Detection of a Membrane-associated Protein on Detached Membrane Ribosomes in \textit{Staphylococcus aureus}

By LARS-ÅKE ADLER* AND STAFFAN ARVIDSON

Department of Bacteriology, Karolinska Institutet, S-104 01 Stockholm, Sweden

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Membrane ribosomes from \textit{Staphylococcus aureus} which were detached from the membrane by extraction with the nonionic detergent Triton X-100 retained a protein (MBRP) with a molecular weight of 60000, which was absent from cytoplasmic ribosomes. MBRP was detected and quantified by immunological methods. When membrane ribosomes were dissociated into 50S and 30S subunits, MBRP remained associated with the 50S particle. MBRP was found both on membrane ribosomes and in the cytoplasm in roughly equal amounts. When added to Triton X-100-solubilized protoplasts, antibodies to MBRP produced immunoprecipitates which contained a complex of MBRP and three other proteins with molecular weights of 71000, 46000 and 41000. Four proteins with the same molecular weights as those of the MBRP complex were found associated with membrane ribosomes. The proteins of molecular weight 71000, 60000, 46000 and 41000 seemed to be present in stoichiometrically equivalent amounts in the complex.

INTRODUCTION

Secreted proteins in both eukaryotic (Palade, 1975) and prokaryotic (Baty et al., 1981; Randall & Hardy, 1977; Varenne et al., 1978) cells are synthesized on membrane-associated ribosomes. Part of a specific secretion apparatus has been demonstrated in eukaryotic cells, consisting of a cytoplasmic protein–RNA complex (signal recognition particle, SRP), which binds to the signal sequence of the nascent secretory protein and induces a translational arrest (Walter & Blobel, 1980, 1982), and a membrane-bound docking protein, which releases the translation arrest when the polysome reaches the membrane (Meyer et al., 1982). SRP could also direct the secretion of a prokaryotic exoprotein in a eukaryotic \textit{in vitro} translation/secre tion system, indicating that a similar mechanism is operating in prokaryotic cells (Müller et al., 1982). Indirect evidence for a protein-secretion apparatus in bacteria also comes from the isolation of several classes of secretion-deficient mutants from \textit{Escherichia coli}, mapping at different locations on the \textit{E. coli} chromosome (Wanner et al., 1979; Dassa & Boquet, 1981). The postulation of a bacterial SRP is also consistent with results recently obtained by Hall et al. (1983), who found a mutation within the hydrophilic segment of the lambda receptor signal sequence, which resulted in an early stop in the translation of the lambda receptor RNA. Recently Horiuchi et al. (1983\textit{a}, \textit{b}) and Marty-Mazars \textit{et al.} (1983) demonstrated a protein with a molecular weight of 64000 in ribosome-bearing membranes that was covered on the cytoplasmic surface by the ribosomes. This protein, which was found both in the cytosol and in the membranes, was suggested to participate in the initiation of protein secretion, as has been shown for SRP (Horiuchi \textit{et al.}, 1983\textit{b}).

In the present study the association of membrane proteins with membrane-bound (i.e. exoprotein-synthesizing) ribosomes has been investigated using \textit{Staphylococcus aureus}. This organism secretes large amounts of extracellular proteins, which together constitute over 30\% of

\textit{Abbreviations:} MBRP, membrane-bound ribosome protein; SRP, signal recognition particle.
the total protein synthesis (Abbas-Ali & Coleman, 1977), and it should therefore have a relatively large number of membrane-associated ribosomes. Our concept was that membrane-bound ribosomes which were gently dissociated from the membrane with a nonionic detergent might retain one or more of the membrane proteins involved in the binding of exoprotein-synthesizing ribosomes to the membrane. A similar approach was used by Kreibich et al. (1978), when they identified two ribosome-binding proteins (ribophorines) in the endoplasmic reticulum.

METHODS

Bacterial strain and cultivation conditions. Staphylococcus aureus V8 was used throughout this study. Precultures were grown overnight in Tryptic Soy Broth (Difco). Bacteria from 50 ml of the preculture were used to inoculate 250 ml CCY-medium (Arvidson et al., 1972) in a 5-l baffled shake-flasks, which were incubated for 4 h at 37 °C on a rotary shaker. At this time 200 μg chloramphenicol ml⁻¹ (Parke Davis, Pontypool, Gwent, UK) was added and the culture was poured onto 50 ml frozen buffer A (10 mM-Tris/HCl, pH 7:4; 10 mM-magnesium acetate; 50 mM-Na₂Cl).

All further handling, except for protoplasting, was at 4 °C.

Preparation of protoplasts. Cells were centrifuged at 7000 g for 10 min, resuspended in buffer A with 200 μg chloramphenicol ml⁻¹ and repelleted. Protoplasts were prepared by incubating the bacteria at 37 °C for 15 min in buffer A (1/10 the original culture volume) containing 1:1 m-sucrose, 50 μg chloramphenicol ml⁻¹ and 100 μg lysostaphin ml⁻¹ (Schwarz-Mann, Orangeburg, NY, USA). Whole cells and debris were removed by centrifugation at 5000 g for 10 min. Protoplasts were sedimented at 20 000 g for 30 min and washed once in buffer A containing 1:1 m-sucrose and 50 μg chloramphenicol ml⁻¹. Protoplasts were lysed by dilution in 30 ml 10 mM-Na₂HPO₄, 10 mM-magnesium acetate, 50 mM-KCl, pH 7:4 (pH was adjusted by adding 1 m-HCl).

The membrane suspension was sonicated twice for 10 s with an MSE 100 W ultrasonic disintegrator, and the membranes were pelleted at 27 000 g for 30 min. The supernatant was centrifuged at 150 000 g for 2 h in a Beckman SW 50.1 rotor to sediment the cytoplasmic ribosomes. The membrane pellet was washed twice in phosphate buffer and suspended in buffer A containing 4% (w/v) Triton X-100 (Sigma) to solubilize the membrane and release the membrane-associated ribosomes. After centrifugation at 15 000 g for 10 min to remove large particles the ribosomes were collected by ultracentrifugation at 150 000 g for 2 h. These ribosomes will be referred to as membrane ribosomes.

Both membrane and cytoplasmic ribosomes were further purified by chromatography on Sepharose 2B (Pharmacia) as described by Smith et al. (1978). Fractions of the ribosome peak were pooled and pelleted at 150 000 g for 2 h.

Preparation of membrane antigen. Protoplasts were prepared as described, except for the omission of chloramphenicol. The protoplasts were lysed by suspension in buffer B (10 mM-Na₂HPO₄, 10 mM-magnesium acetate, pH 7:4), containing 1 μg DNAase ml⁻¹ (Sigma) and 1 μg RNAase ml⁻¹ (Sigma). Membranes were washed twice in buffer B, then twice in phosphate/EDTA buffer (10 mM-Na₂HPO₄, 10 mM-Na₂EDTA, pH 7:4; pH was adjusted by adding 1 M-NaOH) and finally once in phosphate/EDTA buffer containing 0.5 M-KCl.

Preparation of antisera. Freund’s complete adjuvant containing 5 mg protein was injected into rabbits subcutaneously in the neck in five portions. After 3 weeks, and then at 5 week intervals, the same amount of protein in Freund’s incomplete adjuvant was injected intramuscularly, and 10 d after each intramuscular injection, 50-40 ml of blood was collected from the ear vein.

Antiserum towards individual antigens were prepared by injecting isolated immunoprecipitates from crossed immunoelectrophoresis gels. The precipitates were cut out and homogenized in adjuvant with a Teflon pestle homogenizer. The precipitates from four gels were injected each time, as described above.

Antibodies were purified according to Harboe & Ingild (1973), except for omission of the final chromatography step. Concentration was performed in a Minicon B-cell (Amicon Corp., Lexington, Mass., USA) to a protein concentration of 50-100 mg ml⁻¹.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins to be analysed were denatured by boiling for 2 min in 2% (w/v) sodium dodecyl sulphate (SDS), 0.1 M-di-dithiothreitol, 0.08 M-Tris/sulphate buffer, pH 6-1, 10% (w/v) glycerol and 0.005% (w/v) bromophenol blue. Electrophoresis was performed at 35 mA for 4 h, in a slab gel apparatus described by Studier (1973) in a 7.5-15% (w/v) polyacrylamide gradient gel using a discontinuous buffer system according to Neville (1971), with the separation gel buffer at pH 9-18.

Alternatively, electrophoresis was done in a Protean dual-slab cell (Bio-Rad) using the buffer system of Laemmli (1970). Samples were electrophoresed for 5 h at 15 mA in a 12% (w/v) polyacrylamide gel at pH 8-8.

Crossed immunoelectrophoresis (CIE). CIE was done as described by Owen & Salton (1975) in 1% (w/v) Sea Kem Me agarose (Marine Colloids Div., FMC Corporation, Rockland, Me., USA) containing 0.024 M-barbitual buffer, pH 8-6, and 1% (w/v) Triton X-100. Gels (3-5 ml) were cast on 5 x 5 cm glass plates. Samples were applied to wells
concentration of 500 pg ml\textsuperscript{-1} each. After standard. Protein concentrations were determined by the method of Lowry, with bovine serum albumin as temperature with agitation. The Sepharose was washed three times in PBS buffer before adding the solubilized Antibodies (200\(\text{pl}\)). After washing eight times with 1 ml PBS, the pelleted sample was denatured in 40\(\mu\text{l}\). CIE of a mixture of both antigens against the MBRP antiserum (data not shown).

Quantification of antigens by rocket immunoelectrophoresis. The amount of MBRP in various subcellular fractions was determined by rocket immunoelectrophoresis according to Weeke (1973), with the gel containing 1\% (w/v) Triton X-100. Each sample was quantified by cutting out paper copies of rockets and weighing them.

Analysis of MBRP on rapidly prepared membrane ribosomes. Protoplasts from 50 ml culture were suspended in 50 ml buffer A with 0-2\(\mu\text{g}\) DNAase ml\textsuperscript{-1} (RNAase-free, Worthington), and centrifuged through a 10 ml cushion of 1 m-sucrose in buffer A at 27000\(\times\) g for 40 min. The pellet was solubilized in 5 ml buffer A with 2\% (w/v) Triton X-100, and insoluble material was removed by centrifugation at 27000\(\times\) g for 20 min. The supernatant was further centrifuged at 150000\(\times\) g for 45 min and 0-1 ml of the suspended pellet was run in a 15-30\% (w/v) sucrose gradient at 150000\(\times\) g for 80 min. Fractions of 12 drops were collected from the bottom of the tube, and absorption was measured at 260 and 280 nm. Fractions were dialysed against distilled water, lyophilized and analysed by rocket immunoelectrophoresis against MBRP-antiserum, as described.

Radiolabelling of total protein. To obtain satisfactory incorporation of radiolabelled amino acids, cells were grown in a synthetic medium. Cells from 10 ml preculture were inoculated into 50 ml AAM-medium (Rudin et al., 1974), with the following modifications: the pH was 7-2, and glycine and serine were added to a final concentration of 500\(\mu\text{g}\) ml\textsuperscript{-1} each. After 4 h cultivation, 25 \(\mu\text{Ci}\) (925 kBq) \[^{14}\text{C}\] protein hydrolysate (Amersham) was added, and the culture was grown for another 2 h.

Immunoprecipitation. Radiolabelled protoplasts were solubilized in PBS buffer (0-0 15 M-phosphate, 0-83 M-NaCl, pH 7-2) with 4\% (w/v) Triton X-100. Debris was removed by centrifugation at 20000\(\times\) g for 20 min. Antibodies (200 \(\mu\text{l}\)) were coupled to 50 \(\mu\text{l}\) protein-A Sepharose CL-4B (Pharmacia) in PBS buffer for 1 h at room temperature with agitation. The Sepharose was washed three times in PBS buffer before adding the solubilized protoplasts. The mixture was agitated for 1 h at 4\(^\circ\)C in the presence of 1 mm-phenylmethylsulphonyl fluoride (Sigma). After washing eight times with 1 ml PBS, the pellet sample was denatured in 40 \(\mu\text{l}\) preparation buffer for SDS-PAGE electrophoresis as described.

Autoradiography. Fluorography with sodium salicylate (Merck) was performed as described by Chamberlain (1979).

Assays. RNA was determined by the orcinol method (Herbert et al., 1971) using yeast RNA (Sigma) as standard. Protein concentrations were determined by the method of Lowry, with bovine serum albumin as standard. Malate dehydrogenase was assayed as oxaloacetate-dependent oxidation of NADH by the method of Yoshida (1965). Succinate dehydrogenase was determined as described by Hederstedt et al. (1979) using the synthetic electron acceptor 2,6-dichloroindophenol with phenazine methosulphate as electron carrier.

RESULTS

Comparison of cytoplasmic and membrane ribosomes by CIE

The antiserum raised against the highly purified membranes gave a maximum of approximately 40 visible immunoprecipitates when run against Triton X-100-solubilized \textit{S. aureus} membranes (Fig. 1\textit{c}). The antigen contained less than 1\% RNA relative to protein and was therefore considered as essentially free of ribosomes. Membrane ribosomes which were released from the membranes by Triton X-100 and then purified by repeated ultracentrifugation and Sepharose 2B chromatography were shown to retain at least one membrane antigen detected by CIE against the membrane antiserum (Fig. 1\textit{b}). This antigen (MBRP) was released from the ribosomes by 0-5 M-KCl or with 1\% (w/v) deoxycholate in buffer A. No MBRP was found on cytoplasmic ribosomes purified by the same procedures (Fig. 1\textit{d}). A monospecific antiserum against MBRP was obtained by immunizing rabbits with the immunoprecipitates from CIE gels similar to those seen in Fig. 1\textit{b}). CIE of Triton X-100-solubilized protoplasts against this antiserum gave only one visible immunoprecipitate as seen in Fig. 1\textit{d}). This precipitate was identical to that obtained with the high-salt or deoxycholate wash from the membrane ribosomes, as revealed by CIE of a mixture of both antigens against the MBRP antiserum (data not shown).
Sucrose gradient centrifugation of membrane ribosomes

Membrane ribosomes were subjected to sucrose gradient centrifugation in ribosome-preserving buffer A, and each fraction was analysed for MBRP by rocket immunoelectrophoresis against the monospecific MBRP antiserum. MBRP closely followed the sedimentation pattern of the ribosomes. Because the gradient was deliberately overloaded in order to be able to detect MBRP, a typical ribosome/polysome sedimentation profile was not obtained (data not shown). However, that the $A_{280}$-absorbing material in the peak fractions represented ribosomes was shown in parallel experiments where the starting material was treated with RNAase before the gradient centrifugation (data not shown) or where the magnesium concentration during sucrose gradient centrifugation was lowered, thereby dividing the ribosomes into their subunits (Fig. 2). Dissociation of the ribosomes into 50S and 30S subunits resulted in a change in the position of MBRP which now sedimented together with the 50S subunit (Fig. 2). This indicated that MBRP was really bound to the ribosomes and did not simply co-sediment with them. The fact that MBRP sedimented slightly faster than the 50S peak indicated that MBRP was not bound to every 50S particle, and that particles associated with MBRP were heavier (see below).

Analysis of cytoplasmic and membrane ribosomes by SDS-PAGE

SDS-PAGE of membrane ribosomes and cytoplasmic ribosomes revealed several qualitative and quantitative differences. At least four proteins were missing from the cytoplasmic ribosome...
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Fig. 2. Sedimentation pattern of membrane-bound ribosomes and MBRP in sucrose gradient centrifugation: ○, absorbance at 280 nm; ●, relative amount of MBRP; 8 A_{280} units of ribosomes were suspended and centrifuged in buffer A with a low Mg^{2+} concentration (1 mM).

Fig. 3. SDS-PAGE of (a) cytoplasmic and (b) membrane ribosomes. Samples containing 40 μg protein were applied to the gel, and electrophoresis was performed according to Neville (1971). Molecular weight markers were bovine serum albumin (68000), ovalbumin (43000), carbonic anhydrase (29000), trypsin inhibitor (20100) and lysozyme (14300), indicated by pointers.

preparation, and three proteins were present in significantly lower amounts (Fig. 3). From this experiment it could not be determined which protein was MBRP. SDS-PAGE/CIE of membrane ribosomes against the MBRP antiserum showed one single immunoprecipitate of a protein with an apparent molecular weight of 60000 (see Fig. 4). No immunoprecipitate was seen with proteins from cytoplasmic ribosomes (data not shown). The molecular weight of MBRP was confirmed by immunoprecipitation of a high-salt wash of radiolabelled membrane ribosomes. One single band with a molecular weight of 60000 was obtained (data not shown).
Immunoprecipitation of MBRP from Triton X-100-solubilized radiolabelled protoplasts

When $^{14}$C-labelled protoplasts were solubilized with Triton X-100 and immunoprecipitated with protein A-coupled antibodies against MBRP, four peptides with apparent molecular weights of 71 000, 60 000 ($=\text{MBRP}$), 46 000, and 41 000 were recovered (Fig. 5a). Since [U-$^{14}$C]-protein hydrolysate was used for radiolabelling, it could be concluded from the autoradiogram that these proteins appeared at approximately equal amounts in the precipitate. The same four proteins seemed to be present on rapidly prepared membrane ribosomes (Fig. 5b). As judged from the staining, these proteins also appeared in approximately equimolar amounts.

Cellular distribution of MBRP

The distribution of MBRP between the cytoplasm and the membrane is represented in Table 1. Of the total MBRP, 30–32% was found in the cytoplasmic fraction after osmolysis in a ribosome-preserving buffer and 40–43% in the membrane fraction. Sonication of the membranes in a buffer with a low Mg$^{2+}$ concentration (1 mM) resulted in the release of 20–33% of the total MBRP, leaving only 10–20% in the membranes. This treatment also resulted in the release of most of the ribosomes, measured as RNA. Sonication released only 7% of the membrane-bound enzyme succinate dehydrogenase, indicating that the integrity of the membranes was not seriously disturbed. After sonication, the membrane fraction was almost completely free of malate dehydrogenase, which is a cytoplasmic enzyme.

Centrifugation of the cytoplasmic fraction at 105 000 g for 2 h to sediment the ribosomes revealed that 15–18% of the MBRP in the cytoplasm sedimented together with the ribosomes. The total recovery of MBRP in the subcellular fractions was only about 75% of that found in Triton X-100-solubilized protoplasts.

DISCUSSION

Membrane-bound ribosomes which were detached by solubilization of the membrane with Triton X-100 retained one protein (MBRP) which was detected with an antiserum against ribosome-free membranes (Fig. 1b). This supported the hypothesis that this protein was a component of a protein-secretion apparatus that remained associated with the ribosomes after gentle extraction of the membrane with nonionic detergent.
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Fig. 5. (a) Autoradiogram of $^{14}$C-labelled proteins precipitated with the MBRP antiserum. The immunoprecipitates were separated by SDS-PAGE according to Laemmli (1970). (b) SDS-PAGE of the peak fraction from sucrose gradient centrifugation of rapidly prepared membrane ribosomes. Electrophoresis was performed according to Neville (1971). Molecular weight standards were as in Fig. 3.

Table 1. Distribution of MBRP relative to a cytoplasmic enzyme, malate dehydrogenase, and to a membrane enzyme, succinate dehydrogenase

<table>
<thead>
<tr>
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<th>Malate dehydrogenase</th>
<th>Succinate dehydrogenase</th>
<th>MBRP</th>
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<tbody>
<tr>
<td>Cytoplasmic fraction</td>
<td>0.86</td>
<td>&lt;0.01</td>
<td>0.30-0.32</td>
</tr>
<tr>
<td>Membrane fraction</td>
<td>0.14</td>
<td>0.99</td>
<td>0.40-0.43</td>
</tr>
<tr>
<td>Membranes after sonication at 1 mM-Mg$^{2+}$</td>
<td>0.02</td>
<td>0.92</td>
<td>0.10-0.20</td>
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Marty-Mazars et al. (1983) recently identified six proteins in the ribosome-bearing domains of Bacillus subtilis membranes, which all sedimented together with the ribosomes after extraction of the membranes by Triton X-100, and at least one of which was considered to be involved in protein secretion (Horiuchi et al., 1983b). MBRP, which had an apparent molecular weight of
60000 in SDS-PAGE, was attached to the membrane ribosomes even after extensive washing with Triton X-100 and subsequent Sepharose 2B chromatography and sucrose gradient centrifugation. Since MBRP was not found on equally purified cytoplasmic ribosomes we assumed that MBRP was specifically associated with membrane ribosomes and might therefore be part of a protein-secretion apparatus. Sucrose gradient centrifugation of dissociated ribosomes indicated that MBRP was bound to the 50S subunit (Fig. 2). This sedimentation profile also indicated that 50S subunits with MBRP sedimanted slightly faster than the 50S subunit alone. This was consistent with the finding that MBRP was associated with three other proteins in roughly equimolar amounts (Fig. 5b), which together would increase the mass of the 50S particle by approximately 10%. The association of MBRP with the 50S particle further supports the idea that it may be engaged in protein secretion, since the nascent peptide exit site and also the membrane-binding site are located on the 50S subunit (Bernabeu & Lake, 1982).

The fact that MBRP was originally detected on membrane ribosomes with an antiserum raised against ribosome-free membranes suggested that MBRP was a membrane protein. However, when the subcellular distribution was investigated we found that large amounts of MBRP were present both in the cytoplasm and in complexed membranes (i.e. membranes including ribosomes). Most of the MBRP in the membranes was associated with the ribosomes and was released together with them upon sonication at low Mg2+ concentration (Table 1) or extraction with Triton X-100 (quantitative data not shown). In contrast, MBRP in the cytosol was not associated with the ribosomes, although a minor part (15-18%) sedimented together with the ribosomes at 150000g (Table 1). Whether this portion of MBRP was really bound to the ribosomes was not tested. However, MBRP sedimenting with cytoplasmic ribosomes disappeared when the ribosomes were purified by Sepharose chromatography, indicating a much weaker association than with membrane ribosomes.

The presence of MBRP both on membrane ribosomes and in the cytoplasm suggests that MBRP may participate in protein secretion in a way similar to that of the signal recognition particle (SRP) of eukaryotic cells which oscillates between the ribosomes and the membranes (Walter & Blobel, 1982). Horiuchi et al. (1983a, b) have recently found a protein (CM-protein, mol. wt. 64000) in B. subtilis membranes which was protected from proteolysis by the ribosomes, and which was also present in large amounts in the cytoplasm. A similar distribution was also found for a protein (SecA, mol. wt 92000) involved in secretion in E. coli (Oliver & Beckwith, 1982).

A very interesting finding was that antibodies against MBRP precipitated a complex of four different proteins from Triton X-100-solubilized protoplasts (Fig. 5a). Since the antiserum against MBRP was prepared by immunizing rabbits with immunoprecipitates cut out from CIE gels (see Fig. 1b), it was possible that the antigen (i.e. immunoprecipitate) contained all these four proteins, and hence the MBRP antiserum was not monospecific. However, immunoprecipitation of the antigen, used for preparation of the CIE gels, with the final MBRP antiserum yielded only one protein band on SDS-PAGE with an apparent molecular weight of 60000 (data not shown). This antiserum was raised against the material released by sodium deoxycholate from purified membrane-ribosomes. It could thus be concluded that the MBRP antiserum was monospecific, and that most of the MBRP in the cell was complexed with three other proteins.

Analysis of rapidly prepared membrane-bound ribosomes by SDS-PAGE revealed the presence of four proteins in roughly equimolar amounts with the same molecular weight as those of the MBRP-complex (Fig. 5b). Since the polypeptides of molecular weight 71000, 46000 and 41000 were absent on extensively purified membrane ribosomes (Fig. 3b) it may be concluded that MBRP was more firmly bound to the ribosomes than to the other three proteins of the MBRP-complex, and that the complex was probably bound to the ribosomes via the MBRP.

Horiuchi et al. (1983a) reported that antibodies to a membrane protein (mol. wt 64000) that was covered by secreting ribosomes immunoprecipitated a complex of several proteins. Antibodies to the staphylococcal MBRP (mol. wt 60000) gave, with Triton X-100-solubilized protoplasts of B. subtilis, an immunoprecipitate which contained four proteins with apparent molecular weights of 64000, 62000, 43000 and 40000 (Adler & Arvidson, 1984). We believe that the protein of molecular weight 64000 studied by Horiuchi et al. (1983a, b) corresponds to the
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MBRP described here, and that the complex they observed is analogous to the MBRP-complex. Further work will be required to determine the function of this complex in protein secretion.

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REFERENCES


