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TRANSPORT AND PROCESSING OF STAPHYLOCOCCAL ENTEROTOXIN A

by

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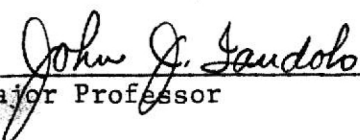
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INTRODUCTION

The enterotoxins which are a group of extracellular proteins produced by Staphylococcus aureus, a Gram positive bacterium, are responsible for staphylococcal food poisoning (7). Of the six distinct enterotoxins that have been purified, enterotoxin A (SEA), is the most frequent cause of staphylococcal gastroenteritis (20). It is not known if these proteins have any additional roles during infection.

Until recently little direct information existed on the transmembrane transport of the enterotoxins. Early evidence suggested a lipoprotein/protease release mechanism for SEA and enterotoxin B (SEB). However, this model was based on extracellular accumulation of enterotoxin and did not present direct evidence for a lipoprotein intermediate (8). Recently, Tweten and Iandolo have identified a larger, membrane-bound, kinetic precursor to SEB that is not a lipoprotein (80). They suggested that SEB was similar to periplasmic and outer membrane proteins of Escherichia coli that are initially synthesized as precursor proteins which have an additional amino terminal extension or signal sequence. These precursors are transiently associated with the inner membrane of E. coli prior to removal of the signal sequence. The signal sequence is then proteolytically removed and the mature protein is released from the membrane.

The present study was undertaken to determine if SEA, like SEB and E. coli periplasmic and outer membrane proteins, could demonstrate a precursor-product relationship. The organism chosen for the analysis was S. aureus 13N2909, a mutant of S. aureus strain 100, that produces high levels of SEA (34).

The results obtained from this study showed that SEA is initially synthesized as a larger molecular weight precursor. This precursor is

proteolytically processed and the newly processed mature SEA is temporarily sequestered in the cell wall before its release into the extracellular environment. Additional studies demonstrated that high concentrations of energy uncouplers stopped both processing and synthesis.

LITERATURE REVIEW

A. Demonstration of Precursor Forms

Study of the mechanism by which proteins are transported across the cell membrane began 28 years ago with Palade's observation that animal cells contained both free and membrane-bound ribosomes (60). Subsequently, some eukaryotic secretory proteins were found to be synthesized on membrane-bound polysomes (61, 81) while cytoplasmic proteins were synthesized on free polysomes (70, 64, 35). To explain how the membrane-bound and free ribosomes differed in their location and in their selection of mRNA, a difference in ribosome structure was proposed. However, no such difference could be detected. Therefore, Blobel and Sabatini (11) proposed that the mRNA for secretory proteins contained a sequence in the amino terminal coding region which signaled the cellular machinery to initiate extracellular transport. They did not describe the nature of this signal except that it occurred early in the coding sequence for the amino terminal residues. Independently of Blobel and Sabatini, Milstein et al. (53) observed that in vitro translation of immunoglobulin light chains from myeloma cell mRNA yielded a protein 3000 daltons larger than the extracellular form. This larger precursor was converted to a size equivalent to the extracellular form when homologous membranes from the myeloma cells were added during translation (21). An additional sequence was present on the amino terminus of the light chain that was proteolytically removed in the maturation process. These findings provided the first evidence that proteins destined for secretion have a unique amino terminal sequence that was removed after it had served its function of initiating transport of the protein. After the discovery of this unique sequence, the presence of signal sequences in many other eukaryotic proteins was confirmed (36, 38, 45, 49, 68, 69).

Yamamoto and Lampen were the first to report that a secreted prokaryotic protein was synthesized as a larger precursor (86). They found that the extracellular penicillinase of B. licheniformis was initially produced as a larger, membrane-bound precursor. Subsequently, Inouye and Beckwith reported that the product of in vitro synthesis of E. coli alkaline phosphatase, a periplasmic protein, was larger than the periplasmic form (39). Their experiments also showed that the addition of membrane fractions could bring about a reduction in the size of the synthesis product to that of the mature form. Subsequently, a number of periplasmic and outer membrane proteins of E. coli were described that were initially synthesized as precursors (62, 67).

Sequence determinations (55, 59, 57) revealed that the signal sequence was composed of approximately 15-30 mostly hydrophobic amino acids located at the amino terminus. Also the signal sequence contained 1-3 charged amino acids (usually lysine or arginine) within the first five residues.

B. Models for Protein Export

Various models for the mechanism of protein export have been presented. However, it is probable that no single model will be applicable to the export of all bacterial proteins. Two of the more prominent models are described below.

According to the "signal hypothesis" proposed by Blobel and co-workers (9, 12), proteins of Gram negative bacteria are transferred vectorially across the membrane. As a protein destined for export is being synthesized, the signal sequence would emerge from the ribosome and would bind to the membrane resulting in attachment of the polysomes (synthesizing this protein) to the membrane. According to the original model, this binding is suggested to recruit membrane proteins which, together with ribosomal

components, constitute a membrane pore through which the growing polypeptide chain is cotranslationally extruded. After its extrusion to the noncytoplasmic face of the membrane, the signal sequence is removed. For periplasmic proteins, the remainder of the protein would continue its passage and finally be secreted. In the case of some inner membrane proteins, the passage may not be completed and the protein would remain embedded in the membrane.

The signal hypothesis envisions that the polypeptide chain has crossed the plane of the membrane vectorially and in an extended form. According to this model, the role of the signal sequence is to mediate the initial events of export, that is, to bring the synthesizing ribosomes to the membrane, facilitating the formation of an export pore. However, in no case have the prokaryotic pore proteins invoked by the signal hypothesis been identified.

A very different model that does not require an ordered vectorial export process nor any special cellular export machinery has been proposed by Wickner (85). The "membrane trigger hypothesis" emphasizes the rate of self assembly of proteins in mediating their own export. The hypothesis allows for synthesis of a protein molecule to be completed before its export begins. The role of the signal sequence is to promote folding of the newly made precursor into a conformation that is soluble and competent for export. Upon reaching the membrane the protein is "triggered" into a new conformation allowing it to spontaneously insert into, or through, the phospholipid bilayer unaided by any sort of pore. Cleavage of the signal sequence would facilitate this conformational change and render it irreversible. Although it is not directly stated in the hypothesis (85) that posttranslational processing would occur, this type of processing is implied.

Eukaryotes and prokaryotes appear to share common mechanisms for certain aspects of protein export. The most striking evidence for this similarity came from studies in which the gene for a eukaryotic secreted protein, preproinsulin, was introduced into E. coli. The eukaryotic signal sequence of preproinsulin served to promote transfer of the polypeptide across the prokaryotic cytoplasmic membrane (76). The signal peptide was cleaved at the correct position to yield proinsulin (75). Ovalbumin, another eukaryotic secreted protein, can also be secreted by E. coli (33). Less extensive experiments suggested that when the gene for prokaryotic β -lactamase was introduced into yeast cells, the β -lactamase was correctly processed (65).

Much of the understanding of protein export has come from eukaryotic studies where the initial events of export can be reproduced in vitro (10, 12, 9). The powerful genetic tools available in E. coli have facilitated the selection and analysis of mutants that have allowed further dissection of the process of protein export. Therefore, most of the following information is from studies in prokaryotic systems.

C. Gene Fusions in the Study of Secretion

Genetic studies verified that the signal sequence functioned to promote the transport of proteins. Using Casadaban's method of in vivo gene fusion (16), Silhavy et al. (72) were able to fuse the β -galactosidase gene (lacZ) to the signal sequence of the lamB gene which codes for an outer membrane protein that functions as a porin for transport of maltose and maltodextrins. Also the lamB gene product serves as the receptor for bacteriophage λ .

The fusion resulted in the production of a hybrid protein comprised of the amino terminal half of the lamB protein fused to a major functional

portion of β -galactosidase. It was thought that by placing the lamB signal sequence at the amino terminus of the lacZ gene, the lamB signal sequence would promote transport of β -galactosidase to the outer membrane of E. coli. The results showed that some of the synthesized fusion protein was transported to the outer membrane. This study suggested that the signal sequence could direct the transport of a normally cytoplasmic protein; but it was not conclusive evidence since the hybrid protein contained a large amount of lamB sequence following the signal sequence.

Moreno et al. (54) obtained lamB-lacZ fusions that fused the lacZ gene directly to the DNA coding for the lamB signal sequence. The results showed that the hybrid protein remained cytoplasmic. Thus, in this case, the signal sequence was not sufficient for the entire transport process.

The subcellular location of other E. coli genes fused to lacZ has also been examined. One class of malF-lacZ fusions produced a hybrid protein localized to the inner membrane, which is where the malF gene product normally resides (71). In strains with malE-lacZ (4) fusions, β -galactosidase activity was found in the inner membrane and not in the periplasm. The malE gene codes for the maltose binding protein (MBP) which is normally found in the periplasm. In no case has any hybrid protein containing β -galactosidase been detected in the periplasm. One explanation for the properties of these fusions is that initiation of secretion occurred but was aborted. β -galactosidase included within its sequence certain amino acids that cannot pass through the cytoplasmic membrane, thus the hybrid protein became stuck in the membrane (82).

In summary, gene fusion studies have established that in certain cases, a cytoplasmic protein can be guided to the cell envelope and also that a functional signal sequence is required but not necessarily sufficient (54) to mediate this event.

D. Amino Acid Changes in Signal Sequence Mutants

A general procedure for selecting mutants altered in the signal sequence of a secreted protein has been one of the major outgrowths of the gene fusion technique. The phenotype of one of the classes of lamB-lacZ fusion proteins was used to directly select for mutations in the signal sequence. The lamB gene can be induced by growth on maltodextrans since the lamB gene is under transcriptional control of the maltose operon. Class III lamB-lacZ fusions conferred a Mal^S phenotype (6) which was expressed as cell lysis after induction of the maltose operon. The Mal^S phenotype resulted because large amounts of the hybrid protein jammed the export machinery. The hybrid protein inserted into the membrane without being effectively transported thus disrupting the membrane (4). Mutations that prevent the cell from initiating the secretion process relieved the Mal^S phenotype, yielding Mal^R bacteria. Mal^R mutant derivatives which still produced the hybrid protein were selected. In all cases 100% of the hybrid protein in these Mal^R mutant strains was found in the cytoplasm. The mutations mapped very early in the hybrid gene, in a region corresponding to the coding regions for the signal sequence of λ receptor.

Mal^R derivatives have also been obtained from malE-lacZ fusions. These fusion proteins also remain cytoplasmic and their mutations mapped early in the hybrid gene (4). The mutations have been crossed into the wild type malE (3) or lamB (30) genes by genetic recombination. The lesions mapped early in their respective genes and resulted in accumulation of the larger molecular weight precursor form of MBP and λ receptor. These results demonstrated that early mutations in the malE and lamB genes prevented export of the hybrid protein.

These mutations have been analyzed further by DNA sequencing (5, 29). All four of the lamB point mutations and four of the five malE point

mutations produced changes in the signal sequence from hydrophobic or weakly hydrophilic amino acids to charged residues. These mutations strongly support the contention that the hydrophobicity of this region is critical for export. One of the mutations, a leucine to proline change may prevent export of the MBP by altering the secondary structure of the signal sequence. However, a recent review (52) cited that mutants of lamB have been isolated that introduced charged amino acids into the hydrophobic core of the signal sequence without serious effect on lamB export (Emr and Silhavy, unpublished results).

Koshland (43) has reported two mutations that dramatically affect export of the TEM β -lactamase encoded by Tnl. The mutations were obtained by site specific mutagenesis and were isolated by classical genetic techniques. In one of the mutants, an arginine residue replaced a hydrophobic amino acid in the signal sequence and resulted in the accumulation of β -lactamase precursor in the cytoplasm. In another mutant, three amino acids at the boundary between the charged amino-terminal region and the hydrophobic region were changed. This resulted in a shorter hydrophobic core, due to the introduction of a charged residue. Export of β -lactamase occurred in this case, but at a much slower rate than in the wild type. Three other mutants with neutral or hydrophilic amino acids substituted for hydrophobic amino acids in the signal sequence affected processing, but not export of β -lactamase.

Not all disruptions of the hydrophobic core have dramatic effects on export. A mutation was fortuitously obtained in the prolipoprotein gene that introduced a charged amino acid into the signal sequence (46). In this strain the major portion of the prolipoprotein is found in the outer membrane, indicating that export has occurred (47, 48). However, only

prolipoprotein is found, thus showing that the mutation affects processing and that export is not dependent on processing.

Strains carrying mutations in the positively charged amino terminal region of the signal sequence of λ receptor have been isolated (66). Two of these mutations affect translational initiation, probably by inducing secondary structure in the mRNA which interfered with ribosome binding. One mutation that changed arginine to serine in the charged region resulted in a 75% reduction in the amount of mutant receptor found in the outer membrane. When this mutation was crossed into a lamB-lacZ fusion protein, its synthesis was completely inhibited (29). These results suggested that the charged amino acids were somehow signaling the ribosomal machinery to continue synthesis of the protein.

Based on the results above, Hall and Schwartz (37) have proposed that translation and export of λ receptor protein are coupled. They suggested that there is a 'stop translation' sequence in mRNA that halted continued translation of the protein until the basic amino acids, located at the extreme amino terminus, were bound to a hypothetical membrane protein. When this occurred, a 'continue translation' message is given to the ribosome complex by the hypothetical membrane protein. However, there is no direct evidence, at this point, for a 'stop translation' sequence in the mRNA or a membrane receptor protein in prokaryotes.

E. Role of the Mature Portion of a Protein in Export

According to the signal hypothesis (9, 12), the mature portion of a protein is transported passively through the membrane after the signal peptide has initiated the vectorial transfer process. In contrast, the membrane trigger hypothesis (85), requires a particular tertiary conformation, determined by the entire protein sequence before export can begin.

In this case, the carboxy terminus of a protein would play an active role in its export. However, in all cases examined so far, the evidence has shown that deleting from 10% to 50% of the C-terminus of the exported protein does not affect export or processing.

Translation products of amber mutants of E. coli examined by Ito and Beckwith (41) are transported even when two-thirds of the carboxy terminus is missing. Also, nonsense fragments of an arginine-binding protein have been found in the periplasm (17) and a premature termination product of an outer membrane constituent, ompA protein, was still capable of export and localization into the outer membrane (14). Similar results have been obtained by Koshland and Botstein (44) in an examination of a series of β -lactamase chain-termination mutants. Both precursor and mature forms of the nonsense fragment are made, indicating that processing showed no requirement for an intact carboxy region. Osmotic shock was performed and in no case were the mature peptides found in the periplasm. Results from cell fractionation studies initially lead to the conclusion that the β -lactamase fragments were in the cytoplasm and not secreted. However, subsequent analysis of the location of these peptides indicated that they could be digested by externally added trypsin. Therefore, these β -lactamase peptides must have traversed the inner membrane but remained membrane associated. Thus, it appears that the carboxy terminus of β -lactamase is necessary to promote stability or release from the membrane, but is not required for the protein to be exported or processed.

F. Discrete Export Sites

Evidence for discrete export sites within the cytoplasmic membrane was found by using the gene fusion technique. When a malE-lacZ fusion was induced with maltose, secretion of the hybrid protein was initiated but not

completed. This resulted in the hybrid protein remaining associated with the cytoplasmic membrane. The synthesis of this hybrid protein resulted in severe inhibition of processing and of proper localization of other noncytoplasmic proteins (4). Similar results were obtained with a lamB-lacZ fusion strain (fusion class that contained one-half of the lamB gene) induced with maltose (72).

Ito et al. suggested that specific sites in the cytoplasmic membrane became progressively occupied by the hybrid protein (malE-lacZ or lamB-lacZ) resulting in an inhibition of normal localization and processing of periplasmic and outer membrane proteins (40). If this is a correct interpretation, then most of the periplasmic and outer membrane proteins share a common step in localization before the polypeptide becomes accessible to the processing enzyme. Furthermore, there may be only a limited number of sites in the cytoplasmic membrane where proteins can be exported.

G. Other Elements of the Export Machinery

One of the cellular components involved in processing and transport of noncytoplasmic proteins in Gram negative bacteria is the peptidase which removes the signal sequence. Activities capable of processing the precursors of fl coat protein, alkaline phosphatase, lipoprotein, lamB protein, MBP have been described (18, 19, 39, 50, 51, 63). It is not presently known whether there is a single enzyme or a set of enzymes with either broad specificities or specificity for each precursor protein. Several unique processing enzymes instead of a common one have been suggested in E. coli because precursor cleavage sites have different DNA sequences. However, this does not exclude the possibility that a single enzyme is responsible for processing of all precursors. If the enzyme is indeed a common one, it probably would not recognize specific sequences but

be a part of a complex which would present the cleavage site to the enzyme at the proper time.

Genetic studies have indicated several functions involved in the transport process that map outside the gene for the transported protein. The first report of a mutation that affected secretion was made by Wanner et al. (83). The mutation, called perA caused a reduced amount of alkaline phosphatase in the periplasmic space. The level of transcription of the alkaline phosphate gene was not affected in this mutant. Also, the precursor of the alkaline phosphatase did not accumulate in the cytoplasm, suggesting that secretion was affected. The protein profile of the perA mutant when compared with that of a wild type strain demonstrated that seven periplasmic and three outer membrane proteins were also present in reduced amounts in the mutant's periplasm. Although the direct cause of the reduced levels of these proteins was not determined, the study did indicate that posttranscriptional events could affect the synthesis of secreted proteins.

Another mutant has been isolated in which the appearance of several exported proteins was simultaneously affected. The mutational lesion in this strain mapped at minute 22 on the E. coli chromosome in a gene called expA (25). This mutant was identified on the basis of decreased enzyme activity of two periplasmic acid phosphatases. Ten periplasmic and three outer membrane proteins were found in decreased amounts in the cell envelope of the expA mutant, however, its inner membrane protein profile was indistinguishable from that of a wild type strain. It is not known whether the lack of the affected proteins is the result of a transcriptional or posttranscriptional defect.

The involvement of ribosomal proteins in the transport process in E. coli has been demonstrated by Emr et al. (28). They have identified several suppressor mutations that restored export in strains carrying signal sequence mutations in the lamB protein. Mapping experiments showed that the suppressor phenotype was the result of mutations in any of at least three different chromosomal loci. One class of suppressor mutations, containing the largest number of independent isolates, mapped in the major ribosomal gene cluster very close to the rapE locus. All of the known gene products of this operon which have been transcribed and translated in vitro are ribosomal proteins (42). This class of suppressors phenotypically suppressed all known export deficient mutations internal to the signal sequence region of the lamB gene. These results suggested that at least one ribosomal protein (perhaps 5S) may play an important role in the export of λ receptor protein to the outer membrane.

A mutant of E. coli which was able to transport and localize the cytoplasmic protein, elongation factor 2 (EF2), in the outer membrane has been identified by Dombou (27). The mutation responsible for the sucrose-dependent, spectinomycin resistant phenotype of this mutant occurred in either ribosomal protein S4 or S5. This mutation may be very similar to Emr's suppressor mutation discussed above. Both Emr's and Dombou's results strongly suggested that ribosomal proteins play an important role in the export process in E. coli.

Oliver and Beckwith (58) have identified a mutation called secA (standing for secretion defective). The secA mutant strain grew normally at 30°C. However, at 42°C it was unable to grow on any medium, formed filaments, and accumulated precursors of many proteins, including MBP, alkaline phosphatase, ompF protein and lamB protein. The mutation was not strictly conditional since a small amount of precursor was detected

even at 30°C. Not all exported proteins were affected in the secA mutant, since a number of periplasmic proteins were still properly localized at the nonpermissive temperature.

The secA gene is located at one end of a cluster of genes involved in cell division and envelope biosynthesis. Other previously known mutations that mapped in this gene cluster have no effect on protein export. Since these other mutants did not accumulate precursors, it was unlikely that the export defect in the secA mutant was a secondary effect of blocked cell division.

One explanation for the properties of the secA mutation was that it caused alteration of a protein component of the cell's export machinery. However, the possibility that the physiological state of the membrane had been altered as a result of the mutation cannot be excluded. Thus, exactly how the secA mutation affected transport is not understood.

It has been demonstrated by Smith (73) that alkaline phosphatase and diptheria toxin were cotranslationally secreted in vitro into inverted inner membrane vesicles made from E. coli. These proteins were processed to their mature form. When the outer (cytoplasmic) surface of the vesicles was subjected to protease treatment prior to initiation of translation, both alkaline phosphatase and diptheria toxin were made but were not secreted into vesicles or processed. However, sequestration and processing occurred if protease was added after the ribosomes were vesicle bound and translation was initiated. This experiment indicated that inner membrane proteins that are on the cytoplasmic surface of the membrane may be required for polysome attachment and/or subsequent export of proteins.

The export machinery may consist of several different proteins which could be involved. Currently, only signal peptidase and possibly a ribosomal

protein have been implicated in the export process. The secA mutant's defective gene product may be directly involved in the export process and thus define components of the export process. The perA and expA mutants are not well understood. In these mutants either a transcriptional or posttranscriptional defect may account for the decreased amounts of certain noncytoplasmic proteins.

H. Protein Transport in Gram Positive Bacteria

Protein transport in Gram positive bacteria has not been examined as closely as protein transport in Gram negative bacteria. A few early studies in Gram positive bacteria proposed models for transport based on indirect evidence. Berkeley et al. (8) observed that quinacrine and cerulenin could prevent the accumulation of staphylococcal enterotoxin A (SEA) in growing cultures of S. aureus. Cerulenin inhibited fatty acid synthesis (23) and quinacrine inhibited the B. licheniformis penicillinase processing enzyme (78). Based on these data, it was proposed that SEA was transported by a lipid intermediate/protease release mechanism. Production of extracellular staphylocoagulase was also inhibited by cerulenin and quinacrine (32). However, these investigators raised doubts about a lipoprotein intermediate because of nonspecific effects of cerulenin. Instead they proposed that the cell bound staphylocoagulase was the precursor to the extracellular coagulase. Accumulation of staphylococcal enterotoxin B (SEB) in the extracellular environment was inhibited when cerulenin was added to growing cultures. However, cerulenin did not suppress accumulation of SEB in concentrated, nongrowing cells (1). The serine protease inhibitor tosyl-lysyl-chloromethyl ketone (TLCK) also prevented accumulation of SEB in growing cultures (2). These early studies concentrated on the accumulation of secreted proteins and did not present any direct evidence for

lipoprotein intermediates or precursor proteins. The inhibitory effects of cerulenin on SEA, SEB, and staphylocoagulase may be indirect since cerulenin has recently been shown to inhibit synthesis or assembly of several outer membrane proteins of E. coli, none of which is a lipoprotein (13).

Precursor proteins have been identified for the penicillinases of B. licheniformis (19) and S. aureus (57). These precursor proteins can be processed and secreted into the extracellular environment or be processed, associated with lipid, and remain membrane-bound (57, 74, 56).

Recently, Tweten and Iandolo (79, 80) have identified a larger membrane-bound form of SEB. This larger form of SEB was demonstrated to be the kinetic precursor to extracellular SEB. It was also found that once SEB was processed and released from the membrane, it was transiently associated with the cell wall before being released into the extracellular environment. Neither protease nor high salt concentration removed the SEB associated with the cell wall. However, this cell wall associated SEB was released by removal of the cell wall with lysostaphin. From these data, Tweten and Iandolo suggested that SEB was temporarily sequestered in the cell wall before being released to the extracellular environment (80). The cell wall may be needed for the transport to function correctly since it has been shown that L-forms of SEB-producing S. aureus did not produce SEB. However, L-forms of SEA-producing S. aureus did produce SEA (22).

Two larger precursors to staphylococcal alpha toxin have been identified by Tweten, Christianson, and Iandolo (ms. in preparation). These precursors cannot be detected in a pulse-chase analysis, indicating that they are rapidly processed. However, 2,4 dinitrophenol appeared to inhibit processing and allowed the two alpha toxin precursors to accumulate on the membrane while very little extracellular alpha toxin was formed. Presently,

it is not known if the two alpha toxin precursors are due to two processing events or if the larger precursor is shortened by an endogenous protease, unrelated to precursor processing. Preliminary evidence has shown that newly synthesized alpha toxin was also temporarily sequestered in the cell wall before being released to the extracellular environment.

I have studied the transmembrane transport of SEA. By in vivo pulse-chase analysis, I was able to identify a kinetic precursor to SEA (pSEA). Tryptic peptide mapping was used to examine the relationship of pSEA and SEA. I also investigated the effects of two uncouplers of the proton motive force on processing and synthesis of pSEA. Lastly, I analyzed the cellular location of pSEA and SEA during transport.

MANUSCRIPT

Transport and Processing of Staphylococcal Enterotoxin A

ABSTRACT

A larger molecular weight form of staphylococcal enterotoxin A was shown to be the kinetic precursor to extracellular enterotoxin A by in vivo pulse-chase analysis. The tryptic peptide maps of the [³H]-labeled precursor and enterotoxin A were identical, thus confirming that the two proteins were structurally related. Low concentrations of 2,4 dinitrophenol and carbonyl-cyanide-m-chlorophenyl hydrazone inhibited processing of the precursor, and as the concentration of these two uncouplers was increased, synthesis of the precursor toxin was also inhibited. Subcellular fractionation revealed that mature enterotoxin A was temporarily associated with the cell wall before being released into the extracellular environment. A part of the cell associated SEA was extracted with a high salt solution at 0-2°C suggesting that the mature protein may be retained by the cell via an ionic interaction.

INTRODUCTION

Staphylococcal enterotoxin A (SEA), the most frequent cause of staphylococcal gastroenteritis (5), is an extracellular protein produced by various strains of S. aureus. The physical properties of SEA have been reported (1), but limited information is available that addresses the secretion of this toxin. One study, based on indirect evidence, does present a model for the transmembrane transport of SEA. Berkeley et al. demonstrated that cerulenin, an inhibitor of fatty acid synthesis, and quinacrine, an inhibitor of the B. licheniformis penicillinase-releasing protease, prevented accumulation of SEA in growing cultures (2). Based on this evidence, these investigators suggested that SEA was secreted by a lipid intermediate/protease release mechanism. Their hypothesis was dependent upon variations in the accumulation of extracellular SEA and did

not present any direct evidence for a lipoprotein intermediate. Recently, cerulenin has been shown to inhibit synthesis or assembly of several E. coli outer membrane proteins, none of which are lipoproteins (3). Thus, the inhibiting activity of cerulenin on SEA was indirect and not necessarily indicative of a lipoprotein intermediate.

Recently Tweten and Iandolo (14, 15) have identified a larger, membrane bound form of staphylococcal enterotoxin B (SEB), that was shown to be the precursor to extracellular SEB. These investigators also proposed that SEB was transiently sequestered in specialized regions of the cell wall before being released into the extracellular environment (15).

Enterotoxins A and B appear to be quite similar in their chemical structure and in their clinical symptoms of intoxication. However, there are differences in the biosynthesis and regulation of these two toxins (1, 12). This study focused on the transport characteristics of SEA, which were compared to those of SEB. We demonstrated a precursor of extracellular SEA and showed that uncouplers of the membrane potential affect SEA and SEB differently. Evidence will be presented that SEA, like SEB, is transiently associated with the cell wall before its release into the extracellular environment.

MATERIALS AND METHODS

Organism. S. aureus 13N2909 (obtained from Anna Johnson-Winegar, Fort Detrick, Frederick, Maryland) a mutant of S. aureus 100, that produces high levels of SEA (9), was used for all experiments described.

Maximal SEA production. An overnight culture grown at 37°C in 4% N-Z-amine A (Sheffield Products, Memphis, TN) 0.2% yeast extract (Difco Laboratories, Detroit, Mich.) 0.2% glucose, and 0.75% MEM vitamins (GIBCO Laboratories, Grand Island, NY), pH 6.7, was diluted 1:50 into the same growth medium.

The culture was then incubated at 37°C with agitation. Samples (1 ml) were taken every hour for 10 h and the optical density was determined at 655 nm. The cells were removed by centrifugation and the spent medium was saved. The cells were then washed with 1 ml of fresh growth medium and recentrifuged. The two growth medium supernatants were combined and dialyzed overnight against distilled water. The samples were then lyophilized and prepared for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis was carried out as described for SEB (15).

Protein blotting. After electrophoresis the proteins were electroblotted by the procedure of Towbin (13) to nitrocellulose sheets for 2 h at 1.0-1.2 amps. Additional protein-binding sites on the nitrocellulose were blocked by incubating the blot in blocking buffer (1 mM tris (hydroxymethyl) aminomethane-hydrochloride, 0.2 mM EDTA, 5 mM NaCl, 0.1% NaI, 3% hemoglobin (Sigma), pH 7) for 1 h at 37°C. Next, the blot was incubated at 25°C for 6 h in blocking buffer plus 50 µg of purified rabbit anti-SEA immunoglobulin G (IgG) (rabbit anti-SEA sera was the generous gift of Anna Johnson-Winegar, Ft. Detrick, Frederick, MD). The blot was then washed (14) five times for 5 min. each time with 200 ml of 0.1 M tris (hydroxymethyl) aminomethane-hydrochloride, pH 7 containing 1% NaCl to remove the unbound antibody. After washing, the blot was incubated for 12-16 hours at 25°C in blocking buffer plus staphylococcal protein A (Sigma) labeled with ^{125}I . Protein A was iodinated by the chloramine T method of Krause and McCarty (10). The specific activity of the iodinated protein A was approximately 3×10^6 cpm/µg. Approximately 2×10^5 cpm/ml of labeled protein A was sufficient for a 15 x 12 cm blot. After probing with protein A the blot was again washed as already described. Autoradiograms of the blot were then prepared by exposure to Kodak X-OMAT AR X-ray film for 12-18 h.

To evaluate the amount of radioactivity in each band, the autoradiogram was used as a template and the SEA bands were cut from the blot. The bands were emulsified in a toluene-based scintillation cocktail and counted by liquid scintillation (15).

Labeling conditions. For all labeling experiments, *S. aureus* 13N2909 cells were grown overnight at 37°C and diluted 1:50 into the growth medium described. The culture was incubated with agitation until the optical density at 655 nm was 3.8-4.0 (at which point SEA production was maximal). Approximately 100 ml of culture were centrifuged at 25°C and then the cells were washed two times with defined medium 4 (containing 2.08% of a mixture of 18 amino acids) described by Wu and Bergdoll (16). The cell pellet was then cold shocked with 25 ml of ice cold hypotonic buffer (10 mM Na₂HPO₄, 20 mM MgSO₄, pH 6.5) (14) to deplete the amino acid pools. After recentrifugation, the cell pellet was resuspended in 25 ml of medium 4 without leucine, isoleucine, and valine and incubated at 37°C for 15 min. with vigorous shaking to deplete any remaining intracellular leucine, isoleucine, and valine. The cells were repelleted and resuspended in 1.0 ml of medium 4 without leucine, isoleucine, and valine and were equilibrated at 37°C. All three of these amino acids were omitted to increase labeling efficiency because they are all interconverted in the same biosynthetic pathway and they are relatively abundant in SEA (1). The cellular proteins were then pulse-labeled with L-[4,5-³H] leucine, (130-190 Ci/m mol; Amersham, Chicago, IL) at 37°C.

For pulse-chase experiments, the cells were pulse-labeled for 2 min. with a total of 500 µCi (0.5 ml) of [³H] leucine and then chased by diluting the resuspended cells 1:2 in 1.5 ml of medium 4 containing 30 mg leucine, 10 mg isoleucine, and 10 mg valine. After the addition of chase,

0.5 ml samples were withdrawn at various times. The samples were placed immediately in tubes containing 1 ml crushed ice and were held in an ice bath. The lower temperature stopped the incorporation of [^3H] leucine and the processing of pSEA to SEA.

For the inhibition of processing by carbonyl cyanide-m-chlorophenyl hydrazone (CCCP) and 2,4 dinitrophenol (DNP) the cells were prepared and pulse-labeled in the manner described above. The cells were then chased with an equal volume of medium 4 containing the inhibitor at 2x the final concentration. The final concentrations used for CCCP were 10 μM , 100 μM or 1000 μM and for DNP were 1 mM, 10 mM, 50 mM, 100 mM.

Immunoprecipitation of pSEA and SEA. Each sample (0.5 ml of cells in medium and 1.0 ml ice) was lysed by the addition of 50 μl of hypertonic buffer (10 mM NaH_2PO_4 , 20 mM MgSO_4 , 3.45 M NaCl, pH 6.5), 500 μg of lysostaphin and 500 μg of DNase I (Sigma). Lysis was continued for 12-16 h at 0°C . The lysate was then diluted 1:2 with solubilization buffer (0.1 M tris (hydroxymethyl) aminomethane-hydrochloride, pH 8, containing 0.5% deoxycholate, 2.0% Nonidet P40, and 1.5 M urea) (14) and the remaining insoluble debris was removed by centrifugation at 30,000 x g for 10 min. The supernatant was incubated with 25 μg purified rabbit anti-SEA IgG for 12-16 h at 2°C to complex the SEA and pSEA. The antigen-antibody complexes were then precipitated by adding 250 μl affinity-purified goat anti-rabbit IgG immobilized on nylon beads (AMF Biologicals Diagnostics, Seguin, TX). Incubation was then continued for an additional 12-16 hours at 2°C .

The immunoprecipitates were collected by centrifugation and washed once with 10 ml of solubilization buffer diluted 1:2 with distilled water. After recentrifugation the immunoprecipitates were washed with 10 ml borate-saline buffer (0.05 M boric acid, 0.2 M NaCl, pH 8.0). The solid

phase antibody was dissociated from the antigens by boiling in 1.0 ml of 1% SDS for 15 min. The nylon beads were removed by centrifugation and the supernatant containing the [3 H]-labeled pSEA and SEA was lyophilized.

Subcellular fractionation of cells. Subcellular fractionation was performed as outlined by Tweten and Iandolo (15). All of the procedures were carried out at 0-2°C unless otherwise stated. Extracellular proteins were separated from pulse-labeled cells by centrifugation at 30,000 x g for 20 min. The medium was saved, and the cells were washed in 2 ml hypotonic buffer (13) to remove any residual SEA. The cells were then repelleted and the supernatants from both centrifugations were combined. The extracellular SEA was then immunoprecipitated. After removal of the extracellular SEA, protoplasts were formed by resuspending the cell pellet in 1 ml of hypotonic buffer containing 50% sucrose, 1 mg lysostaphin, 50 μ l hypertonic buffer and 500 μ g of DNase I. Protoplast formation took approximately 4 h at 0°C.

The proteins removed by digestion of the cell wall were separated from the protoplasts by centrifugation through a 50% sucrose solution in an SW 50.1 rotor (Beckman Instruments, Palo Alto, Calif.) at 58,000 x g for 90 min. The proteins released from the cell wall were at the top of the tube. The sucrose present with the cell wall proteins was reduced by dialysis and the SEA was immunoprecipitated.

The protoplasts were resuspended in 1 ml of hypotonic buffer and sonicated. Next 500 μ g lysostaphin, 50 μ l hypertonic buffer and 500 μ g DNase I were added and the suspension was incubated overnight at 0°C. A two step (50%-75% w/v) sucrose gradient was used to separate soluble proteins from membrane proteins. After centrifugation at 58,000 x g for 2 h in a SW 50.1 rotor, the membrane fraction was collected at the interface of the 2 sucrose layers. The soluble proteins were located at the top of

the gradient and the unlysed cells and protoplasts pelleted through the sucrose.

The membrane fraction was collected and resuspended in solubilization buffer. Before removing insoluble material by centrifugation at 30,000 x g for 10 min., the membrane fraction was sonicated briefly and diluted 1:2 with distilled water. The solubilized membrane proteins were then immunoprecipitated.

The soluble protein fraction was collected and recentrifuged at 100,000 x g for 90 min. in a SW 50.1 rotor. The pellet was treated as described for the membrane fraction and immunoprecipitated. The supernatant was also immunoprecipitated.

SDS-PAGE. The lyophilized immunoprecipitated proteins were prepared for SDS-PAGE and electrophoresis was carried out as described for SEB (15). The gels were prepared for fluorography (11), dried, and exposed to Kodak X-OMAT AR X-ray film for 12-18 h at -70°C.

Peptide mapping. The [³H]-labeled bands of pSEA and SEA were located by using the autoradiogram of the gel as a template. The bands were then cut from the gel. To remove the residual 2,5-diphenyloxazole (PPO) used for gel fluorography, the excised bands were washed 3x in 10 ml of distilled water for 30 min. The rehydrated gel slices were then washed in 10 ml of 100% dimethylsulfoxide for 1 h and finally they were washed 2x in 10 ml of 25% isopropanol and 10% methanol in distilled water.

Each gel slice was suspended in 1 ml of 50 mM ammonium bicarbonate (pH 7.8) containing 0.05 mg/ml of trypsin (2x crystallized; Millipore Corp., Bedford, Mass.). After incubation at 37°C for 24 h, the solution containing the tryptic peptides was withdrawn and lyophilized. The tryptic peptides were then suspended in electrophoresis buffer (acetic acid-formic

acid-water, 3:1:16 [vol./vol.]. Approximately 100,000 cpm of each digest was spotted onto a 20 cm x 20 cm thin layer cellulose plate (Eastman Kodak chromatogram, no. 13255). Electrophoresis (from anode to cathode) was carried out at 50 V/cm until the tracking dye (1% pyronine Y) migrated 14 cm. The cellulose plate was then dried and chromatographed in the second dimension in pyridine-butanol-acetic acid-water, 25:32.5:5:20 (vol./vol.) until the solvent front migrated 14 cm. EN³HANCE Spray (New England Nuclear, Boston, Mass.) was sprayed on the cellulose plate and the plate was wrapped in cellophane and exposed to X-ray film at -70°C up to 2 weeks.

RESULTS

Identification of a precursor to SEA. In order to determine when SEA was maximally produced by S. aureus 13N2909, samples were taken periodically from a growing culture. The supernatants were analyzed for toxin by SDS-PAGE and electroblotting. The length of incubation time at which 13N2909 was maximally producing SEA was determined by calculating the difference in the amount of SEA produced between consecutive samples. The largest difference in radioactivity between samples was taken as the point where maximal production of SEA occurred. Maximal production of SEA determined by this method corresponded with an optical density of 3.8-4.0 (Fig. 1).

S. aureus 13N2909 was grown to this optical density and analyzed by a pulse-chase experiment to establish a precursor-product relationship for SEA. The rationale for this type of experiment was to preferentially label the precursor form of SEA by exposure to short pulses with [³H] leucine. By hypothesis, the precursor form would be of a larger molecular weight than the mature product. Thus, the precursor would be the slower migrating form on an SDS polyacrylamide gel. After increasing incubation time with the unlabeled amino acid (chase), stoichiometrically related amounts of

radioactivity should disappear from the precursor and appear in the mature product.

Whole cell lysates of 13N2909 from a pulse-chase experiment demonstrated a precursor-product relationship (Fig. 2). The larger molecular weight form of SEA was converted to the mature form within 1 min. The molecular weight of the SEA precursor (pSEA) was approximately 35,900 (Fig. 3), whereas that of SEA was 34,700. This corresponded to a molecular weight difference of about 1200 or a peptide extension of 10-12 amino acids.

Two dimensional tryptic peptide mapping was used to examine the relationship between pSEA and SEA by other than immunological methods. Extracellular SEA contained 16 distinct [³H]-labeled tryptic peptides (Fig. 4a). The precursor of extracellular SEA contained the 16 peptides found in the mature SEA map (Fig. 4b). This confirmed that these two proteins were related. A mixed peptide map that contained the peptides from both pSEA and SEA also showed the 16 common [³H]-labeled tryptic peptides (Fig. 4c).

Localization of pSEA and SEA. When the extracellular proteins from pulse-labeled S. aureus 13N2909 were removed and the cells lysed, a large amount of mature SEA fractionated as a soluble or cytoplasmic protein (Fig. 5A, lane 3). However, when protoplasts were formed first, a significant amount of mature SEA appeared in the cell wall fraction (Fig. 5B, lane 2). When the extracellular and cell wall associated SEA was examined by pulse-chase analysis we found that the levels of cell wall associated SEA decreased proportionately, as the extracellular SEA increased (Fig. 6). These results correlate with those obtained from similar studies with SEB (15).

A portion of the cell wall sequestered mature SEA was released when intact cells were shaken vigorously in a 1 M NaCl solution at 0°C for 1 h

(Fig. 7). This result is in contrast to those reported for SEB. Both high salt concentration and proteinase K had no effect on release of SEB from the cell wall (15).

The above results suggest that SEA, like SEB, is temporarily sequestered in the cell wall before release into the extracellular environment.

A significant amount of pSEA and SEA fractionated as soluble or cytoplasmic proteins (Fig. 5B, lane 3) when protoplasts were lysed. The same result was observed when whole cells were lysed, but in this case a portion of the mature SEA fractionating as soluble proteins should be considered as cell wall proteins. Nevertheless, both pSEA and SEA were present to some extent as soluble or cytoplasmic proteins. We thought that the pSEA may have been trapped in small membrane vesicles during the isolation procedure and therefore remained cytoplasmic. Therefore, we collected the top of the gradient where the soluble pSEA and SEA were located, and recentrifuged at $100,000 \times g$ to pellet any particulate material. We found that only a small amount of mature SEA pelleted (Fig. 5A, lane 4; Fig. 5B, lane 4). If cells and protoplasts were gently syringed instead of sonicated during the lysis procedure to avoid formation of small membrane vesicles, identical results were obtained (data not shown). Thus, it appears that the pSEA and SEA found in the cytoplasm were not associated with a particulate fraction in these preparations.

Inhibition of processing. Processing of some *E. coli* precursors can be inhibited by uncouplers of membrane potential. The conversion of pSEA to SEA was inhibited when the final concentration of CCCP was $10 \mu\text{M}$ and $100 \mu\text{M}$ (Fig. 8, lanes 3 and 4). In addition DNP at final concentrations of 1 mM , 10 mM and 50 mM also stopped processing of pSEA to SEA (Fig. 8, lanes 6, 7 and 8). In comparison with the uninhibited chase control (Fig. 8,

lane 2) the accumulation of pSEA in samples containing CCCP at 10 μ M and 100 μ M and DNP at 1 mM, 10 mM and 50 mM demonstrated that processing was inhibited. Higher concentrations of CCCP and DNP appeared to inhibit de novo protein synthesis as well as processing (Fig. 9, lanes 5 and 9). Some mature SEA was present in all of the samples because the inhibitor was added after a 2 min. pulse and some SEA was already formed by this time. Furthermore, when pulse-labeled cells were chased with approximately 50 mM DNP (without any unlabeled amino acids), processing was inhibited and synthesis of pSEA was steadily reduced (Fig. 9). On the other hand, processing of the SEB precursor (pSEB) to SEB was effectively inhibited and translation continued in the presence of 50 mM DNP.

DISCUSSION

We have demonstrated a kinetic precursor to extracellular SEA by in vivo pulse-chase analysis in S. aureus 13N2909. Within 5s of the pulse substantially more mature SEA was present than pSEA. This suggested to us that the additional sequence of pSEA must be removed very rapidly during transport. Currently, it is unknown whether this is the result of rapid posttranslational processing of the full length precursor or cotranslational processing of incompleated chains.

Two dimensional peptide mapping proved the homogeneity of pSEA and SEA since these two proteins shared 16 peptides. If SEA was derived from pSEA by cleavage of a signal sequence, we would expect the two proteins to have a large number of peptides in common. We also expected at least one additional peptide on the pSEA map to account for the difference in molecular weight. However, we did not observe any additional [³H]-labeled peptides for pSEA. Since the difference between pSEA and SEA is only 10-12 amino acids, it is possible that there are no leucine, isoleucine or valine

residues in the peptide extension of pSEA. Therefore, no [^3H]-labeled peptide(s) representing the peptide extension would be visible. The other possibility is that a distinctive pSEA peptide(s) had the same mobility as one of the 16 SEA peptides. Nevertheless, 16 major peptides are similar and indicate that pSEA and SEA are related.

SEA, like SEB, was found to be transiently associated with the cell wall. A similar phenomenon has also been observed with staphylococcal alpha toxin (Tweten, Christianson and Iandolo, ms. in preparation) suggesting that this occurrence may be involved in the general transport process for extracellular proteins of S. aureus. It may be that SEA, SEB, and alpha toxin are sequestered in the cell wall as part of a special mechanism to facilitate their transport through the thick, highly crosslinked cell wall of S. aureus (4). Previous studies with L-forms derived from SEA⁺ cells did produce SEA, but L-forms derived from SEB⁺ cells did not produce SEB (6). Therefore, a careful examination must be made of mutants which are defective in various aspects of cell wall synthesis before the significance of the cell wall in the overall transport of SEA, SEB, and alpha toxin can be determined.

Part of the cell wall associated SEA was released by 1 M NaCl indicating that it might be retained by ionic interactions. The cell wall fraction of SEB was not removed by 1 M NaCl suggesting that it is not retained by ionic interactions (15). Also, the cell wall associated SEA was not permanently associated with the cell wall since we showed that during a pulse-chase experiment, the cell wall associated SEA was released into the extracellular environment. A similar experiment with SEB gave the same results (15).

Why some of the precursor fractionated as a soluble or cytoplasmic protein is currently unknown. The pSEA may fractionate as a soluble or

cytoplasmic protein because it was loosely associated with the inside of the cell membrane as a first step in secretion. Such a situation could entail nonspecific binding of the signal sequence to the membrane which would be followed by lateral movement and subsequent recognition by the secretion site. The nonspecifically bound precursor proteins may then be loosely attached to the membrane and become detached during the lysis procedure.

The mature SEA that fractionated as soluble or cytoplasmic proteins could be cell wall associated SEA that was released when partially protoplasted cells were lysed. Another possibility for observing pSEA and SEA in the cytoplasm is that the full length precursor is translated and processed in the cytoplasm.

Low concentrations of DNP and CCCP inhibited the conversion of pSEA to SEA and increasing concentrations of these inhibitors appeared to stop both processing and synthesis of pSEA. These same energy uncouplers have been shown to inhibit processing of SEB (15) and several E. coli precursors (7, 8). The results suggested a role for membrane potential in the secretion of proteins. However, caution must be exerted in concluding that inhibition of processing by uncouplers implies that an energized membrane is required for processing because these compounds perturb membranes and have more than one effect (8). Also it is not clear which step in export is the energy consuming step.

The evidence presented here indicates that SEA is initially synthesized as a precursor. Processing occurs at some stage to give mature SEA. The mature SEA is temporarily associated with the cell wall before release into the extracellular environment.

FIGURE LEGENDS

Figure 1. Determination of maximal SEA production by S. aureus 13N2909. Samples (1 ml) were taken from a S. aureus 13N2909 culture every hour for 10 h and the absorbance at 655 nm was determined. The cells were removed by centrifugation and the spent medium was prepared for SDS-PAGE. Electrophoresis was carried out and then the proteins were electroblotted from the gel to nitrocellulose paper. The blot was blocked with hemoglobin, probed with anti-SEA IgG and ^{125}I protein A. The autoradiogram of the blot was used as a template to excise the SEA bands. Each band was then counted by liquid scintillation. \circ absorbance at 655 nm; Δ calculated difference in the amount of SEA produced between consecutive samples.

Figure 2. Pulse-chase analysis of pSEA. S. aureus 13N2909 cells (1.5 ml) were pulsed with 500 μCi of [^3H] leucine at 37°C. After 2 min., the suspension was diluted 1:2 with 30 mg leucine, 10 ml isoleucine, and 10 mg valine for chase. Samples (0.5 ml) were withdrawn from the cell suspension at various times after the addition of the three unlabeled amino acids and processed as described in the text. Total pSEA and SEA were immunoprecipitated from each sample and analyzed by SDS-PAGE. Shown is an autoradiogram of the gel depicting the [^3H] leucine-labeled pSEA (p) and mature SEA (m) found in samples taken at 5, 30, 60, 120, 240, 480 s post-chase (lanes 1-6, respectively).

Figure 3. Molecular weight plot of pSEA and SEA. From left to right, the circles indicate the following markers: bovine serum albumin (66,000 dal), ovalbumin (45,000), trypsinogen (24,000), beta-lactoglobulin (18,400), and lysozyme (14,300). \circ , pSEA; Δ , SEA.

Figure 4. Two dimensional peptide maps of pSEA and SEA. [^3H] leucine-labeled pSEA and SEA were digested with trypsin and the tryptic peptides were separated by thin layer electrophoresis followed by chromatography. Autoradiograms are shown of [^3H] leucine-labeled tryptic peptides from: A, extracellular SEA; B, pSEA; C, mixture of peptides from both SEA and pSEA. Peptides common to both proteins are numbered 1-16. Electrophoresis was from left to right (anode to cathode) and chromatography was from bottom to top.

Figure 5. Localization of pSEA and SEA. Pulse-labeled (2 min.) cells were fractionated into their subcellular components, and the pSEA and mature SEA were evaluated as described in the text. A, fractionation of intact cells. Lanes: 1, extracellular SEA; 2, control showing no SEA released from intact cells after removal of the extracellular SEA (compare with B, lane 2, SEA released after digestion of the cell wall with lysostaphin); 3, pSEA and SEA released by lysis of the intact cells; 4, SEA pelleted from the lysate of intact cells; 5, membrane-associated pSEA and SEA. B, fractionation of protoplasts. Lanes: 1, extracellular SEA; 3, pSEA and SEA released by lysis of the protoplasts; 4, SEA pelleted from the lysate of the protoplasts; 5, membrane-associated pSEA and SEA. The positions of pSEA (p) and SEA (m) are indicated.

Figure 6. Localization of pSEA and SEA during a pulse-chase experiment. The samples taken during a pulse-chase experiment were separated into extracellular and cell wall fractions. The SEA was immunoprecipitated and analyzed by SDS-PAGE. Symbols: \circ , extracellular SEA, Δ , cell-associated SEA released by removal of the cell wall, \bullet , total radioactivity in both fractions.

Figure 7. Partial release of cell-associated SEA in the presence of 1M NaCl. Duplicate samples of pulse-labeled cells were fractionated into their subcellular components. Lanes: 1 and 5, extracellular SEA; 2, SEA released from cells by incubation in hypotonic buffer for 1 h at 0°C; 6, same as 2, except 1M NaCl was included; 3 and 7, SEA released by lysing cells washed with hypotonic buffer (lane 3) or 1M NaCl (lane 7); 4 and 8, membrane-associated SEA from cells washed with hypotonic buffer (lane 4) or 1M NaCl (lane 8). The positions of pSEA (p) and SEA (m) are indicated.

Figure 8. Precursor processing in the presence of CCCP and DNP. Cells were pulsed with [^3H] leucine for 2 min. and then chased with 30 mg leucine, 10 mg isoleucine and 10 mg valine in 3 ml of reaction mixture for an additional 10 min. in the presence of the inhibitors. The total pSEA and SEA were then immunoprecipitated and analyzed by SDS-PAGE. Lanes: 1, products labeled after a 2 min. pulse; 2, products labeled after a 10 min. chase with leucine, isoleucine and valine; 3-9, cells chased with leucine, isoleucine and valine plus inhibitor; 3, 10 μM CCCP; 4, 100 μM CCCP; 5, 1000 μM CCCP; 6, 1 mM DNP; 7, 10 mM DNP; 8, 50 mM DNP; 9, 100 mM DNP. The positions of pSEA (p) and mature SEA (m) are shown.

Figure 9. Inhibition of processing and synthesis of pSEA by DNP. Cells were pulsed for 2 min. with 500 μCi [^3H] leucine and then chased with 50 mM DNP. An autoradiogram is shown of mature SEA (m) and its precursor (pSEA) immunoprecipitated from samples taken at 60, 120, 240, 480, 980 s (lanes 1-5, respectively) after the addition of DNP.

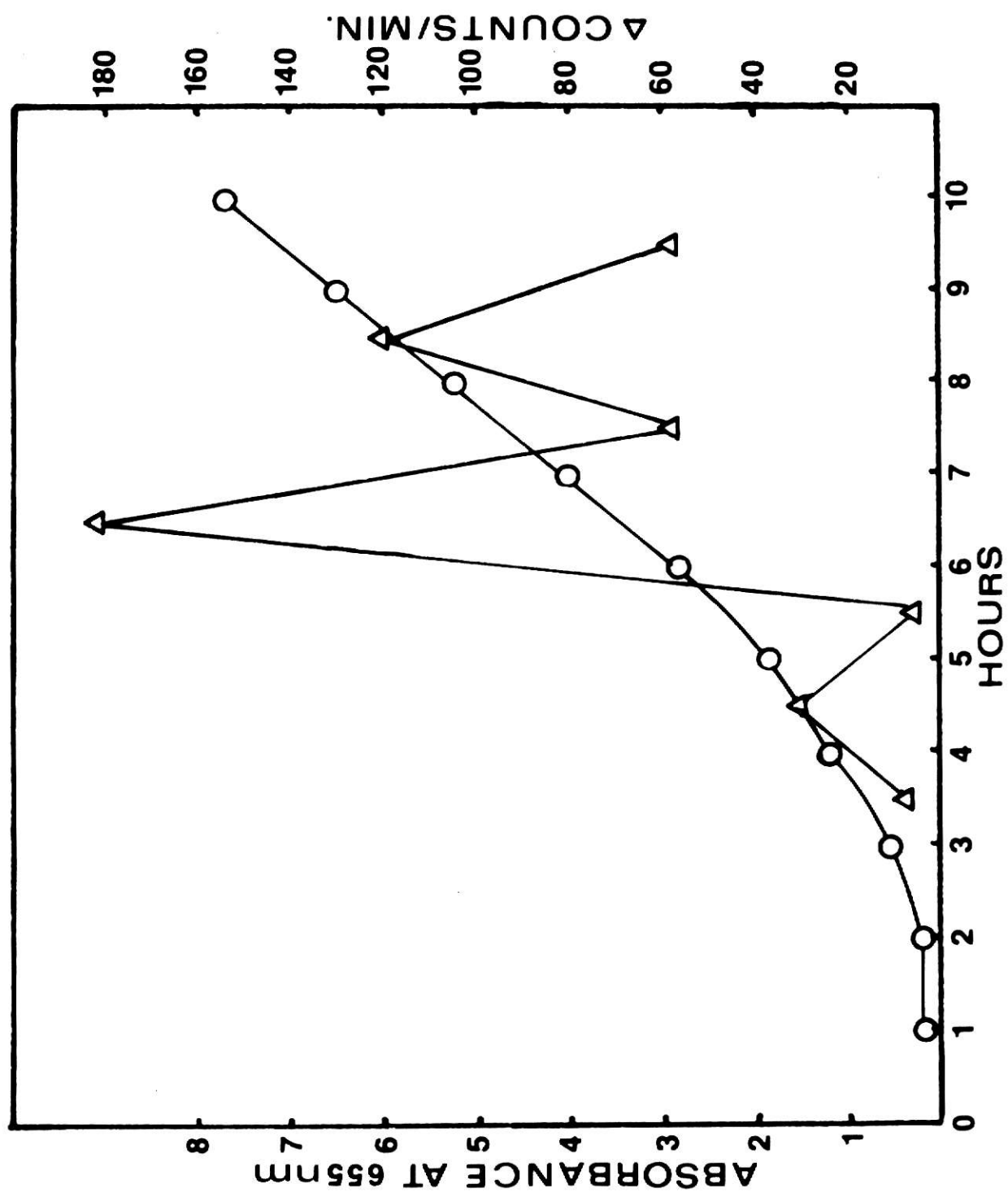
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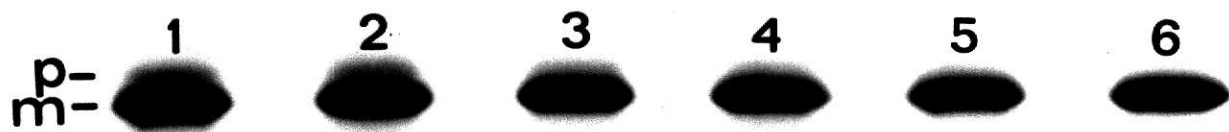
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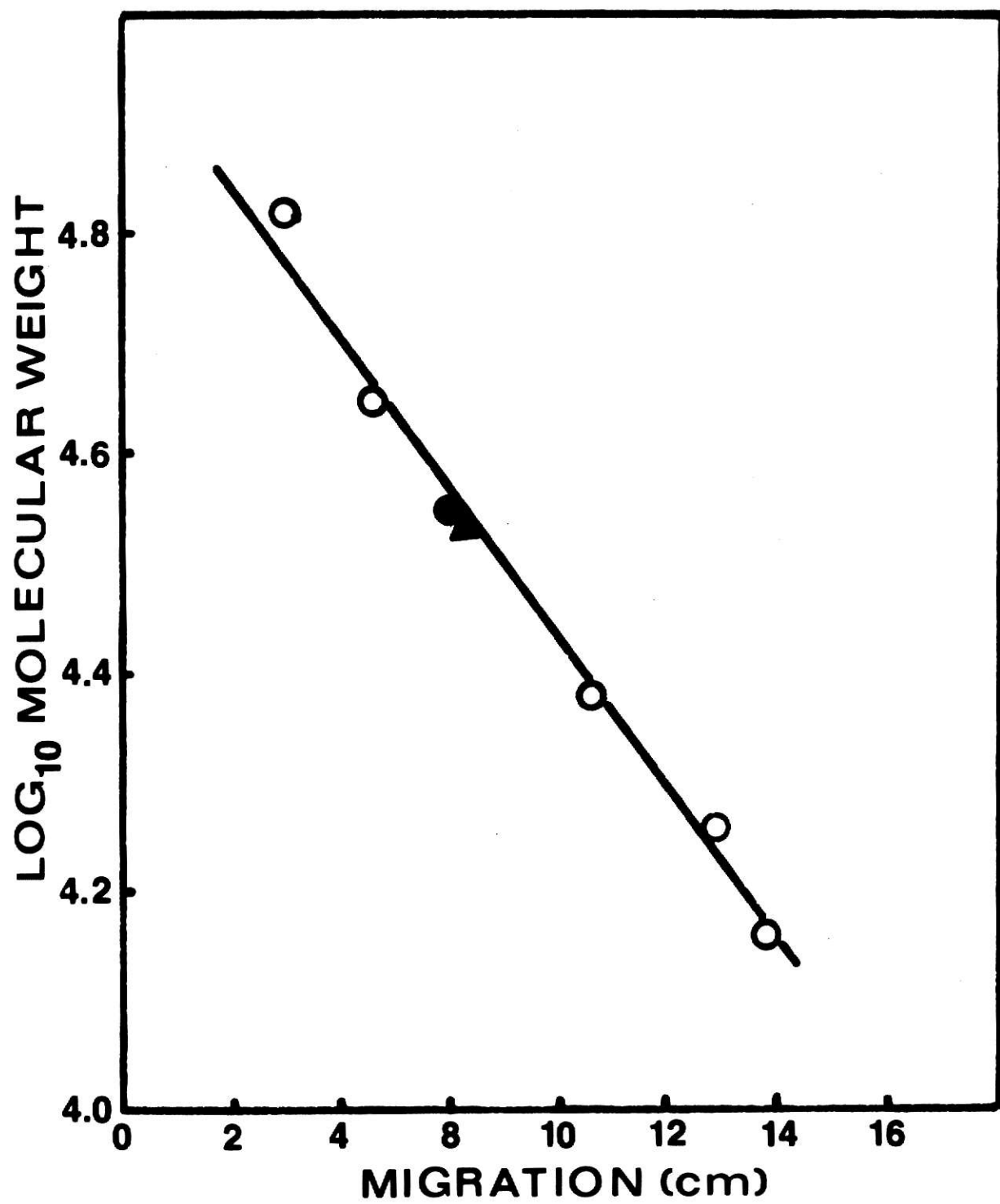
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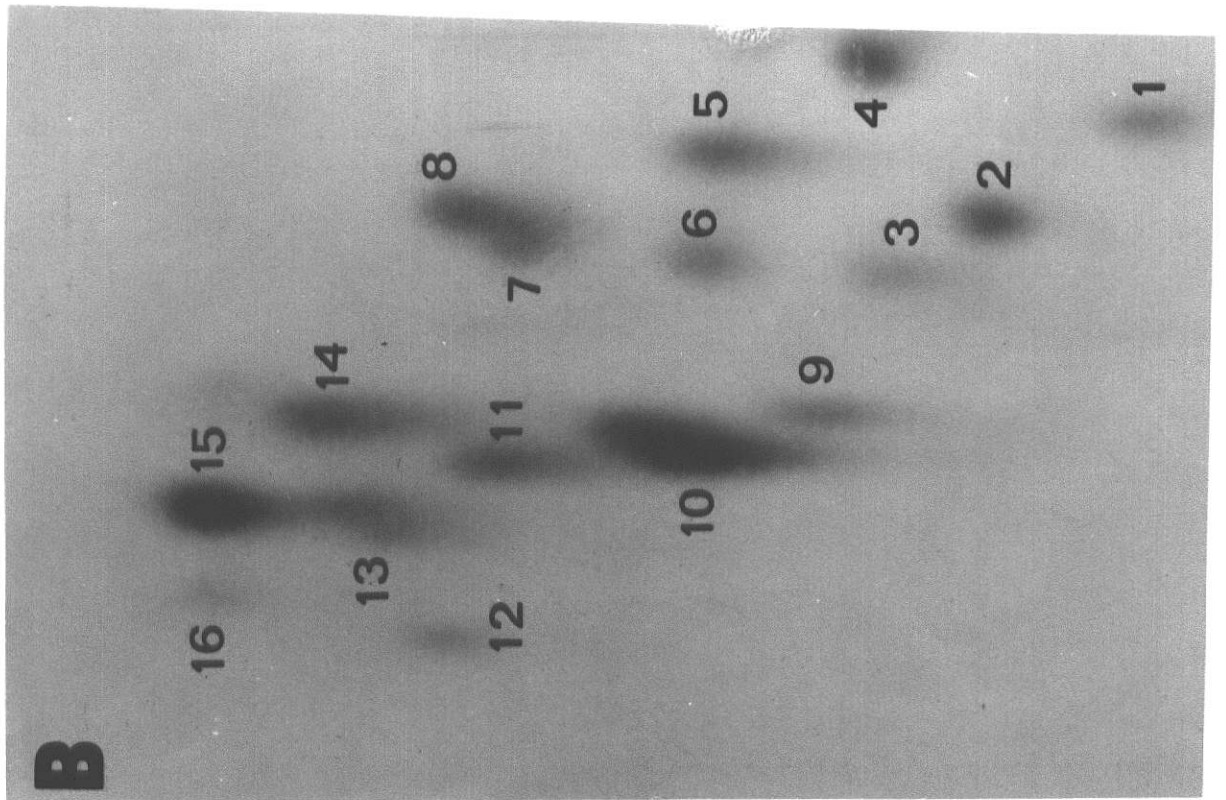
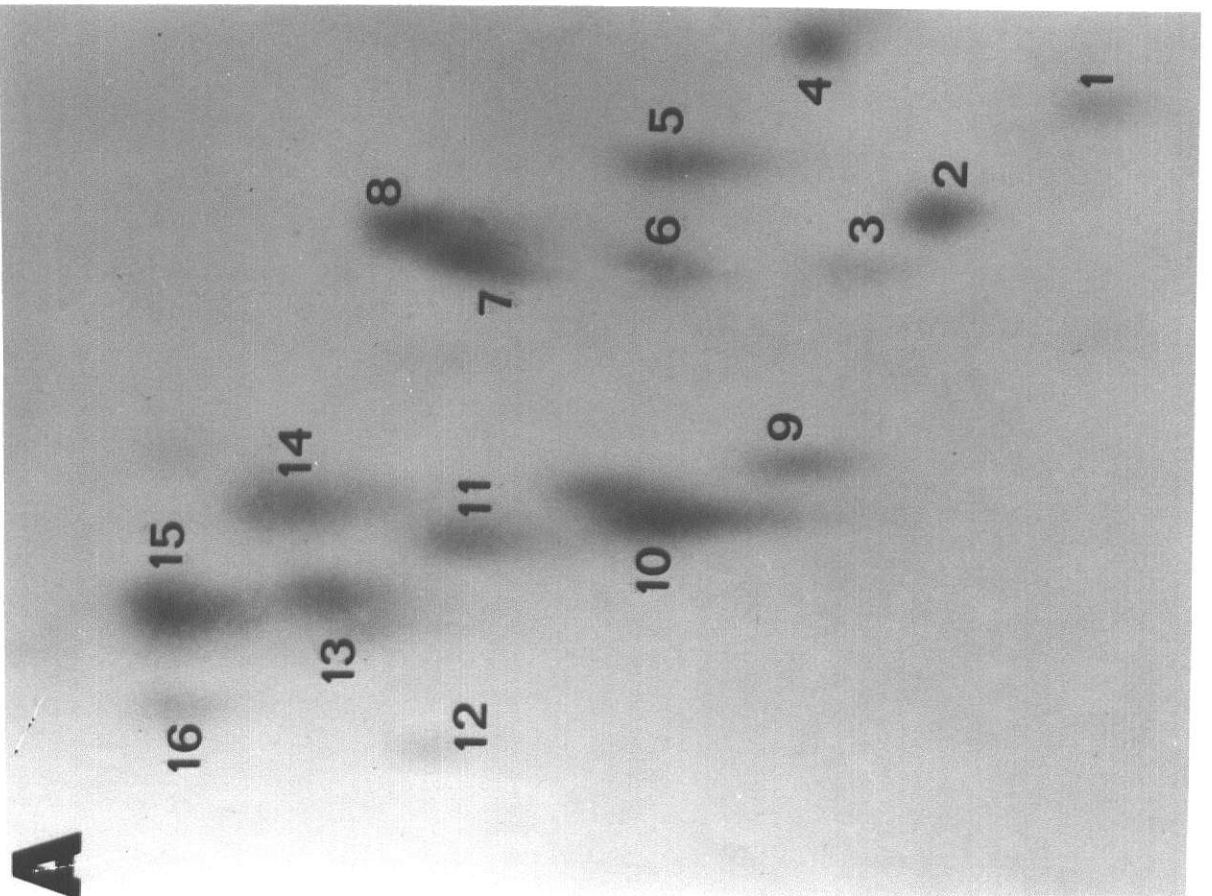
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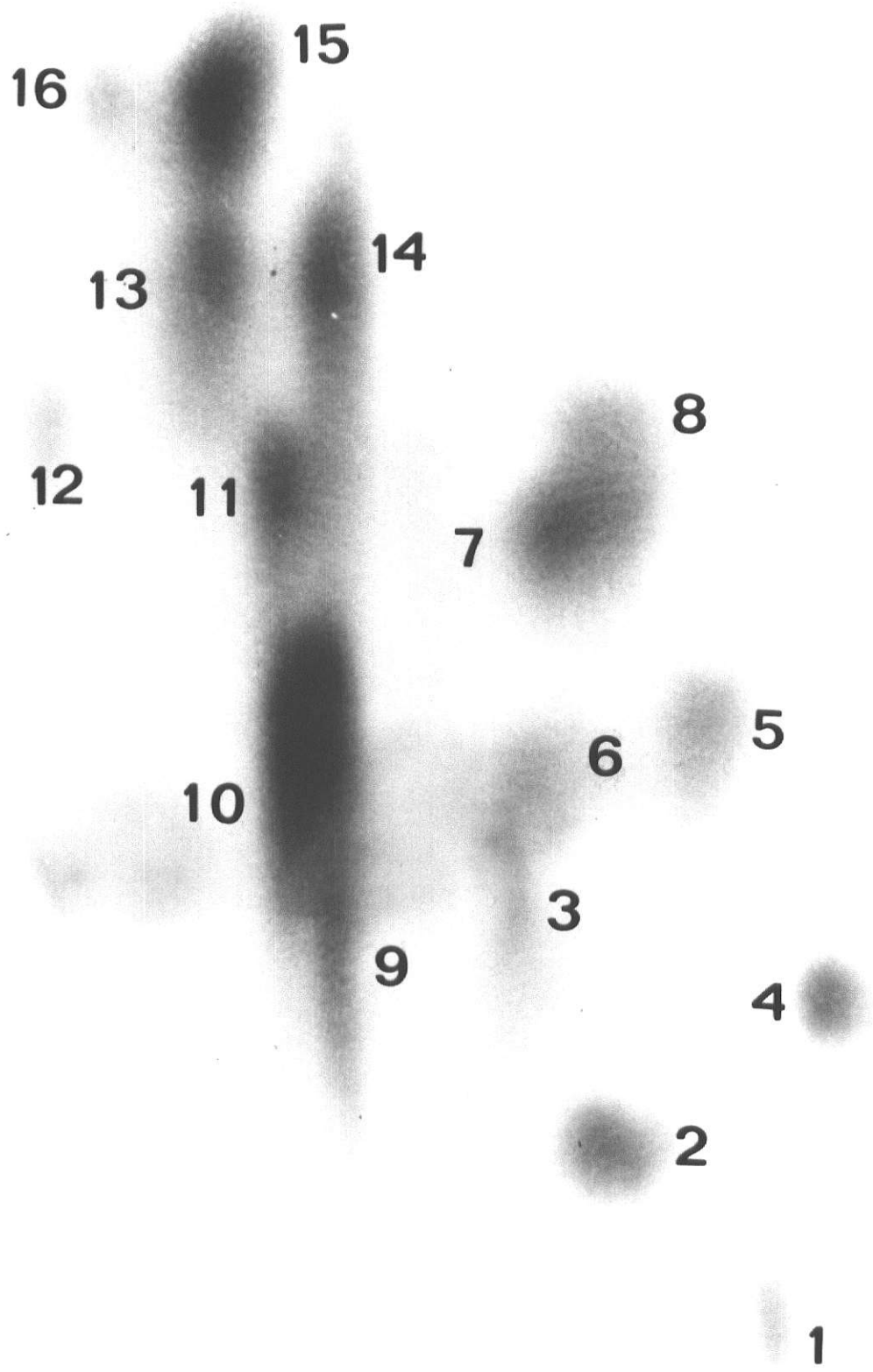
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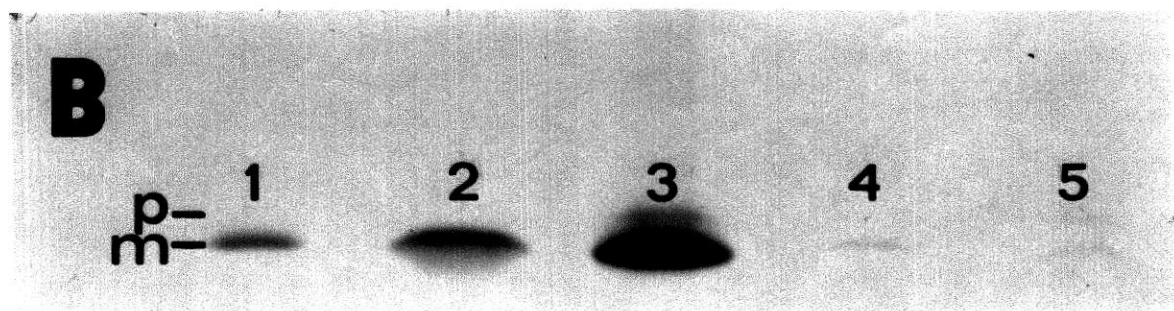
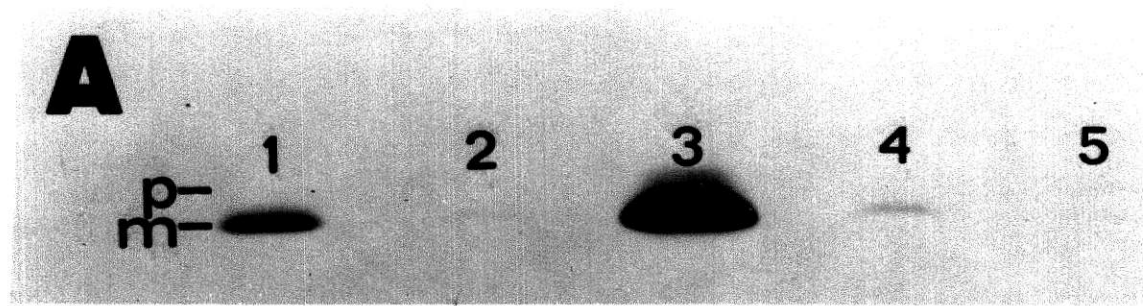


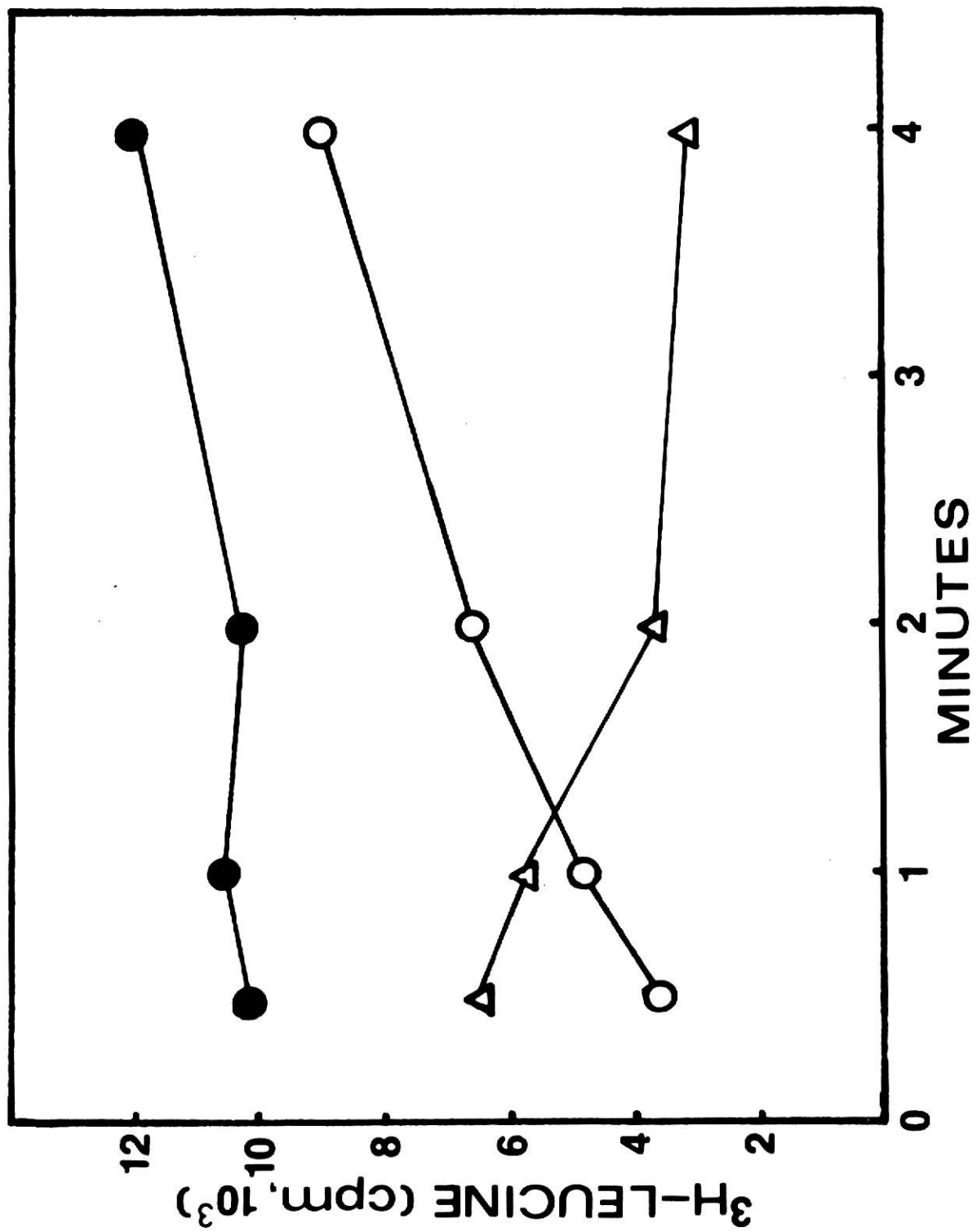


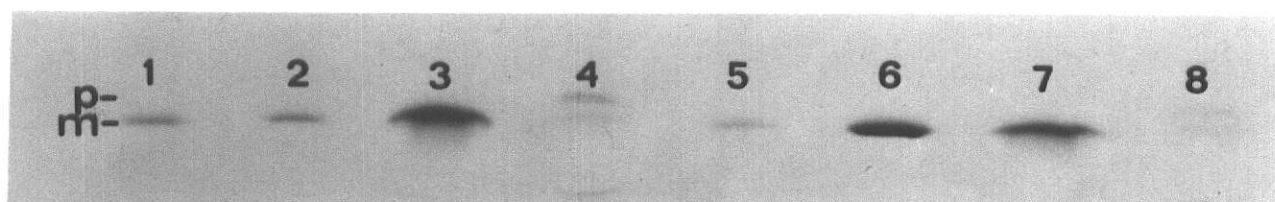


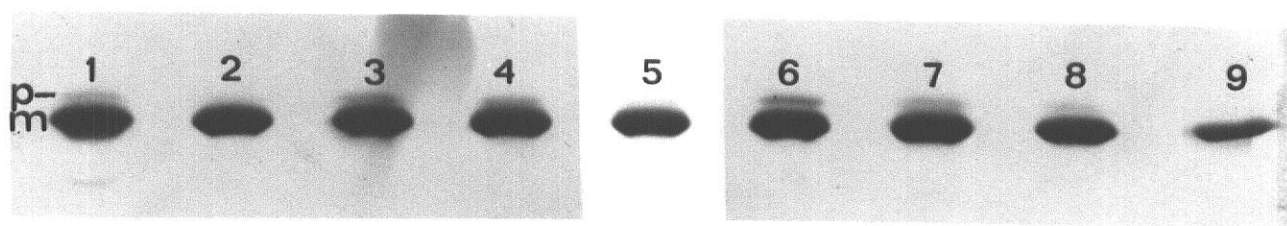


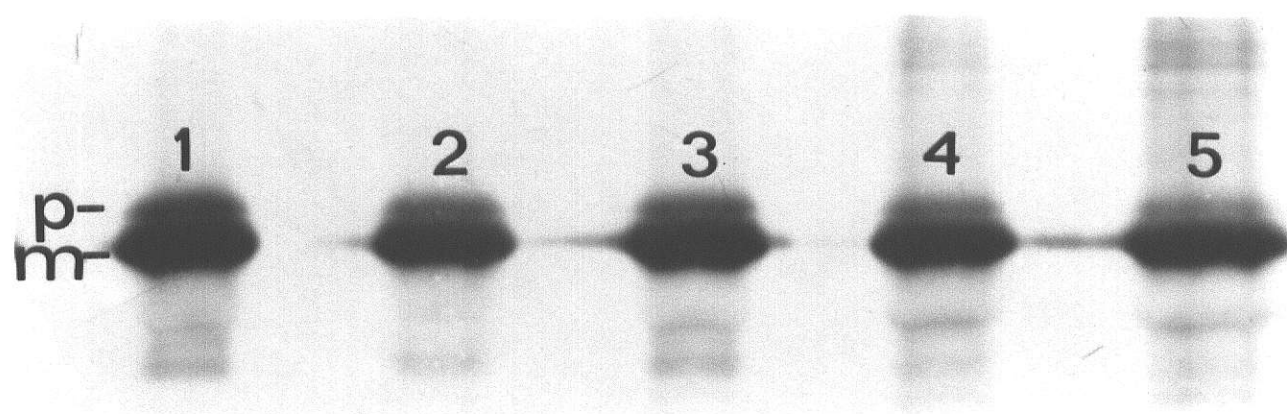
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FINAL DISCUSSION

Until recently, little direct evidence existed for precursors to any of the extracellular proteins in S. aureus. In the past 2 years, Tweten and Iandolo (79, 80) have identified a kinetic and structural precursor to staphylococcal enterotoxin B (SEB). In addition, two precursors to staphylococcal alpha toxin have also been detected (Tweten, Christianson, and Iandolo, ms. in preparation). In the preceding manuscript, the transport and processing of staphylococcal enterotoxin A (SEA) was examined.

The method that we first used to detect a larger molecular weight precursor to SEA was by the electroblot method of Towbin (77). When membrane proteins from SEA producing S. aureus strains such as S6, S6R, FRI 100, and 742 were separated on a sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and electroblotted to nitrocellulose paper, no larger molecular weight precursor could be detected. These SEA⁺ cells produce SEA at very low levels, approximately 1-5 µg/ml (20). Therefore, if a precursor to SEA existed on the membranes, it was at a very low concentration and thus was not detected by the electroblot procedure.

To establish a precursor-product relationship for SEA, we next tried a pulse-chase analysis. The rationale for this type of experiment was to preferentially label the precursor for SEA by exposure to short pulses with a radioactive amino acid. By hypothesis, the precursor form would be of a larger molecular weight than the mature product. Thus, the precursor would be the slower migrating form on a SDS-PAGE. After increasing incubation time with the unlabeled amino acid (chase) stoichiometrically related amounts of radioactivity should disappear from the precursor and appear in the mature product.

Using the above strategy, we performed a pulse-chase experiment with S. aureus strain S6 by using [³⁵S] methionine to pulse label and 50 mg of

unlabeled methionine to chase. Since SEA only contains 3 methionine residues, we did not obtain adequate labeling of SEA with [^{35}S] methionine. Therefore a pulse-chase analysis with S6 was tried using [^3H] leucine to pulse and 30 mg unlabeled leucine, 10 mg unlabeled isoleucine, and 10 mg unlabeled valine to chase. The cells were pulsed with [^3H] leucine in the absence of leucine, isoleucine, and valine and then were chased with these same amino acids. This was done to increase labeling efficiency because these amino acids are all interconverted in the same biosynthetic pathway and are relatively abundant in SEA (7). The results of the pulse-chase analysis did not demonstrate a precursor. Instead the results showed us that S6 produced many proteins which were cross reactive with our anti-SEA immunoglobulin G (IgG).

We next obtained a culture of a high SEA⁺ producing strain, S. aureus 13N2909. This organism is a mutant of S. aureus 100 that produces approximately 57 $\mu\text{g/ml}$ SEA and very few (if any) other extracellular proteins (34). When membranes from 13N2909 were analyzed by the electroblot procedure (77), no larger molecular weight form of SEA was detected. The reasons for this result will be explained in greater detail later. However, when 13N2909 cells were used in a pulse-chase analysis, a larger molecular weight precursor was detected and was chased into the mature form of SEA. In all pulse-chase analyses with 13N2909, the cells were pulsed with [^3H] leucine and chased with 30 mg unlabeled leucine, 10 mg unlabeled isoleucine, and 10 mg unlabeled valine for the reasons discussed above.

In order to confirm that the larger molecular weight precursor to SEA (pSEA) and SEA were structurally related, two dimensional tryptic peptide mapping was done. Peptide maps of pSEA and SEA demonstrated that the two proteins were related because the maps were identical. Both the map of pSEA and SEA showed 16 distinct [^3H] leucine peptides. Molecular weight

determination by SDS-PAGE revealed that the molecular weight of pSEA was 35,900 and that of SEA was 34,700. This corresponded to a difference in molecular weight of approximately 1200 or a peptide extension of 10-12 amino acids. The small difference in molecular weight of pSEA and SEA may help explain why the peptide maps were identical. Since the difference between the two proteins is 10-12 amino acids, it is conceivable that there is no leucine, isoleucine, or valine present in the peptide extension of pSEA. The other possibility is that the additional pSEA peptide(s) had the same mobility as one of the 16 SEA peptides. Nevertheless, 16 major [³H] leucine peptides are similar and indicate that pSEA and SEA are related.

To examine the subcellular location pSEA and SEA, pulse-labeled cells were fractionated into extracellular, cell wall, membrane, and cytoplasmic fractions. When this was done, a large amount of SEA was found associated with the cell wall. Washing the cells at 0°C with a 1M NaCl solution removed a part of the SEA from the cell suggesting that the SEA may be held by an ionic interaction. During a pulse-chase experiment, the levels of cell wall associated SEA decreased concomitantly as extracellular levels increased. Thus, the cell wall associated SEA was only transiently sequestered within the cell wall. It is possible that SEA is sequestered in the cell wall in specific regions as part of a special mechanism to facilitate its transfer through the thick, highly crosslinked cell wall of S. aureus (15). However, other investigators showed that L-forms derived from SEA producing cells did produce SEA (22). Therefore, it will be necessary to obtain mutants which are defective in the various aspects of cell wall synthesis in order to elucidate the significance of the cell wall associated SEA in the overall transport of SEA.

The subcellular fractionation procedure also revealed that there were approximately equal amounts of pSEA and SEA associated with the membrane. The hydrophobic signal sequence of pSEA would promote membrane association. Although the additional sequence on SEA has not been confirmed as a signal sequence, it is a reasonable assumption since all prokaryotic precursors sequenced have a hydrophobic signal sequence. The mature SEA which is associated with the membrane may be SEA that is locked into the membrane when the transport process is interrupted, after cell disruption. However, this membrane associated SEA may be nonspecifically associated with the membrane.

A substantial portion of pSEA and SEA fractionated as soluble or cytoplasmic proteins. The presence of mature SEA in the cytoplasmic fraction may be cell wall associated SEA which is released when partially protoplasted cells are lysed. The pSEA may fractionate as soluble or cytoplasmic protein because it was loosely associated with the inside of the cell membrane. Such a situation would entail nonspecific binding of the signal sequence to the membrane which would be followed by lateral movement and subsequent recognition by the secretion site. The nonspecifically bound precursor proteins may be loosely attached to the membrane and become detached during the lysis procedure. The other possibility for observing pSEA and SEA in the cytoplasm is that the full length precursor is translated and processed in the cytoplasm. To date no other precursor protein, that I am aware of, has been identified that is translated and processed in the cytoplasm.

The inability to detect a precursor from the membrane fraction of 13N2909 by the electroblot method (77) may be explained by the fractionation data discussed. Most of the pulse-labeled pSEA was detected in the cytoplasm, not in the membrane fraction. Since only membrane fractions

were analyzed by the electroblot method (77), the small amount of pSEA present probably was not detected.

Precursor processing of several E. coli proteins (26, 24, 31) was sensitive to uncouplers of the proton motive force but not sensitive to inhibitors of ATP synthesis which do not affect the proton motive force (84). These results suggested that an energized membrane was required for processing. However, recent in vitro evidence demonstrated that only E. coli leader peptidase and phospholipid vesicles were required for processing of M13 procoat (84). These results may not apply to secreted proteins since M13 procoat is an integral membrane protein. Nevertheless, uncouplers of the proton motive force such as carbonyl-cyanide-m-chlorophenyl-hydrazone (CCCP) and 2,4 dinitrophenol (DNP) at concentrations of about 100 μ M CCCP and lower and about 50 mM DNP and lower inhibited the conversion of pSEA to SEA. When a final concentration of approximately 50 mM DNP was used to inhibit pulse-labeled cells, conversion of pSEA to SEA was stopped and also synthesis of pSEA was inhibited. It was evident that synthesis of pSEA was affected since pSEA did not accumulate with time even though processing was stopped. However, these results do not confirm that an energized membrane was required for processing since CCCP and DNP have pleomorphic effects on membranes.

Early indirect evidence suggested that SEA was transported by a lipoprotein intermediate (8). To examine the possibility that SEA was a lipoprotein, 13N2909 cells were pulsed with [14 C]-palmitate. When lipoproteins of S. aureus were labeled with [3 H]-palmitate, the isotope was incorporated into the plamitate containing lipid of membrane-bound penicillinase (57). Thus, if [14 C]-palmitate was incorporated into pSEA, it would suggest that pSEA was derivatized with a lipid. However, no label

was incorporated into pSEA suggesting that if pSEA did contain a lipid, it was different from the penicillinase lipid.

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TRANSPORT AND PROCESSING OF STAPHYLOCOCCAL ENTEROTOXIN A

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ABSTRACT

The transmembrane transport of staphylococcal enterotoxin A (SEA) was investigated. A larger molecular weight form of SEA was shown to be a kinetic precursor to extracellular SEA by in vivo pulse-chase analysis. The conversion of this precursor SEA (pSEA) was achieved by proteolytic shortening or processing of pSEA during transport. The molecular weight of pSEA was determined to be 35,900 and that of SEA to be 34,700. Two dimensional tryptic peptide maps of the [³H]-labeled precursor and enterotoxin A were identical, thus confirming that the two proteins were also structurally related. Low concentrations of energy uncouplers, such as 2,4 dinitrophenol and carbonyl-cyanide-m-chlorophenyl hydrazone inhibited processing of the precursor toxin, and as the concentration of these two uncouplers was increased, synthesis of pSEA was also inhibited. Subcellular fractionation revealed that mature SEA was temporarily associated with the cell wall before being released into the extracellular environment. A part of the cell associated toxin was removed with a high salt solution at 0-2°C suggesting that the mature protein may be retained by the cell via an ionic interaction.