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A Protein-Conducting Channel in the Endoplasmic Reticulum

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Summary

The existence of a protein-conducting channel in the endoplasmic reticulum membrane was demonstrated by electrophysiological techniques. Pancreatic rough microsome (RM) vesicles were fused to one side (*cis*) of a planar lipid bilayer separating two aqueous compartments of 50 mM salt. This exposed the cytoplasmic surface of the RMs, with its attached ribosomes, to the *cis* chamber. Addition of 100 μM puromycin to the *cis* side caused a large increase in membrane conductance, presumably the result of puromycin-induced clearance of nascent protein chains from the lumen of protein-conducting channels. When puromycin was added at low concentrations (0.33 μM), single channels of 220 pS were observed. These closed when the salt concentration was raised to levels at which ribosomes detach from the membrane (150–400 mM), indicating that the attached ribosome keeps the channel in an open conformation. A mechanism for a complete cycle of opening and closing of the protein-conducting channel is suggested.

Introduction

Many proteins are unidirectionally integrated into or translocated across cellular membranes either during or shortly after their synthesis. The signal hypothesis was proposed as a conceptual framework for the experimental analysis of these processes (Blobel and Sabatini, 1971; Milstein et al., 1972; Blobel and Dobberstein, 1975; Blobel, 1980; Walter et al., 1984). Its tenets are as follows: First, each protein translocated across (or integrated into) a distinct cellular membrane contains a signal sequence; second, this sequence is membrane specific; third, recognition of this sequence and its subsequent targeting are mediated by a signal sequence–specific recognition factor and a cognate receptor on target membranes; and fourth, translocation across the membrane occurs through a proteinaceous channel. A fifth tenet is that, for proteins to become integrated into the membrane, a stop transfer sequence in the polypeptide opens the protein-conducting channel to the lipid bilayer and displaces the polypeptide from the channel's aqueous environment (Blobel, 1980).

Signal sequence–specific recognition factors and cognate receptors have been identified. Evidence for a protein-conducting channel has, however, remained indirect. Specifically, nascent peptide chains in various stages of translocation across the endoplasmic reticulum (ER) were accessible to aqueous perturbants (Gilmore and Blobel, 1985), evidence consistent with their being located in a proteinaceous channel. Also, short nascent chains were resistant to protease treatment after detergent solubilization of microsomal membranes. This is because individual chains are presumably protected by their location in a tunnel of the ribosome and in a channel in the microsomal membrane (Connolly et al., 1989).

We have used electrophysiological techniques to test directly for protein-conducting channels. These techniques are sensitive enough to reveal individual ion-conducting channels and should readily reveal the presence of presumably larger protein-conducting channels. We previously fused rough microsomes (RMs) of pancreatic ER to planar lipid bilayers (Simon et al., 1989). We observed large channels with conductances of 60, 80, and 115 picosiemens (pS) in 45 mM potassium glutamate—conductances significantly larger than those of ion-conducting channels. Moreover, a large 115 pS channel was seen when inverted vesicles (InVs) from Escherichia coli were fused with planar lipid bilayers. Given that both RMs and InVs can translocate protein, the 115 pS channel is a candidate for a protein-conducting channel. However, no evidence implicated these channels directly in protein translocation. Indeed, the number of channels observed when only one RM vesicle fused to the bilayer was unexpectedly low (Simon et al., 1989). A single RM vesicle contains scores of ribosomes, each potentially synthesizing and translocating a polypeptide chain. We suggested that channels occupied by a nascent polypeptide might not be able to simultaneously conduct ions (Simon et al., 1989). Such channels might be detectable only if they could be purged of nascent polypeptides and if they could be kept open after polypeptide evacuation.

Puromycin, an adenosine derivative, is the classic reagent for uncoupling a nascent polypeptide from its ribosome-bound peptidyl–tRNA. Being an analog of aminoacyl–tRNA, it is covalently incorporated into the carboxy-terminal of a nascent protein chain (Skogerson and Moldave, 1968; Traut and Monro, 1964; Pestka, 1974). The reaction is catalyzed by the peptidyltransferase activity of the ribosome, and the resulting peptidyl–puromycin is released from the ribosome. In intact cells, after incorporation of puromycin into nascent secretory proteins, the peptidyl–puromycin is released from the ribosome, translocated across the ER membrane into the lumen, and then secreted (Siuta-Mangano and Lane, 1981). This indicates that the translocation apparatus can conduct nucleoside derivatives.

Puromycin's effect on RMs has been studied in detail. After formation, peptidyl–puromycin is vectorially transported into the vesicular lumen (Redman and Sabatini, 1966). Efficient coupling of puromycin to nascent chains occurs at salt concentrations as low as 25 mM KCl (Adelman et al., 1973). Ribosomes remain attached to the membrane at this concentration, and they dissociate only at concentrations higher than 100 mM (Adelman et al., 1973). This makes it experimentally possible to separate puromy-

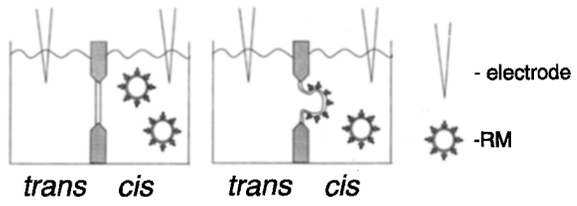

Figure 1. Topology of the Experiment

A diagram illustrating the topological orientation of the microsomes, ribosomes, and the bilayer. The *cis* chamber refers to the side to which the RMs are added. Upon fusion of an RM to the bilayer, the cytosolic surface of the microsome, with its attached ribosomes, faces the *cis* chamber. The *trans* side is equivalent to the lumenal side of the ER. This illustration is not to scale. The diameter of each RM is 0.2 μm, and the diameter of the bilayer is 1 mm.

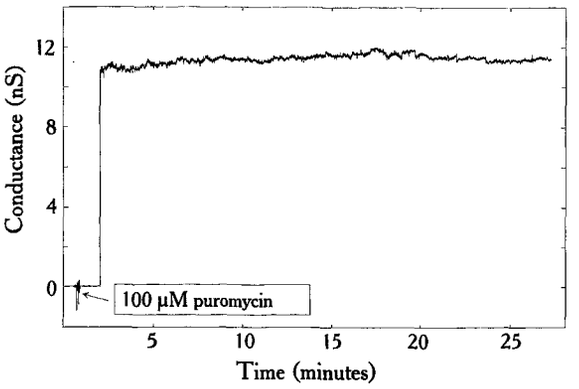

Figure 2. Puromycin-Revealed Ion Channels

Approximately 0.5 μl of RMs (0.02 A_{280} U/ml) were pressure injected from a micropipette into the space adjacent to the *cis* face of the bilayer (Niles and Cohen, 1987; Simon et al., 1989). In the presence of an osmotic gradient, RMs fuse to the bilayer within seconds, which resulted in conductance increases that were stable for hours (Simon et al., 1989). The *cis* compartment was then perfused to eliminate the osmotic gradient and unfused RMs. A 15 μl aliquot of 20 mM puromycin (Boehringer-Mannheim Biochemicals) was added to the *cis* chamber (final concentration 100 μM puromycin) at the arrow, while both chambers were being stirred with two magnetic stir bars (stirring the solutions causes a small increase in the electrical noise). A sharp increase in membrane conductance was observed within 45 s of addition of puromycin to the bath.

cin-induced chain release from subsequent detachment of the ribosome. This appeared important, because keeping the ribosome attached might be essential for keeping the protein-conducting channel open after the puromycin-induced release of the nascent chain at low salt concentrations (Blobel and Dobberstein, 1975).

In this paper we describe and characterize protein-conducting channels in the ER that are revealed by puromycin and that close after detachment of ribosomes with high salt.

Results

Puromycin-Revealed Channels

A planar lipid bilayer was formed in the hole of a Teflon partition separating two aqueous compartments containing 45 mM potassium glutamate. (Glutamate was used as the anion, because it is impermeable to most channels, but should easily permeate a protein-conducting channel. This was of concern, because the ER is the site of synthesis for all plasma membrane ion channels. If these were active in the ER, they would otherwise be observed in our experiments.) RMs were fused to one side of the bilayer, referred to as the *cis* side, exposing their cytosolic surface and attached ribosomes to the *cis* chamber. Their lumenal surface was exposed to the *trans* chamber (Figure 1).

Puromycin, when added to the *cis* chamber, is expected to form peptidyl–puromycin, which should be released to the *trans* chamber. As a result, the protein-conducting channel should be cleared of its nascent chain and, if it remains open, should be able to conduct ions. Indeed, as shown in Figure 2, 45 s after addition of puromycin there was a sharp increase in membrane conductance. This conductance reached a plateau of 10 ns over the next minute or two and remained stable for the next 45 min.

There are only two known loci for puromycin action, both of which depend upon the similarity between puromycin and an amino acid bound to something else: a subgroup of aminopeptidases that cleave aminoacyl–β-naphthylamides that are competitively inhibited by puromycin (Brecher and Suszkiw, 1969; Suszkiw and Brecher, 1970; Hersh, 1981); and the ribosomal peptidyltransferase, for which the normal substrate is aminoacyl–tRNA (Skogerson and Moldave, 1968; Traut and Monro, 1964; Pestka, 1974). Nonetheless, the conductance increase could also have been the result of puromycin's acting as a nonspecific ionophore. Three types of experiments were performed to address this possibility. The outcomes of all three strongly suggest that its effect is not that of a nonspecific ionophore.

First, 100 μM puromycin was added to both the *cis* and *trans* solutions bathing a planar bilayer that did not contain fused RMs. No change in membrane conductance was seen (data not shown). Therefore, the puromycin-induced conductance increase required the presence of RMs.

Second, 100 μM puromycin was added to the solution bathing the *trans* side of the lipid bilayer containing fused RMs. The *trans* side contains the exposed lumen of the RMs (Figure 1). Coupling puromycin to the nascent chain cannot occur on this side. However, if puromycin acts as a nonspecific ionophore for fused RMs, it would cause a conductance increase. Such an increase was not observed after a 10 min incubation (Figure 3). When puromycin was subsequently added to the *cis* side of the same bilayer, a large conductance increase was observed (Figure 3). This *cis*/*trans* specificity demonstrates that puromycin reveals a conductance only when applied to the ribosomal side of the membrane.

Third, after RM fusion, EDTA was added to the *cis* side prior to the addition of puromycin. EDTA blocks the action of peptidyltransferase (Traut and Monro, 1964). Hence, formation of peptidyl–puromycin and subsequent channel clearance and membrane conductance increase should not occur. In fact, a conductance increase did not occur (data not shown), again arguing against puromycin's acting as a nonspecific ionophore on RMs.

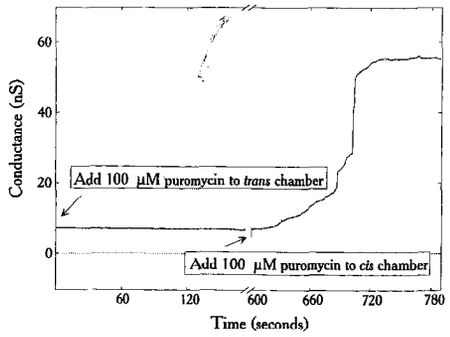

Figure 3. Specificity of Puromycin Action

RMs were fused with the planar bilayer, as described in Figure 2, and the osmotic gradient was eliminated by the addition of 150 μl of 6 M urea, 45 mM potassium glutamate, 5 mM HEPES–KOH (pH 7.5) to the *trans* compartment. At the first arrow, 15 μl of 20 mM puromycin (as described in Figure 2) was added to the *trans* chamber (lumenal side of the RMs). After 10 min, 15 μl of 20 mM puromycin was added to the *cis* chamber (cytosol side of the RMs). A substantial increase of membrane conductance was observed only after the addition of puromycin to the *cis*, ribosomal, face of the bilayer (see Figure 1). The conductance increase, as in the experiment in Figure 2, occurred over a 1–2 min period and then plateaued (note the difference in time scale between the two figures).

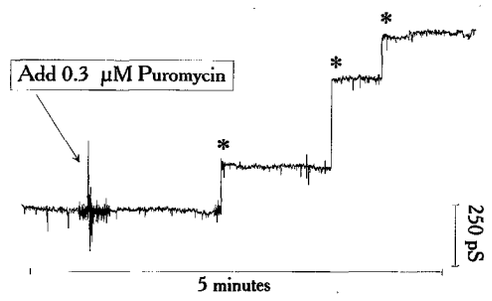

Figure 4. Single Puromycin-Revealed Channels

RMs were fused with the planar bilayer as described in Figure 2, with the exception that the solution bathing the membrane was 50 mM KCl, 5 mM HEPES–KOH (pH 7.5), 3 mM $MgCl_2$. The 300 mM urea on the *cis* side provided the osmotic gradient. Immediately after the first sign of RM fusion to the membrane, the *cis* chamber was perfused with 20 ml of 50 mM KCl, 5 mM HEPES–KOH (pH 7.5), 3 mM $MgCl_2$ to eliminate the osmotic gradient and flush out unfused RMs. After addition of 5 μl of 200 μM stock of puromycin, discrete, consecutive jumps in the conductance of 220 pS, 440 pS, and 220 pS were observed (at asterisks). At faster time resolution, the 440 pS jump resolves into two discrete steps.

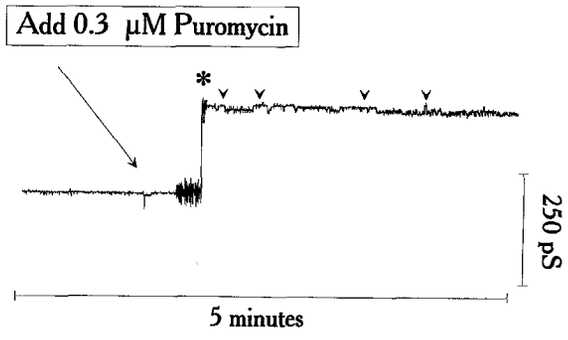

Figure 5. A Single Puromycin-Revealed Channel

RMs were fused with a bilayer, as described in Figure 2. However, after the first opening of a puromycin-revealed 220 pS channel (at asterisk) the *cis* solution was perfused, as above, to remove free puromycin. Some of the small chloride channels that can be seen are marked by the arrowheads.

Taken together, these data strongly suggest that when protein-conducting channels are occupied by nascent polypeptide chains, they are not freely permeable to ions. However, when puromycin releases peptide chains, the channels become unplugged and can then serve as a conduit for ions.

Individual Protein-Conducting Channels

The large conductance increase revealed after 100 μM puromycin was added to the *cis* side of the membrane did not allow analysis and characterization of single protein-conducting channels. To that end, we lowered the concentration of puromycin to 0.33 μM to slow the rate of chain release. About 1 min after the addition of puromycin to the *cis* side of a bilayer containing fused RMs, the conductance increased in one discrete step of 220 pS (Figure 4, steps at asterisks). Minutes thereafter, a second step increase of 440 pS (which at higher time resolution is seen as two discrete steps) and a third step increase of 220 pS were observed (Figure 4). This suggests consecutive stepwise clearance first of only one, then of an additional two, and finally, of a fourth protein-conducting channel in the fused RMs. These channels remained open for many minutes until we further perturbed the system (as described below).

To analyze only a single channel, the *cis* chamber was perfused after the first puromycin-induced clearance of a channel, to remove unreacted puromycin and to inhibit further clearance of other protein-conducting channels (Figure 5). This single 220 pS channel also remained open.

The channels shown in Figures 4 and 5 were recorded in the presence of 50 mM KCl instead of 50 mM potassium glutamate. This enables small, 10 pS chloride channels to be seen; they look like small boxcars riding atop of the large, puromycin-revealed channel (arrowheads in Figure 5). The conductance of a channel is a function of the ions that pass through it. The puromycin-revealed channels were 220 pS in 50 mM KCl, 5 mM HEPES–KOH. This was reduced to 180 pS in 50 mM potassium glutamate, 5 mM HEPES–KOH and increased to 360 pS in 50 mM potassium glutamate, 50 mM HEPES–KOH, indicating that the channel is permeable to both glutamate (MW 175.5) and HEPES (MW 238.3). The mobility of glutamate in solution is approximately 25% that of chloride. Thus, the measured conductance is lower, even though the channel is freely permeable to glutamate. Neither the conductance nor the gating of the channel was affected by changing the magnitude or polarity of the voltage across the membrane. The conductance of the channel increased almost linearly with increases of ion concentration (Figure 6).

Closing of Protein-Conducting Channel

Formation of peptidyl–puromycin and its subsequent vectorial discharge into the lumen of the ER proceed efficiently

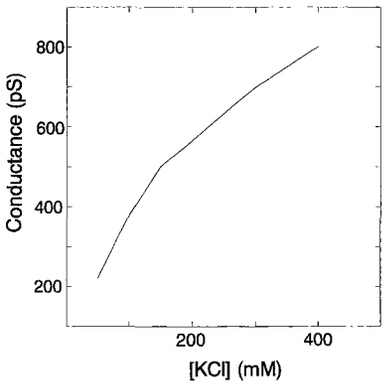

Figure 6. Effect of Salt on Channel Conductance

The single-channel conductance of the puromycin-revealed channels is plotted as a function of the concentration of KCl.

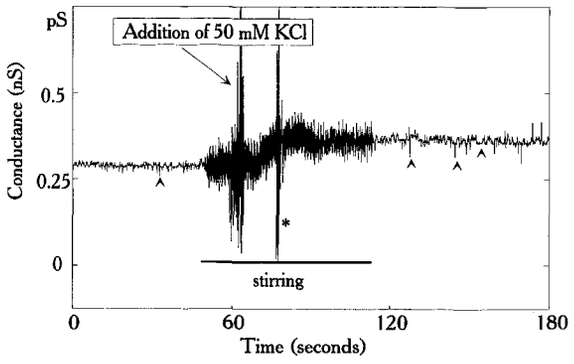

Figure 7. Effect of Raising the KCl Concentration on Channel Activity

RMs were fused to a bilayer, the osmotic gradient was eliminated, and puromycin was added and removed as described in Figure 4. The KCl concentration was then increased a 50 mM step, by the addition of 50 μl aliquots of 3 M KCl to both the *cis* and *trans* solutions while both solutions were being stirred. It was necessary to vigorously stir the solutions during the addition of KCl; otherwise the membranes broke. Thus, starting just before the addition of KCl and lasting for a few minutes after, there is a substantial increase in the electrical noise from stirring the two chambers. KCl was added to the chambers at the first large transient in the recording (at the arrow). As the KCl diffused toward the membrane, increasing the number of charge carriers available to flow through the channel, there was a substantial increase in the conductance. At the second large electrical transient (asterisk), 50 μl aliquots were removed from both the *cis* and *trans* solutions to return the volumes to 3 ml. There is an increase both in the total membrane conductance and in the size of the small chloride channels, which at this sweep speed look like small hairs on the record (arrowheads). The membrane conductance continued to increase with further additions of KCl (data not shown). In the presence of KCl (rather than potassium glutamate), there was a greater variability in the magnitude of the background membrane conductance. This was due to varying numbers of potassium channels and, especially, chloride channels that have fused to the bilayer.

at KCl concentrations below 100 mM. Subsequent dissociation of ribosomes, however, occurs only at KCl concentrations between 100 and 1000 mM (Adelman et al., 1973). All previous experiments were deliberately carried out at low salt concentrations to keep the ribosomes in place. We next investigated whether removing the ribosomes at high salt concentrations would affect the protein-conducting channel. These experiments were done in the presence of KCl rather than potassium glutamate, enabling us to monitor the effects of raising the KCl concentration on both the small chloride channel and the large puromycin-revealed channel simultaneously (see Figure 5). After revealing a single protein-conducting channel with puromycin, we perfused the *cis* chamber to remove unreacted puromycin. The salt concentration was then raised 50 mM, by the addition of 3 M KCl to both the *cis* and the *trans* chambers. This resulted in a gradual increase in membrane conductance, reflecting the time course for KCl to diffuse toward the membrane and to increase the number of charge carriers flowing through the channels (Figure 7). Raising the salt concentration from 50 to 100 mM had no other noticeable effect on the channel's behavior.

The salt concentration was increased in additional 50 mM steps. When it reached 300 mM, one of the puromycin-revealed channels closed (Figure 8, top panel). Other puromycin-revealed channels closed at 150 mM KCl (Figure 8, middle panel), or 400 mM KCl (Figure 8, bottom panel). One channel did not even close at 600 mM salt (data not shown). We do not know if this particular channel would have closed if the membrane had been observed longer or if the salt concentration had been raised yet higher. However, it has been reported that only 75% of the ribosomes are removed from the membrane, even in higher salt concentrations (Adelman et al., 1973).

These data demonstrate that keeping the ribosome attached is essential for keeping the channel open after formation and clearing of peptidyl–puromycin from the channel at low salt concentration. Thus, at low salt concentration, the closing of the protein-conducting channel can be experimentally separated into two distinct steps: release of the nascent chain and detachment of the ribosome.

Discussion

The results presented in this paper provide compelling evidence for the existence of protein-conducting channels in the ER. Figure 9 summarizes some of its features. The tunnel in the large ribosomal subunit through which the nascent chain exits (Lake, 1985; Yonath et al., 1987) is shown to be aligned with the lumen of the protein-conducting channel. This is based on the observation that nascent chains in RMs are relatively resistant to protease treatment (Sabatini and Blobel, 1970; Connolly et al., 1989).

The protein-conducting channel is not freely permeable to ions when occupied by a translocating polypeptide. Therefore, the nascent chain, which projects into the ER lumen, is shown to fill the channel. A protein-conducting channel would have to be large enough to accommodate two strands (i.e., a loop) of a translocating polypeptide (Perara and Lingappa, 1985; Shaw et al., 1988). It seemed surprising that the channel could be large enough to allow a polypeptide loop to move across and yet not simultaneously let ions move freely across. However, the protein-conducting channel would not have to fit snugly around the polypeptides to restrict ionic movement. Ions normally

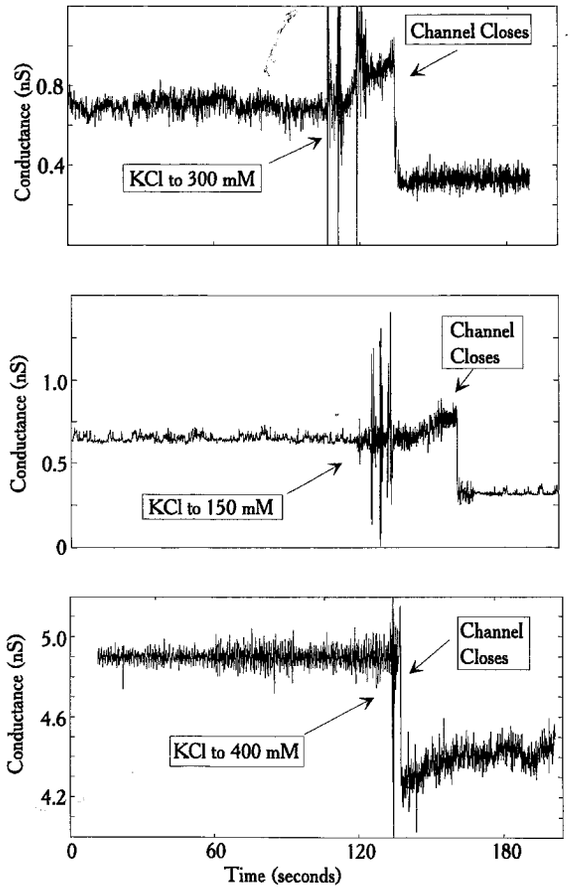

Figure 8. Closure of Puromycin-Revealed Channels

The KCl concentration was increased in 50 mM steps, as described in Figure 7. After several such steps, the single, large, puromycin-revealed channels closed. The channels usually closed as the salt concentration was increasing near the membrane. In the experiment shown in the top panel, the channel closed as the concentration reached 300 mM (from 250 mM). In the experiment shown in the middle panel, the channel closed as the concentration reached 150 mM (from 100 mM). This is the same channel whose opening was shown in Figure 5. In the experiment shown in the bottom panel, the channel closed as the concentration was first being raised above 350 mM (to a final concentration of 400 mM). Thus, in the bottom panel, most of the increase of conductance through the potassium and chloride channels is observed to occur after the closing of the puromycin-revealed channel. The top two panels are from experiments in which a single puromycin-revealed channel was opened. The bottom panel shows the closing of one of the four puromycin-revealed channels whose opening was shown in Figure 4. In all experiments, the size of the closing step is proportional to the salt concentration (see Figure 6).

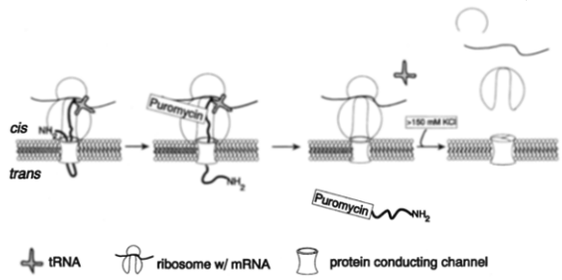

Figure 9. Model for Puromycin-Revealed Protein-Conducting Channels

As shown in the four panels, this model is as follows. In the first panel, ions are unable to freely pass through the protein-conducting channel when a nascent chain is being translocated. Second, puromycin is incorporated into the carboxy-terminal of the nascent chain by the ribosomal peptidyl transferase. Third, peptidyl–puromycin is released from the ribosome and translocated across the bilayer. This unplugs the protein-conducting channel, allowing ions to freely pass through. Finally, raising the salt concentration releases the ribosome from the membrane, allowing the protein-conducting channel to close.

migrate in a hydrated state with a radius of 10 Å (Mullins, 1959; Hille, 1984). The energy of hydration is too large for them to shed their water shells readily. Ion channels facilitate dehydration through the use of specific reactive groups. Hydrated ions may not readily pass a protein-conducting channel lacking such groups when the channel is occupied by a translocating peptide. Only when the protein-conducting channel is cleared by the formation of peptidyl–puromycin and subsequent vectorial discharge into the ER lumen is it detected as a large ion-conducting channel of 220 pS (Figures 4 and 5 and Figure 9, third panel). It is quite possible that, as sections of the polypeptide backbone containing small side chains move through the channel, some ions slip through as well. However, a 220 pS conductance, at a membrane potential of −50 mV, corresponds to 68 million ions per second. The slippage of ions around a translocating polypeptide, if it occurs, is likely to be orders of magnitude slower and would not be detected by these techniques.

The protein-conducting channel closed when the salt concentration was raised high enough (150–400 mM) to dissociate the ribosomes (Figure 8 and Figure 9, fourth panel). This suggests that the ribosome may directly interact with a subunit(s) of the protein-conducting channel, keeping it in an open configuration. We do not know whether the ribosomal tunnel and the membrane channel are so tightly coupled to each other that ions must flow through both or whether ions can flow between the ribosome-membrane junction to enter the protein-conducting channel. It is also possible that closing of the channel may be regulated by some other factor that, like the ribosomes, can only be removed from the membrane by salt after chain release.

Physiological Gating of the Channel

What are the physiological signals that regulate the opening and closing of this channel? Premature chain termination by puromycin and closure of the channel by high salt concentrations are nonphysiological conditions. In vivo, chain termination is effected by termination codons and release factors. It is not yet known whether the ribosome detaches from the ER membrane after each round of chain translocation and, if so, how it would be accomplished. Nevertheless, the observed closure of the protein-conducting channel after artificial chain release and ribosome detachment makes physiological sense. If the protein-conducting channel did not close after each round of translocation, ions and small metabolites could indiscriminately enter the lumen of the ER and be lost from the cell via the secretory pathway. Likewise, if metabolites and ions were

compartmentalized into the ER, they would leach out into the cytoplasm. Proteins can be translocated across many membranes posttranslationally. Clearly, in these cases, the ribosome is not needed to keep the channel open. However, the channel cannot close as long as it is occupied by a translocating chain. The ribosome, or some other cytosolic factor, may be facilitating translocation by stabilizing the protein-conducting channel in an open conformation. Similarly, there are many examples of ion channels that cannot close when their lumina are occupied. Local anesthetics (Schwarz et al., 1977), impermeant ions (Marchais and Marty, 1979; Van Helden et al., 1977), and tetraethylammonium (Armstrong, 1971) all insert into the lumen of ion channels, thereby keeping them from closing.

If, in fact, the protein-conducting channel of the ER closes after each round of translocation, it follows that it opens for each new round. To prevent leakage, the opening is probably as tightly linked to the initiation of chain translocation as closure is linked to its termination. The conditions necessary for opening this channel are the conditions necessary for initiating polypeptide translocation. The nascent ER-targeted signal sequence is first recognized by SRP. The complex of the ribosome, mRNA, SRP, and signal sequence is then targeted to the ER through the SRP receptor. This is accompanied by displacement of the signal sequence from SRP (Gilmore and Blobel, 1983) in a GTP-dependent reaction (Connolly and Gilmore, 1989; Poritz et al., 1990).

What are the sufficient conditions for opening the channel? The simplest scenario is that the signal sequence itself serves as the ligand to open the channel. After its GTP-dependent displacement from SRP, it would bind to a subunit of the protein-conducting channel. Alternatively, the SRP receptor—or the ribosome—could serve as the ligand, following conformational changes that might be linked to either binding of SRP to its receptor or displacement of SRP from either its receptor or the ribosome.

What is the relationship between the puromycin-revealed protein-conducting channels (Figures 4–8) and the large channels of 60, 80, and 115 pS seen in the absence of puromycin (Simon et al., 1989)? It is possible that the latter channels are unrelated to protein translocation and are involved in transport of metabolites or ions. Alternatively, they may represent subconductances of a protein-conducting channel that has been teased into partially opened states (see the section on physiological gating above). The 60, 80, and 115 pS channels opened more frequently in the presence of GTP and closed more frequently in the presence of GTPγs (Simon et al., 1989), evidence consistent with the finding that binding and hydrolysis of GTP play a role in protein translocation (Connolly and Gilmore, 1989).

Comparison with Other Channels

Although protein-conducting channels have not been described previously, they need not be that different from some of the well-characterized ion channels. Conductance through the potassium channel is blocked when it is occupied by a flexible peptide region at its aminoterminal (Armstrong, 1971; Hoshi et al., 1990; Zagotta et al., 1990).

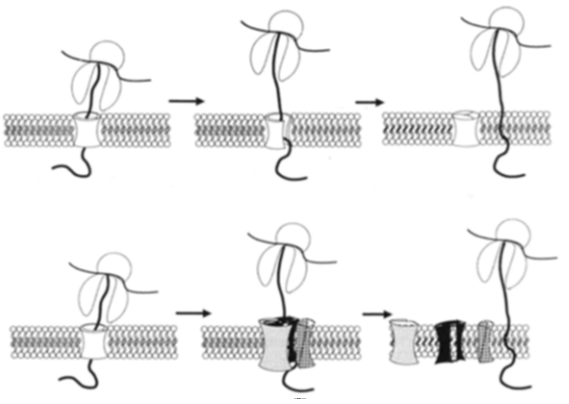

Figure 10. Model for the Integration of Transmembrane Proteins

Two different hypotheses could explain how a nascent polypeptide chain could be displaced from the channel's aqueous lumen and into the hydrophobic core of the bilayer. After recognition of a stop transfer sequence (Blobel, 1980) in the translocating polypeptide, the translocating polypeptide could slip through a gap in between two subunits of the protein-conducting channel (top panel), or the subunits of the protein-conducting channel could disassemble (bottom panel).

Inactivation of the sodium channel is believed to occur by a similar mechanism (Armstrong and Bezanilla, 1977). Similarly, ions cannot freely flow through a protein-conducting channel when it is translocating a nascent peptide (see the beginning of Discussion). The potassium channel is postulated to have a hydrophobic binding pocket inside the mouth of its pore (Armstrong, 1969); likewise, a protein-conducting channel may have a site to bind loosely the short hydrophobic core of the signal sequence. Furthermore, like many other channels, the protein-conducting channel is likely to function like a ligand-gated channel (see the section on channel gating above).

Integration of Transmembrane Proteins

A protein-conducting channel, like an ion channel, would need to open in only one dimension—perpendicular to the bilayer—to allow proteins to move across. However, to translocate and integrate the domains of an integral transmembrane protein, it would need to open in two dimensions. First, it must open perpendicularly to the membrane to allow a domain of the protein to pass through (Figure 10). Then it must open into the plane of the membrane to allow another domain, which is to become a transmembrane segment, to move laterally into the lipid environment. This means that the protein-conducting channel must be able to recognize a "stop transfer sequence" that signals a latent transmembrane segment (Blobel, 1980).

How can the transmembrane domain leave the channel? It has been suggested that the protein-conducting channel consists of subunits that dissociate, leaving a transmembrane segment embedded in the bilayer (Figure 10, bottom panel) (Blobel, 1980). Alternatively, the protein-conducting channel may remain as an intact structure (Singer et al., 1987) that allows the transmembrane segments to slip between two of its subunits (Figure 10, top panel). In this capacity, this channel could be like the monazomycin channel, which is permeable to charged qua-

ternary ammonium ions with long (C_{12}) alkyl chains. The charged groups pass down through the lumen of the channel, and it has been suggested that the hydrocarbon chains slide though the hydrophobic region at the interfaces between the subunits (Heyer et al., 1976).

Role of Lipids in Translocation

An alternative to the idea of a proteinaceous channel is the proposal that the translocating chains move directly through the hydrophobic core of the lipid bilayer (Von Heijne and Blomberg, 1979; Engelman and Steitz, 1981). This notion was prompted by the observation that many signal sequences targeted to the ER or prokaryotic plasma membrane have little primary sequence homology other than a stretch of hydrophobic amino acids (Gierasch, 1989; Engelman and Steitz, 1981; Von Heijne and Blomberg, 1979; Wickner, 1980; Chou and Kendall, 1990). Compatible with this hypothesis are the observations that chemically synthesized signal peptides change to an α-helical conformation in a hydrophobic environment (Briggs et al., 1989; Cornell et al., 1989) and that they can effect nonbilayer structures in lipids (Killian et al., 1990). This has led to several suggestions that phospholipids play an essential role in translocation, either by directly interacting with the signal sequence (Briggs et al., 1989; Nesmeyanova and Bogdanov, 1989) or by forming a pore of lipids in a hexagonal phase structure (Killian et al., 1990). However, the observation that signal peptides can interact with lipids does not mean that signal sequences do interact with lipids. Indeed, the first interaction of ER-addressed signal sequences is not with membrane lipids but with a protein—the 54 kd subunit of SRP (Krieg et al., 1986; Kurzchalia et al., 1986). It is argued that if the signal sequence interacts directly with the lipid bilayer, increasing the hydrophobicity of the signal sequence should increase the efficiency of translocation (Chou and Kendall, 1990). But increasing the length of hydrophobic amino acids and α-helical content sometimes increases (Chou and Kendall, 1990) and other times decreases (Yamamoto et al., 1990) protein translocation. This suggests that the net hydrophobicity is not the factor that determines whether a polypeptide can cross the hydrocarbon barrier. Other arguments against a primary role for lipids in protein translocation have been recently reviewed (Singer, 1990).

Still, it was important to examine whether lipids could affect the observed puromycin-revealed channels. When a vesicle fuses with a planar lipid bilayer, the lipids of the vesicle rapidly exchange with those of the significantly larger (8000×) planar bilayer (Woodbury and Miller, 1990). We repeated the experiments reported in this paper using bilayers formed from PE (E. coli):PS (bovine brain) (1:1); PE:PC (egg) (1:1); diphytenol PC (synthetic lipid 20:0); PC; PE:diphytenol PC (1:1); and PE:diphytenol PC:cholesterol (2:2:1 or 1:1:1). The experimental results were indistinguishable from one another, indicating that the channels are insensitive to bulk lipid (data not shown). If there are any essential lipids for translocation, they would have to be tightly bound to the translocation site. We believe that the protein-conducting channel is formed of proteins, because its discrete size seems inconsistent with a nonbilayer lipid structure and because it is insensitive to changes of membrane lipid.

Candidate Proteins

There are a number of proteins that have been identified in the endoplasmic reticulum that could be the protein-conducting channel. One candidate is the "signal sequence receptor complex" (SSR) (Wiedmann et al., 1987). The α subunit of this hetero-oligomeric complex of integral membrane proteins could be cross-linked to the signal sequence (hence its name), as well as to downstream peptides of the translocating chain (Wiedmann et al., 1989; Krieg et al., 1989) (hence its proposed role as a subunit of a channel-forming protein). Since the channel appears to be kept open by attachment of the ribosome, ribosome receptors are also candidates for the channel protein(s). An integral ER membrane protein of 180 kd has been proposed to be a ribosome receptor (Savitz and Meyer, 1990). Cleavage of the signal sequence is temporally coupled to translocation. The signal peptidase complex is a hetero-oligomer of five integral membrane proteins and is also a candidate for (or may be a part of) a protein-conducting channel (Evans et al., 1986). It is also possible that the channel protein(s) has not yet been identified and isolated. We hope through purification and reconstitution of these candidates to identify the channel protein(s).

Implications for Other Systems

A number of different biochemical pathways have been identified for signal sequence–mediated protein movement across membranes. It is not yet known to what extent they use similar biophysical mechanisms nor whether the systems that use similar schemes are phylogenetically related or have evolved analogous strategies. However, all of these systems have to solve the same biophysical problem of moving a long, often hydrophilic, polypeptide across a hydrocarbon barrier.

The protein-conducting channel of the ER is likely to be a prototype of similar channels for protein translocation across the prokaryotic plasma membrane (from the cytoplasm to the periplasmic space in the gram-negative bacteria), across the inner mitochondrial membrane (from the matrix to the intermembrane space), across the chloroplast inner membrane (from the stroma to the intermembrane space), and across the thylakoid membranes (from the stroma to the intradisc space). It has been suggested that all of these membranes are evolutionarily related to the prokaryotic plasma membrane (Blobel, 1980). The signal sequences targeted to these membranes appear to be similar and, to some extent, interchangeable.

A second class of transporters move polypeptides across two membranes. In the case of signal sequence–mediated translocation from the cytoplasm to the matrix of mitochondria or to the stroma of chloroplasts, the existence of two aligned channels in the inner and outer organelle membrane has been proposed (Blobel, 1980).

Clearly distinct from these lipid bilayer-embedded channels is the "transporter" of the nuclear pore complex. Molecules of up to 100 Å in diameter can freely permeate the pore complex without a targeting signal (Paine et al., 1975;

Peters, 1983; Jiang and Schindler, 1986). With such a signal, gold particles of up to 230 Å can be transported (Dworetzky et al., 1988; Dworetzky and Feldherr, 1988). This suggests that molecules would not need to unfold for translocation. This is in contrast to the lipid-embedded channels (described above) and the ATP-binding cassette (ABC) transporters (described below), for which proteins must be in an "unfolded" state to translocate across the membrane.

In addition, membranes contain protein transporters that are representatives of the large ABC family. Such proteins may transport peptides from the cytoplasm to the lumen of the ER for binding to MHC I molecules (Deverson et al., 1990; Trowsdale et al., 1990). The determinants for transport are not known. ABC proteins are involved in the transport of certain proteins across bacterial membranes (Blight and Holland, 1990; Gilson et al., 1990) and the eukaryotic plasma membrane (Kuchler et al., 1989; McGrath and Varshavsky, 1989). The transporters are often referred to as "pumps." This implies that they expose their lumen sequentially to each side of the membrane. However, when transporting a polypeptide of 1,000 amino acids (e.g., hemolysin), the lumen of the transporter must be open to both sides of the bilayer. It therefore appears that distinct mechanisms have evolved to move proteins across membranes. Although the lipid-embedded transporters are unlikely to be structurally homologous, it will be of interest to investigate to what extent they utilize common mechanisms.

Experimental Procedures

Bilayer Formation

Planar phospholipid bilayers of bacterial phosphatidylethanolamine (PE) and bovine phosphatidylserine (PS) (20 mg/ml each in decane [Fluka]) were made as described previously (Mueller et al., 1962; Mueller et al., 1963; Mueller and Rudin, 1969; Simon et al., 1989) across a 1 mm hole in a KEL-F partition separating two chambers. All of the lipids used for these experiments were either purified or chemically synthesized lipids from Avanti-Polar (Birmingham, AL). The lipids are stored as stock solutions (10% [w/v]) in chloroform with argon gas (to reduce oxidation). Bilayer-forming solutions are made by pipetting volumes (usually 100 μl) into a glass test tube with a Teflon-lined stopper. The chloroform is evaporated with argon, the tube is evacuated for 20 min, and the lipids are resuspended in the appropriate solvent. The experiments described here were repeated with mixtures of: PE:PS, 10:10 mg/ml; PE:PS, 20:10 mg/ml; PE, 20 mg/ml; soybean phosphatidylcholine (PC), 20 mg/ml; chemically synthesized diphytenoylphosphatidylcholine (DPPC), 20 mg/ml; DPPC:PE:PS, 10:10:5 mg/ml; and DPPC:PE, 20:20 mg/ml. In addition, each of the above solutions was made with 5 mg/ml cholesterol. The two chambers initially held 3 ml of *trans* solution (45 mM potassium glutamate, 5 mM HEPES–KOH [pH 7.5], 3 mM $MgCl_2$). A bilayer was only used if, after initial formation, it had a stable conductance of 10 pS (10^9 Ω·cm²) and a capacitance of 10 nF (~1 μF/cm²) for at least 20 min. After bilayer formation, the *cis* chamber was made hyperosmotic by the addition of 150 μl of 6 M urea, 45 mM potassium glutamate, 5 mM HEPES–KOH, 3 mM $MgCl_2$ to a final concentration of 300 mM urea. All experiments were repeated with the *cis* chamber initially hyperosmotic, with 3.0 ml of *cis* solution (250 mM sucrose, 45 mM potassium glutamate, 5 mM HEPES–KOH [pH 7.5], 3 mM $MgCl_2$). The results obtained with urea and sucrose were indistinguishable from each other. When PS was included in the lipid-forming solution, 10 mM $CaCl_2$ was added to the *cis* solution. After fusion of the RMs, the $CaCl_2$ was perfused out.

Fusion of the RMs

RMs were prepared as described previously (Walter and Blobel, 1983) and were stored in 20 μl aliquots at −80°C in 250 mM sucrose, 50 mM triethanolamine–HCl [pH7.5], 1 mM dithiothreitol at a concentration of 50 A_{280} U/ml (determined in 1% SDS). Approximately 0.5 μl of RMs (0.02 A_{280} U/ml) were pressure injected (Picospritzer II, General Valve Corporation, NJ) from a micropipette into the space adjacent to the *cis* face of the bilayer (Niles and Cohen, 1987; Simon et al., 1989). In the presence of an osmotic gradient, RMs fuse to the bilayer within seconds, which results in conductance increases that were stable for hours (Simon et al., 1989). After fusion, the *cis* compartment was then perfused with 20 ml of *trans* solution to eliminate the osmotic gradient and unfused RMs. This was accomplished with a homemade device of two 20 ml syringes connected plunger-to-plunger (C. Miller, personal communication).

Addition of Reagents

Stock solutions of 20 mM puromycin–HCl (Boehringer-Mannheim Biochemicals and Calbiochem-Behring) were adjusted to pH 7.5 with KOH and stored frozen in 20 μl aliquots. Puromycin was added to the chambers by hand with an Eppendorf pipette while both chambers were being stirred with two magnetic stir bars (stirring the solutions causes a small increase in the electrical noise).

Electrophysiology

The voltage across the bilayer was applied via two calomel electrodes, which are connected to the *cis* and *trans* compartments via glass capillary tubes filled with 150 mM KCl in 1.5% agar. The voltage was imposed and the current measured with a homemade voltage clamp using an operational amplifier (AD515 Analog Devices, Norwood, MA) with feedback resistors of 10^8 (0.5%), 10^9 (0.5%), or 10^{10} (1%) ohms (K and M Electronics Inc., Springfield, MA). The *trans* chamber was grounded, and all voltages were recorded for the *cis* chamber relative to *trans*. The current required to impose the voltage across the bilayer was simultaneously stored on a chart recorder (Kipp and Zonen BD-41, Delft, Holland), digitized (Neuro-Corder Model DR-390, Neurodata Corp., NY), and stored on a video tape recorder (Panasonic). Data were imported into an IBM-compatible computer via a digitizing board (Scientific Solutions, 125 kHz—part of the TL-1 DMA interface of PClamp, Axon Instruments, Foster City, CA). The conductance was plotted as the current divided by the applied voltage.

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