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A 64-kilodalton membrane protein of *Bacillus subtilis* covered by secreting ribosomes

(ribosome-binding protein/membrane protein topography/protein secretion apparatus)

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ABSTRACT **The complexed (ribosome-bearing) membrane fraction of *Bacillus subtilis* contains several proteins (CM-proteins) that are virtually absent from the ribosome-free fraction and hence might be components of the apparatus of protein secretion. We have determined, by trypsin digestion and by labeling with a nonpenetrating reagent (diazoiodosulfanilic acid), the accessibility of four of these proteins on the two surfaces of the membrane, as exposed either in protoplasts or in inverted membrane vesicles. The 68-kilodalton protein is a transmembrane protein and the 45-kilodalton protein faces only the external surface, whereas the 31-kilodalton protein is inaccessible from either side. Of particular interest is the 64-kilodalton protein: it can be digested by trypsin, and can bind antibody, on the cytoplasmic surface, but only after the ribosomes have been released. This protein is thus evidently a component of the apparatus of protein secretion, closely covered by secreting ribosomes. Whether the other CM-proteins are also involved in protein secretion is uncertain.**

Earlier work in this laboratory has shown that in lysates of *Bacillus subtilis* the disrupted membrane can be separated by density into a complexed fraction (carrying ribosomes) and a free fraction (without ribosomes) (1, 2). It seemed possible that comparison of these fractions might identify the components of the apparatus of protein secretion, if they were present only in the complexed fraction. In fact, the complexed fraction was found to contain at least six immunologically distinct major electrophoretic bands (CM-proteins, for complexed membrane proteins) that were virtually absent from the free membrane fraction (1, 2). However, because the free membrane fraction also contains unique major bands (1), it seems clear that proteins involved in protein secretion are not the only ones to be separated in this fractionation. It may also be relevant that the rough microsomes of animal cells contain only two major bands, of 65 and 63 kilodaltons (kDa), absent from the smooth microsomes (3).

To shed further light on the functions of the CM-proteins we have determined their accessibility on the two surfaces of the membrane, using antibodies, trypsin, and a nonpenetrating reagent, diazoiodosulfanilic acid (DSA). The results provide strong evidence that one CM-protein, of 64 kDa, is part of the apparatus of protein secretion, because it is tightly covered by ribosomes on the cytoplasmic surface of the membrane. It may thus be a ribosome-binding protein. However, that term is not yet justified, for the protein may, alternatively, be bound only to the nascent secreted chain, while the active secretion of that chain draws the ribosome close to the membrane.

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MATERIALS AND METHODS

The strain of *B. subtilis*, conditions of growth, and antibodies were as described (1, 2). Labeled cells were prepared by growth in the presence of [^{35}S]methionine for two generations before harvesting (at *ca.* 6×10^8 cells per ml). Cells were converted to protoplasts as described (1, 2).

Cell Fractionation. The total membrane and the complexed, derived, and free membrane fractions were prepared as described (1), except that the protoplasts were not isolated before lysis, and they were lysed in a French press. Briefly, the lysates were centrifuged in a biphasic sucrose gradient and the complexed membrane was collected at the interface. To prepare the derived membrane, complexed membrane was exposed to 0.01 mM Mg^{2+} and the resulting less dense membrane fraction, freed of ribosomes, was collected.

Immunoprecipitation. As previously described in detail (2), the sample was dissolved in 1% $NaDodSO_4$, diluted 1:10, and incubated with an excess of IgG from rabbit antiserum to each CM-protein, and the complex was adsorbed on formalin-fixed *Staphylococcus aureus* A. The precipitate was analyzed by gel electrophoresis.

Treatment of Protoplasts with Trypsin. Labeled protoplasts [100 μCi of [^{35}S]methionine in 50 ml of culture (1 Ci = 3.7×10^{10} Bq)] were suspended in 1.3 ml of cold buffer A [10 mM Tris·HCl, pH 7.6/50 mM KCl/10 mM $Mg(OAc)_2$] containing 20% sucrose and chloramphenicol at 100 μg/ml. A portion of this suspension (0.4 ml) was incubated for 60 min at 0°C with trypsin (2 mg/ml) and then mixed with 10 ml of soybean trypsin inhibitor (2 mg/ml) in cold buffer B (50 mM Tris·HCl, pH 7.4/150 mM NaCl) with 20% sucrose. The protoplasts were collected by centrifugation (25,000 × *g*, 30 min), washed with 10 ml of buffer B with sucrose, and solubilized in 0.5 ml of buffer B with 1% $NaDodSO_4$ held at 100°C for 3 min. After the traces of debris had been removed, portions were analyzed.

Treatment of Complexed Membrane with Insolubilized Trypsin. Freshly isolated complexed membrane (160 μg of protein, 3.5×10^7 cpm), from cells labeled as above, was suspended in 0.15 ml of buffer A and incubated for 60 min at 25°C, with several intervals on a Vortex mixer, with 50 μl of a suspension of insoluble trypsin on beads (5 units when assayed with benzylarginine ethyl ester). The reaction was terminated by adding 1.0 ml of cold buffer B with trypsin inhibitor (2 mg/ml). After solubilization of the membrane, followed by centrifugation to remove beads and debris, the supernatant was analyzed.

Abbreviations: kDa, kilodalton(s); DSA, diazoiodosulfanilic acid; CM-proteins, proteins unique to complexed membrane.

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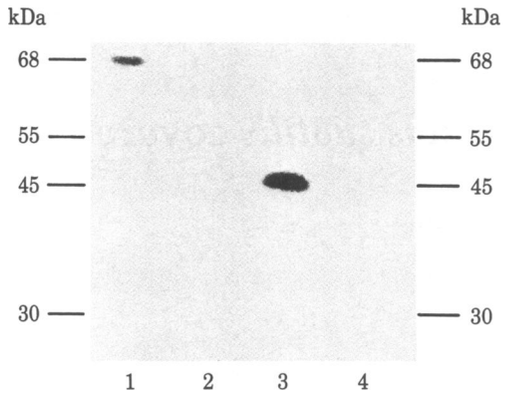

FIG. 1. Extracellular labeling of CM-proteins in protoplasts by [^{125}I]DSA. The protoplasts were labeled with [^{125}I]DSA, washed, lysed, and subjected to immunoprecipitation as described in the text. Equal portions of the lysate were used for each antiserum, and the immunoprecipitates were electrophoresed and fluorographed (7-day exposure). Antibodies used were lane 1, anti-68-kDa protein; lane 2, anti-64-kDa protein; lane 3, anti-45-kDa protein; and lane 4, anti-31-kDa protein. Positions of marker proteins are indicated on the sides.

Preparation of Diazo[^{125}I]iodosulfanilic Acid ([^{125}I]DSA). [^{125}I]Iodosulfanilic acid (New England Nuclear) was converted to the diazonium salt according to the supplier's instruction manual, by incubating 1.0 mCi for 5 min on ice with 5 μl of 0.05 M $NaNO_2$ and 5 μl of 0.1 M HCl, and then neutralizing with 50 μl of 10 mM sodium phosphate buffer (pH 7.4). The [^{125}I]-DSA thus formed was added immediately to the reaction mixtures described below.

[^{125}I]DSA Labeling of Protoplasts. Protoplasts prepared from 40 ml of culture were resuspended in 1.0 ml of buffer C [10 mM sodium phosphate, pH 7.4/10 mM $Mg(OAc)_2$, 100 μg of chloramphenicol per ml/20% sucrose]. To this suspension was added freshly prepared [^{125}I]DSA (0.5 mCi, final concentration about 0.1 μM). After 15 min on ice, with several intervals on a Vortex mixer, the protoplasts were diluted with 10 ml of the same buffer, pelleted by a brief centrifugation, and washed three more times with buffer. The protoplasts (1.8×10^7 cpm) were treated with 1% $NaDodSO_4$ at 100°C for 3 min and then immunoprecipitated.

[^{125}I]DSA Labeling of Complexed Membrane Fraction. Complexed membrane (3.5 mg of protein) was suspended in 0.2 ml of modified buffer C, with 0.15 M NaCl instead of the sucrose, and freshly prepared [^{125}I]DSA (0.2 mCi) was added. After 15 min on ice, with several mixings, the suspension was diluted with 4.7 ml of the same buffer and centrifuged at 42,000 rpm for 1 hr in a Beckman SW 50.1 rotor. The pellet (3×10^7 cpm) was resuspended in 1.0 ml of buffer B, solubilized in 1% $NaDodSO_4$, and immunoprecipitated.

^{125}I-Labeled Antibody. Antibody was labeled with radioactive iodine by the chloramine-T method of Greenwood *et al.* (4), as modified by Horiuchi *et al.* (5). The final preparation contained 4×10^4 cpm/μg. This treatment did not destroy more than 10% of the antibody activity, determined by immunoprecipitation.

Reagents. [^{125}I]Iodosulfanilic acid (1,000 Ci/mmol) and [^{35}S]-methionine (1,000 Ci/mmol) were from New England Nuclear. Trypsin, insoluble trypsin (attached to beads of crosslinked agarose), and trypsin inhibitor were obtained from Sigma. The beads were washed extensively with buffer A before use. All other chemicals were of reagent grade.

RESULTS

Accessibility of CM-Proteins on the Surface of the Membrane. To test for the accessibility of four CM-proteins on the external membrane surface, we treated protoplasts with [^{125}I]-DSA, which attaches covalently to histidine or tyrosine residues and does not penetrate into protoplasts of *B. subtilis* (6). After this treatment the membrane was isolated, solubilized with $NaDodSO_4$, and incubated with antibody, and the precipitates were subjected to $NaDodSO_4$ gel electrophoresis. As Fig. 1 shows, the immunoprecipitated 45-kDa protein was labeled heavily and the 68-kDa protein more weakly, while no radioactive 64-kDa or 31-kDa protein was recovered. Intact cells treated with [^{125}I]DSA yielded similar results (data not shown). In control tests with membrane solubilized by Triton X-100 all four proteins were labeled by [^{125}I]DSA.

As a further test for the accessibility of these four proteins on the external surface, protoplasts were treated with trypsin and the membrane was recovered and analyzed. This treatment did not alter the 64-, 45-, or 31-kDa bands, but it converted

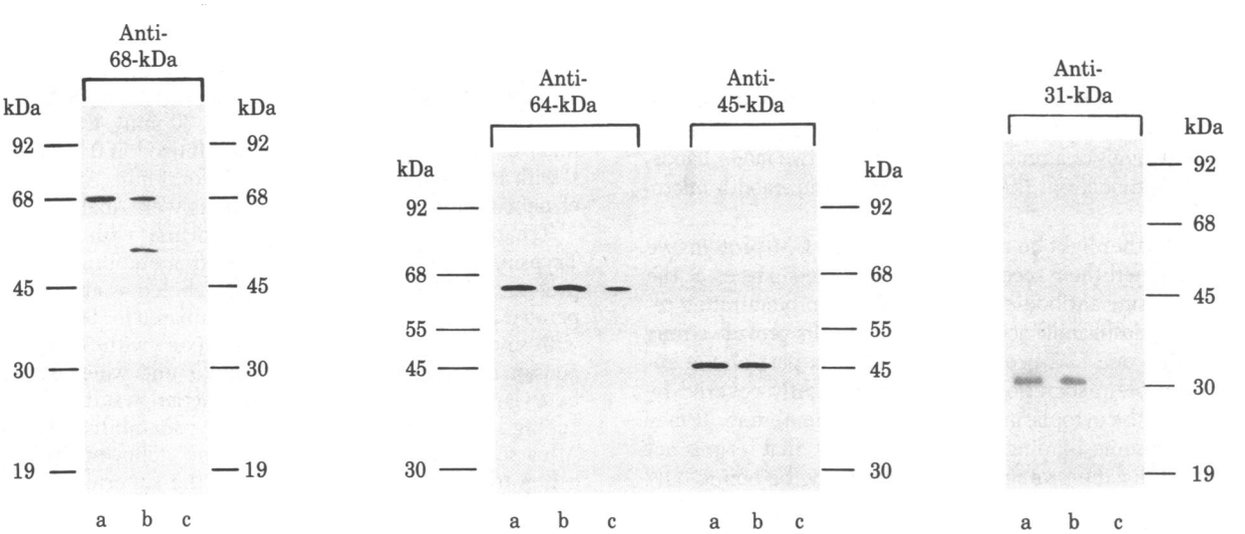

FIG. 2. Effect of trypsin treatment on CM-proteins in protoplasts. Protoplasts labeled with [^{35}S]methionine were exposed to soluble trypsin under the different conditions described below. Equal samples were then treated with each antibody (designated at the top of the figure), and the immunoprecipitates were analyzed. Lanes b, incubation with trypsin for 60 min at 0°C before addition of trypsin inhibitor; lanes c, same, but in presence of 1% Triton X-100; lanes a, control, with trypsin added after trypsin inhibitor, just before solubilization.

much of the 68-kDa protein to a smaller product (about 50 kDa), which was still precipitable by anti-68-kDa antibody and was retained in the membrane (Fig. 2). This cleavage clearly occurred on the protoplast surface and not during lysis, for when the addition of trypsin was delayed until just before solubilization with $NaDodSO_4$ there was no cleavage (Fig. 2, lane a). As a control for the negative reactions the proteins were solubilized with Triton X-100 (as described above for DSA) and incubated with trypsin: all were digested, leaving no immunoprecipitable fragments. In the experiment of Fig. 2 this procedure left some 64-kDa molecules undigested, but digestion was complete in another experiment (see Fig. 4, lane d).

For the 68-, 64-, and 31-kDa proteins in the protoplast membrane the reactions with trypsin agree with the accessibility or inaccessibility to DSA, but the 45-kDa protein reacted only with DSA and not with trypsin. Evidently a surface region of this protein is accessible but lacks a sequence susceptible to trypsin.

Accessibility of CM-Proteins on the Cytoplasmic Surface of the Membrane. To test the accessibility of the CM-proteins on the inner surface of the plasma membrane, the complexed membrane fraction from lysates, believed to consist largely of inside-out vesicles, was treated with [^{125}I]DSA, as described above for the protoplasts. Labeling was heavy for the 68-kDa protein but not detectable for the 45- and 31-kDa proteins (Fig. 3). The 64-kDa protein was labeled weakly (Fig. 3) or not at all in various experiments, suggesting a limited accessibility.

The complexed membrane fraction was also treated with trypsin, both soluble (as above) and in an insolubilized form (attached to agarose beads). The results were essentially the same with the two forms of the enzyme. As Fig. 4 shows, the 64-, 45-, and 31-kDa proteins were not cleaved (lanes b). In contrast, the 68-kDa protein was partly digested, but a larger fragment was removed than at the protoplast surface, leaving a residual fragment of about 40 kDa in the membrane (Fig. 4, lane c). The difference in cleavage of the 68-kDa protein in protoplasts and in vesicles establishes this protein as a transmembrane protein.

Effect of EDTA on Accessibility of the 64-kDa Protein to Trypsin. We have previously observed that the 64-kDa protein is more readily extracted than other CM-proteins from the complexed membrane by acid or alkali (2), which suggested that it is superficially located. Moreover, its slight and variable labeling by treatment of vesicles with [^{125}I]DSA, noted above, located it on the cytoplasmic surface. Its failure to be digested in vesicles by trypsin could be due to lack of the necessary amino acid residues in its exposed region or to protection by the ribosomes complexed with these vesicles.

To test for the latter possibility complexed membrane was suspended in a buffer lacking Mg^{2+} and containing 10 mM EDTA. This treatment presumably released all the ribosomes on the outside of the vesicles. The effect on accessibility was dramatic: insolubilized trypsin eliminated about 75% of the 64-kDa protein molecules in one experiment and 100% in another, as judged by the density of the immunoprecipitated band in a fluorogram (Fig. 4, lane c). Moreover, digestion was extensive, because no residual fragments could be detected in the membrane by immunoprecipitation, in contrast to the residue left after digestion of the transmembrane 68-kDa protein.

After removal of the ribosomes by EDTA, trypsin still failed to digest the 45- and 31-kDa proteins in vesicles (Fig. 4, lanes c). This result fits the negative reactions with DSA (Fig. 3).

Accessibility of CM-Proteins in Derived Membrane to Trypsin. In the preceding experiment the reactivity of the 64-kDa protein in the presence of EDTA might have been due not simply to release of ribosomes but to a conformational effect of EDTA on the membrane. To eliminate this possibility we pre-

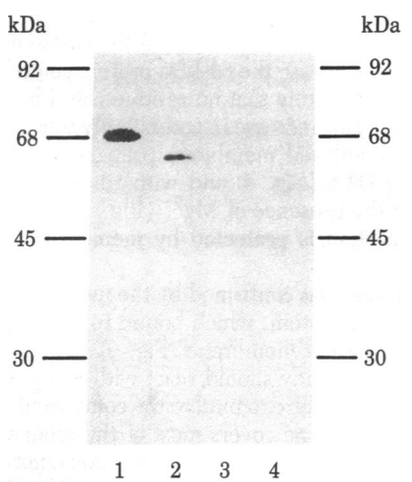

FIG. 3. Labeling of CM-proteins in complexed vesicles by [^{125}I]DSA. The complexed membrane fraction was labeled and analyzed as described in the text. Equal portions were precipitated with antibodies to lane 1, 68-kDa protein; lane 2, 64-kDa protein; lane 3, 45-kDa protein; and lane 4, 31-kDa protein. The fluorograms were obtained after 10-day exposure.

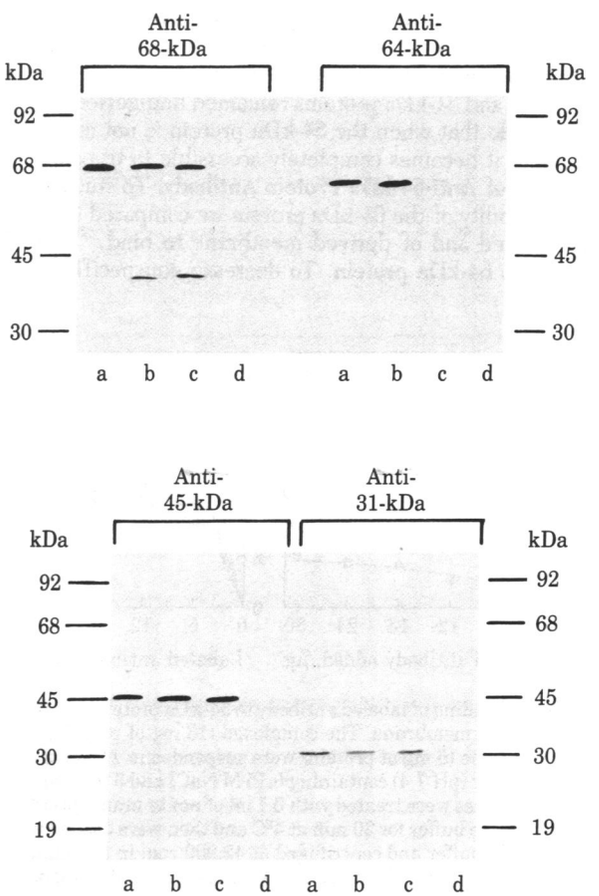

FIG. 4. Effect of EDTA on reaction of complexed vesicles with trypsin. Complexed membrane was exposed to trypsin under the different conditions described below. Equal samples were then solubilized, treated with each antibody, and analyzed. Lanes b, incubation with insolubilized trypsin for 60 min at 25°C before addition of trypsin inhibitor; lanes c, 10 mM EDTA added before incubation; lanes d, 1% Triton X-100 added before incubation; lanes a, control, with trypsin added after trypsin inhibitor, just before solubilization.

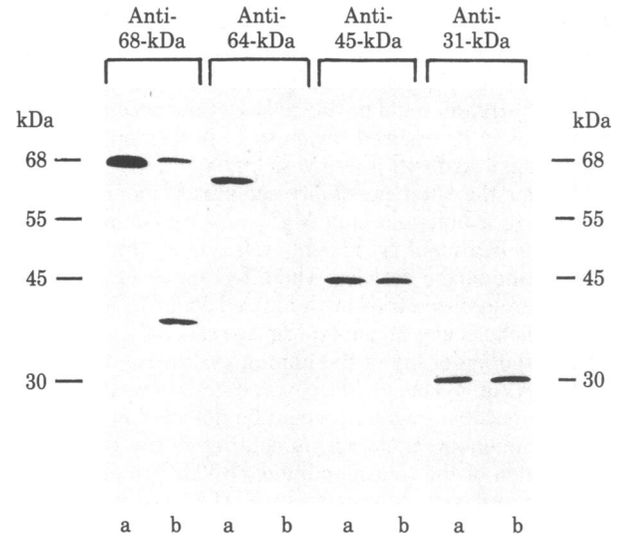

FIG. 5. Treatment of derived membrane vesicles with trypsin. [^{35}S]-Methionine-labeled derived membrane, freed of ribosomes by low Mg^{2+} and isolated from complexed membrane, was incubated with (lanes a) or without (lanes b) insolubilized trypsin and was analyzed as in Fig. 4.

pared derived membrane, freed of ribosomes, in the usual way—i.e., exposure to low Mg^{2+}, separation in a sucrose gradient from the residual complexed membrane, and then restoration of Mg^{2+} (10 mM). Again, the 64-kDa product was completely digested, and the 45- and 31-kDa proteins remained undigested (Fig. 5). We conclude that when the 64-kDa protein is not covered by a ribosome it becomes completely accessible to trypsin.

Binding of Anti-64-kDa Protein Antibody. To study further the accessibility of the 64-kDa protein we compared the ability of complexed and of derived membrane to bind ^{125}I-labeled antibody to 64-kDa protein. To decrease nonspecific binding

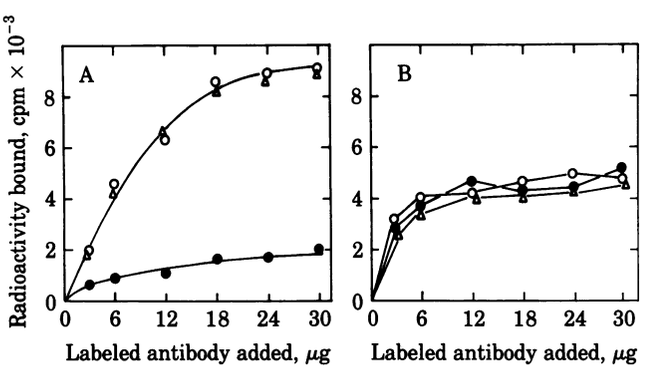

FIG. 6. Binding of labeled antibody to 64-kDa protein to complexed and to derived membrane. The complexed (16 mg of protein) and derived membrane (6 mg of protein) were suspended in 1.0 ml of 50 mM Tris·HCl buffer (pH 7.4) containing 0.15 M NaCl and 5 mM $Mg(OAc)_2$. Both membranes were treated with 0.1 ml of nonimmune IgG (4.5 mg/ml) in the same buffer for 20 min at 4°C and then were diluted with 3.9 ml of ice-cold buffer and centrifuged at 42,000 rpm in a Beckman SW 50.1 rotor for 1 hr at 4°C. The pellets were resuspended in 1.0 ml of the same buffer. Derived membrane (0.3 mg of protein) (*A*) and complexed membrane (0.8 mg of protein, about half ribosomal) (*B*) were mixed with various amounts of ^{125}I-labeled antibody to 64-kDa protein in 0.25 ml of buffer. After incubation for 30 min at 25°C, 4.5 ml of ice-cold buffer was added. The membrane was recovered, washed twice with buffer by centrifugation, and solubilized, and radioactivity was measured. ^{125}I-Labeled antibody to 64-kDa protein was added alone (○), or with nonimmune IgG at 2.4 mg/ml (△), or with unlabeled antibody to 64-kDa protein at 2.4 mg/ml (●).

the preparations were pretreated with nonimmune IgG. As Fig. 6 shows, the derived membrane bound radioactivity in a dose-dependent manner, and the binding was greatly reduced by an excess of unlabeled antibody to 64-kDa protein but not by nonimmune IgG. With the complexed membrane, in contrast, the observed binding was nonspecific, because it was not reduced by unlabeled antibody. It is thus clear that the ribosomes on the complexed membrane protect the 64-kDa protein not only from access to trypsin but also from access to antibody.

In the control measurements in Fig. 6 the complexed membrane showed distinctly more nonspecific binding of labeled protein (in the presence of unlabeled antibodies) than a comparable sample of derived membrane. Evidently the chloramine-T treatment of the antibody has yielded radioactive products that bind to ribosomes.

DISCUSSION

The topography of four CM-proteins in the membrane of *B. subtilis* has been examined by exposing the external surface (in protoplasts) and the cytoplasmic surface (in vesicles) to the nonpenetrating radioactive reagent [^{125}I]DSA, or to trypsin, and then precipitating with specific antibodies. One protein, of 68 kDa, was found to span the membrane: it could be labeled with [^{125}I]DSA, or partly digested by trypsin, on either surface. Moreover, the treatment with trypsin left fragments of different sizes (Figs. 2 and 4) in the membrane of protoplasts or of vesicles, thus confirming the expectation that the vesicles exposed predominantly their cytoplasmic surface.

The 45-kDa CM-protein was found to face only the external surface, where it could be labeled by [^{125}I]DSA but did not provide a substrate for trypsin. The 31-kDa protein could not be labeled or digested on either surface. After solubilization by a mild, nonionic detergent all the CM-proteins could be digested by trypsin completely (i.e., to immunologically undetectable fragments), and so their limited reactivity in the membrane evidently reflects their limited accessibility.

The results obtained with the 64-kDa protein were particularly interesting. Because this protein is more easily extracted than the others (2), and because it is also present in the cytosol (2), it might be expected to have a superficial location on the cytoplasmic surface. Indeed, as expected, on the protoplast surface it did not react with either DSA or trypsin. Moreover, on the cytoplasmic surface of the vesicles it reacted only slightly with DSA, and not with trypsin, until the ribosomes were released. After this release the 64-kDa protein could be digested by trypsin so completely that no residue could be detected in the membrane. This increase in accessibility was demonstrated both with a complexed membrane preparation tested in the presence of EDTA (Fig. 4) and with ribosome-free derived membrane in the presence of Mg^{2+} (Fig. 5). It is evident that the 64-kDa protein is protected by membrane-associated ribosomes.

This conclusion was confirmed by the use of ^{125}I-labeled antibody to 64-kDa protein, which bound to derived membrane but not to complexed membrane (Fig. 6). Moreover, because this polyclonal antibody should react with many determinants on the antigen, its failure to bind to the complexed vesicles suggests that the ribosome covers most of the otherwise exposed surface of the molecule, though this protection is not tight enough to exclude a reagent of low molecular mass (DSA, 297 daltons).

In some membrane preparations treated to release ribosomes all the 64-kDa molecules were digested by trypsin (Figs. 4 and 5), but in others some molecules persisted. The labeling of the 64-kDa protein by [^{125}I]DSA in intact vesicles was also variable. It thus appears that preparations may vary in the frac-

tion of vesicles that are inverted or in the fraction of sites covered by ribosomes.

It is formally possible that ribosomes might be protecting the 64-kDa protein not by direct contact but by contact with another site in the membrane, inducing a conformational change, just as a ligand bound to a ribosome can inhibit binding of other ligands at a distance. However, the fluidity of the membrane, in contrast to the relative rigidity of the ribosome, makes such an indirect action unlikely. The effect of the ribosomes therefore provides strong evidence that the 64-kDa protein is part of an apparatus of secretion, localized at its cytoplasmic end and normally covered by a ribosome. It is of interest to note that the ability of inner membrane vesicles of *Escherichia coli* to sequester secreted proteins is destroyed by treatment by trypsin, but only after the ribosomes have been released (7).

Genetic studies in *E. coli* have also revealed two proteins involved in protein secretion: mutations in *secA* and *prlA* alter protein translocation (8, 9). The secA protein, 92 kDa, has been found in both the membrane and the cytoplasm (8). In animal cells Sabatini, Kreibich, and co-workers (3, 10, 11) have identified two membrane glycoproteins, 65 and 63 kDa, that appear to play a role in the binding of ribosomes and hence were called "ribophorins." These proteins are present in rough but not smooth microsomes; they can be crosslinked to the attached ribosomes; and they remain with an aggregate of ribosomes after most of the membrane proteins are removed by a nonionic detergent. However, in contrast to the tight coverage of the bacterial 64-kDa protein by ribosomes, in eukaryotic rough microsomes the two ribophorins are fully accessible to lactoperoxidase/I_2, and one of them is also accessible to trypsin (10). Moreover, whereas in bacteria release of nascent chains by puromycin results in release of ribosomes from the membrane (6, 12), in the eukaryotic system the ribosomes remain bound after puromycin treatment, and even added free ribosomes may bind to membrane (13), unless a high concentration of salt is added.

The presence of the 64-kDa protein in the cytosol (2) as well as in the membrane suggests that it may cycle on and off the ribosome and the membrane in the initiation of secretion, as has been shown for the signal recognition particle of animal cells (14–17). More recent work on eukaryotic cells has identified another membrane protein as a "docking" or receptor protein (17, 18), which binds the signal recognition particle on the secreting ribosome. It is not clear whether the bacterial 64-kDa protein, or any of the other CM-proteins described here, is similar in function to this protein or to the ribophorins. The bacterial and the eukaryotic systems seem likely to be very similar, because a eukaryotic protein, initiating with a eukaryotic signal sequence, can be secreted by a recombinant bacterium (19), and a bacterial signal sequence can be recognized by the eukaryotic signal recognition particle and membrane receptor (20).

Because ribosomal protein S1 of *E. coli* has a molecular mass of 63 kDa, and because it is more readily released than most ribosomal proteins, it seemed important to be sure that the 64-kDa protein is not its homologue in *B. subtilis*. Comparison of free and complexed ribosomes of this organism has shown a 60-kDa protein (presumably S1) in both preparations, distinct from the 64-kDa component present only in the complex (1). Moreover, the distinction has been confirmed immunologically (M. Caulfield, personal communication).

The 64-kDa protein offers promise of leading to additional components of the machinery of protein secretion in bacteria, because under appropriate circumstances its antibody precipitates not only that protein but a complex of proteins associated with it (unpublished data).

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