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Localization and Quantitation of Proteins Characteristic of the Complexed Membrane of *Bacillus subtilis*

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We prepared antibodies to four proteins (molecular weights, 68,000, 64,000, 45,000, and 31,000) that are characteristic of the complexed (ribosome-bearing) fraction of the membrane of *Bacillus subtilis* and found that these proteins are immunologically distinct. Quantitation by immunoprecipitation confirmed that the ribosome-free membrane fraction contains much lower concentrations of these four proteins than the complexed-membrane fraction. The 64-kilodalton protein appeared to be attached more loosely than the other proteins, since it was more readily extracted from the membrane. In addition, this protein was also present in the cytosol in an even greater amount than in the membrane. The 68-, 64-, and 31-kilodalton proteins are present in cells in stoichiometrically equivalent amounts.

In the accompanying paper (3) we show that in lysates of *Bacillus subtilis* a complexed-membrane fraction (vesicles carrying ribosomes) can be separated by sucrose gradient centrifugation from a free-membrane fraction and that at least six major electrophoretic bands (i.e., proteins) are virtually unique to the complexed-membrane fraction (CM proteins). In this study we used antibodies to four of these proteins to quantitate their distribution more precisely. Our results confirm that these proteins are much more concentrated in the complexed-membrane fraction than in the free-membrane fraction. In addition, one of the proteins, the 64-kilodalton (kd) protein, is also present in the cytosol.

MATERIALS AND METHODS

The strain of *B. subtilis*, the conditions of growth, and the gel electrophoresis procedure used are described in the accompanying paper (3). Cells were labeled by growth for two generations in the presence of [^{35}S]methionine and in the presence of [^{3}H]oleic acid, where noted below.

Cell fractionation. The complexed- and free-membrane fractions and the "derived" membrane fraction (complexed-membrane fraction freed of ribosomes) were prepared as described in the accompanying paper (3).

To fractionate protoplasts into total membrane, free ribosomes, and cytosol, the lysate from 200 ml of culture in 4 ml of cold buffer A (10 mM Tris-hydrochloride [pH 7.6], 50 mM KCl, 10 mM magnesium acetate, 1 mM phenylmethylsulfonyl fluoride, 1 mM *o*-phenanthroline) containing 100 μg of chloramphenicol per ml and 1 mM dithiothreitol was centrifuged at 3,000 × *g* for 5 min to remove intact cells and cell debris. To recover additional, trapped membrane, the pellet was resuspended in 4 ml of buffer A and centrifuged again. The combined supernatants were centrifuged at 45,000 rpm for 1 h in an SW50.1 rotor, and the resulting sediment was washed by centrifugation in 5 ml of buffer A and was used as the total-membrane fraction (including ribosomes complexed with membranes). The supernatant from the SW50.1 centrifugation was layered onto 1 ml of 2.0 M sucrose in buffer A and was centrifuged at 45,000 rpm for 4 h. The resulting pellet was used as the free-ribosome fraction, and the total layer above the interface was used as the cytosol fraction.

The total-membrane and ribosome fractions were each suspended in 2.0 ml of buffer B (50 mM Tris-hydrochloride [pH 7.6], 0.15 M NaCl). The cytosol (about 8.4 ml) was concentrated to about 1.0 ml by lyophilization, dialyzed against buffer B, and adjusted to a final volume of 2.0 ml with the same buffer.

Preparation of antibodies. To isolate proteins for immunization complexed membrane solubilized with sodium dodecyl sulfate (SDS) as described in the accompanying paper (3) was electrophoresed on a preparative polyacrylamide slab gel (thickness, 3 mm) and stained. The bands of interest were cut out with a razor blade, pooled, and homogenized in 1 ml of cold 10% trichloroacetic acid–30% ethanol in a 4-ml glass-Teflon homogenizer, until they were completely disintegrated. After centrifugation in a Sorvall SS-34 rotor for 10 min at 10,000 rpm, the supernatant was discarded. Extraction with trichloroacetic acid-ethanol was repeated four times, and the resulting pellet, which was completely decolorized, was shaken with 1 ml of 0.1 N NaOH for 2 to 4 h at 37°C and then centrifuged. This extraction procedure was repeated three more times. The combined extracts were lyophilized, dis-

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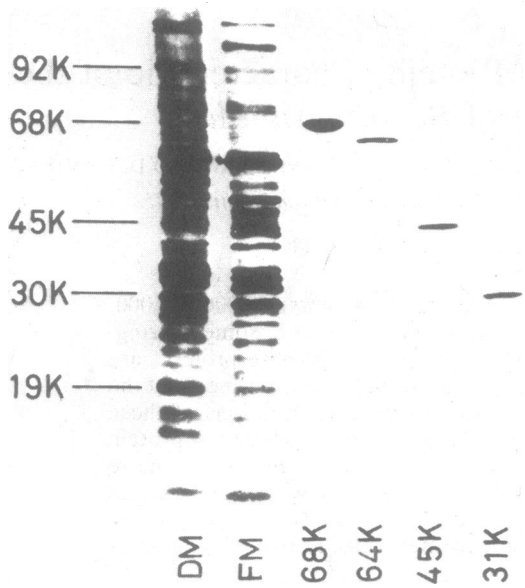

FIG. 1. Specificities of antisera raised against four CM proteins. Cells were grown for two generations in 200 ml of medium supplemented with 0.02% Casamino acids, 100 μCi of [^{35}S]methionine, and 19 other amino acids (0.15 mM each), and samples of the solubilized total membrane were immunoprecipitated with the four antisera and analyzed as described in the text. The patterns of the derived membrane (DM) and free-membrane (FM) fractions from the same cells before immunoprecipitation are presented for comparison.

solved in 1 ml of 0.1 M sodium phosphate (pH 7.4) containing 0.1% SDS, and dialyzed against the same buffer. Electrophoresis produced only a single band for each preparation, at the expected position.

Each protein (200 to 500 μg in 1 ml) was emulsified by sonication with an equal volume of Freund complete adjuvant (Difco Laboratories) and was injected into the footpads of a rabbit. After 10 days at least four similar injections were made subcutaneously in the back (at 2-week intervals). Blood was taken from the ear vein 10 to 14 days after each of the later injections. Antibodies were assayed by immunoprecipitation as described below. The immunoglobulin fraction was purified from the antisera by two ammonium sulfate fractionations and DEAE-cellulose chromatography.

Immunoprecipitation. Samples were solubilized with 1% SDS at 100°C for 3 min unless specified otherwise. After centrifugation the supernatant was diluted 10-fold with immunoprecipitation buffer (50 mM Tris-hydrochloride [pH 7.4], 0.15 M NaCl, 5 mM EDTA, 0.5% Nonidet P-40 or 0.5% Triton X-100, 0.01% NaN_3). Ovalbumin was also added to a final concentration of 200 μg/ml. Portions were mixed with each antibody in an amount shown to be sufficient to precipitate all of the corresponding antigen. After 30 min on ice, enough *Staphylococcus aureus* A cells were added to adsorb all of the immunoglobulin G, and 30 min later the complex was pelleted by centrifugation and washed three times with immunoprecipitation buffer supplemented with 0.1% SDS. The immunoprecipitate was then analyzed by gel electrophoresis, as described below.

Reagents. [^{35}S]methionine and [^{3}H]oleic acid were obtained from New England Nuclear Corp. Other chemicals were of reagent grade.

RESULTS

Immunological evidence for four distinct CM proteins. Because *B. subtilis* is rich in proteases, it seemed possible that some of the many CM proteins might be cleavage products of others. To test for this possibility and to facilitate quantitative studies and large-scale preparation, we prepared antibodies to four of the CM proteins (the 68-, 64-, 45-, and 31-proteins). To do this, each band was cut out from multiple gels, extracted, pooled, and injected into rabbits (see above).

To test the uniqueness of the four antigens and the specificities of the antibodies, the total-membrane proteins labeled with [^{35}S]methionine were solubilized by adding SDS, and samples were incubated with each antibody. When the resulting immunoprecipitates were analyzed by SDS gel electrophoresis followed by fluorography, each antiserum was found to precipitate only the protein of the expected molecular weight, without any cross-reacting proteins (Fig. 1). It was evident that the four bands of particular interest were antigenically distinct from each other and from all other membrane proteins. However, one band may well have contained more than one protein species.

Since the antisera did not precipitate the two larger CM proteins (the 130- and 92-kd proteins), it is clear that these larger proteins are not aggregates of any of the four smaller ones, as initially seemed possible. Accordingly, antibodies against these two proteins were also prepared, and each antibody was found to precipitate only the corresponding band from solubilized membrane (data not shown).

Quantitation of immunoprecipitated proteins. In the accompanying paper (3), we show that in *B. subtilis* membranes analyzed by gel electrophoresis, six protein bands from the complexed-membrane fraction (CM proteins) are virtually absent from the free-membrane fraction. To study quantitatively the distribution of four of these proteins in cells, we initially scanned the densities of the electrophoretic bands after staining with Coomassie brilliant blue or after fluorography of proteins containing [^{35}S]methionine. To increase the specificity of the measurement and to permit application of the measurement to cell fractions other than membranes, we subsequently added an immunoprecipitation step.

In the procedure finally adopted, the material

to be analyzed was immunoprecipitated and analyzed as described above. With each antibody the specific band constituted about one-third of the total immunoprecipitated radioactivity; much of the remainder, which represented nonspecific adsorption, appeared at the bottom of the gel (M_r, <12,000). Since the level of radioactivity of the band was often too low for accurate direct counting, we measured the radioactivity in arbitrary units by scanning the fluorogram and cutting out and weighing the peak areas. The reliability of this method was tested with ^{14}C-labeled bovine serum albumin applied to a gel in varying amounts. As Fig. 2 shows, the peak area of this compound was proportional to sample size over a broad range. All reported analyses were within that range.

Comparison of four CM proteins (the 68-, 64-, 45-, and 31-kd proteins) in complexed-membrane, derived membrane, and free-membrane fractions. Equal amounts of free-membrane fraction and derived membrane fraction (freed of ribosomes) (in terms of ^{35}S), were analyzed by the method described above. As Table 1 shows, the free-membrane fraction contained only one-tenth as much 68- and 64-kd proteins as the derived membrane fraction, and the ratio was about one-third for the 45- and 31-kd proteins.

The yield of derived membrane fraction from complexed-membrane fraction was small and variable, and some of the membrane proteins might have been lost during the conversion. Accordingly, for further studies we used the complexed-membrane fraction instead of the derived membrane fraction. Because the ribosomes in this fraction also contained ^{35}S, the cells were also labeled with [^{3}H]oleic acid as a basis for measuring the amount of membrane in a sample (Fig. 3). (A control experiment with cells labeled with [^{3}H]oleic acid alone showed that this radioactivity could not be contaminating the bands of protein separated by electrophoresis.) The relative concentrations of the CM proteins in the complexed- and free-membrane fractions (Table 2) were about the same as the relative concentrations observed in derived membrane and free-membrane fractions (Table 1).

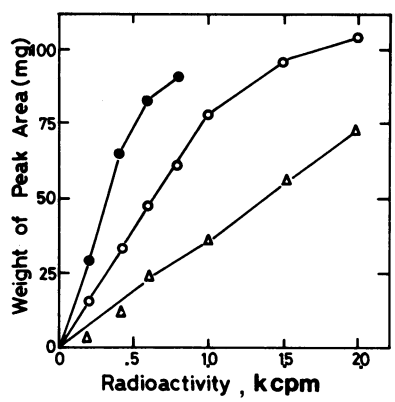

FIG. 2. Relationship of weight of peak area scanned from a fluorogram strip to the amount of radioactive protein loaded on the gel. Bovine serum albumin was labeled by reductive methylation with [^{14}C]formaldehyde and sodium cyanoborohydride (1). The labeled protein (7.6×10^5 cpm/mg) was applied to a gel slot in the amount indicated on the abscissa, and after electrophoresis the gel was subjected to fluorography. The amount of labeled protein was measured by weighing the peak area after the resulting fluorogram strips were scanned at 540 nm with a Shimazu model CS-900 dual-wavelength scanner. Films were exposed for 24 h (△), 48 h (○), or 72 h (●).

TABLE 1. Quantitation of four CM proteins in derived membrane and in free-membrane fractions[a]

Protein	Membrane fraction	[^{35}S]methionine in sample (cpm, × 10^3)	Wt of peak area from fluorogram (mg)	Free/derived ratio
68 kd	Derived	28	26.3	0.07
	Derived	14	12.5	
	Free	28	1.8	
64 kd	Derived	65	26.1	0.10
	Derived	32	11.8	
	Free	65	2.7	
45 kd	Derived	65	35.1	0.38
	Free	65	13.5	
31 kd	Derived	65	47.1	0.32
	Free	65	15.2	

[a] The amounts of the 68-, 64-, 45-, and 31-kd proteins were determined as described in the legend to Fig. 3.

In Fig. 3 the fluorographic peak area of each band is plotted against the amount of [^{3}H]oleic acid in the sample used for immunoprecipitation. The slopes of the plots were used to determine as accurate an estimate of the concentrations of the CM proteins as possible, and these data, normalized to the value for the 68-kd protein, were combined with the M_r values to calculate the relative molar concentrations of the four proteins in the complexed-membrane fraction. As Table 2 shows, the molar ratio of the 31-kd protein to the 68-kd protein is compatible with a 1:1 stoichiometry, whereas there appeared to be about one-half as much 45-kd protein and one-third as much 64-kd protein.

Test for CM proteins in the cytosol and ribosomes. To determine whether subcellular fractions other than the membrane fractions contain

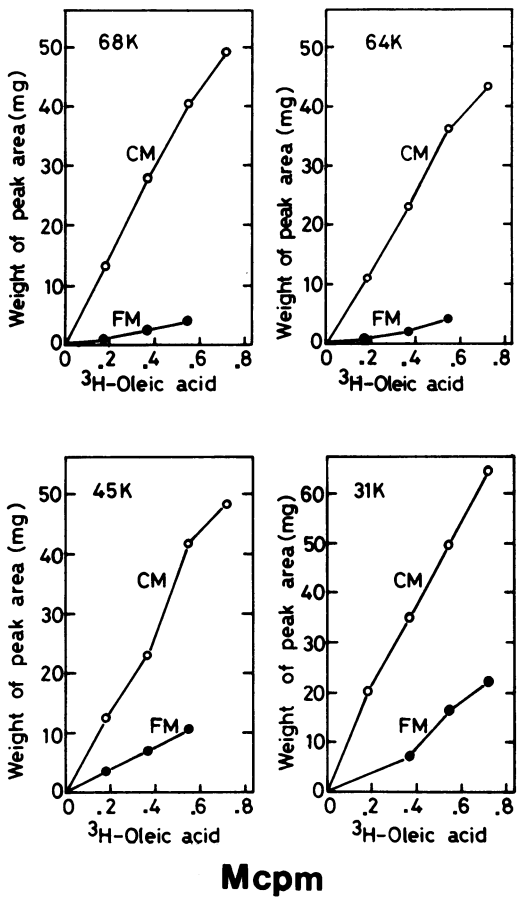

FIG. 3. Fluorometric quantitation of immunoprecipitated CM proteins in complexed- and free-membrane fractions. Cells were grown as described in the legend to Fig. 1, but with 50 μCi of [^{35}S]methionine and 400 μCi of [^{3}H]oleic acid. After harvesting and lysis, the membrane preparation was separated into a complexed fraction and a free fraction; the ratios of ^{3}H to ^{35}S were 5.2 and 7.8, respectively. Samples of varying size (measured in terms of ^{3}H) were subjected to immunoprecipitation with each antibody, followed by SDS gel electrophoresis and fluorography. The fluorograms were analyzed as described in the legend to Fig. 2. The concentration of each protein was calculated from the slope and is shown in Table 2 in terms of the weight of the peak area per unit of membrane. CM, Complexed-membrane fraction; FM, free-membrane fraction.

any of the four CM proteins which we studied (or any cross-reacting proteins), cells were converted to protoplasts and separated into total membrane, (including membrane-bound ribosomes), free ribosomes, and cytosol. As Fig. 4 shows, the cytosol yielded no detectable band with the 68-, 45-, or 31-kd protein antiserum, but with the 64-kd protein antiserum it formed a precipitate that yielded a single radioactive band, at 64 kd. A comparison of fractions from equal amounts of lysate showed that there was about twice as much of this protein in the cytosol as in the membrane (Fig. 5).

Figure 4 also shows that the free ribosomes from the cytosol yielded no detectable specific immunoprecipitate.

Immunological comparison of the 64-kd protein in the presence and absence of detergent. In the experiment described above the antiserum was raised with SDS-solubilized membrane protein, and the cytosol, like the membrane, was tested for the 64-kd protein in the presence of SDS. Since SDS drastically alters the conformation of proteins, we wanted to determine whether in the absence of detergent, the 64-kd protein in the cytosol in its native state would also react with our antibodies. In addition, we observed that the 64-kd membrane protein could also be solubilized by a nonionic detergent, Triton X-100, which should have had little effect on the native conformation of the protein, and so we also tested the membrane and the cytosol in the presence of Triton X-100 without SDS. We used

TABLE 2. Quantitation of CM proteins in complexed- and free-membrane fractions[a]

Protein	Membrane fraction	Wt (arbitrary units)[b]	Free/complexed ratio	Wt relative to 68-kd protein wt (%)[c]	Molar ratio relative to 68-kd protein in complexed-membrane fraction (%)[d]
68 kd	Complexed	7.43	0.09	100	100
	Free	0.69			
64 kd	Complexed	6.38	0.11	30	32
	Free	0.69			
45 kd	Complexed	6.94	0.28	35	53
	Free	1.88			
31 kd	Complexed	9.34	0.27	46	95
	Free	2.64			

[a] These data were derived from the immunoquantitation data in Fig. 3.

[b] Weights of peak areas (in milligrams per 10^5 cpm of [^{3}H]oleic acid) were calculated from the slopes of the lines in Fig. 3.

[c] Corrected for differences in exposure time. Similar results were obtained from gels with the same exposure time.

[d] Corrected for molecular weights, assuming random distribution of methionine in the proteins.

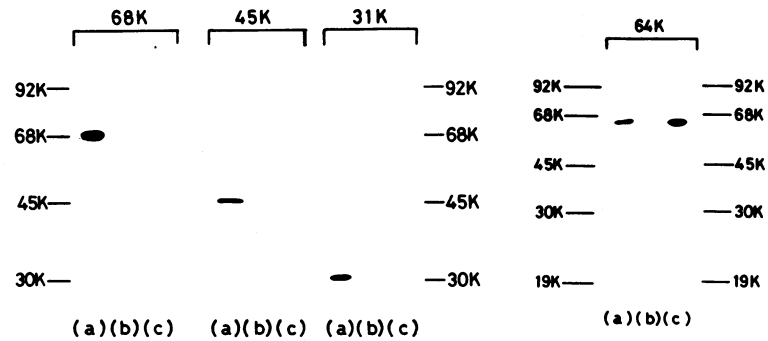

FIG. 4. Subcellular localization of four CM proteins in *B. subtilis* cells. From cells grown in the presence of [^{35}S]methionine, total membrane, ribosomes, and cytosol fractions were separated. These fractions were treated with SDS, immunoprecipitated with each antibody (indicated at the top), and electrophoresed. Lanes a, Total membrane; lanes b, ribosomes; lanes c, cytosol. Except for the band at 64 kd, no immunoprecipitate band was observed in the ribosome and cytosol fractions even when the size of the sample was increased 10-fold.

an amount of antibody that yielded, after preliminary titration, maximal precipitation of the 64-kd protein from SDS-solubilized membrane. All immunoprecipitates were washed with an SDS solution as usual (see above), to eliminate any proteins that might have been complexed with the 64-kd protein in the membrane or the cytosol.

As Figure 6 shows, the Triton X-100 extract of membranes yielded about the same amount of specific precipitate as the SDS extract. Moreover, the cytosol without detergent or with Triton X-100 yielded an immunoprecipitated band which was indistinguishable from that obtained with SDS. Evidently, the SDS-solubilized 64-kd protein and the native forms (both in the cytosol and in the membrane) expose the same major immunological determinants.

Extraction of CM proteins. To test the strength of attachment of four CM proteins, the complexed-membrane fraction was suspended in water, which did not remove any of the four proteins, and was treated with acid or alkali, which have been used widely to distinguish integral from peripheral membrane proteins (7). Table 3 shows that both of these reagents extracted the 64-kd protein to a moderate extent, but they did not significantly remove the other three proteins. Thus, it appears that the four CM proteins which we studied are all integral membrane proteins, but that the 64-kd protein is bound somewhat more loosely than the others. Treatment of the complexed-membrane fraction with KCl at concentrations up to 0.5 M had no effect on the contents of the proteins.

DISCUSSION

In the accompanying paper (3), we show that disrupted membranes of *B. subtilis* can be separated by biphasic sucrose density gradient centrifugation into a complexed-membrane fraction (carrying ribosomes) and a free-membrane fraction, which have very different protein compositions. In the present work four of the electrophoretic bands characteristic of the complexed-membrane fraction (CM proteins) were used to raise antibodies, and these antibodies showed that each of the bands was immunologically distinct and did not cross-react with any other membrane protein. Two additional CM proteins (the 130- and 92-kd proteins) have also been found to be immunologically distinct; the distribution of these proteins in cells is under investigation.

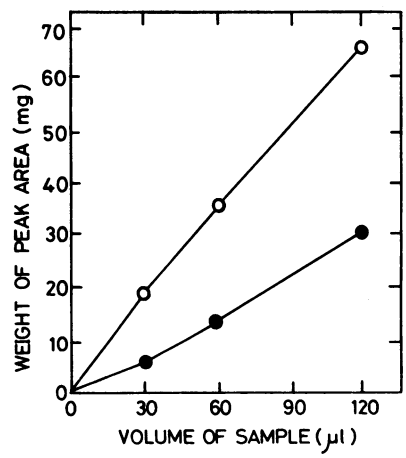

FIG. 5. Quantitation of the 64-kd protein in membrane and cytosol fractions. The total membrane and cytosol were diluted equally (see text), different volumes were treated with anti-64-kd protein antibody, and the precipitates were analyzed as described in the legend to Fig. 3. Symbols: ●, Total membrane ○, cytosol.

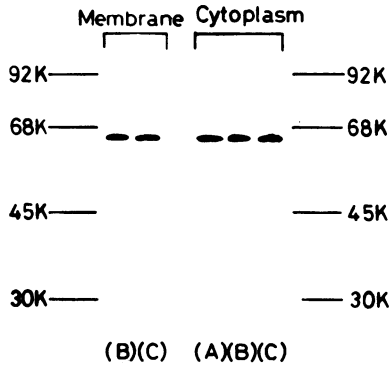

FIG. 6. Immunological comparison of the membrane and cytosol forms of the 64-kd protein. The membrane and cytosol fractions of cells labeled with [^{35}S]methionine were treated either with 1% Triton X-100 for 30 min at 4°C with several mixings or with 1% SDS at 100°C for 3 min, followed by centrifugation to remove insoluble material. The Triton X-100 solutions were immunoprecipitated with anti-64-kd protein antibody in the presence of 1% Triton X-100, whereas the solutions obtained with 1% SDS were diluted 10-fold with immunoprecipitation buffer and then incubated with anti-64-kd protein antibody in a final SDS concentration of 0.1%. Immunoprecipitates were washed with buffer containing 0.1% SDS and were analyzed by gel electrophoresis and fluorography. Lane A, Cytosol, untreated; lanes B, supernatant after treatment with 1% Triton X-100; lanes C, supernatant after treatment with 1% SDS.

Immunological quantitation of these proteins confirmed that the complexed- and free-membrane domains in cells separate rather cleanly during lysis, even though they probably break up into vesicles along arbitrary lines. Indeed, it is noteworthy that the free-membrane fraction contained only one-tenth as much 68- and 64-kd proteins as either the complexed-membrane fraction (standardized in terms of [^{14}C]oleic acid) or the derived membrane fraction (freed of ribosomes and standardized in terms of [^{35}S]methionine). This low value suggests that the actual free-membrane domains in cells may entirely lack these proteins and that their presence in the free-membrane fraction reflects imperfect separation from complexed-membrane domains during lysis.

The free-membrane fraction contained somewhat more of the 45- and 31-kd proteins (about 30% of the concentrations in the complexed-membrane fraction). This result suggests that several CM proteins are not all present in the membranes as a stable, fixed complex and that the 45- and 31-kd proteins are more often present in or adjacent to free domains. Alternatively, these two bands may contain more than one protein; one component may be unique to the complexed-membrane fraction, and the other(s) may not be.

In the complexed-membrane fraction we found a 1:1 molar equivalence for the 68- and 31-kd proteins (Table 2), along with about one-half as much 45-kd protein and one-third as much 64-kd protein. Conceivably, these proteins might all be parts of an apparatus of secretion that contains single copies of some components and multiple copies of others. Thus, the more abundant component(s) could form a porin-like channel (reviewed in reference 6), and the less abundant protein could perform other functions in secretion. On the other hand, it also seems quite possible that the CM proteins are not all involved in protein secretion. So far, such a function has been strongly supported only for the 64-

TABLE 3. Release of proteins from the complexed-membrane fraction by alkali and acid

Prepn[a]	Amt of:[b]			
	68-kd protein	64-kd protein	45-kd protein	31-kd protein
Control I[c]	5.76 (100)[d]	4.23 (100)	4.88 (100)	5.74 (100)
Control II[e]	5.58 (97)	3.98 (94)	4.64 (95)	5.62 (98)
NaOH (0.04 N)	5.18 (90)	3.36 (79)	4.39 (90)	5.11 (89)
NaOH (0.1 N)	4.88 (85)	2.74 (65)	4.15 (85)	4.77 (83)
Acetic acid (0.5 N)	4.72 (82)	2.87 (68)	4.16 (85)	4.64 (81)

[a] Freshly prepared complexed-membrane fraction was suspended in water on ice. To 1.0 ml of this suspension (approximately 1.1×10^6 cpm), NaOH or acetic acid was added. After 30 min at 4°C with several vortexings, 3.0 ml of ice-cold water was added, and the membranes were recovered and analyzed.

[b] The amounts of the proteins retained in the membrane are expressed as weights of the peak areas (in grams, $\times 10^{-2}$) obtained from the fluorogram strips, as described in the legends to Fig. 2 and 3. The times of exposure were as follows: 4 days for the 68-kd protein and 10 days for the other proteins (64, 45, and 31 kd).

[c] In control I, the same amount of the complexed-membrane fraction used for each experiment was directly subjected to determination without any treatment.

[d] The numbers in parentheses are percentages.

[e] In control II, the same amount of the complexed-membrane fraction was processed like each experimental sample, except that water was substituted for acid or alkali.

kd protein, since it is covered by a ribosome on the cytoplasmic surface of the membrane (S. Horiuchi, P. C. Tai, and B. D. Davis, Proc. Natl. Acad. Sci. U.S.A., in press).

The use of antibodies further showed that the 64-kd protein is more easily extracted from the membrane than the other proteins. In addition, the anti-64-kd protein antibody also precipitated a protein of the same size from the cytosol, where it was present in an even larger amount than in the membrane (Fig. 4 and 5). These two proteins are probably identical, since no large excess of antibody to the membrane protein was required for extensive precipitation of the cytosol protein. It is interesting that the sum of the membrane and cytosol pools of the 64-kd protein yields a 1:1 stoichiometry relative to the 68- and 31-kd proteins in the membrane, although whether this result is coincidental or significant remains to be seen. The presence of large amounts of the 64-kd protein in both the membrane and the cytosol suggests that these two pools may exchange, and work is in progress to test this possibility. Studies with *E. coli* mutants have revealed another protein, Sec A, that is involved in secretion, and this protein is also found (in lysates at low ionic concentrations) in both cytoplasmic membranes and cytosol (5).

In the experiments described here the anti-64-kd protein immunoprecipitate from either the Triton X-100-solubilized membrane or the cytosol was washed with SDS to increase the specificity of the reaction. However, the antibody provided even more interesting results when the immunoprecipitate from the Triton X-100-solubilized membrane was prepared without SDS. This immunoprecipitate was found to contain a complex of the 64-kd protein with several other proteins, which therefore also seem likely to be involved in protein secretion (M. Caulfield, P. C. Tai, and B. D. Davis, manuscript in preparation). Further work will be required to determine whether this complex is analagous to the signal recognition particle of animal cells (8–10), which attaches ribosomes carrying an emerging signal sequence to the receptor protein in the membrane (2, 4) and which is believed to cycle between the cytosol, free polysomes, and the membrane.

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