Identification of a region in the S1 subunit of pertussis toxin that is required for enzymatic activity and that contributes to the formation of a neutralizing antigenic determinant

(truncation/NAD glycohydrolase/ADP-ribosyltransferase/monoclonal antibodies)

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Communicated by Norman Davidson, March 16, 1988 (received for review November 5, 1987)

ABSTRACT The S1 subunit of pertussis toxin possesses two regions (homology boxes), each spanning 8 residues, that are nearly identical in sequence to similarly located regions in the enzymatically active A fragments of two other ADP-ribosylating toxins: cholera toxin and *Escherichia coli* heat-labile toxin. This observation suggests a functional role for one or both of these regions in enzymatic activity. We have examined the role of one of these regions, located near the amino terminus of the S1 subunit, by using a high-level recombinant expression system and progressive truncation of the gene sequence encoding the amino terminus of the molecule. A series of six truncated, recombinant proteins were produced at high levels in *E. coli* and examined for their enzymatic and antigenic properties. The three molecules that lacked most or all of the homology box delimited by amino acid residues 8 and 15 lacked detectable enzymatic activity. All of the three molecules in which the box was retained exhibited detectable activity. Only those recombinant molecules that possessed the homology box reacted with a neutralizing and passively protective monoclonal anti-S1 antibody. These findings identify the region of homology located near the amino terminus of S1 as an apparent enzymatic subsite and a potentially important antigenic determinant.

Pertussis toxin (PTX), also known as pertussigen, lymphocytosis-promoting factor, and islet-activating factor, is an oligomeric protein produced by *Bordetella pertussis*. PTX has been found to exert a variety of biological effects and is believed to occupy a central role in the pathogenesis of whooping cough (reviewed in refs. 1 and 2). Purified PTX is also capable of conferring protection to mice against lethal challenge with live *B. pertussis* (3). Accordingly, recent efforts to produce acellular vaccines for pertussis have included chemically inactivated (detoxified) PTX as a major component of such vaccines (4, 5).

PTX is a hexameric protein (M_r 117,000) composed of five dissimilar subunits termed S1 (M_r 28,000), S2 (M_r 23,000), S3 (M_r 22,000), S4 (M_r 11,700), and S5 (M_r 9300), which are represented in a molar ratio of 1:1:1:2:1 in each toxin molecule (6). PTX appears to exert many of its biological effects in eukaryotic cells through the ADP-ribosylation of membrane-bound guanine nucleotide-binding regulatory components (N_i or G_i) of the adenylate cyclase complex (7, 8). The enzymatic activity of PTX is associated with the S1 subunit. The remaining subunits, comprising the "B oligomer," appear to be responsible for the binding of PTX to target-cell receptors (9, 10).

The nucleotide sequence and genetic organization of the *B. pertussis* PTX operon have been elucidated (11, 12). Knowledge of the DNA sequence of the operon has facilitated the expression of the genes (cistrons) encoding the individual subunits in *Escherichia coli* (13–16). This ability allows genetic studies designed to identify portions of the toxin subunits involved in toxic and immunogenic activity. Such information might then permit the construction and expression of mutant toxin molecules that lack enzymatic and toxic activities but that retain immunogenicity. This strategy would avoid potential problems associated with chemical inactivation of purified pertussis toxin, including residual toxic activity and destruction of antigenic epitopes (4, 17).

Two regions of the S1 molecule, each consisting of 8 amino acid residues, possess sequence homology with the "A" fragments of cholera toxin (CTX) and *E. coli* heat-labile toxin (HLT) (11). Since all three toxins ADP-ribosylate guanine nucleotide-binding proteins, we reasoned that one or both of these conserved regions might play a role in enzymatic activity. We have examined the role of one of these two regions, a sequence of 8 amino acids delimited by residues 8 and 15,[¶] referred to here as the "homology box," by using progressive exonucleolytic digestion of the gene encoding the S1 subunit to yield a series of S1 molecules with amino-terminal deletions (truncations). Analyses of the recombinant products revealed that the homology box is required for enzymatic activity and that the amino acids of the box contribute to the formation of a potentially important antigenic determinant.

MATERIALS AND METHODS

Materials. DNA-modifying enzymes were purchased from New England Biolabs, Bethesda Research Laboratories, Boehringer Mannheim, and International Biotechnologies (New Haven, CT). All enzymes were used according to the suppliers' recommendations. PTX was purified from *B. pertussis* by the method of Sekura *et al.* (18). The S1 subunit of PTX was partially purified (>95%) by chromatography over CM-Sepharose (19). ATP, GTP, thymidine, dithiothreitol, and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) were obtained from Sigma; [*carbo*- 14 C]NAD (44 mCi/mmol; 1 Ci = 37 GBq), from Amersham; and [*adenylate*- 32 P]NAD (20–30 Ci/mmol), from New England Nuclear. The preparation of anti-S1 monoclonal antibodies B2F8 and 1B7 has been described (20, 21).

Abbreviations: PTX, pertussis toxin; CTX, cholera toxin; HLT, *Escherichia coli* heat-labile toxin.

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[¶]The numbering system used herein begins with Asp-1 of the processed, mature form of the S1 subunit.

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Anti-PTX serum was obtained from rabbits injected subcutaneously with glutaraldehyde-treated PTX (3). Transducin was purified from membranes of bovine retinal rod outer segments as described by Baehr *et al.* (22).

Plasmids and Bacterial Strains. The construction of expression plasmid pPTXS1/2 (Fig. 1), which contains the bacteriophage λ P_L promoter, a synthetic ribosomal binding site, and the DNA sequence encoding the S1 subunit minus the leader sequence, will be described elsewhere (23). *E. coli* FM5 cells were derived from *E. coli* K-12 and contain an integrated gene encoding a temperature-sensitive λ phage repressor cI857 (24). Manipulations of DNA, vector construction, and bacterial transformation were performed by standard methods (25).

BAL-31 Digestion and Construction of S1 Expression Vectors. The expression plasmid pPTXS1/2 was opened at the initiation codon by digestion with *Nde* I restriction endonuclease and then treated with BAL-31 exonuclease ("slow" species, International Biotechnologies) under standard conditions (26). At various times up to 180 min, aliquots were removed from the reaction mixture and incubated for 15 min at 65°C to stop the reaction. Each aliquot was treated with Klenow fragment of *E. coli* DNA polymerase I to repair BAL-31-digested ends (25) and then digested with *Sst* I. The resultant DNA fragments were then separated by electrophoresis in an agarose gel, and the 3- to 3.5-kilobase fragments were isolated by electroelution (27) onto NA-45 DEAE-membranes (Schleicher & Schuell). The gel isolation procedure allowed determination of the sizes of the BAL-31-generated fragments from each timed aliquot and removal of the apposing arm of the expression plasmid. To replace this arm, which carries the P_L promoter and the ribosome binding site, the analogous region was isolated from the parent vector (pCFM4722) by treatment with *Sst* I and *Hpa* I followed by agarose gel electrophoresis and electroelution. The isolated arm was then ligated with the individual BAL-31-generated 3- to 3.5-kilobase DNA isolates from pPTXS1/2.

Expression of Recombinant Plasmids Containing Truncated S1-Subunit Genes and Preparation of Recombinant Products. *E. coli* FM5 cells were transformed with the truncated S1 expression plasmids and plated on kanamycin-containing agar. After incubation at 28°C, colonies representing various times of BAL-31 digestion were selected, replica-plated, and grown in small liquid cultures at 28°C before induction at 42°C for 4 hr. In this expression system the full-length, processed S1 is synthesized and segregated into intracellular inclusion bodies (23). Those cultures exhibiting inclusions were identified, and the matching replica-plated colonies were subjected to flask-scale (1 liter) fermentation at 42°C. At various

times, samples from each culture were removed and examined by NaDodSO₄/PAGE (28) for the presence of inducible proteins of appropriate size. Cells from those cultures identified as producing truncated proteins of interest were sedimented by centrifugation, resuspended in 1 mM dithiothreitol, and subjected to lysis in a French pressure cell at 10,000 psig. The inclusion bodies and other sedimentable material were collected by centrifugation at 17,400 $\times$ *g* for 20 min. The pellets were then suspended in 4 M urea and washed twice by centrifugation at 12,000 $\times$ *g* for 30 min. The pellets were extracted in phosphate-buffered saline (20 mM sodium phosphate/150 mM NaCl, pH 7.4) containing 8 M urea and 1 mM phenylmethylsulfonyl fluoride. The extracts were centrifuged at 12,000 $\times$ *g* for 30 min, and the supernatant fractions containing the recombinant truncated proteins were dialyzed against phosphate-buffered saline with 1 mM phenylmethylsulfonyl fluoride. Dialysis resulted in the formation of precipitates containing the truncated proteins. For some assays the resultant suspensions were used directly after solubilization by dilution. Alternatively, the precipitates were collected by centrifugation at 14,000 $\times$ *g* for 30 min and then resolubilized in 8 M urea/50 mM Tris-HCl, pH 8.0. Any insoluble material was removed by centrifugation. Stock solutions containing S1 protein at 100 μ g/ml in 4 M urea/50 mM Tris-HCl, pH 8.0 were stored at -20°C and diluted into the appropriate assay buffer for analysis.

Immunoblot Analyses. Electrophoretic transfer to nitrocellulose was performed as described by Burnette (29). Antibodies bound to electroblotted proteins were detected with appropriate anti-IgG reagents coupled to horseradish peroxidase, using 4-chloro-1-naphthol as the chromogenic substrate.

NAD Glycohydrolase and ADP-Ribosyltransferase Assays. NAD glycohydrolase activity was measured as the ability to catalyze the release of nicotinamide from NAD (30). Reaction mixtures (50 μ l) containing 30 μ M [*carboxyl*-¹⁴C]NAD (0.06 μ Ci), 20 mM dithiothreitol, 1.0% (wt/vol) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 100 μ M ATP, 50 μ g of ovalbumin, 50 mM Tris-HCl (pH 8.0), and 0.25–0.5 μ g of recombinant protein were incubated at 30°C for 3 hr. The amount of labeled nicotinamide released was determined essentially as described by Carroll *et al.* (30).

ADP-ribosyltransferase activity was determined as the ability to catalyze the transfer of ADP-ribose from [*adenylate*-³²P]NAD to purified transducin (31). Reaction mixtures (40 μ l) containing 33 μ M [*adenylate*-³²P]NAD (0.8–1.0 μ Ci), 10 mM thymidine, 100 μ M ATP, 100 μ M GTP, 10 mM dithiothreitol, 0.5 mM dipalmitoyl phosphatidylcholine, 1 or 4 μ g of transducin, 50 mM Tris-HCl (pH 8.0), and recombinant proteins were incubated at 37°C for 30 min. For electrophoretic analysis the reactions were terminated by the addition of 5 μ l of a 10-mg/ml solution of bovine serum albumin and 50 μ l of ice-cold 10% (wt/vol) trichloroacetic acid. Precipitates were allowed to form for 1 hr on ice and then sedimented at 12,000 $\times$ *g* for 10 min at 4°C. The pellets were washed twice with cold 5% trichloroacetic acid containing 0.1% phosphoric acid and once with ether. The pellets were then solubilized in electrophoresis sample buffer (27) and heated to 95°C for 5 min before analysis by NaDodSO₄/PAGE and autoradiography. For quantitative determinations, the reactions were terminated by the addition of 10 μ l of ice-cold 6 M guanidine hydrochloride containing ovalbumin at 5 mg/ml. The mixtures were then applied to 1-inch squares of filter paper that had been impregnated with 10% trichloroacetic acid/0.1% phosphoric acid. The filters were washed and air-dried, and the radioactivity on each was determined by liquid scintillation spectrometry.

Mouse Protection Studies. Cohorts of 20 female CFW mice were immunized intraperitoneally at 24 days of age with 20 μ g (total protein) of recombinant protein preparations emulsified in complete Freund's adjuvant. At 10 and 20 days after the

FIG. 1. Expression plasmid pPTXS1/2. The plasmid contains the gene encoding the full-length, processed (lacking the leader sequence) form of S1 with a methionine residue at the amino terminus. The positions of the origin of replication (*ori*) and the kanamycin-resistance gene (*Kan^r*), which provides a means of selecting transformants, are indicated.

primary immunization, the mice received 20- μ g booster doses of antigen in incomplete Freund's adjuvant and in phosphate-buffered saline, respectively. Thirty days after the primary inoculation, the mice were challenged by intracerebral injection of a lethal dose of *B. pertussis* strain 18323 (30,000 colony-forming units). Control cohorts included mice that received three injections of phosphate-buffered saline alone and mice that received commercially available diphtheria/tetanus/pertussis vaccine (Lederle Laboratories). Serum was obtained from each mouse 1 day prior to challenge and was analyzed for anti-PTX antibodies by ELISA and for the ability to inhibit PTX-mediated clustering of Chinese hamster ovary (CHO) cells (32).

Other Analytical Procedures. DNA sequencing was performed by the primer-extension, chain-termination method (33, 34). Relative amounts of recombinant products were determined by quantitative densitometric scans of polyacrylamide gels stained with Coomassie brilliant blue R-250. Protein determinations were performed by the method of Bradford (35) or by the bicinchoninic acid (BCA) method (36), with bovine serum albumin as the standard.

RESULTS

Expression of Truncated S1-Subunit Genes. Six expression clones that produced truncated S1 proteins of interest were selected for analysis. The products of these clones, termed 6A, 35A, 39A, 33B, 2B, and 3B, were found to be produced at levels representing 40–80% of the inclusion-body proteins. The amino-terminal sequences of the six truncated S1 analogs were deduced from their DNA sequences and are illustrated in Fig. 2. Two of these molecules, 39A and 33B, were of particular interest because they bracketed a sequence of 7 noncontiguous amino acids that define a region of homology (homology box) that is present in the enzymatically active A fragments of both CTX and HLTX (shaded boxed regions in Fig. 2).

Enzymatic Activities of the Truncated S1 Subunits. Initial determinations of enzymatic activity employed suspensions of precipitated recombinant S1 analogs. After solubilization by dilution, the proteins were assayed for the ability to catalyze the ADP-ribosylation of purified transducin. Only those recombinant S1 proteins that contained the homology region (6A, 35A, and 39A) expressed ADP-ribosyltransferase activity as determined qualitatively by autoradiography (Fig. 3). A similar pattern of activity was obtained when the NAD glycohydrolase activity of such preparations was measured (data not shown). To avoid potential difficulties associated with incomplete or differential solubilization of the precipitated recombinant proteins, the enzymatic activity of each was determined after solubilization with urea (Table 1). NAD glycohydrolase activity was detected only among the

FIG. 3. ADP-ribosyltransferase activity of the truncated, recombinant S1 products. Reaction mixtures contained 0.5 μ g of S1 analog (as indicated above each lane) and 1.0 μ g of transducin. Control reaction mixtures contained either buffer (–) or 2.0 μ g of PTX. Reaction products were analyzed by NaDodSO₄/12.5% PAGE followed by autoradiography. Positions and molecular weights of standard proteins run in the same gel are indicated at left. Arrow indicates the position of the M_r 39,000 α subunit of transducin.

recombinant S1 proteins that possessed the region of homology (6A, 35A, and 39A). The specific activities of the recombinant subunits 6A and 35A were lower than that of authentic S1 and ranged from 10% to 20% of the activity of the authentic protein as determined in several experiments. Nevertheless, the activity of the recombinant molecules—including 39A, which exhibited a much reduced specific activity—was reproducibly detectable. Quantitation of the ADP-ribosyltransferase activity of the solubilized proteins revealed a virtually identical pattern of activity, with the exception of the appearance of a distinct gradient of diminishing activity that corresponded to increasing truncation (Table 1). Finally, mixing experiments with purified S1 failed to reveal enzyme-inhibitory activity in preparations of truncated subunit 33B, 2B, or 3B (data not shown). These results firmly establish a correlation between the presence of the homology box and the expression of enzyme activity.

Reactivity of Truncated S1 Subunits with Monoclonal and Polyclonal Antibodies. Monoclonal antibody 1B7 inhibits PTX-mediated ADP-ribosyltransferase activity as well as other biological activities of PTX, including the CHO-cell clustering effect and islet activation (35). Further, 1B7 can confer passive protection in mice against intracerebral and aerosol challenge with *B. pertussis* (21). When used to probe immunoblots, 1B7 was found to react with only those S1 analogs possessing the homology box (6A, 35A, and 39A) and not with those lacking it (33B, 2B, and 3B) (Fig. 4B). The epitope for 1B7 appears to reside within the area of the

FIG. 2. Amino-terminal sequences of the truncated S1 proteins. The amino acid sequence of each product (shown here in standard one-letter symbols) was deduced from the DNA sequence. The construction of the expression vectors results in the presence of a Met-Val fusion dipeptide at the beginning of each truncated molecule. The shaded portions of the S1 sequence indicate residues that are identical to those in the analogous regions of CTX and HLTX.

Table 1. NAD glycohydrolase and ADP-ribosyltransferase activities of truncated S1 proteins

S1 protein	NAD glycohydrolase*	ADP-ribosyltransferase†
6A	0.85 ± 0.21	26,018 ± 4,753
35A	0.87 ± 0.05	5,620 ± 771
39A	0.24 ± 0.05	420 ± 177
33B	0.02 ± 0.06	6 ± 10
2B	0.0 ± 0.0	46 ± 80
3B	0.03 ± 0.10	117 ± 203
Purified S1	9.71 ± 0.13	45,859 ± 1,662

Values obtained from reactions containing substrate and buffer alone were subtracted from the experimental values prior to calculation.

*NAD glycohydrolase activity was determined in samples containing 250 ng of urea-solubilized recombinant S1 or purified S1. Values are the means of triplicate determinations ± standard deviations and are expressed as pmol of nicotinamide released per min per µg of protein.

†ADP-ribosyltransferase activity was determined with 4 µg of purified transducin as substrate and 50 ng of urea-solubilized recombinant S1 or purified S1 as enzyme. Values represent the total acid-precipitable radioactivity (cpm) ± standard deviation and are the means of triplicate determinations.

homology box defined by amino acid residues 8–14. Another anti-S1 monoclonal antibody, B2F8, reacted with protein 6A, only weakly with protein 35A, and not at all with molecules that were further truncated (data not shown). Rabbit anti-PTX serum exhibited a pattern of reactivity similar to that of monoclonal antibody 1B7 (Fig. 4A). A virtually identical pattern of reactivity was observed when the antibodies were tested by ELISA using plates coated with the recombinant subunit preparations (data not shown).

Immune Response of Mice to Truncated S1 Analogs. When mice were immunized with preparations containing recombinant protein 6A, 39A, or 33B and subsequently were challenged intracerebrally with a lethal dose of *B. pertussis*, no significant increase in survival was observed relative to control groups that received phosphate-buffered saline alone (data not shown). In contrast, mice that received immunizing doses of commercial diphtheria/tetanus/pertussis vaccine exhibited a mean survival >50%, over the course of several experiments. Surprisingly, none of the sera from mice immunized with the recombinant proteins were capable of inhibiting PTX-mediated clustering of CHO cells, despite the observation that the mice immunized with each S1 analog developed significant serum titers of antibodies against PTX as measured by ELISA (data not shown).

To further verify that the truncated products lacking the homology box (e.g., 33B) contained the appropriate S1 sequences, sera from mice that had been immunized with truncated recombinant products 6A and 33B were used in immunoblot analysis. The sera reacted with all of the truncated products, as evidenced by the dominant immunoreactive bands at appropriately decreasing molecular weight that are visible on the *E. coli* protein background (Fig. 4C and D). More important, both of these sera reacted specifically with the S1 subunit of PTX.

DISCUSSION

Using progressive amino-terminal truncation of the S1 subunit, we have obtained evidence for the involvement of a specific region of this molecule in the enzymatic activities of PTX. This region also appears to contain at least a portion of an antigenic determinant that is capable of eliciting antibodies in mice that protect against *B. pertussis* infection and that neutralize toxin activity. The region referred to here as the homology box spans amino acids 8–15 of the mature (processed) S1 molecule and is identical in 7 of 8 residues to a

FIG. 4. Reaction of recombinant, truncated S1 molecules with monoclonal and polyclonal antibodies in immunoblots. Aliquots containing equal amounts of recombinant S1 (≈1.0 µg) or PTX (1.5 µg) were subjected to NaDodSO₄/12.5% PAGE and electroblotted to nitrocellulose. Parallel blots were then incubated with rabbit anti-PTX (RαPTX) serum (A), with monoclonal antibody 1B7 (B), or with serum obtained from mice immunized with recombinant proteins 6A (C) or 33B (D). Bound antibodies were detected with peroxidase-linked antibodies. Arrow indicates the position of S1 subunit of PTX in each blot.

sequence found in the amino-terminal regions of the A fragments of CTX (residues 6–13) and HLTX (residues 6–13) (11, 12, 38).

The specific enzyme activities of the nearly full-length truncated S1 analogs (6A and 35A) were lower than that of purified S1 derived from PTX. The reduced activity of the truncated subunits might be attributable to irreversible, partial denaturation as a result of segregation into inclusion bodies or of repeated solubilization with urea during preparation for analysis. However, the progressive decrease in specific ADP-ribosyltransferase activity that accompanies increasing truncation might also suggest that relatively small deletions or substitutions in the amino-terminal region near the homology box have a significant effect on enzymatic activity. It is possible that the amino-terminal Met-Val substitution (in place of Asp-Asp) contained in the truncated subunit 6A (Fig. 3) accounts for its reduced activity. Similarly, proximal effects associated with the deletion of the 6 amino acids and their replacement with a Met-Val dipeptide might also account for the rather dramatic reduction (by a factor of 50–100) in enzyme activity that was observed in the case of 39A.

Although we have not eliminated the possibility that deletion of the amino-terminal residues results in important structural perturbations in other remote sites, the most straightforward interpretation of the present data is that the region of homology constitutes part of the enzymatic active center and may function in catalysts *per se* or in substrate binding. The present results also predict that the removal or alteration of the analogous regions in CTX and HLTX would have a similar effect on enzyme activity.

The epitope for monoclonal antibody 1B7 resides, either entirely or in part, within the homology box. This finding suggests that removal of this box to eliminate enzymatic activity of PTX could have the unwanted effect of reducing, or even eliminating, its protective immunogenicity. It might also be predicted that monoclonal antibody 1B7 would crossreact with CTX or HLTX. We have not been able to demonstrate a reaction between 1B7 and CTX. However, Burns *et al.* (39) have shown that antibodies prepared against

a peptide corresponding to residues 6–17 of the S1 subunit are capable of binding to the A fragment of CTX, as judged by both immunoblotting and ELISA. This finding suggests a structural similarity between the two toxins in the region of homology and, further, suggests a functional role for the region in the activities of the two toxins.

Rabbit anti-PTX serum yielded a pattern of reactivity that was essentially the same as that of monoclonal antibody 1B7, suggesting that the amino-terminal region contains an immunodominant epitope(s). However, mice immunized with either of the recombinant proteins 6A and 33B, the latter lacking the homology box, produced antibodies that appeared to bind to all of the recombinant S1 molecules. This finding may indicate an essential difference between the antibody response of animals immunized with whole PTX and that of animals immunized with isolated S1, relating either to the chemical treatment (glutaraldehyde) of PTX used for immunization or to differential immunologic processing of S1 and PTX.

This study and others (14, 15) have shown that individual recombinant subunits of PTX are incapable of conferring protection in mice against intracerebral challenge with live *B. pertussis*. Therefore, it appears that it will be necessary to use a specifically altered recombinant S1 subunit (and perhaps other altered subunits) to create an inactive, chimeric holotoxin for use as a constituent of an acellular vaccine for whooping cough.

We thank J. L. Cowell for providing *B. pertussis* strain 18323, W. Baehr for assistance in the purification of transducin, and T. Jones and his colleagues at Amgen Development, Inc., for the design and synthesis of oligonucleotides. We gratefully acknowledge the technical assistance of L. Mutch, S., Northcutt, S. Smith, and P. Coggsell. We thank R. Evans, G. Hettrick, and J. Heuston for providing the graphics and B. Kester for assistance in the preparation of the manuscript. This work was done while W. C. held a National Research Council–National Institutes of Health research associate-ship. The investigations were supported in part by a National Institutes of Health Small Business Innovation Research Grant 1 R43 AI23566-01 to W.N.B.

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