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Transport and Processing of Staphylococcal Alpha-Toxin†

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Two larger precursors to staphylococcal alpha-toxin were identified and partially characterized. Both precursor proteins were present on the cell membrane at very low levels and appeared to be rapidly processed to the mature form. Dinitrophenol inhibited processing such that the two precursors accumulated in the membranes, whereas little extracellular (mature) alpha-toxin is formed. The peptide maps of the ^{35}S-labeled peptides from extracellular alpha-toxin and the two precursors were almost identical. The larger precursor protein contained four additional peptides and the smaller precursor protein contained three additional peptides not found in the extracellular toxin.

Staphylococcal alpha-toxin is a membrane-damaging toxin secreted by many strains of *Staphylococcus aureus*. It has been shown to preferentially lyse the erythrocytes of various mammalian species, induce spastic paralysis of smooth muscle, and disrupt liposomes (2). However, despite the fact that it is the most intensely studied of the staphylococcal toxins, its exact role in pathogenesis has never been defined.

Six and Harshman (3) purified alpha-toxin and found two closely related but electrophoretically distinguishable forms of the toxin (i.e., charge isomers) that also differed slightly in molecular weight. Nevertheless, they determined the minimum molecular weight of alpha-toxin to be approximately 28,000. Subsequently, Cassidy and Harshman (1) were able to isolate a single form of the toxin and attributed the variability in size to the action of endogenous proteases.

Although several studies (for a review, see reference 2) have examined the biochemical nature and mode of action of alpha-toxin, none have addressed the extracellular transport of this protein toxin. We now report the results of our experiments on the transport of alpha-toxin and compare these results with earlier studies on the transport of staphylococcal enterotoxin B (5).

MATERIALS AND METHODS

Bacterial strains, media, and lysis conditions. *S. aureus* S6 was used for all experiments described. Overnight inocula, defined media, preparation of the cells for in vivo pulse-labeling with [^{35}S]methionine, and cellular fractionation procedures have been described previously (5).

Briefly, whole cells were separated from the medium by centrifugation and lysed with lysostaphin (100 μg/ml) in hypotonic buffer (10 mM Na_2HPO_4, 20 mM $MgSO_4$, pH 6.5). The membranes were then harvested by centrifugation of the lysates through a solution of 50% sucrose in hypotonic buffer. Under these conditions, the membranes formed a pellet, and the cytoplasmic contents remained on top of the sucrose shelf. The membrane pellets were suspended in solubilization buffer (4), sonicated briefly, and diluted 1:2 with distilled water. Insoluble debris was removed by centrifugation, and the supernatant containing the solubilized membrane proteins was subjected to immunoprecipitation.

Immunoprecipitation of alpha-toxin. All procedures involving the immunoprecipitation of alpha-toxin from pulse-chase samples or subcellular components were carried out as described for enterotoxin B (5) with the following modifications. Rabbit anti-alpha-toxin immunoglobulin G (IgG; the generous gift of S. Harshman, Vanderbilt University, Nashville, Tenn.) was used as the primary antibody, and the antigen-antibody complexes were precipitated by the addition of goat anti-rabbit IgG immobilized on nylon beads (AMP Biological and Diagnostic Products Co., Seguin, Tex.).

SDS-PAGE. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as follows. Immunoprecipitates and acetone-washed trichloroacetic acid precipitates of cell lysates, membranes, or media were suspended in 20 μl of sample buffer (36 mM sodium phosphate buffer [pH 7.2], 4% SDS, 14% glycerol, 0.1% phenol red) and boiled for 5 min. Electrophoresis was carried out in 0.8-mm gels with a stacking gel consisting of 5% acrylamide–0.26% bisacrylamide and a resolving gel of 17% acrylamide–0.38% bisacrylamide. After impregnation of the gels with 2,5-diphenyloxazole, the labeled proteins were visualized by autoradiography against Kodak X-OMAT AR film at −70°C for 1 to 24 h. The details of these procedures are contained in reference 5.

Peptide mapping. The ^{35}S-labeled alpha-toxin and its precursors were isolated from long SDS-PAGE gels. To separate the alpha-toxin precursors sufficiently to

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allow peptide mapping, solubilized membranes were electrophoresed on 25-cm resolving gels. Slices of the dried gels containing the labeled proteins were excised (after locating the bands by autoradiography) and rehydrated for 1 h at room temperature in distilled water. The 2,5-diphenyloxazole used for fluorography of the gels was removed by shaking the rehydrated gel slices for 1 h in 100% dimethyl sulfoxide and then for 1 h in a solution consisting of 10% methanol and 25% isopropanol and finally in distilled water. Elution and tryptic digestion of the proteins was carried out by incubating gel slices for 24 h at 37°C in 2 ml of a 50 mM NH_4HCO_3 buffer (pH 7.8) containing 50 μg of trypsin (recrystallized twice; Millipore Corp., Bedford, Mass.). The supernatant solution containing the tryptic peptides was separated from the gel material by centrifugation and lyophilized. The peptides were finally dissolved in electrophoresis buffer and spotted on a thin-layer cellulose plate (Kodak Chromogram no. 6064). Electrophoresis was carried out at 50 V/cm at 10°C in a buffer consisting of acetic acid-formic acid-water (3:1:16). Chromatography in the second dimension was in pyridine-butanol-acetic acid-water (25:32.5:5:20) (4).

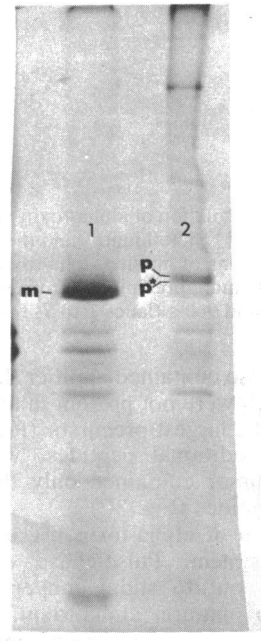

FIG. 1. Membrane-bound precursors of alpha-toxin. *S. aureus* S6 cells were suspended in 1.5 ml of medium and labeled for 10 s with 500 μCi of [^{35}S]methionine and then chased for 10 min with the addition of 50 mg of unlabeled methionine in the presence and absence of DNP (final concentration, 50 mM). An autoradiogram is shown of the proteins from lysostaphin-induced lysates of whole cells that were immunoprecipitated with anti-alpha-toxin antibody and separated by SDS-PAGE. Lane 1, proteins specific for anti-alpha-toxin precipitated in the absence of DNP; lane 2, proteins precipitated in the presence of DNP. The positions of mature alpha-toxin (m) and of the two precursors (p and p*) are shown.

RESULTS

Initial attempts to demonstrate a precursor to alpha-toxin were carried out as reported for enterotoxin B (5). Whole cell lysates were prepared during the period of maximum toxin production and subjected to immunoprecipitation. However, when this preparation was subjected to SDS-PAGE (Fig. 1), a larger precursor to alpha-toxin could not be detected. This was an unexpected result because alpha-toxin synthesis and enterotoxin B synthesis are temporally coincident in *S. aureus* S6, and a precursor to enterotoxin B has been readily detected in this type of experiment (5).

When dinitrophenol (DNP) was used to inhibit precursor processing of alpha-toxin, two larger proteins were isolated by precipitation with anti-alpha-toxin antibody (Fig. 1). The larger of the two proteins (p) had a molecular weight estimated by SDS-PAGE of 44,750, whereas the small protein (p*) had a molecular weight of 44,000. In this gel system, extracellular alpha-toxin migrated with an apparent molecular weight of 42,900, which was considerably larger than the minimum molecular weight of 28,000 reported by Six and Harshman (3). Harshman (personal communication) also has observed considerable variation in the migration of alpha-toxin on SDS gels that is due to the composition and pH of the buffer system. Alpha-toxin preparations obtained from S. Harshman also migrated with an apparent molecular weight of 42,900 in our system.

These proteins were shown to accumulate with time in the presence of DNP (Fig. 2), whereas levels of mature (extracellular) alpha-toxin did not substantially increase. In Fig. 2, both precursor proteins appear as a single band due to radioactive blurring consequent to accumulation of the proteins in the shorter (14 cm) gels. However, they could be separated adequately on long (25 cm) gels, which were used when the precursors were purified for peptide mapping.

Peptide analysis. The ^{35}S-labeled tryptic peptides of p and p* were compared by two-dimensional peptide mapping (Fig. 3). Extracellular alpha-toxin contained at least 10 distinct tryptic peptides (Fig. 3A) labeled with [^{35}S]methionine. Since alpha-toxin contains a minimum of six methionine residues based on amino acid analysis (3), some of these peptides could be due to incomplete digestion with trypsin. Both of the precursors (Fig. 3B and C) contained all 10 peptides found in the extracellular alpha-toxin map, confirming the relationship between these proteins. The tryptic digests of the two precur-

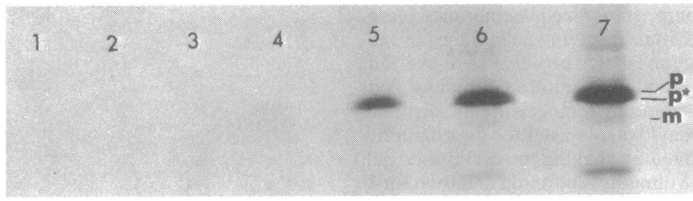

FIG. 2. Accumulation of alpha-toxin precursors. Cells were suspended in 1.5 ml of medium and labeled for 15 s with 500 μCi of [^{35}S]methionine and then chased with 50 mg of unlabeled methionine in the presence of 50 mM DNP. The positions of mature alpha-toxin (m) and its precursors (p and p*) are marked on the autoradiogram. Proteins were immunoprecipitated from lysostaphin-induced whole cell lysates. Samples were taken at 5, 30, 60, 120, 240, 480, and 960 s (lanes 1 to 7, respectively) after the addition of DNP.

sor proteins also contained another three to four peptides which were not present in the extracellular toxin. The largest precursor (Fig. 3B) contained four additional peptides, whereas the smaller precursor contained only three of the four extra peptides (Fig. 3C).

Identification of alpha-toxin precursors in an uninhibited system. Pulse-chase experiments were carried out to show conversion of the precursor to mature alpha-toxin. However, when alpha-toxin was immunoprecipitated from whole cell lysates taken during the course of the experiment, a larger precursor was not seen (data not shown). Since the level of mature alpha-toxin greatly exceeded that of the precursors, a different strategy was needed for analysis. We eliminated the comparatively large amount of extracellular mature toxin by purification of the membranes and analyzed only the membrane-associated alpha-toxin. The two precursor proteins were observed (Fig. 4). Thus, the two alpha-toxin precursors detected by DNP inhibition of processing could also be identified in uninhibited cells.

In this experiment (Fig. 4), we also detected a protein that was larger than the two previously identified precursors (c). Evidently this protein did not accumulate in the presence of DNP since it was not detected under those conditions (Fig. 2). However, when the tryptic peptides of this protein were analyzed, only four ^{35}S-labeled peptides were found (data not shown). Three of the peptides corresponded closely to tryptic peptides 1, 4, and 5 of mature alpha-toxin (Fig. 3A), but the fourth peptide was not related to any of the 10 peptides of alpha-toxin. At this time, the relationship of this protein to alpha-toxin is uncertain.

DISCUSSION

Both alpha-toxin and enterotoxin B are secreted by *S. aureus* S6, yet the temporal aspects of the processing of their respective precursors

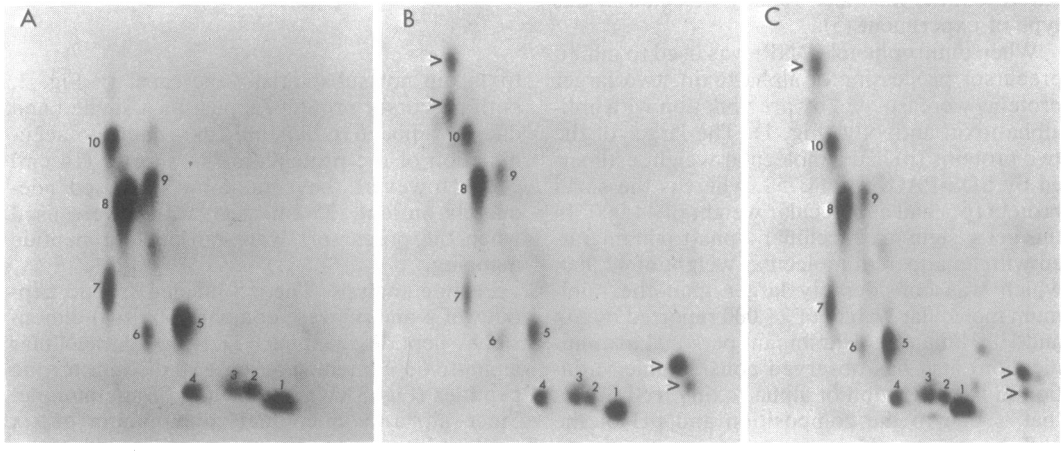

FIG. 3. Two-dimensional peptide mapping analysis of alpha-toxin and its precursors. Protein bands corresponding to [^{35}S]methionine-labeled alpha-toxin and its precursors were separated on 25-cm SDS-PAGE gels, excised from dried gels, and digested with trypsin. The peptides were separated by thin-layer electrophoresis (left to right) followed by chromatography in the vertical dimension. Autoradiograms are shown of the tryptic peptides from (A) extracellular alpha-toxin, (B) precursor protein p, and (C) precursor protein p*. Peptides common to all three proteins are numbered 1 through 10, and peptides unique to the precursors are labeled by arrows.

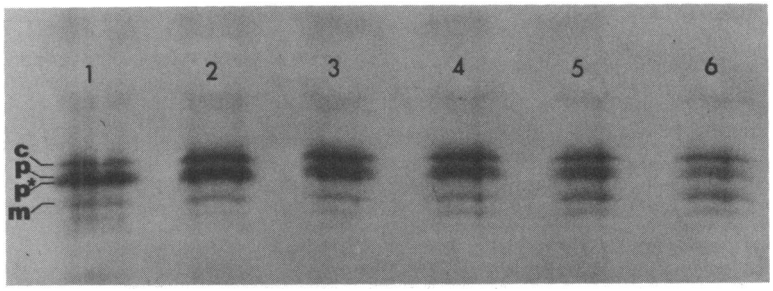

FIG. 4. Pulse-chase analysis of membrane-associated alpha-toxin. Membranes purified from cell samples taken during a pulse-chase experiment were solubilized and analyzed for alpha-toxin and its precursors by immunoprecipitation and SDS-PAGE. An autoradiogram is shown of the alpha-toxin-related proteins immunoprecipitated from the membranes of cells taken at 30, 60, 120, 240, 480, and 960 s post chase. The positions of mature alpha-toxin (m), the two precursors (p and p*), and a protein which cross-reacts with the anti-alpha-toxin antibody (c) are shown.

differ. Although small amounts of alpha-toxin precursors exist as membrane-bound species, it is evident that a larger precursor to alpha-toxin is not present on the cell membrane to any significant degree. In contrast, a larger precursor to enterotoxin B is readily detectable under the same conditions (5). To demonstrate the presence of a larger precursor to alpha-toxin, processing was inhibited by DNP. This compound had been previously shown to inhibit processing of the enterotoxin B precursor, but it did not totally inhibit synthesis, and the precursor accumulated in the cell membrane (5). In this study, two larger proteins which cross-reacted with antiserum to alpha-toxin were shown to accumulate in the presence of DNP, whereas very little extracellular (mature) alpha-toxin was formed. These data indicated that DNP inhibited the processing of the alpha-toxin precursor since (i) very little mature alpha-toxin was formed and (ii) these proteins reacted with anti-alpha-toxin antibody.

The relationship of these two larger proteins to mature alpha-toxin was confirmed by comparing the ^{35}S-labeled tryptic peptides obtained from each. All 10 peptides of the mature toxin were present in the peptide maps of the two precursors. In addition, four ^{35}S-labeled peptides were present in the peptide map of the larger precursor that were not present in mature alpha-toxin. The smaller precursor contained three of the four additional peptides found in the large precursor.

Both of the precursors were also found at low levels on the membranes of normal, uninhibited cells, supporting the idea that the two larger proteins identified after DNP inhibition are present normally in vivo. Thus, alpha-toxin was apparently synthesized with an additional sequence which was removed before its appearance in the extracellular environment. Presumably, the additional sequences found on the alpha-toxin precursors were due to a signal peptide, although confirmation of this will require sequencing of these proteins.

Why two larger precursors are detected in DNP-inhibited and normal cells is currently unknown. Possibly the smaller protein is an intermediate step in the conversion of the larger protein to mature alpha-toxin. Then the alpha-toxin precursor may require at least two proteolytic processing events to form mature alpha-toxin. However, we cannot rule out shortening of the larger precursor by the action of nonspecific endogenous proteases unrelated to precursor processing.

The additional sequences must be removed very rapidly during transport because only very small amounts of alpha-toxin precursors accumulate on the membrane. However, if these proteins were converted to mature alpha-toxin via post-translational processing, then the rate of conversion, as judged from pulse-chase experiments of uninhibited cells, would be too slow to account for the appearance of mature alpha-toxin. Alternatively, these precursors may disappear from the membrane because of proteolytic degradation, but this would mean that synthesis of a full-length precursor is a nonproductive step in the synthesis of alpha-toxin. In either case, most of the mature alpha-toxin produced probably does not arise directly from processing the full-length precursor. The additional sequences are more likely removed cotranslationally (before the termination of synthesis) instead of post-translationally. Furthermore, if the signal peptide is removed cotranslationally, then a significant share of the incomplete alpha-toxin chains should lack signal peptides. We are currently attempting to verify this by examining incomplete chains for the presence of a signal sequence.

ACKNOWLEDGMENT

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