments of Pgp were found not to be anchored in membranes. We have shown that charged amino acids flanking internal putative TM segments are important in controlling the membrane orientation of these TM segments (Zhang et al., 1995).

Previously, we showed that membrane insertion of TM3 of hamster Pgp differs in rabbit reticulocyte lysate (RRL) and wheat germ extract (WGE) translation systems (Zhang & Ling, 1995). In RRL, the TM3 inserts into membranes in a $N_{in}-C_{out}$ orientation after membrane insertion of TM1 and TM2. However, TM3 does not insert into membranes in the WGE system, although membrane integration of TM1 and TM2 proceeds normally. It was not known what factor(s) caused this difference between RRL and WGE, and we investigated further to identify the factor in this study. We found that the failure of TM3 to insert into membranes in WGE is associated with salt-stripped wheat germ ribosomes. We conclude that specific ribosomal factors are important for the reinitiation of membrane insertion of internal TM segments, and thus for the proper folding of Pgp.

MATERIALS AND METHODS

Materials. Wheat germ was obtained from Arrowhead Mills (Hereford, TX). Rabbit reticulocyte lysate and wheat germ extract translation systems, SP6 RNA polymerase,

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RNasin, ribonucleotides, RQ1 DNase, canine pancreatic microsomal membranes, and *Hin*dIII were purchased from Promega. Sephadex G-75, puromycin hydrochloride, deoxycholate, and Triton X-100 were obtained from Sigma. Sepharose CL-6B and the cap analog $m^7G(5')ppp(5')G$ were purchased from Pharmacia. [^{35}S]Methionine and Amplify were purchased from DuPont and Amersham, respectively. Molecular weight markers for electrophoresis of proteins and RNAs were from GIBCO-BRL.

Preparation of Crude Wheat Germ Extract. Preparation of crude WGE was performed with a modified procedure described previously by Anderson et al. (1983). Briefly, 20 g of sieved wheat germ was ground with an equal weight of acid-washed sea sand in a prechilled mortar for 60 s. The mixture of ground wheat germ and sea sand was then transferred into a centrifuge tube, mixed thoroughly with 40 mL of buffer A [20 mM HEPES/KOH, pH 7.6, 100 mM KCl, 1 mM $Mg(OAc)_2$, 2 mM $CaCl_2$, and 1 mM DTT], and centrifuged at 30000*g* for 20 min. After centrifugation, the thick upper layer of fatty acid was removed. The supernatant was collected and separated on a Sephadex G-75 column (2.5 cm × 40 cm) preequilibrated in buffer B-120 [20 mM HEPES/KOH, pH 7.6, 5 mM $Mg(OAc)_2$, 1 mM DTT, and 120 mM KCl]. The elution was performed using buffer B-120, and the excluded fractions were collected, pooled, and centrifuged for 20 min at 30000*g*. The supernatant was then centrifuged at 100000*g* for 1 h, and the pellet was discarded. The supernatant was designated crude wheat germ extract (WGE) and stored in aliquots at −70 °C.

Fractionation of Crude Wheat Germ Extract by Centrifugation. The crude WGE was centrifuged at 150000*g* for 1 h using a Beckman Optima Ultracentrifuge. The top three-fourths of the supernatant was collected, designated S1, and stored at −70 °C. The bottom one-fourth of the supernatant was discarded. The pellet was resuspended in buffer B-120 and designated P1. Portions of P1 were saved (stored at −70 °C), and the remainder was diluted with an equal volume of buffer B-1200 (same as B-120, but containing 1.2 M KCl) to achieve a final KCl concentration of 0.6 M. The mixture was incubated on ice for 30 min and then centrifuged at 150000*g* for 5 h. The top three-fourths of the supernatant was collected, concentrated using Centricon-50, washed once with buffer B-120, designated S2, and stored at −70 °C. The bottom one-fourth of the supernatant was discarded. The final pellet was resuspended in buffer B-120 or buffer B-50 (same as B-120, but containing 50 mM KCl) to a protein concentration of ∼40 mg/mL, designated P2, and stored at −70 °C.

Exposure of P2 to Various Treatments. (1) Detergents. Treatment of P2 with detergents was performed as described previously (Archambault de Vencay et al., 1991). Deoxycholate and Triton X-100 were added to P2 to final concentrations of 1% and 0.5%, respectively, and incubated on ice for 30 min. The mixture was then separated by chromatography on a Sepharose CL-6B column, and eluted proteins were detected by determining the OD_{280nm} on a Shimadzu UV-160 spectrophotometer. Peak fractions (only one peak at OD_{280nm} was observed) were collected and concentrated by centrifugation at 150000*g* for 1 h. The pellet was resuspended in buffer B-120 for further analysis.

(2) pH. Samples containing 200 μL of P2 in buffer B-120 were dialyzed overnight against 20 mL of buffer C [5 mM $Mg(OAc)_2$, 1 mM DTT, 120 mM KCl, and 20 mM HEPES] at pH 5.0, 6.0, 7.6, 8.0, or 9.0. After dialysis, the pH of each fraction was measured, and the treated fractions were used for further analysis.

(3) Temperature. Two hundred microliters of P2 in buffer B-120 was incubated for 30 min at 0, 30, 37, 45, or 65 °C and then used for further analysis.

(4) Mg^{2+}. Two hundred microliters of P2 in buffer B-120 containing $Mg(OAc)_2$ was dialyzed overnight against 20 mL of buffer B-120 without $Mg(OAc)_2$. $Mg(OAc)_2$ was added back to a final concentration of 5 mM to a portion of the dialyzed P2. Both the dialyzed and reconstituted P2 samples were used for further analysis.

Turbidimetric Measurements. Determination of the turbidity change of P2 caused by Mg^{2+} was performed as described previously (Sperrazza et al., 1980). Ten microliters of P2 (14.9−24.2 mg/mL protein) was mixed with 90 μL of buffer B-120 containing various concentrations of $Mg(OAc)_2$ as indicated in the figure legends and incubated at 37 °C for 10 min. The OD_{310nm} was determined using a Shimadzu UV-160 spectrophotometer.

Determination of P2 Composition. Quantitation of protein content was performed using Bio-Rad reagents and bovine serum albumin as a standard. The protein composition of P2 was determined using 12.5% SDS−PAGE stained with Coomassie Brilliant Blue. RNA in P2 was isolated by extracting 3 times with phenol−chloroform, precipitating with ethanol, and quantifying by determining the OD_{260nm}. The RNA composition of P2 was examined using a 1% agarose−formaldehyde gel stained with ethidium bromide.

Estimate of the Size of the Active Component(s) in P2 by Gel Filtration. A 0.5 mL sample containing 3.5 mg of P2 in buffer B-50 was applied onto a Sepharose CL-6B column (1 cm × 100 cm) preequilibrated with buffer B-50. Fractions of 1 mL were collected. The void volume was determined using blue dextran 2000. Thyroglobulin and ferritin (Pharmacia) were used as protein size markers.

Isolation of Ribosomes from Drug-Sensitive and -Resistant Cells. Parental (SKOV3) and multidrug-resistant (SKOV/VLB) cells were grown to confluency in α-MEM containing 10% fetal bovine serum. SKOV/VLB cells were maintained in the presence of 1 μg/mL vinblastine. Confluent cells were collected, washed with phosphate-buffered saline, lysed, and fractionated by centrifugation as described previously (Zhang et al., 1996). The 100000*g* supernatant was then centrifuged again at 150000*g* for 3 h, and the pellet (ribosomes) was resuspended in buffer B-120 and stored in −70 °C.

In Vitro Transcription and Translation. In vitro transcription and translation of the Chinese hamster Pgp-N3 (R207V/K210D) construct was performed as described previously (Zhang et al., 1993, 1995). For analysis of the effects of WGE or P2 on the topogenesis of TM3, 6 μg of protein from these fractions was added to the translation mix to a final concentration of 10% (v/v). The translation reaction was then initiated by addition of RNA transcripts. Membrane-associated translation products were isolated by centrifugation and analyzed using SDS−PAGE and fluorography as described previously (Zhang & Ling, 1991).

RESULTS

TM3 of Pgp Does Not Insert into the Membrane in the Presence of WGE. We previously reported that the TM3 in a truncated Chinese hamster Pgp molecule (Pgp-N3) synthesized by the RRL system has two different membrane

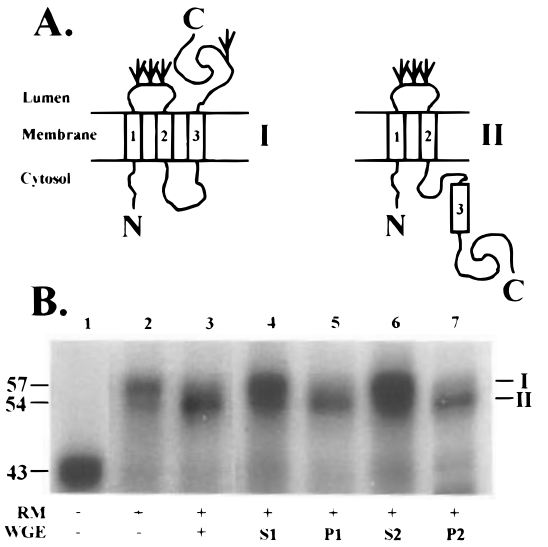

FIGURE 1: Salt-resistant high-speed pellet of WGE inhibits the insertion of TM3 into membranes. (A) Two topological models for a truncated Pgp molecule with three TM segments (Pgp-N3). In topology I, the long C-terminal domain is located in the lumen of microsomal vesicles. In topology II, this domain and TM3 are in the cytoplasm. The branched symbols represent oligosaccharide chains, and the numbered rectangles denote putative TM segments. (B) Insertion of TM3 into membranes is inhibited by a salt-resistant, high-speed pellet. A mutant Pgp-N3 transcript (Zhang et al., 1995) was used to direct translation in RRL in the absence (lane 1) or presence (lanes 2−7) of microsomal membranes. The translation reaction in lanes 3−7 was performed in the presence of 6 μg of proteins of crude WGE (lane 3), WGE supernatant (lane 4), WGE pellet (lane 5), WGE supernatant after 0.6 M KCl treatment (lane 6), or WGE pellet after 0.6 M KCl treatment (lane 7). The 57-kDa band corresponds to topology I, the 54 kDa-band to topology II. The nonglycosylated Pgp-N3 is 43 kDa.

topologies (Figure 1A; Zhang et al., 1993). In topology I, TM3 reinitiated membrane insertion and was located within the membrane, resulting in a glycosylated C-terminal reporter in the microsome lumen. In topology II, TM3 did not insert into the membrane and was located in the cytoplasm with the C-terminal reporter. Molecules with these two different foldings can be distinguished on SDS−PAGE by the different sizes caused by the different glycosylation patterns. Only topology II was produced in WGE (Zhang & Ling, 1995).

In this study, we used a mutant Pgp-N3 (R207V/K210D) for which more of the polypeptide was in topology I compared with the wild-type Pgp-N3, presumably because of the changes of charged amino acids (Zhang et al., 1995). As shown in Figure 1B, in the RRL system a precursor of 43 kDa was translated from the Pgp-N3 (R207V/K210D) transcript in the absence of microsomes (Figure 1B, lane 1). In the presence of microsomes, glycosylated proteins of 57 and 54 kDa were produced. They represent topologies I and II, respectively. Addition of 6 μg of WGE to the RRL translation system significantly decreased the generation of the 57-kDa protein (topology I) and increased the generation of the 54-kDa protein (topology II) (Figure 1B, lane 3). This result suggests that membrane insertion of TM3 decreased in the presence of WGE and that the insertion of TM1 and TM2 into the membrane was apparently not affected. These observations are consistent with our previous studies using a commercial WGE (Zhang & Ling, 1995).

The Active Fraction in WGE Is Associated with a Salt-Resistant Pellet Fraction (P2). To determine whether the active fraction in crude WGE is associated with large complexes, we performed a centrifugation of WGE at 150000*g* for 1 h (see Materials and Methods) and determined the inhibitory activity of both supernatant (S1) and pellet (P1) fractions (inhibitory activity refers to the ability to decrease the generation of topology I). As shown in Figure 1B, the P1 fraction decreased the generation of topology I molecules (Figure 1B, lane 5), whereas the S1 fraction did not (Figure 1B, lane 4). In our previous study, the inhibitory activity was found in both the soluble and pellet fractions (Zhang & Ling, 1995). This discrepancy may be due to the different methods used for preparation of WGE and/or the less stringent centrifugation conditions used in the previous study. We next treated the P1 fraction with 0.6 M KCl. The salt-stripped pellet (P2) was separated from the solubilized fraction (S2) by centrifugation for 5 h at 150000*g*. Again, the inhibitory activity was found in the pellet fraction (P2) (Figure 1B, compare lanes 6 and 7).

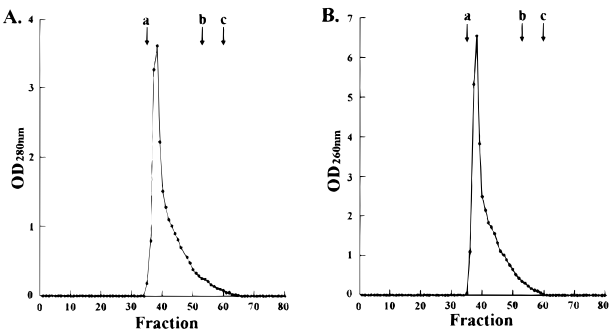

FIGURE 2: Active component of P2 is a complex of high molecular weight. P2 was subjected to gel-filtration chromatography using Sepharose CL-6B (column: 1 cm × 100 cm). Fractions of ~1 mL were collected and monitored by the absorption at OD_{280nm} (panel A) or OD_{260nm} (panel B). The void volume was estimated using blue dextran (arrow a). Arrows b and c indicate the peaks for elution of thyroglobulin (669 kDa) and ferritin (440 kDa), respectively.

P2 Is a Large Complex Consisting of both RNA and Protein. To estimate its size, P2 was subjected to gel filtration on Sepharose CL-6B, which excludes globular proteins of $>4 \times 10^6$ daltons. As shown in Figure 2A, a single peak of OD_{280nm} was observed. This peak eluted immediately following the void volume indicator blue dextran. This suggests that P2 is a large complex with a size slightly smaller than 4×10^6 daltons. Absorption at 260 nm was superimposable with that at 280 nm (Figure 2B).

To determine whether P2 contains nucleic acids, phenol and chloroform extraction of P2 was performed as described under Materials and Methods. The nucleic acid fraction was analyzed by formaldehyde−agarose gel electrophoresis. As shown in Figure 3A, two major distinct RNA bands were observed, corresponding to the 25S and 18S ribosomal RNAs of wheat germ. This result suggests that P2 contains ribosomes. To determine the protein composition of P2, the fraction was solubilized and separated on a SDS−PAGE. The proteins in P2 are mostly in the size range of 15−60 kDa (Figure 3B, lane 3). The RNA:protein ratio (w/w) was about 0.96:1 (see Table 1).

P2 Consists of Wheat Germ Ribosomes. The above studies suggest that P2 consists of wheat-germ ribosomes. To buttress this conclusion, we performed a functional study using RRL that does not contain ribosomes. RRL was centrifuged for 5 h at 150000*g*, and the pellet (RRL P1)

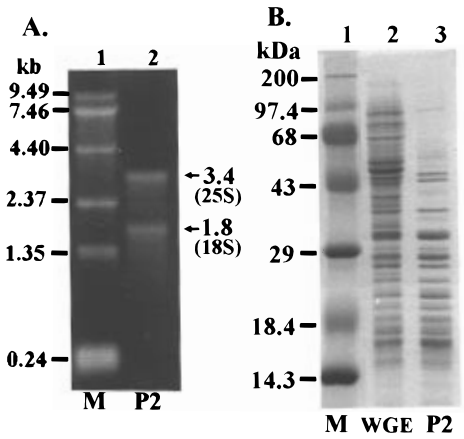

FIGURE 3: P2 contains both RNA and protein. (A) P2 contains rRNA. RNA was isolated using phenol/chloroform extraction and ethanol precipitation and analyzed on a formaldehyde−agarose gel. Lane 1 contained the RNA standards (BRL). Lane 2 was loaded with 1.6 μg of RNA isolated from P2. (B) P2 contains proteins. Fifteen micrograms of protein of crude WGE (lane 2) or P2 (lane 3) was separated on a 12.5% SDS−PAGE. Lane 1 was loaded with denatured protein size markers (BRL).

containing ribosomes and the supernatant [RRL(S/N)] were separated. The RRL(S/N) lost its translation ability (Figure 4A, lane 1), and translation ability was recovered by reconstitution of RRL(S/N) with RRL P1 (Figure 4A, lane 2). This finding suggests that the RRL(S/N) does not contain ribosomes. Addition of WGE P2 to the RRL(S/N) also restored translation (Figure 4A, lane 3). Addition of RRL P1 generated mostly topology I molecules, whereas addition of WGE P2 generated only topology II molecules. Thus, the topology determinant(s) coisolate(s) with ribosomes.

To determine whether the active molecules in P2 can be extracted with detergent, we treated P2 with a mixture of 1% Triton X-100 and 0.5% deoxycholate on ice for 30 min as described under Materials and Methods. We found that the detergent-treated P2 was still able to inhibit the generation of the topology I molecules (data not shown). Thus, the inhibitory activity of P2 is resistant to extraction by Triton X-100/deoxycholate and is a part of the wheat germ ribosomes.

The Effect of P2 on the Insertion of TM3 into Membranes Is Cotranslational. To determine whether the effect of P2 on the insertion of TM3 into membranes is co- or posttranslational, we added P2 at the beginning (B) or end (E) of each translation. Addition of P2 at the beginning of translation inhibited the generation of topology I molecules (Figure 4B, lane 4). However, generation of topology I molecules was not affected by P2 added after termination of translation by puromycin (Figure 4B, lane 2). When P2 was added at the beginning of translation, addition of puromycin had no effect on the generation of topology I molecules (Figure 4B, lane 3), eliminating the possibility of interference by puromycin. Thus, we conclude that the effect of P2 on the generation of topology I molecules occurs cotranslationally, which is consistent with the conclusion that ribosomes are involved in controlling the membrane insertion of TM3.

The Inhibitory Activity of P2 Requires Mg^{2+}. It is known that the function of ribosomes requires Mg^{2+} (Sperrazza & Spremulli, 1983). We next determined whether the P2 requires Mg^{2+} to inhibit the generation of topology I molecules. P2 was prepared in the presence or absence of $Mg(OAc)_2$ [designated P2($+Mg^{2+}$) and P2($-Mg^{2+}$), respectively], and its inhibitory activity was determined. Then, P2($+Mg^{2+}$) was dialyzed against buffer that did not contain Mg^{2+}, and the inhibitory activity was determined again.

P2($+Mg^{2+}$) inhibited the generation of topology I molecules (Figure 5A, lane 3), whereas P2($-Mg^{2+}$) did not (Figure 5A, lane 5). In the control reaction, buffers with or without Mg^{2+} did not affect the generation of topology I molecules. Dialysis of P2($+Mg^{2+}$) against buffers containing no Mg^{2+} inactivated the inhibitory activity of P2 (Figure 5A, lane 7), and the inhibitory activity could not be recovered after addition of 5 mM Mg^{2+} (Figure 5A, lane 8). P2($-Mg^{2+}$) dialyzed against a buffer containing 5 mM Mg^{2+} also failed to inhibit the activity (Figure 5A, lane 6). These results suggest that the inhibitory activity of P2 (a) requires Mg^{2+} and (b) is irreversibly lost upon removal of Mg^{2+}.

We also measured turbidity for both P2($+Mg^{2+}$) and P2($-Mg^{2+}$) as described under Materials and Methods. The turbidity of P2($+Mg^{2+}$) decreased with decreasing concentrations of Mg^{2+}, and that of P2($-Mg^{2+}$) increased with increasing concentrations of Mg^{2+} (Figure 5B). These observations are consistent with the reported properties of wheat germ ribosomes (Sperrazza et al., 1980).

The Inhibitory Activity of P2 Is Sensitive to Temperature and pH. We next determined whether the inhibitory activity of P2 is temperature-sensitive. Equal amounts of P2 were first treated at 0, 30, 37, 45, or 65 °C for 30 min. Six micrograms of the treated P2 was then mixed with RRL prior to the translation reaction. The inhibitory activity of P2 was lost after pretreatment at 65 °C (Figure 6A, lane 4), suggesting that the active component in P2 is heat-labile.

To investigate the pH sensitivity of the active component in P2, equal amounts of P2 were dialyzed overnight against buffer C at pH 5.0, 6.0, 7.6, 8.0, or 9.0 (see Materials and Methods). After dialysis, the pH change of the P2 fractions was determined. Six micrograms of the dialyzed P2 was then added to the RRL translation mixture. The activity of P2 was lost only when P2 was dialyzed against pH 9.0 (Figure 6B, lane 4). Buffer C alone at pH 9.0 did not affect the generation of topology I molecules (data not shown). Thus, P2 activity is sensitive to alkaline pH.

Membrane Insertion of Pgp Sequences Translated by Ribosomes from Drug-Sensitive and -Resistant Mammalian Cells. The above studies suggest that the cotranslational membrane insertion of TM3 of Pgp differs with different ribosomes. Since Pgp is normally overexpressed in drug-resistant mammalian cells, it is interesting to determine whether ribosomes from drug-sensitive and -resistant cells have different effects on the folding of Pgp. To determine what topology TM3 of Pgp adopts when translated by ribosomes from drug-sensitive and -resistant mammalian cells, we isolated ribosomes from a parental human ovarian cancer cell line, SKOV3, and its drug-resistant derivative, SKOV/VLB, cell line (Bradley et al., 1989). These ribosomes were supplemented in the ribosome-stripped RRL translation system, and the insertion of TM3 into membranes was analyzed. As shown in Figure 7, ribosomes from both SKOV3 and SKOV/VLB cells supported the generation of mainly the topology I proteins (lanes 4 and 5), the same as RRL ribosomes (lane 2). Only the wheat germ ribosomes generated the topology II proteins (lane 3). This result suggests that ribosomes in drug-sensitive and -resistant cells

Table 1: Comparison of Characteristics between P2 and Ribosomes

properties	P2	ribosome	references
size (daltons)	$<4 \times 10^6$	3.9×10^6	Cammarano et al. (1972)
		3.8×10^6	Nieuwenhuysen & Caluwaert (1978)
RNA composition	3.4kb and 1.8 kb	25S (3.4 kb) and 18S (1.8 kb)	Cammarano et al. (1972)
protein composition	15−60 kDa	<60 kDa	Bielka, 1982
RNA/protein ratio (w/w)	0.96:1	0.97:1	Cammarano et al. (1972)
turbidity[a]	Mg^{2+} dependent	Mg^{2+} dependent	Sperrazza et al. (1980)
function[b]	Mg^{2+} required	Mg^{2+} required	Sperrazza & Spremulli (1983)

[a] The turbidity of both P2 and ribosomes was determined at OD_{310nm}. [b] The function of P2 denotes its activity to inhibit the membrane insertion of TM3 of Pgp. The function of ribosomes denotes their activity to synthesize polyphenylalanine.

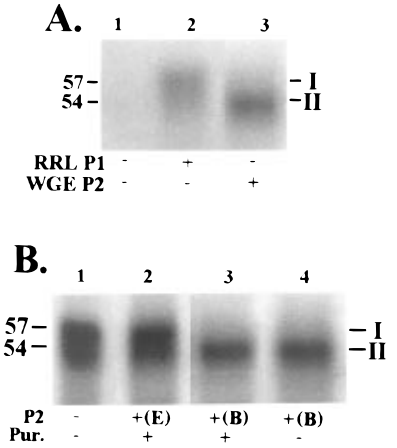

FIGURE 4: Activity of inhibiting TM3 insertion into membranes is associated with wheat germ ribosomes. (A) Translations in RRL devoid of ribosomes. RRL was centrifuged to remove ribosomes. The supernatant [RRL(S/N)] was used in a translation assay supplemented with buffer only (lane 1), P1 of RRL (RRL P1) (lane 2), or P2 of WGE (WGE P2) (lane 3) at 250 μg/mL. (B) The effect of P2 on insertion of TM3 into the membrane is cotranslational. P2 was added at the beginning (B) (lanes 3 and 4) or end (E) of the translation reactions. Translations in lanes 2 and 3 were terminated by addition of puromycin (Pur.). Lane 1 contains no P2 and puromycin. I = topology I; II = topology II.

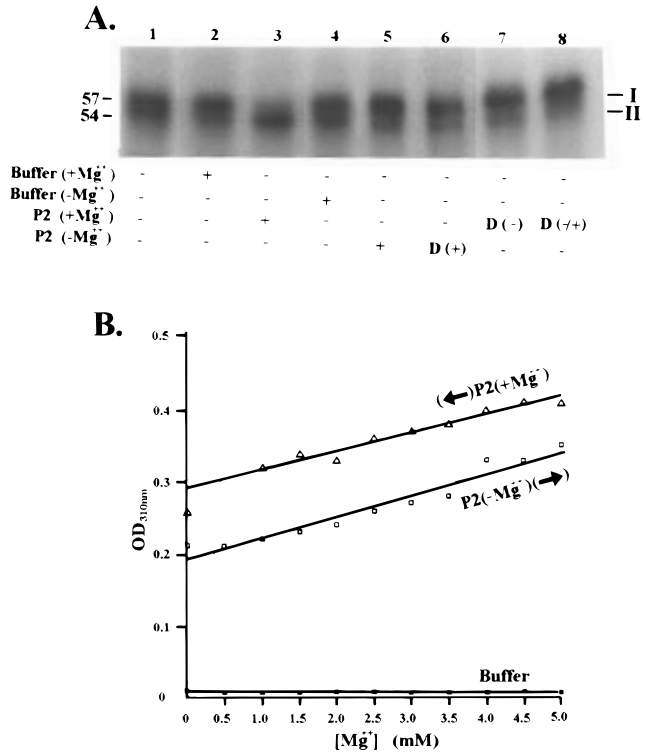

FIGURE 5: Requirement of Mg^{2+} for P2 activity and turbidity. (A) P2 activity requires Mg^{2+}. P2 was prepared in the absence or presence of Mg^{2+} and subjected to dialysis in the presence or absence of Mg^{2+}, respectively. Six micrograms of these P2 fractions was then added to translation reactions. Lanes 3 and 5 are translations supplemented with P2 prepared in the presence [P2-($+Mg^{2+}$)] and absence of Mg^{2+} [P2($-Mg^{2+}$)], respectively. Lanes 6 and 7 are translations supplemented with P2($-Mg^{2+}$) or P2-($+Mg^{2+}$) dialyzed against a buffer with or without Mg^{2+}, respectively. Lane 8 is a translation supplemented with Mg^{2+}-reconstituted P2($+Mg^{2+}$) after removal of Mg^{2+} by dialysis. Lanes 2 and 4 are control translations supplemented with buffers including (+) and excluding (−) Mg^{2+}, respectively. Lane 1, no P2 or buffer. I = topology I; II = topology II. (B) Turbidity caused by P2 is dependent on Mg^{2+} concentration. P2($+Mg^{2+}$) or P2($-Mg^{2+}$) was diluted in buffers containing different concentrations of Mg^{2+}, and the OD_{310nm} was then determined. Open triangles represent P2-($+Mg^{2+}$) (0.24 μg of proteins) diluted in buffers with decreasing concentrations of Mg^{2+}. Open squares represent P2($-Mg^{2+}$) (0.45 μg of proteins) diluted in buffers with increasing concentration of Mg^{2+}. The filled symbols represent a control with buffers only. Arrows indicate that the Mg^{2+} concentration was decreasing or increasing. The data points were fitted to a straight line using linear regression analysis.

behave the same in regulating the membrane insertion of Pgp sequences.

DISCUSSION

In this study, we further characterized the translocation of TM3 of Pgp into the membrane by examining the effects of different translation systems on the process. A salt-resistant, high-speed (150000*g*) pellet fraction (P2) containing both RNA and protein from WGE inhibited insertion of TM3 into membranes. The P2 fraction apparently consists of ribosomes.

The properties of P2 (Table 1) are consistent with the known properties of ribosomes. The active component of P2 was estimated by gel-filtration chromatography to be slightly smaller than 4×10^6 daltons. This value is consistent with a molecular mass of 3.9×10^6 daltons for plant ribosomes (Cammarano et al., 1972) and of 3.8×10^6 daltons for *Artemia salina* (brine shrimp) ribosomes (Nieuwenhuysen & Clauwaert, 1978). Second, the RNA composition of P2 is also consistent with wheat germ ribosomal RNAs (Cammarano et al., 1972). Third, the protein composition of P2 falls in the range expected for wheat germ ribosomes (Bielka, 1982). Fourth, the weight ratio between RNA and protein content in P2 is also consistent with the reported data of ribosomes (Cammarano et al., 1972). Fifth, both the activity in P2 and the activity in ribosomes show similar sensitivity to thermal and alkaline treatment. Finally, Mg^{2+} is required for the function of both P2 and ribosomes. Removal of Mg^{2+} irreversibly inhibits their activity. Taken together, these data demonstrate that the inhibitory activity of P2 is associated with wheat germ ribosomes.

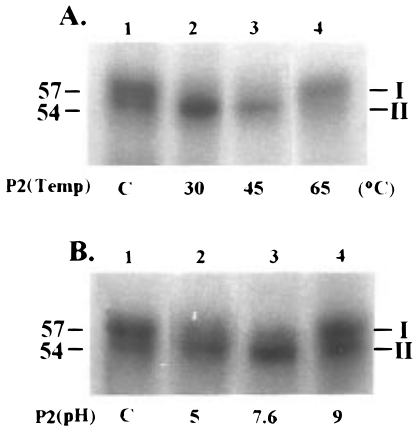

FIGURE 6: Characterization of P2. (A) P2 activity is heat labile. P2 was treated for 30 min at 0, 30, 37, 45, or 65 °C, and then 6 μg of the heat-treated P2 was added to the translation reaction. Representative samples treated at 30 °C (lane 2), 45 °C (lane 3), or 65 °C (lane 4) were shown. Lane 1, no P2. (B) P2 activity is sensitive to high pH. P2 was dialyzed overnight at 4 °C against a buffer with different pH. Six micrograms of P2 from each treated sample was then added to the translation reaction. Representative samples treated at pH 5 (lane 2), pH 7.6 (lane 3), or pH 9 (lane 4) were shown. Lane 1, no P2. I = topology I; II = topology II.

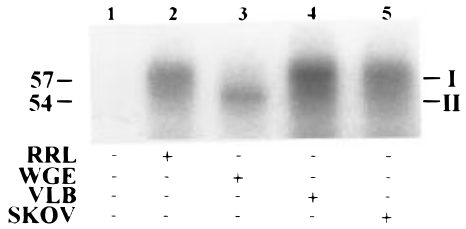

FIGURE 7: Translation and insertion of TM3 by SKOV3 and SKOV/VLB ribosomes. Ribosomes were prepared from parental SKOV3 and drug-resistant SKOV/VLB cell lines and were used to support translation of TM3 in ribosome-stripped RRL system. Lanes 1 and 2 are controls supplemented with buffer only and RRL ribosomes, respectively. Lane 3 is a reaction supplemented with wheat germ ribosomes. Lanes 4 and 5 are reactions supplemented with SKOV/VLB (VLB) and SKOV3 (SKOV) ribosomes, respectively. Ribosomes were supplemented to a final concentration of 250 μg/mL in each reaction. I = topology I; II = topology II.

Our observations suggest that wheat germ ribosomes function differently from mammalian ribosomes in regulating the membrane insertion and folding of Pgp sequences. We cannot yet rule out the possibility that another complex was coisolated with wheat germ ribosomes and is responsible for the inhibitory activity. However, protein contamination of ribosomal preparations was unlikely because (a) the RNA:protein ratio in P2 was consistent with data reported for purified ribosomes (Table 1), and protein contamination would make the ratio significantly lower; and (b) the inhibitory activity of P2 is resistant to high salt and detergent extraction (Sperrazza et al., 1980). In addition, the fact that the inhibitory effect of P2 was exerted cotranslationally indicates that ribosomes are likely to be involved.

Several recent studies suggest that ribosomes may be involved in the topogenesis of membrane proteins. Borel and Simon (1996) showed that the integration of Pgp sequences into membranes cannot occur while the ribosomes are still attached to the nascent peptide. These ribosome-attached peptides are likely still trapped in the protein-conducting channel located on the ER membrane. Membrane integration occurs only after the synthesis of peptide fragments is complete and ribosomes have been released from nascent peptides.

Studies of apolipoprotein B (apo B) by Hegde and Lingappa (1996) suggested that ribosomes and the membrane-conducting channel interact tightly to ensure that nascent secretory proteins are shielded from the cytoplasm during transfer into the endoplasmic reticulum. During the membrane translocation of apo B protein, ribosomes go through several stages of tight and loose association with the protein-conducting channel generated by pausing signal sequences in the nascent peptide (Hegde & Lingappa, 1996).

In this study, generation of the topology I was assayed using the glycosylation of a reporter polypeptide as a criterion. Thus, it is possible that the inhibition in generation of the topology I protein by wheat germ ribosomes was due to the inhibition of glycosylation of the reporter. However, we believe that this alternative is highly unlikely for the following reasons: (a) glycosylation of the first loop linking TM1 and TM2 in Pgp-N3 molecules was not inhibited by wheat germ ribosomes (see Figure 1); (b) no reporter of Pgp-N3 translated by wheat germ ribosomes in the WGE system was found in the lumen of microsomal membranes by proteolysis assay (Zhang & Ling, 1995); (c) glycosylation of pre-pro-alpha factor was not inhibited by wheat germ ribosomes (unpublished observation); and (d) WGE is widely used for topology and biogenesis studies of many membrane and secretory proteins, and no inhibition of glycosylation has been reported for the WGE system [e.g., see Spiess et al. (1989) and Lopez et al. (1990)]. Therefore, we believe that the inhibition in generation of the 57-kDa protein was likely due to the inhibition of membrane insertion of TM3 into membranes.

We propose that ribosomes are important for the reinitiation of membrane insertion of internal TM segments in a polytopic membrane protein such as Pgp. The insertion of a polytopic protein into the membrane may involve many steps of tight and loose association between ribosomes and the protein-conducting channel located in the ER membrane. Alternatively, the WGE ribosomes may lack determinants present in mammalian (e.g., RRL or SKOV3) ribosomes that are required for the redocking function. It is also possible that the important topology determinants are only weakly associated with WGE ribosomes and lost during the ribosome isolation process. We think this possibility unlikely because (a) the topology I was also inhibited when translation was performed in crude wheat germ extract without ribosome isolation (Zhang & Ling, 1995) and (b) the same ribosome isolation buffer B-120 was used in isolating the mammalian ribosomes used in this study (see Figure 7), and no inhibition of topology I was observed with these ribosomes (i.e., the P1 from wheat germ and mammalian cells have different roles in regulating the folding of TM3). Lastly, the effect of WGE ribosomes on membrane folding of Pgp may be due to the weak interaction between wheat germ ribosomes and dog pancreatic microsomes for the reinitiation of membrane insertion by TM3. However, this weak interaction may provide us with a powerful tool to investigate how ribosomes participate in the reinitiation event of membrane integration of polytopic proteins. It is also currently unknown whether the functional difference between WGE and mammalian (rabbit and human) ribosomes in controlling the topology of Pgp has any evolutionary significance.

Testing ribosomes from other species (e.g., brine shrimp, fungi, and bacteria) will help address this issue.

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