INFECTION AND IMMUNITY, Apr. 1987, p. 963–967
0019-9567/87/040963-05$02.00/0

Vol. 55, No. 4

Expression and Immunological Properties of the Five Subunits of Pertussis Toxin

A. NICOSIA, A. BARTOLONI, M. PERUGINI, AND R. RAPPUOLI*

Sclavo Research Center, 53100 Siena, Italy

Received 4 September 1986/Accepted 7 January 1987

Pertussis toxin, a protein composed of five different subunits, is responsible for the pathogenicity of *Bordetella pertussis* and is the main component of a new vaccine against whooping cough. The genes coding for the five subunits, recently cloned and sequenced, are organized as an operon. We approached the problem of expression of the five genes in *Escherichia coli* and, although we obtained high levels of transcription of the native pertussis toxin genes, the amount of proteins produced was very low or undetectable. To obtain suitable expression of each of the five subunits, we fused their genes to the gene coding for the DNA polymerase of MS2 in the expression vector pEx31. A total of 5 to 30 mg of purified fusion proteins could be obtained from 1 liter of culture. The purified fusion proteins were used to immunize rabbits to obtain sera against each of the five subunits. These sera, although able to recognize the toxin in an enzyme-linked immunosorbent assay and the corresponding subunits in Western blots, were not able to protect CHO cells from the action of pertussis toxin. Mice immunized with the five subunits were not protected from an intracerebral challenge with *B. pertussis*. Subunits S2 and S3, which are 67% homologous, were shown to cross-react immunologically. The fused subunit S1 was able to ADP-ribosylate transducin as efficiently as the native pertussis toxin.

Pertussis toxin (PT), a protein released into the culture supernatant by virulent *Bordetella pertussis* (14, 18, 20), is a major virulence factor of this microorganism (21, 23) and is the main component of a new acellular vaccine against whooping cough (17). PT is composed of five different subunits (20) and, as in the case of diphtheria and cholera toxins, its toxicity is due to the ADP-ribosylation of target proteins in eucaryotic cells (2, 6). The proteins which are ADP-ribosylated by PT are a family of GTP-binding proteins involved in the transduction of signals across the cell membrane (5, 6, 10, 22). Like diphtheria and cholera toxins, PT can be divided into two functionally different moieties: A, which is composed of the enzymatically active subunit S1, and B, which is composed of subunits S2, S3, S4, and S5 arranged in two dimers, D1 (S2 + S4) and D2 (S3 + S4), held together by S5 (20). The B moiety binds the receptors on the surfaces of eucaryotic cells and allows the entry of the enzymatically active subunit S1 into the cells.

The genes coding for the five subunits of PT, recently cloned and sequenced, have been shown to be clustered in an operon (8, 12). Subunits S2 and S3 were found to have 67% homologous amino acid sequences. Four of the five genes have been unequivocally identified by amino-terminal sequencing of the mature subunits, while S5 has been only tentatively identified on the basis of amino acid composition (12). Detoxified PT or a monoclonal antibody against subunit S1 has been shown to be protective in animal models or the CHO assay (11, 16).

Separate expression of each of the five subunits in *Escherichia coli* should furnish useful reagents (subunits and sera) for studying the role of each subunit in the function of PT and therefore provide the basis for the construction of new vaccines against whooping cough. In this paper, we describe the expression of each of the five subunits as fusion proteins in *E. coli* and the production of polyvalent sera against them. The sera against the fusion proteins were able to recognize the native PT in an enzyme-linked immunosorbent assay (ELISA) and the corresponding subunits in Western blots but were not able to neutralize the activity of the toxin in vitro. The above-described reagents allowed us to definitely identify the gene coding for subunit S5 and to establish that subunits S2 and S3 are immunologically cross-reactive. We also show that the fused subunit S1 maintains the same enzymatic activity as the native PT.

MATERIALS AND METHODS

Plasmids and bacterial strains. The 4,696-base-pair (bp) clone containing the five genes coding for PT has been described by Nicosia et al. (12). The numbers of nucleotides we refer to in this paper are those adopted in reference 12. *E. coli* K-12 ΔH1 Δ*trp* (15), which contains a heat-sensitive repressor and plasmid pLC28, was obtained from W. Fiers (Laboratory of Molecular Biology, State University of Ghent, Belgium). Plasmid vectors pEx31 and pEx34 were obtained from Mo-Quen Klinkert (Center for Molecular Biology, Heidelberg, Federal Republic of Germany) and are derivatives of pEx29 (7, 19). Manipulation of DNA and RNA was performed by standard procedures (9).

Production and purification of the fused proteins. Cultures (10 ml) were grown overnight at 30°C in LB medium (9), diluted to 400 ml in fresh LB medium, and grown for 2 h at 30°C and for 2.5 h at 42°C. The bacteria were then harvested and suspended in 3 ml of 25% sucrose–10 mM Tris (pH 8)–1 mM EDTA. Lysozyme (100 μl of a 40-mg/ml solution) and 0.8 ml of 0.5 M EDTA were then added. After incubation at 37°C for 30 min, we added 8 ml of lysis buffer (1% Triton X-100, 50 mM Tris [pH 6], 63 mM EDTA) and kept the mixture in ice for 15 min and at 37°C for 30 min. Cells were then sonicated and centrifuged, and the pellet was suspended in 5 ml of 1 M urea, left at 37°C for 30 min, centrifuged, and solubilized in 5 ml of 7 M urea. The enriched fusion proteins in 7 M urea were then mixed with 2 volumes of sodium dodecyl sulfate (SDS) loading buffer (19) and loaded on a preparative SDS-polyacrylamide gel. After electrophoresis, the fusion proteins were visualized by staining for 10 min with Coomassie blue, sliced out of the gel, and

* Corresponding author.

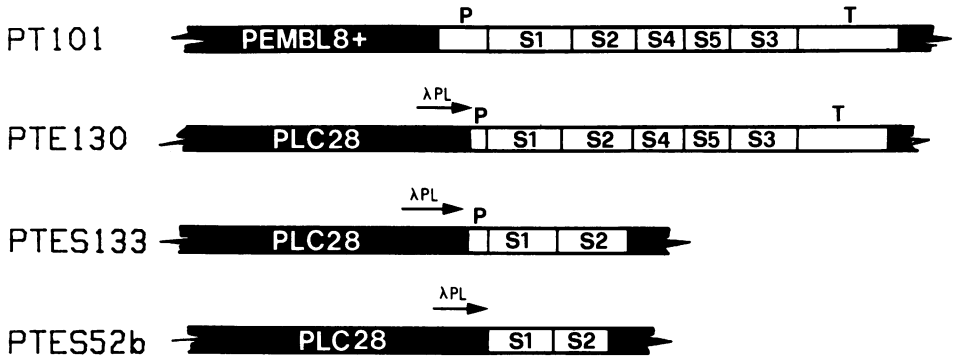

FIG. 1. Clones constructed for the expression of the native PT genes. PT101: *Eco*RI fragment from bp 1 to 4696 cloned in the plasmid vector pEMBL8$^+$ (12). PTE130: *Kpn*I-*Bam*HI restriction fragment from bp 435 and 4574 containing the five genes and their promoter cloned into the *Eco*RI site of plasmid pLC28. PTES133: *Kpn*I-*Sma*I fragment from bp 435 to 2064 containing the genes coding for S1 and S2 and their promoter cloned into the *Eco*RI site of plasmid pLC28. PTES52b: same construction as PTES133 but treated with *Bal* 31 to delete the PT promoter from the 5′ end and place the S1 gene only under the control of the p_L promoter; the fragment consists of bp 484 to 2064.

electroeluted for 24 h into a dialysis bag. The electroeluted proteins were then dialyzed against 0.1 M $NaHCO_3$.

Other methods. Rabbits were immunized subcutaneously with 0.6 mg of fusion protein mixed with an equal volume of complete Freund adjuvant (total volume, 1 ml). After 18 days, they were immunized again as described above but with incomplete Freund adjuvant. On day 27, 1 ml of protein was administered intravenously without adjuvant. Rabbits were bled 10 days later. The ELISA was performed as already described (18) with goat gamma globulins against PT or fetuin as the solid phase. PT was purified as described by Sekura et al. (18), and Western blotting was performed by standard procedures (1). ADP-ribosylation of transducin was performed as described by Manning et al. (10). The activity of PT in CHO cells was determined as described by Hewlett et al. (4).

For intracerebral challenge, 15 mice for each sample were immunized intraperitoneally with 50, 25, and 12.5 μg of protein. Fourteen days later, they were challenged with *B. pertussis* 18323 (5×10^4 cells per mouse). The survival rate was determined 2 weeks after the challenge.

RESULTS AND DISCUSSION

Expression in *E. coli* of the native PT genes. No detectable expression of PT was obtained with plasmid PT101 (12), which contains the entire PT operon cloned in the plasmid vector pEMBL8$^+$ (Fig. 1). In a further attempt to obtain suitable expression of the PT genes, we cloned the entire operon or parts of it into the expression vector pLC28, which contains the strong p_L promoter of bacteriophage lambda. Three of the constructions used to test the expression of the PT genes are shown in Fig. 1. The above-mentioned plasmids were transformed into *E. coli* K-12 ΔH1 Δ*trp* (which contains a temperature-sensitive repressor of the lambda promoter p_L). After growth at 30°C, the cultures were diluted into fresh medium and incubated for 1 h at 42°C. The cells were then harvested by centrifugation and either used to prepare mRNA or lysed by sonication. The sonic extracts and the supernatants were then used to determine the expression of the PT subunits by SDS-polyacrylamide gel electrophoresis, ELISA, Western blotting, and ADP-ribosylation. Dot blot hybridization with a single-stranded RNA probe specific for the S1 gene showed that the amount of mRNA of the PT genes in clones PT101 and PTE130 was very low compared with that in clones PTES133 and PTES52b, which produce at least 15 times more mRNA than *B. pertussis* (Fig. 2). Despite the high level of transcription of PT genes in the latter two clones, none of them produced enough protein to be visualized by SDS-polyacrylamide gel electrophoresis or detected by ELISA. In Western blots, only clone PTES52b produced a faint band comigrating with native S1, indicating that *E. coli* is able to produce this subunit and cleave its leader peptide (data not shown). ADP-ribosylating activity could be detected in lysates of clones PTES133 and PTES52b (Fig. 2). In no case were we able to detect the expression of any subunit other than S1.

From these results, we can conclude that although *E. coli* can efficiently transcribe cloned PT genes, it produces negligible amounts of proteins. This could be due to the instability of the mRNA, translational control, proteolytic digestion of the proteins, or a combination of the above reasons. Since the expression of the native genes in *E. coli* is very poor, alternative strategies are required to obtain suitable expression of each of the five subunits.

Expression and purification of the fusion proteins. The expression vectors used were pEx31 and pEx34 (7, 19),

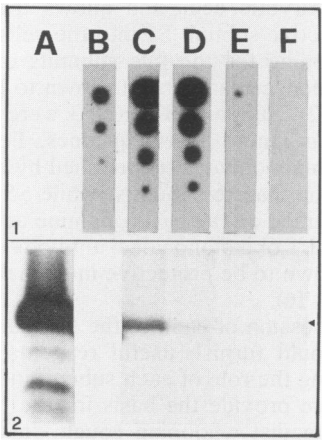

FIG. 2. Expression of the native S1 gene by the clones described in Fig. 1 and detected by dot blot hybridization (panel 1) of RNA with a single-stranded RNA probe specific for the S1 gene (fourfold dilution spots of RNA were used from top to bottom) and ADP-ribosylation (panel 2) of transducin. Lanes: A, 0.5 μg of affinity-purified PT; B, RNA from the Tohama strain of *B. pertussis*; C, PTES52b; D, PTES133; E, PTE130; F, plasmid vector pLC28.

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PTE255 (S1)

Fragment Sau3a-Xba1 (612-1317) cloned in PEX34b/BamH1-Xba1

       EcoR1              BamH1
       ______             ______
....gg gaa ttc gcg cga ccg GAT CCT CCC GCC A....
...... glu phe ala arg pro ASP PRO PRO ALA......


PTE211 (S2)

Fragment Sau96-Sma1 (1433-2064) cloned blunt in PEX31a/BamH1-blunt

      EcoR1             (BamH1-Sau96)
      ______            ___________
....gga att cgg gcg acc gga tcG GCC TCC ACG CCA......
....gly ile arg ala thr gly ser ALA SER THR PRO......
                                 ---


PTE221 (S3)

Fragment SpH1-Dde1 (3014-3628) cloned blunt in PEX34c/BamH1-blunt

      EcoR1            (BamH1-Sph1)
      ______           ________
....gg aat tcg cgc gac cgg atc CGC ACG GCC CAG GCC GTT GCA CCA....
...... asn ser arg asp arg ile ARG THR ALA GLN ALA VAL ALA PRO....
                               ------------------


PTE240 (S4)

Fragment BstN1-BstN1 (2151-2600) cloned blunt in PEX31b/BamH1-blunt

      EcoR1             (BamH1-BstN1)
      ______            _________
....g gaa ttc gcg cga ccg gat cTG GCC GAC GTT CCT....
......glu phe ala arg pro asp LEU ALA ASP VAL PRO....
                               -------


PTE230 (S5)

Fragment Aat2-SnaB1 (2558-3210) cloned blunt in PEX31a/BamH1-blunt

       EcoR1            (BamH1-Aat2)
       ______           ______
...g gga att cgc gcg acc gga tcC GCC GGC TTG CCG....
.....gly ile arg ala thr gly SER ALA GLY LEU PRO....
                               -------
```

FIG. 3. Summary of the constructions by which each of the five genes of PT were fused in frame to the gene of MS2 DNA polymerase in the expression vector pEx31 or pEx34 (19). The sequences of the vectors (lowercase letters) were fused in the *Bam*HI site to the genes coding for the five subunits (uppercase letters). The constructions of subunits S2, S3, S4, and S5, in addition to the sequences of the mature subunits, contain some amino acids deriving from the leader peptide. These amino acids are underlined. The restriction sites in parentheses above the constructions are those sites which were used for cloning and were destroyed in this process.

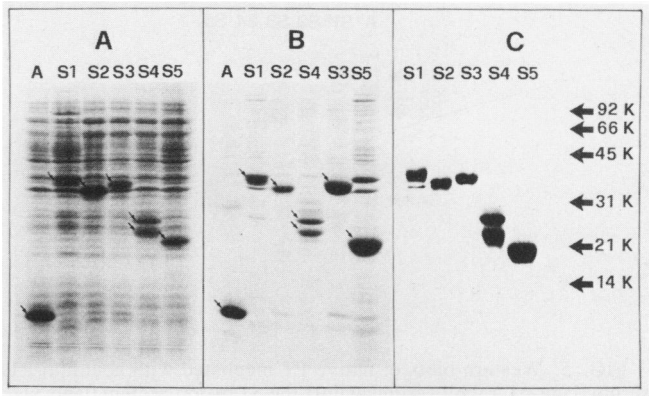

FIG. 4. Expression and purification of the fusion proteins. (A) SDS-polyacrylamide gel electrophoresis of total cell lysates; lane A represents the MS2 DNA polymerase made with the plasmid vector pEx31A. (B) Partially purified fusion proteins (urea extracts). See panel A for description of lane A. (C) Electroeluted fusion proteins.

which contain a fragment of DNA coding for the N terminus of the gene coding for the MS2 DNA polymerase under the control of the p_L promoter of phage lambda. The genes coding for the five subunits of PT were cloned into the above-mentioned vectors as shown in Fig. 3.

Figure 4A shows total cell lysates of the *E. coli* strains producing each of the five subunits. The fusion proteins could be visualized on SDS-polyacrylamide gels and accounted for approximately 5% (S1) to 20% (S5) of the total proteins. Each fusion protein was recognized in Western blots by goat anti-PT serum. In the case of S4, we obtained two bands, and one was probably a proteolytic fragment of the other. In no case were we able to obtain a single band from S4. Following lysis, partial purification, and solubilization with 7 M urea, the fusion proteins were greatly enriched (Fig. 4B). They could be loaded on a preparative SDS-polyacrylamide gel and further purified by electroelution (Fig. 4C). We obtained from 5 to 30 mg of purified proteins from 1 liter of culture; this material was used to immunize mice and rabbits.

Nonprotective purified subunits. The ability of our antigens to induce immunity against pertussis was tested in vivo by the intracerebral challenge assay and in vitro by the CHO cell assay (4). Mice were immunized with three different doses of each of the five subunits or with a mixture of them and then challenged intracerebrally with virulent *B. pertussis*. No protection was obtained in any case. In a parallel experiment, glutaraldehyde-detoxified PT gave 93% protection. In agreement with this result, rabbit sera raised against the five subunits did not protect CHO cells from the activity of 5 ng of PT per ml, while a rabbit antiserum against the native PT was able to protect the cells at a dilution of 1/150. However, the same rabbit antibodies recognized the native PT in the ELISA and the corresponding subunits in Western blots (Fig. 5). Furthermore, rabbit antibodies against the S1 fusion protein inhibited the ADP-ribosylating activity of PT in an in vitro test.

These results suggest that once the subunits are assembled into the native protein, new conformational antigenic determinants are produced which are essential for the neutralization of PT activity.

The absence of protection of mice and the results of CHO tests of the rabbit antiserum against the S1 fusion protein are in contrast with the results of Sato et al. (16), who observed protection in both assays with a monoclonal antibody against

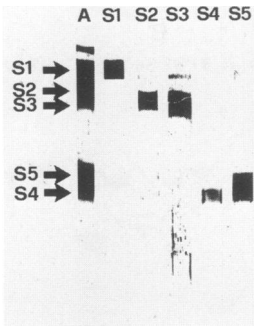

FIG. 5. Western blots of native PT incubated with goat anti-PT serum (lane A), rabbit serum against the S1 fusion protein (lane S1), rabbit serum against the S2 fusion protein (lane S2), rabbit serum against the S3 fusion protein (lane S3), rabbit serum against the S4 fusion protein (lane S4), and rabbit serum against the S5 fusion protein (lane S5).

subunit S1. This result is, however, similar to the finding with diphtheria toxin, where polyclonal anti-fragment A antibodies are not able to neutralize (3, 13), while most of the monoclonal antibodies against fragment A are protective (24).

The availability of polyclonal antibodies against the five subunits has allowed confirmation of the molecular structure of PT proposed on the basis of sequencing of the PT operon (12). The rabbit serum against the S5 fusion protein recognized the native S5 subunit (Fig. 5), providing conclusive evidence that the open reading frame which we had identified as coding for subunit S5 on the basis of amino acid composition (12) is in fact the gene coding for subunit S5.

In agreement with the sequencing analysis, the serum against the S2 subunit recognized both S2 and S3 in Western blots, showing that the two homologous proteins are also immunologically cross-reactive.

Enzymatically active S1 fusion protein. The purified S1 fusion protein (Fig. 4C) was able to ADP-ribosylate transducin in vitro (Fig. 6). The ADP-ribosylating activity was comparable to that of purified PT and, therefore, the MS2 DNA polymerase did not interfere with the enzymatic activity. As in the case of other ADP-ribosylating proteins (e.g., diphtheria toxin fragment A), the fused protein was very stable and maintained its activity after denaturation in urea, boiling, treatment with SDS, and electroelution. The ability to produce large amounts of active S1 is the first step toward the understanding of the structure and function of this enzyme and the identification of its active site, which is a crucial step in the production of nontoxic proteins, should be the basis for the construction of a new, safer vaccine against whooping cough.

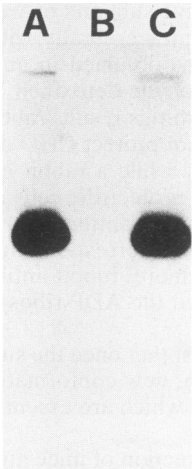

FIG. 6. ADP-ribosylation of transducin by 0.5 μg of native PT (A), 0.5 μg of electroeluted S3 fusion protein (lane B), and 0.1 μg of electroeluted S1 fusion protein (lane C).

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