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Precursor in Cotranslational Secretion of Diphtheria Toxin

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By extracellular labeling of peptides of intact *Corynebacterium diphtheriae*, followed by fractionation of the cells and chain completion by isolated polysomes, it is shown that diphtheria toxin is formed and secreted cotranslationally by membrane-bound polysomes; free polysomes form none. Moreover, when the chains on these polysomes were completed in vitro, in the absence of membrane they were found to include not only diphtheria toxin of a molecular weight of 62,000, but also a larger precursor of a molecular weight of 68,000. The precursor was identified by several properties: immune precipitation; conversion into toxin fragments A and B; adenosine diphosphate ribosyl-transferase activity after activation with trypsin; and cleavage to 62,000 daltons by membrane enzymes. The precursor yields an N-terminal A fragment with a broadened molecular weight distribution, compared with that from authentic toxin, thus supporting the expectation that the extra segment on the precursor is N-terminal.

Corynebacterium diphtheriae appears to secrete diphtheria toxin across the cytoplasmic membrane because cultures can produce large amounts of extracellular toxin without cell lysis. In a preliminary report, Uchida and Yoneda (21) presented evidence that in cell lysates diphtheria toxin is synthesized by the membrane fraction. This finding is consistent with the later demonstration that various secreted proteins are synthesized on membrane-bound polysomes in procaryotes (16, 19), just as in eucaryotes (13). Moreover, many, but not all, secreted proteins are synthesized as a larger precursor with an additional N-terminal leader sequence (9, 22), containing predominately hydrophobic residues (3, 17). The postulated cotranslational secretion of these proteins (2, 9) has been recently established in this laboratory. In *Escherichia coli* spheroplasts (19) or *Bacillus subtilis* protoplasts (18), nascent chains of secretory proteins, still attached to membrane-bound ribosomes, could be labeled extracellularly by reagents that cannot penetrate the cell.

In this communication we use this labeling procedure to show that diphtheria toxin is synthesized on membrane-bound ribosomes, secreted cotranslationally, and initially synthesized as a protoxin with an additional segment in the N-terminal region. Membrane fractions from *C. diphtheriae* or from *E. coli* can process the precursor, even after completion, to the mature form of the toxin.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. The high-yielding toxigenic PW8 strain and the nonlysogenic, nontoxigenic C7(−) strain of *C. diphtheriae* have been previously described (1, 14). *E. coli* 27257 and MRE600 have been previously described (19).

C. diphtheriae was grown in partially deferrated C-Y medium at 34°C with vigorous aeration as previously described (10). For the production of diphtheria toxin, strain PW8 was grown overnight, diluted with fresh C-Y medium to an optical density at 590 nm (OD_{590}) of 2, and incubated until an OD_{590} of 4 was reached. Bacteria were harvested by centrifugation and suspended to an OD_{590} of 8 to 10 in C-Y medium treated with Chelex 100 to further reduce the Fe^{2+} content (10). Samples (4% flask volume) were transferred to Erlenmeyer flasks and shaken at 240 rpm at 34°C. Typically, the production of toxin at maximal rate began within 60 to 90 min. Chloramphenicol (200 μg/ml) was added at 90 to 120 min, the culture was poured over ice, and the cells were quickly harvested by centrifugation.

Extracellular labeling and cell lysis. Bacteria were suspended in 1.0 ml of 10 mM sodium phosphate buffer (pH 7.5) with chloramphenicol, incubated at 0°C for 15 min with 6 μM [^{125}I]diazoiodosulfanilic acid (DSA), washed three times with buffer A [10 mM Tris-hydrochloride (pH 7.6), 50 mM KCl, 10 mM $Mg(C_2H_3O_2)_2$], and resuspended in 10 ml of the buffer with 1 mM dithiothreitol (DTT). Cells were lysed in a French press at 20,000 lb/in^2. Electrophoretically pure DNase (5 μg/ml) was added, and unbroken cells and debris were removed by centrifugation at 3,000 × *g* for 10 min.

Preparation of fractions. Membrane-associated and free polysomes were prepared by chromatography on Sepharose 2B (which gave better separation than the discontinuous sucrose density gradient method

used for *E. coli* lysates [19]). Derived polysomes were prepared by washing the membrane-polysome fraction three times with 1% sodium deoxycholate (DOC) in buffer A, a procedure that effectively removes membrane from polysomes of other bacteria (19). Inner and outer membrane fractions from *E. coli* 27257, and cytoplasmic membrane from *C. diphtheriae*, were prepared by the method of Osborn and Munson (12).

S-100 and S-30 extracts were prepared from *E. coli* MRE600 as previously described (20). Total RNA was prepared by phenol extraction (20) from *C. diphtheriae* PW8 grown and lysed as described above.

In vitro protein synthesis and gel electrophoresis. The polysomes were allowed to finish the nascent chains in an in vitro protein-synthesizing system composed of an *E. coli* S-100 fraction as described previously (20). After incubation at 37°C for 30 min, the ribosomes were removed by centrifugation and the supernatant was analyzed for molecular weight distribution by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as described previously (19). To identify the toxin, we mixed the supernatant (0.1 ml) with 0.05 ml of equine antitoxin serum and incubated it at 37°C for 1 h, added 22 μg of purified diphtheria toxin as a carrier, and further incubated the mixture at 37°C for 1 h. The immune precipitate was processed and analyzed as described previously (19).

Determination of NAD-elongation factor 2 (EF-2) ADP ribosyl-transferase (ADPR-transferase) activity. To make unlabeled diphtheria *tox* gene products in vitro in sufficient quantity for enzymatic studies, we incubated total RNA from the PW8 strain of *C. diphtheriae*, prepared as described above, at 37°C for 1 h in an S-30 protein-synthesizing system from *E. coli* (20). Reaction mixtures (in 0.2-ml portions to obtain maximum activity) contained 20 μCi of [^{3}H]leucine (60 Ci/mmol), an optimal amount of total RNA (8 μg), and other necessary components. The reaction mixtures were pooled (2.0 ml), dialyzed against 10 mM ammonium acetate buffer (pH 7.8), lyophilized, and dissolved in 0.1 ml of sample application buffer (7). For a control, purified diphtheria toxin (4.5 mg/ml) (Connaught Laboratories, Toronto, Canada) was dialyzed against sodium phosphate buffer, pH 7.5. Samples of 0.05 ml of the reaction mixture, or 0.005 ml of the control toxin, were mixed with 0.025 ml of sample application buffer and electrophoresed as described above. Gels were sliced into 2-mm disks, which were transferred separately into microtiter wells (TS-FB-96; Linbro, Hamden, Conn.) and eluted by incubation overnight with 0.2 ml of 10 mM Tris-hydrochloride (pH 8) buffer with 5 mM DTT.

Diphtheria toxin and related polypeptides in the eluates were activated by treatment with 1 μg of trypsin at 37°C for 10 min. Soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, Mo.) was added (20 μg), and the extracts were assayed for ADPR-transferase activity with [^{14}C]adenine dinucleotide (hereafter written [^{14}C]NAD) by the method of Gill and Pappenheimer (7), with wheat germ EF-2 (6) being substituted for rabbit reticulocyte EF-2 in the reaction mixture (7). After 1 h at 37°C, the reaction was stopped by the addition of 5% trichloroacetic acid. The precipitate was collected on Whatman GF/A glass fiber filters, washed, dried, and counted in Econofluor (New England Nuclear Corp., Boston, Mass.) in a liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.).

RESULTS

Cell fractionation and extracellular labeling of membrane-bound polysomes. In iron-limited cultures of *C. diphtheriae* PW8, synthesizing diphtheria toxin at a maximal rate, the polysomes were stabilized by chilling and adding chloramphenicol; the cells were treated with [^{125}I]DSA, lysed in a French press, and fractionated on a Sepharose column. The polysome fractions in *C. diphtheriae* were much more heavily contaminated with membrane/cell wall fragments than those previously prepared from *E. coli* or *B. subtilis* (though the contamination did not interfere with studies on the labeled nascent chains). The membrane-polysome fraction contained ca. 60% of the total cellular label (which was 5% of the ^{125}I added); and the membrane-derived polysomes (prepared by washing three times with 1% DOC) retained a large fraction (50%) of the ^{125}I in the membrane-polysome fraction. Moreover, in control experiments with cells labeled with [^{14}C]oleic acid, the derived polysome fraction contained 20% of the cellular label, and additional washing with Triton X-100 failed to remove this contamination (results not shown). Similarly, the free polysome fraction contained about 20% as much ^{125}I as the derived polysomes (Table 1); because DOC treatment solubilized about 75% of this label, it appears to be largely due to incomplete separation from wall and membrane, rather than to penetration of [^{125}I]DSA into the cell.

Evidence for the attachment of extracellular label to nascent polypeptide chains. The attachment of [^{125}I]DSA to the derived polysomes via nascent polypeptide chains was demonstrated by methods previously described (17). More than 65% of the label was released by treatment with puromycin (plus elongation factor G and GTP), by dialysis against 0.01 mM Mg^{2+}, or by completion of the labeled nascent polypeptide chains in a protein-synthesizing system (with S-100 from *E. coli*). Moreover, the polypeptides released by chain completion are increased in their mean molecular weight as compared to those released by puromycin (Fig. 1). It appeared that much of this material was diphtheria toxin because a major peak was observed with the same apparent molecular weight as the toxin (i.e., 62,000).

Immunological detection of extracellularly labeled peptides related to diphtheria

TABLE 1. *Diphtheria toxin extracellularly labeled with [^{125}I]DSA*

Treatment[a]	Growth conditions	Label released from polysomes (cpm/sample)	
		Precipitated by trichloroacetic acid	Precipitated by antitoxin
Chain completion	$-Fe^{2+}$	23,750	6,270
	$+Fe^{2+}$	19,610	286
Puromycin	$-Fe^{2+}$	21,470	315

[a] Cells grown with limiting Fe^{2+} or with 200 μM Fe^{2+} (designated as $-Fe^{2+}$ or $+Fe^{2+}$) were labeled with [^{125}I]DSA. The labeled derived polysomes (about 30,000 cpm, 1.5 to 2.5 A_{260} units of RNA, in 0.1-ml final volume) were allowed to complete their nascent chains or were treated with puromycin (19), except that phenylmethylsulfonyl fluoride (2 μg/ml) and α-phenanthroline (3 μg/ml) were present in the reaction mixtures and in all subsequent steps. The polysomes were removed by centrifugation; 20 μl of the supernatant fluid was assayed for acid-precipitable radioactivity, and the remainder (180 μl) was incubated with antitoxin (50 μl). Immune precipitates were collected and washed as described in the text. To test for precipitability of the uncompleted labeled chains by antitoxin, we incubated a portion of the polysomes with puromycin (200 μg/ml) as described previously (19).

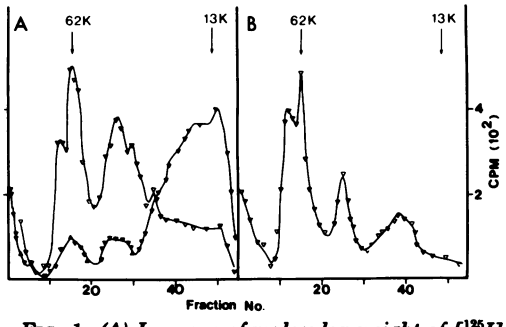

FIG. 1. *(A) Increase of molecular weight of [^{125}I]-DSA-labeled nascent polypeptide chains on completion in an S-100 E. coli protein-synthesizing system. ▲, Puromycin released nascent chains; △, nascent chains completed in vitro. (B) Separation of antitoxin-precipitated completed nascent chains. Both released nascent and completed polypeptide chains were analyzed by polyacrylamide disc gel electrophoresis with 0.1% SDS (18). The arrows are marker proteins: diphtheria toxin (62K) and cytochrome c (13K).*

toxin. Antiserum to diphtheria toxin (plus unlabeled toxin as carrier) precipitated about 30% of the label in the chains that had been labeled extracellularly and then completed in vitro (Table 2). This precipitation is clearly specific. With similar preparations from *C. diphtheriae* grown in the presence of 200 μM Fe^{2+}, which blocks toxin production, the label precipitated by antitoxin did not exceed background. Moreover, without chain completion, the derived polysomes yielded virtually no immune precipitate.

To identify the labeled chains, we washed and dissolved in alkali the immune precipitate from the preparation from toxin-producing cells and fractionated it by electrophoresis on an SDS-polyacrylamide gel, without DTT. Protein peaks were resolved with apparent molecular weights of about 68,000, 62,000, 38,000, and 24,000 (Fig. 1B). The last two have the molecular weights of fragments A and B of cleaved diphtheria toxin, while the presence of a peak at 68,000, heavier than the authentic toxin (62,000), suggested that diphtheria toxin is initially synthesized as a larger precursor.

Identification of the precursor. The secreted chains of toxin evidently lose their signal sequence soon after its protrusion because the predominant product of completion of extracellularly labeled chains has an apparent molecular weight of 62,000 and not 68,000. However, the shorter molecules of the nascent chain population would be more likely to retain the extra precursor segment; these chains also would incorporate more label per chain during completion than the longer nascent chains. In addition, cleavage might be decreased by avoiding the extra manipulation of extracellular labeling. Accordingly, derived polysomes were obtained from unlabeled cells and the chains were labeled by completion in the presence of [^{35}S]methionine. This procedure increased the fraction of label in the 68,000 form (Fig. 2) compared with that observed with extracellular labeling (Fig. 1); other experiments showed an even greater shift toward longer labeled chains. These findings support the inference that the larger species is a precursor of the others.

Because reduction of disulfide bonds by DTT

TABLE 2. *Synthesis of diphtheria toxin exclusively on membrane-associated polysomes*[a]

Determination	Trichloroacetic acid-precipitated protein (cpm/sample)	
	Free polysomes	Membrane-associated polysomes
Total protein	72,600	133,000
Toxin (immune precipitate)	500	23,900

[a] Purified membrane-bound or free polysomes (1.0 A_{260} unit) were allowed to complete their polypeptide chains with [^{35}S]methionine. The products were reacted with antiserum to diphtheria toxin, together with carrier toxin; collected and washed, and evaluated for radioactivity as described in the text. Total protein was precipitated by trichloroacetic acid. The data presented are from a single experiment, which was repeated three times with less than 10% deviation.

separates nicked diphtheria toxin into N-terminal fragment A (24,000 daltons) and fragment B (38,000 daltons) (5), the immune precipitate of the chains completed in vitro was also electrophoresed in the presence of DTT. Both the 68,000- and 62,000-molecular-weight proteins from derived polysomes were greatly diminished, and a sharp peak at 38,000 and a diffuse band at 23,000 to 27,000 daltons appeared (Fig. 2B). Such spreading of the N-terminal fragment A polypeptide (compared with that from diphtheria toxin) would be expected if it were derived both from toxin and from a precursor with an additional N-terminal leader segment.

Enzymatic activity. To further identify the 68,000-molecular-weight peak as a precursor of diphtheria toxin, we tested it as a source of the ADPR-transferase activity characteristic of nicked toxin or fragment A (5). For this purpose RNA from toxin-synthesizing cells was translated in an S-30 extract of *E. coli*, the product was electrophoresed, and the gel was sliced. The proteins eluted from each slice were activated by trypsin, and their NAD-EF-2 ADPR-transferase activity was measured. Control diphtheria toxin showed only the expected peak of activity at 62,000 daltons, while the product of translation in a membrane-diminished system showed a peak at 68,000 as well as at 62,000 daltons (Fig. 3).

Processing of precursor. After completion of the precursor chain in vitro, it could be converted to the mature toxin, molecular weight 62,000, by incubation with cytoplasmic membrane from a nontoxigenic, nonlysogenic *C. diphtheriae* strain, as well as by membrane from the toxigenic PW8 strain. Crude membrane preparations were more active than those that had been separated from wall (by a procedure

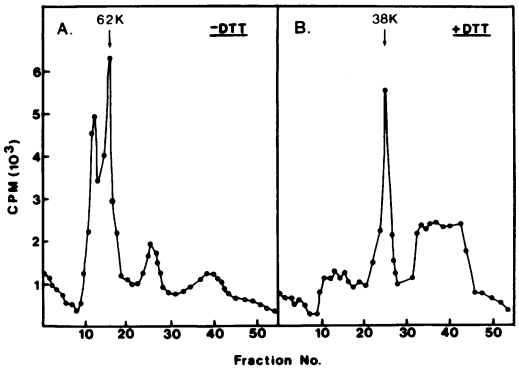

FIG. 2. *Electrophoresis of antitoxin-precipitated completed [^{35}S]methionine-labeled polypeptide chains on 0.1% SDS-polyacrylamide gels in the absence (A) and presence (B) of 5 mM DTT.*

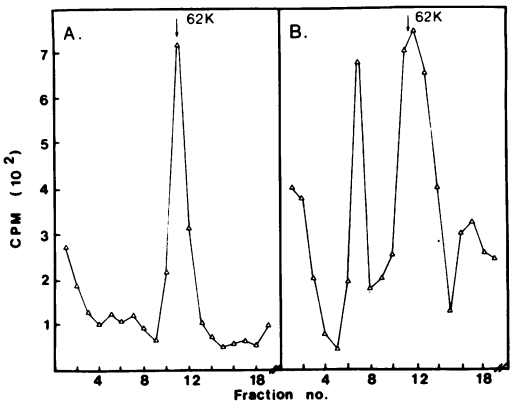

FIG. 3. *NAD-EF-2 ADPR-transferase activity of (A) purified diphtheria toxin- and (B) diphtheria toxin-related polypeptides synthesized in E. coli extracts programmed with RNA extracted from iron-limited C. diphtheriae PW8 after electrophoresis in 0.1% SDS-polyacrylamide gels.*

that involved exposure to EDTA). The outer membrane of *E. coli* was also active. This observation may explain the cleavage of the precursor synthesized in *E. coli* extracts programmed with either RNA (Fig. 1) or β-phage DNA (11).

Exclusive synthesis of toxin on membrane-bound polysomes. The above findings show that membrane-bound polysomes can make diphtheria toxin. Table 2, comparing the products of these and of free polysomes, shows that no toxin is made by the latter.

DISCUSSION

In earlier experiments, we have used the reaction of [^{125}I]DSA with peptide chains to label nascent polypeptides of secreted proteins protruding from spheroplasts of *E. coli* or protoplasts of *B. subtilis*. In the present experiments with *C. diphtheriae*, the lipid-rich wall prevented the formation of satisfactory protoplasts; hence the labeling was performed with intact cells, and the bacteria were disrupted in the French press rather than by osmotic lysis.

Despite the presence of an intact cell wall, a large fraction of the cellular [^{125}I]DSA label (40%) was attached to nascent chains and was recovered with the polysomes derived from the membrane-polysome fraction. Moreover, even though the separation of these polysomes from lipid by DOC was less complete with this organism than it had been with others, the label in the derived polysomes could be shown to be attached to nascent peptide chains because it was released from the ribosomes by several methods (low Mg^{2+}, puromycin, chain completion). In addition, when extracellularly labeled-derived polysomes were allowed to complete

their nascent chains, about 30% of the label in the released product was found in diphtheria toxin, in several molecular forms. The toxin was demonstrated by specific immune precipitation, by molecular weight, and by its characteristic ADPR-transferase activity.

The fraction of cellular label recovered in diphtheria toxin was larger than that observed for secreted proteins in previous experiments with other organisms. One probable reason is that the cells were harvested under Fe^{2+}-limited conditions, where there is little growth and maximal toxin production. In addition, after the extracellular labeling of the cells, pronase removed ca. 90% of the attached label (unpublished data). Because pronase would not be expected to penetrate the wall and reach the whole surface of the membrane, its effectiveness in removing label raises the possibility that [^{125}I]-DSA may be labeling membrane only in localized accessible areas where polypeptide chains are being secreted.

The present experiments further showed that diphtheria toxin resembles many other secreted proteins not only in being secreted cotranslationally but also in being formed as a larger precursor, with an additional N-terminal sequence. The precursor was detected as a peak with an apparent molecular weight of 68,000, along with the toxin peak of 62,000, in the immunoprecipitated products of chain completion of derived polysomes. Like toxin, it yielded, on treatment with trypsin and DTT, a B fragment and an enzymatically active A fragment that transferred an ADPR group to EF-2. The N-terminal location of the extra segment, characteristic of many other secretory proteins, was demonstrated indirectly. Diphtheria toxin can be cleaved by trypsin plus DTT into an N-terminal A fragment and a C-terminal B fragment (15). The fragments obtained from the mixture of toxin plus precursor (formed by derived polysomes), the N-terminal A peak, but not the fragment B peak, was broadened (Fig. 2B). Finally, the presumed precursor function of the 68,000-dalton product was confirmed by showing that its ratio to toxin in the labeled completed chains was increased when the label was incorporated during chain completion (which should heavily label short, unprocessed chains), compared with labeling of extracellular chains (many of which should already have been processed). The cleavage site between segments A and B is accessible in the precursor as well as in toxin.

With some other precursor polypeptides the hydrophobic character of the N-terminal signal segment has been demonstrated by decylagarose chromatography of precursor compared with its product, e.g., with *E. coli* alkaline phosphatase (8) and *B. licheniformis* penicillinase (W. Smith, P.-C. Tai, K. Izui, J. O. Lampen, and B. D. Davis, unpublished data). However, we have not had consistent results with the precursor of diphtheria toxin, perhaps because solution in alkali, after immune precipitation, might have exposed the conformationally sensitive hydrophobic sequence that is known (4) to be present in the B region.

Cells grown with excess Fe^{2+} fail to form toxin, and studies on hybridization of extracted RNA with DNA carrying the *tox* gene provided evidence that Fe^{2+} regulates toxin formation at the level of transcription (10). The present studies provide functional evidence that confirms this conclusion: cells grown in the presence of 200 μM Fe^{2+} failed to yield diphtheria toxin or its precursor among the products formed by membrane-derived polysomes, or among those formed by protein-synthesizing systems programmed with RNA extracted from the organism.

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