Biochemical and Biological Activities of Recombinant S1 Subunit of Pertussis Toxin

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degP-deficient strains of Escherichia coli grown in M-9 medium supplemented with ZnCl2 expressed the recombinant S1 subunit of pertussis toxin (rS1) in a form electrophoretically identical to the authentic S1 subunit. Subcellular fractionation showed that the full-length form of rS1 was membrane associated, while proteolytic fragments of rS1 were present in the periplasm. rS1 was extracted from outer membrane preparations with 8 M urea and purified by gel filtration chromatography. Purified rS1 ADP-ribosylated transducin at a similar molar efficiency relative to authentic pertussis toxin and, when associated with the native B oligomer of pertussis toxin, elicited Chinese hamster ovary cell clustering.

Pertussis toxin (PT; molecular weight, 105,890) is a member of a group of ADP-ribosylating exotoxins. PT is composed of five noncovalently associated subunits designated S1, S2, S3, S4, and S5 (molecular weights, 26,220, 21,920, 21,860, 12,060, and 11,770, respectively) (19). The whole toxin contains one copy of each subunit, except S4, which is present in two copies. PT catalyzes the transfer of the ADP-ribose moiety of NAD to the G regulatory component of the adenylate cyclase complex as well as other G-like proteins. ADP-ribosylation of G reduces the response to inhibitory agents of adenylate cyclase and increases the response to stimulatory agents (8). In the absence of target protein, PT hydrolyzes NAD to ADP-ribose and nicotinamide. Although the in vivo significance of the NAD glycohydrolase reaction is not yet apparent, it is useful for measurement of enzyme activity.

PT is an "A:B" exotoxin. When incubated in 5 M urea, the S1 subunit dissociates from the other five subunits, which maintain a pentameric structure (B oligomer). Ui and co-workers (19) found that the S1 subunit possessed both ADP-ribosyltransferase and NAD glycohydrolase activities in vitro but failed to ADP-ribosylate the G3 target protein in vivo. Upon reassociation of the S1 subunit with the B oligomer, the reconstituted PT expressed in vivo biological activity. Burns et al. (6) have subsequently shown that the carboxy-terminal portion of the S1 subunit was required for binding to the B oligomer.

Two laboratories have cloned and sequenced the PT gene (9, 11), and our laboratories and others have expressed nonfusion forms of the S1 subunit of PT in Escherichia coli (2, 5). Directed mutagenesis on the S1 subunit allowed identification of individual amino acids, including Arg-9, Asp-11, Arg-13, Trp-26, and Glu-129, that were required for expression of ADP-ribosyltransferase activity (1, 3, 4, 12). Our goal toward the production of a recombinant toxoid of PT has been to define the role that these residues play in catalysis and identify amino acid substitutions at these residues which reduce catalytic activity, but retain immunoreactivity. While the biochemical analysis may be performed on the purified S1 subunit or catalytic derivatives, measurement of biological activity requires the introduction of mutations into PT. Two protocols may be used to introduce mutations into PT: (i) the mutations may be inserted directly into the PT operon of Bordetella pertussis (15), which would allow production of mutant PT derivatives by its natural host, or (ii) mutants of the recombinant S1 subunit may be recombined in vitro with native B oligomer to form a mutant hybrid PT (6, 19). In this study, a full-length (nondegraded) form of the S1 subunit (rS1) was purified from E. coli and shown to possess biochemical and biological activities similar to those of the authentic S1 subunit of PT.

MATERIALS AND METHODS

Materials. 125I and 32P[adenylate phosphate]NAD were purchased from Amersham Corp., Arlington Heights, Ill., and Dupont, NEN Research Products, Boston, Mass., respectively. Enzymes used in the manipulation of DNA were purchased from Molecular Biology Resources, Inc., Milwaukee, Wis., or New England BioLabs, Inc., Beverly, Mass. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) molecular weight standards were from Pharmacia, Inc., Piscataway, N.J. Isopropyl-β-D-thiogalactopyranoside was purchased from Research Organics, Inc., Cleveland, Ohio. Protein determinations were made with the protein assay reagent from Pierce Chemical Co., Rockford, Ill., using bovine serum albumin as a standard. Authentic PT was prepared (R.R.) from the culture supernatant of B. pertussis as described previously (13).

Bacterial strains and plasmids. The degP series of Escherichia coli KS272, KS303, KS474, and KS476 were obtained from K. L. Strauch and J. Beckwith (16). ptcα5-1c, which encodes the leader sequence followed in tandem with the mature sequence of the S1 subunit of PT expressed under the regulation of the tac promoter, has been described previously (2). Plasmid manipulations were performed essentially as described in Molecular Cloning (10).

Expression of rS1 in E. coli. (i) Rapid lysis. Overnight cultures of E. coli were diluted 1/100 in 3 ml of M-9 medium (10) supplemented with 0.2% Casamino Acids, 0.2% glycercol, 0.5 mM ZnCl2, and 100 μg of ampicillin per ml. Addition of ZnCl2 yielded a slight precipitate in the M-9 medium and reduced the growth rate of E. coli by about 20%. Cells were incubated at 250 rpm at 30°C. After 2 h, isopropyl-β-D-thiogalactopyranoside (1.0 mM) was added and the incubation was continued for 4 h (cell growth was 0.8 A550 unit). Rapid lysis was performed as follows: 400 μl of
cells was concentrated by centrifugation at 13,400 x g for 1 min, suspended in 40 µl of SDS-PAGE sample buffer containing β-mercaptoethanol, and boiled for 5 min. A 10-µl amount of each sample was subjected to SDS-PAGE (13.5%).

(ii) Subcellular fractionation. Cultures were prepared as described above except that 30-ml cultures were grown. Separation of the periplasmic material was performed by spheroplast formation by using a lysozyme-sucrose solution. This procedure extracts >75% of the periplasmic material, with the retention of >90% of the cytosolic components (2). Lysozyme-treated cells were suspended in 10 ml of 25 mM Tris hydrochloride (pH 7.6) containing 100 mM NaCl, 1 mM MgCl₂, 20 µg of RNase A per ml, and 20 µg of DNase I per ml and broken with a French press. After removal of whole cells by centrifugation at 1,000 x g for 10 min, the cytoplasmic and membrane components were separated by centrifugation at 184,000 x g for 1 h at 4°C. Equal cellular proportions of the periplasmic, cytoplasmic, and membrane fractions were subjected to Western blot (immunoblot) analysis by probing with 0.5 µg of protein A-purified rabbit anti-C180 peptide immunoglobulin G (IgG) per ml followed by 125I-labeled protein A (approximately 3 x 10⁶ cpm, at a specific activity of 4 x 10⁶ cpm/µg of protein A). The C180 peptide is a recombinant protein that corresponds to the first 180 amino-terminal amino acids of the S1 subunit; preparation of anti-C180 peptide IgG has been described before (2).

Purification of rSI. Membrane preparations from a 400-ml culture of E. coli 474(ptaC-1c) were prepared from French press-broken cells. Membranes were suspended in 4 ml of 25 mM Tris hydrochloride (pH 7.6) and subjected to sucrose equilibrium density centrifugation at 141,000 x g for 18 h at 4°C (14). Inner membranes (pigmented) were located at the 0.7 to 1.44 M sucrose interface and outer membranes (opaque) were located at the 1.44 to 2.02 M sucrose interface (14). Western blotting of equal cellular amounts of inner and outer membrane preparations showed that the outer membrane contained >90% of the total membrane-bound rSI. Outer membranes were centrifuged at 184,000 x g for 3 h and suspended in 3 ml of 25 mM Tris hydrochloride (pH 7.6) containing 8 M urea and incubated for 18 h at 4°C. Urea-insoluble material was removed by centrifugation at 190,000 x g for 3 h at 8°C, and the urea-soluble outer membrane material was chromatographed over Sephacryl S200HR equilibrated in 25 mM Tris hydrochloride (pH 7.6) containing 4 M urea (140-ml column). Fractions were collected (3 ml) and analyzed by SDS-PAGE for protein by staining with Coomassie blue or for rSI by Western blotting as described above. Urea-solubilized rSI chromatographed with a retention time slower than the majority of the membrane proteins in the urea-soluble preparation. Fractions containing rSI were pooled, and ammonium sulfate was added to 50% saturation (final concentration). The precipitate was dialyzed into 25 mM Tris hydrochloride (pH 7.6) containing 2 M urea and stored at 4°C. This material was termed the rSI and used for biochemical and biological analyses. Typical yield of purified rSI was about 0.1 to 0.2 mg/liter of cells.

Purification of B oligomer of PT. B oligomer was purified from culture supernatants of a mutant of B. pertussis W28 (81/9E) which secretes into the culture medium only the assembled B oligomer (M. Pizza and R. Rappuoli, manuscript in preparation). B oligomer was purified from the culture medium by affinity chromatography protocols described for PT (13).

ADP-ribosylation of transducin by PT and rSI. The reaction contained the following (in 10 µl): 100 mM Tris hydrochloride (pH 8.0), 2 mM ATP, 20 mM dithiothreitol, transducin, 1 mg of egg albumen per ml, 100 mM [32P]adenylate phosphate]NAD (800 Ci/mm), and 2 to 100 nM rSI or authentic PT. After a 30-min incubation at 37°C, 5 µl of SDS-PAGE sample buffer was added, and the mixture was boiled for 5 min and then subjected to SDS-PAGE. The gel was stained for protein, dried, and then autoradiographed.

CHO cell clustering by PT and hybrid PT. Equimolar amounts of rSI and B oligomer were incubated in 1 M urea at room temperature for 1 h and termed hybrid PT. Authentic PT, rSI, and B oligomer were treated identically. The four preparations were subjected to threefold dilutions in 1.0 mg of egg albumin per ml; they were then added to 1 ml of 10⁴ Chinese hamster ovary (CHO) cells cultured in HAMS F-12 medium supplemented with 10% newborn calf serum in 24-well plates. After 48 h, cells were assayed for clustering morphology (7). The minimum concentration required for elicitation of a clustering response was defined as the dilution of material which yielded a clustering of approximately 50% of the cells. Relative to the minimum concentration required for elicitation of a clustering response, we found that addition of threefold more material showed essentially complete cell clustering and addition of one-third the material showed essentially no cell clustering.

RESULTS AND DISCUSSION

Expression of rSI in degP-deficient strains of E. coli. Earlier, Barbieri et al. reported that when rSI was expressed in E. coli JM103, the peptide was secreted into the periplasm and subjected to proteolytic degradation (2). Subsequently, factors have been identified which influence the proteolytic degradation of rSI. The steady-state level of rSI was greater in degP-deficient strains of E. coli (KS474 and KS476) than in the isogenic strains (KS272 and KS303) (Fig. 1). The majority of the immunogenic material expressed in the degP strains possessed electrophoretic mobility identical to that of the authentic S1 subunit, with only a small amount of rSI degradation observed. E. coli KS474 and KS476 both possess an inactive degP protease which lacks at least one-half of the degP gene and is therefore nonrevertible (Strauch and Beckwith, personal communication), but differ in that KS476 also possesses a truncated form of the lpp outer membrane protein. The lpp mutation gives E. coli KS476 a leaky
phenotype in which a percentage of the periplasmic contents is released into the culture medium (17). Since rS1 was stable in both KS474 and KS476, rS1 stability was independent of the lpp genotype.

In addition to the enhancement of rS1 stability observed upon expression in the degP strains, medium composition also influenced rS1 stability. Steady-state levels of the full-length form of rS1 were increased by cultivation of cells in M-9 medium relative to L broth. Also, supplementing the growth media with 0.5 mM ZnCl₂ increased the steady-state level and final yield of full-length rS1 (data not shown). Enhancement of rS1 stability was achieved with either ZnCl₂ or ZnSO₄, indicating that this effect was zinc mediated. Thus, it appeared that two proteases may be responsible for rS1 degradation: one degP protease and a second protease that was inhibited by zinc. The observation that the addition of zinc to the growth medium of degP mutants carrying ptaS-1c resulted in an increased stability of rS1 was consistent with studies by Strauch and Beckwith (16) which showed that, in addition to degP, other uncharacterized proteases contributed to the turnover of periplasmic and membrane-bound target proteins. Zinc-sensitive proteases have been identified within the periplasm of E. coli (18) and could be responsible for rS1 degradation, but the possibility that an as yet uncharacterized protease was responsible could not be ruled out. The possibility also exists that zinc inhibits residual degP protease activity since the absolute activity of degP mutants has yet to be determined.

Subcellular localization of rS1. Subcellular fractionation was performed by separation of the periplasmic material by using a lysozyme-sucrose solution. This procedure extracts >75% of the periplasmic material, with the retention of >90% of the cytosolic components (2). Lysozyme-treated cells were broken with a French press, and the cytoplasmic and membrane components were separated by ultracentrifugation. rS1 was expressed as several immunoreactive peptides in E. coli JM103 (Fig. 2). One of the larger immunoreactive peptides migrated with a mobility identical to that of the authentic S1 subunit of PT, while the majority of the immunoreactive peptides migrated with apparent molecular weights ranging from 23,000 to 26,000. Subcellular fractionation showed that these immunoreactive peptides were present in the periplasm. These data were consistent with our earlier studies (2). Subcellular fractionation of rS1 expressed in E. coli KS272 was identical to that observed in E. coli JM103. While rS1 was also expressed as several peptides in E. coli KS474, the major immunoreactive peptide migrated electrophoretically with the authentic S1 subunit. Subcellular fractionation showed that this immunoreactive peptide was associated with the cell membrane while the lower-molecular-weight immunoreactive peptides were located in the periplasm (Fig. 2). Upon separation of the membrane fraction into inner and outer membranes by sucrose gradient centrifugation (14), >90% of the rS1 was found to be associated with the bacterial outer membrane. These data suggested the following explanation for the expression of rS1 in E. coli: (i) rS1 was secreted into the periplasm and the leader sequence was removed by host leader peptidase; (ii) the full-length form of rS1 was noncovalently bound to the outer membrane; and (iii) if appropriate proteases were active, rS1 was degraded at its protease-sensitive region (1, 6), which yielded low-molecular-weight peptides that were soluble in the periplasm. Consistent with this model is the observation that the degP phenotype has been shown to reduce the degradation of certain periplasmic fusion proteins and fusion proteins of the membrane-bound maltose-binding protein (16). Gene fusion experiments by Strauch et al. (17) indicated that the gene product of degP was a periplasmic or membrane protease.

Approximately 90% of the rS1 migrated with the oxidized authentic S1 subunit of PT upon SDS-PAGE in the absence of a reducing agent, which indicated that the majority of rS1 was in an oxidized form with an intact disulfide bond (Fig. 3). The remaining 10% of the oxidized rS1 preparation migrated slower than the oxidized authentic S1 subunit, which indicated that this material did not possess an intact disulfide bond and might represent an unfolded peptide. Similar to the
authentic S1 subunit, in the presence of reducing agent, rS1 was converted to a slower-migrating peptide with an apparent molecular mass of 30,000 daltons, consistent with the reduction of the peptide’s disulfide bond. Molar equivalents of oxidized rS1 and the authentic S1 subunit yielded a weaker antigenic signal in the Western blots than the reduced forms of rS1 and the authentic S1 subunit. Visual inspection of Ponceau (Sigma Chemical Co., St. Louis, Mo.)-stained peptides on the nitrocellulose prior to immunoblotting showed that both oxidized and reduced peptides were transferred with similar efficiencies (data not shown).

**ADP-ribosyltransferase activity.** rS1 ADP-ribosylated transducin in vitro. Titration of rS1 and authentic PT showed that the two proteins had essentially the same ADP-ribosyltransferase activity, using purified transducin as the target protein (activities were within 10% relative to protein concentration). Protein concentrations of rS1 and PT used in the determination of ADP-ribosyltransferase activity were adjusted to yield proportional catalytic activity.

**CHO cell clustering.** rS1 was incubated with B oligomer under conditions which allow subunit assembly (6, 19); this mixture was termed hybrid PT. B oligomer used in these experiments did not possess a detectable S1 subunit by Western blot analysis (data not shown). Hybrid PT was about 30% as potent as authentic PT at elicitation of CHO cell clustering (Fig. 4). CHO cell clustering was elicited at 80 pM authentic PT and 250 pM hybrid PT. Neither rS1 nor B oligomer alone elicited CHO cell clustering at 25 nM, the highest concentration tested. The lower activity of hybrid PT, relative to authentic PT, may have resulted from only a percentage of the rS1 assembling with B oligomer or from a lower cytotoxic potential of hybrid PT. One future goal is to establish in vitro conditions which yield quantitative assembly of rS1 with native B oligomer.

Conditions will be established which release rS1 from the outer membrane without the use of denaturing concentrations of urea. This is necessary since one goal is to measure the biological activity of mutants of the S1 subunit which possess reduced catalytic activity. The present purification scheme requires an incubation in 8 M urea to release the peptide from the outer membrane; therefore, the possibility exists that an observed loss in biological activity of mutant rS1 peptides could be due to improper refolding following incubation in urea rather than to an inherent lack of biological activity.

Purification of a biochemically active recombinant S1 subunit represents a step toward our ultimate goal of producing a recombinant toxoid of PT derived solely from antigens produced in E. coli.

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LITERATURE CITED