Immunological Relatedness between *Bordetella pertussis* and Rat Brain Adenylyl Cyclases†

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ABSTRACT: A prokaryotic adenylyl cyclase, secreted by *Bordetella pertussis*, shares a common functional property with eukaryotic adenylyl cyclases, i.e., regulation by the eukaryotic protein calmodulin. Making use of polyclonal antibodies raised against the bacterial adenylyl cyclase and the rat brain adenylyl cyclase catalytic component, respectively, we showed an immunological cross-reactivity between the two enzymes. Furthermore, *B. pertussis* adenylyl cyclase was inhibited and immunoprecipitated by the homologous and one of the heterologous immune sera. These results suggest an evolutionary relationship between the *B. pertussis* enzyme and its eukaryotic counterpart.

Bordetella pertussis, the causative agent of whooping cough, secretes several factors that are thought to be involved in the virulence of this bacterium. One of these factors is an active adenylyl cyclase (Hewlett & Wolff, 1976). *B. pertussis* adenylyl cyclase is released extracellularly and exhibits two unusual properties: it is activated by an eukaryotic protein, calmodulin, which is not known to occur in bacteria (Wolff et al., 1980), and it can invade animal cells, causing unregulated synthesis of cyclic AMP and impairment of cellular functions (Confer & Eaton, 1982). These remarkable properties of *B. pertussis* adenylyl cyclase raised the possibility that this enzyme might have originated from some eukaryotic cyclase that the bacteria picked up during the course of their evolution (van Heyningen, 1982). Since no DNA or amino acid sequences are known for *B. pertussis* and eukaryotic adenylyl cyclases, the hypothesis of a common evolutionary origin of the two enzymes remained an attractive speculation.

Having obtained polyclonal antibodies raised against the purified *B. pertussis* adenylyl cyclase and the rat brain synaptosomal adenylyl cyclase catalytic component, respectively, we checked the above hypothesis by performing immunological cross-reactions between the two enzymes.

In the present paper, we report that the bacterial and brain adenylyl cyclases are immunologically related, thus suggesting a common evolutionary origin of the two enzymes. The fact that calmodulin, an eukaryotic regulatory protein, highly conserved during evolution, stimulates a bacterial protein furthermore suggests that *B. pertussis* adenylyl cyclase could, indeed, be of eukaryotic origin.

Experimental Procedures

Purification of Enzymes. B. pertussis Adenylyl Cyclase. B. pertussis 18323, phase I (type strain ATCC 9797), was grown and harvested as previously described (Ladant et al., 1986). Twenty grams of bacteria was extracted with 4 M urea in buffer A (25 mM Tris-HCl,[1] pH 8, 6 mM $MgCl_2$, and 0.1% Triton X-100). After dialysis for 24 h against buffer A, adenylyl cyclase from 80 mL of "urea extract" (10 units/mL, 4 units/mg of protein) was adsorbed onto 4 mL of Affi-Gel–calmodulin (Bio-Rad) for 6–8 h at 4 °C in a rotary shaker (30 rpm). The gel was washed twice with 50 mL of buffer A; then adenylyl cyclase was desorbed from the gel with 8 M urea in buffer A. Urea was removed by gel filtration of the enzyme preparation on Sephadex G-25 equilibrated with buffer A. Adenylyl cyclase (30–50 μg/mL; specific activity between 1200 and 1600 units/mg of protein) was stored at −20 °C. On SDS–PAGE, the purified preparations contained three bands between 43 and 50 kDa, as has been reported for the extracellular enzyme (Ladant et al., 1986). From 20 g of bacteria one obtained between 150 and 200 μg of pure protein, corresponding to an overall yield of about 40%. One unit of enzyme activity corresponds to 1 μmol of cAMP formed per minute at 30 °C and pH 8. Enzyme activity was measured as previously described (Ladant et al., 1986).

Escherichia coli adenylate kinase was purified according to the method of Bârzu and Michelson (1983). *E. coli* adenylyl cyclase–β-galactosidase hybrid protein (monomer = 175 kDa) was purified according to the method of Ullmann (1984).

Rat Brain Adenylyl Cyclase Catalytic C Component. We have described the purification of a 155-kDa protein, identified as the brain enzyme catalytic C component (Coussen et al., 1985, 1986). We have also identified in brain other adenylyl cyclase catalytic components, of 130 and 105 kDa (Coussen et al., 1986; Monneron et al., 1987). The 155-kDa catalytic component C, but not the other components, has also been purified in other laboratories (Pfeuffer et al., 1985; Yeager et al., 1985). In this study, we used a simplified procedure to obtain the 155-kDa protein. Rat brain synaptosomes (250 mg) were prepared according to the method of d'Alayer et al. (1983). They were incubated for 20 min at 37 °C in the presence of 100 μM guanylyl imidodiphosphate and 5 mM $MgCl_2$ and solubilized at 4 °C in a medium containing 0.9% Lubrol Px and inhibitors of proteases (Coussen et al., 1986). The sample was centrifuged at 45000*g* for 10 min at 4 °C. The Lubrol-soluble extract (120 mg of protein) was adjusted

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[1] Abbreviations: Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; kDa, kilodalton(s).

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to 0.5 M NaCl and 1 mM $MgCl_2$ and incubated overnight at 4 °C with 4 mL of forskolin–agarose on a roller. The resin had been prepared according to the method of Pfeuffer et al. (1985). The resin was washed, and the bound protein was eluted with 100 μM forskolin (Coussen et al., 1986). The adenylyl cyclase activity of this fraction was 12 ± 3 μmol of $cAMP \cdot min^{-1} \cdot mg^{-1}$. (Protein was estimated by densitometry of the gels.) Upon forskolin removal, the enzyme activity was 4-fold stimulated by 1 μM calmodulin. The proteins of the preparation were recovered as described by Coussen et al. (1986) and analyzed by SDS–PAGE. A band at 155 kDa was by far the major component (Figure 2A).

Immunoblots. Proteins separated by SDS–PAGE were transferred to nitrocellulose sheets and incubated with sera as described by Towbin et al. (1979). The immunochemical detection was performed with ^{125}I-labeled protein A. When transferred onto nitrocellulose sheets, the rat brain C component containing fraction showed three major Ponceau red stained protein bands, of 155, 64, and 58 kDa (Figure 2E). The latter two bands were more conspicuous on the Ponceau red stained transfer than in silver-stained gel.

Immunization Procedures. Guinea pig polyclonal immune sera against *B. pertussis* adenylyl cyclase were obtained by immunizing the animals 5 times intravenously at 10-day intervals with pure native or denatured adenylyl cyclase, the latter one obtained by boiling the enzyme preparation for 5 min at 100 °C in the presence of 2% SDS, 5% 2-mercaptoethanol, 3% glycerol, and 50 mM Tris-HCl, pH 7. Native and denaturated enzymes were injected with poly(A)–poly(U) adjuvant (0.4 mg of adjuvant/injection). Guinea pigs were bled before immunization for preimmune serum samples. Ten days after the last booster, sera were collected.

Rabbit polyclonal immune sera against the *B. pertussis* and rat brain C-subunit adenylyl cyclases were prepared according to the method of Jacob et al. (1986). For *B. pertussis* adenylyl cyclase, the 43–50-kDa bands were cut from the blots and injected. For the rat brain C component, rabbits (25 and 26) were immunized with the 155-kDa protein-containing strip. One rabbit (27) was immunized with the 58–64-kDa region of the blotted rat brain preparation.

Inhibition of B. pertussis Adenylyl Cyclase. Sixty microliters of pure adenylyl cyclase (0.1 unit/mL) in buffer C (50 mM Tris-HCl buffer, pH 8.0, 0.1% NP-40, and 0.1 mM $CaCl_2$) supplemented with 10 nM bovine brain calmodulin and 0.1 mg/mL bovine serum albumin was incubated with increasing amounts of immune sera or preimmune sera. After 15 h of incubation at 4 °C, adenylyl cyclase activity was assayed as previously described (Ladant et al., 1986). The activity of samples without added sera was taken as 100%.

Immunoprecipitation of ^{125}I-Labeled B. pertussis Adenylyl Cyclase. A preparation of the purified bacterial adenylyl cyclase, comprising three polypeptides of 43, 45, and 50 kDa, as well as bovine brain calmodulin, present in excess over the enzyme, was subjected to iodination as previously described (Ladant et al., 1986). All four polypeptides were iodinated (2×10^7 cpm per unit of enzymatic activity) (Figure 3B, lane 1). The preparation was incubated for 16 h at 4 °C in a final volume of 60 μL of buffer C containing 1 μM calmodulin, 0.1 mg/mL bovine serum albumin, and preimmune or immune serum as indicated in the text. To each tube was added 50 μL of 50 mM Tris-HCl buffer, pH 8.0, containing 5 mg of protein A–Sepharose. Incubation was continued for 1 h at 4 °C with occasional shaking. The preparation was centrifuged (2000*g*, 5 min, 4 °C). The pellet was washed twice with 0.5 mL of Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl, 0.1 mM $CaCl_2$, and 0.5% NP-40, resuspended in Laemmli sample buffer (Laemmli, 1970), and boiled for 2 min. The preparation was centrifuged (2000*g*, 5 min, 20 °C) and the supernatant applied on 10% SDS–PAGE. After electrophoresis, the gel was dried and autoradiographed at −70 °C.

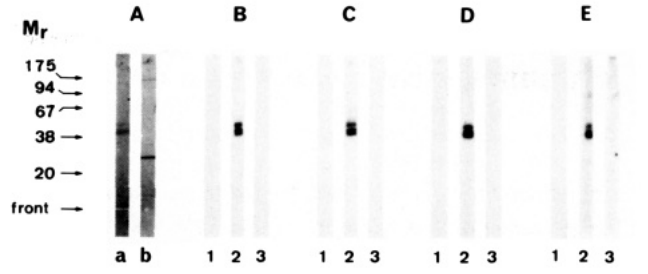

FIGURE 1: Immunochemical detection of the *B. pertussis* adenylyl cyclase by homologous and heterologous immune sera. Purified *B. pertussis* adenylyl cyclase (a, 1, and 2) and a mixture of purified *E. coli* adenylyl cyclase–β-galactosidase hybrid protein and *E. coli* adenylate kinase (b and 3) were separated by SDS–PAGE (10%), transferred to a nitrocellulose sheet, and reacted with sera diluted 1:500. Lanes 1, preimmune sera corresponding to immune sera of lanes 2 and 3. (A) Ponceau red staining of the immunoblotted preparations; (B and C) anti *B. pertussis* adenylyl cyclase sera [(B) obtained in guinea pigs and (C) in rabbit]; (D and E) anti rat brain C component immune sera [(D) 27; (E) 25].

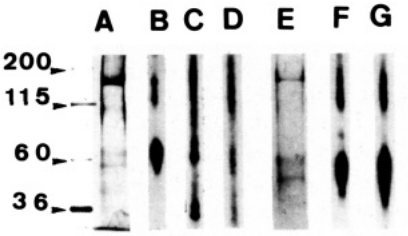

FIGURE 2: Immunoblots of the rat brain adenylyl cyclase catalytic components. The purified enzyme preparations were separated by SDS–PAGE (7.5–10%). Lane A represents the silver-stained protein pattern of the preparation in immunoblots shown in lanes B, C, and D. Lane E represents the Ponceau red stained transfer of the same preparations used in immunoblots in lanes F and G. The immune sera were diluted 1:250. Lane B, immune serum 27 raised against the rat brain 64-kDa component; lanes C and D, guinea pig (C) and rabbit (D) immune sera raised against purified *B. pertussis* adenylyl cyclase; lanes F and G, immune sera raised against the rat brain 155-kDa C component (25 and 26).

Phosphodiesterase Assay. Calmodulin-dependent and -independent phosphodiesterase activities in the rat brain extracts and adenylyl cyclase preparations were measured according to the procedure of Rutten et al. (1973) in an assay medium with or without 1 μM calmodulin (Klee et al., 1979).

RESULTS

Characterization of the Antibodies. Anti *B. pertussis* adenylyl cyclase antibodies recognized in immunoblots of the purified bacterial adenylyl cyclase preparation all three bands of 43, 45, and 50 kDa (Figure 1). Other *B. pertussis* toxins, such as pertussis toxin or filamentous hemagglutinin, were not recognized by the immune sera. The sera raised against the rat brain blotted 155-kDa adenylyl cyclase catalytic component, or against the 64-kDa protein, all recognized the rat brain 155-kDa protein, a 130-kDa protein (not detected on Ponceau red stained transfers), and two components of 58 and 64 kDa (Figure 2B,F,G). The 130-kDa protein probably represents the proteolytic fragment of the 155-kDa catalytic component described earlier (Monneron et al., 1987). The 64-kDa protein content of our final preparation was found to increase, seemingly at the expense of the 155-kDa protein, with the length of the acid precipitation step used to recover the proteins of

the final preparation. The identical recognition pattern given by the antibodies raised against the 155-kDa and the 64-kDa proteins is also suggestive of the structural relationship between these two components. However, since a calmodulin-dependent phosphodiesterase of 64 kDa is abundant in brain synaptosomes (Klee et al., 1979), we checked for its presence in our purified adenylyl cyclase final preparation. Neither calmodulin-dependent nor calmodulin-independent phosphodiesterase activity could be detected. It is, therefore, unlikely that the immune sera contained antibodies directed against phosphodiesterase.

Immunological Cross-Reaction between B. pertussis Adenylyl Cyclase and Brain Adenylyl Cyclase C Components. In order to search for homology between brain and *B. pertussis* adenylyl cyclases, we tested antibodies directed against the two enzymes for cross-reaction in "Western blots". Figure 1 shows that the antibodies directed against the rat brain 155-kDa C component (Figure 1E), as well as the antibodies raised against the brain 64-kDa protein (Figure 1D), gave the same recognition pattern of *B. pertussis* purified adenylyl cyclase as did the homologous antibodies raised against the bacterial enzyme (Figure 1B,C). The preimmune sera gave no detectable signals. These results suggest that antibodies specific for the brain adenylyl cyclase C components specifically reacted with *B. pertussis* adenylyl cyclase. One could argue, however, that the positive sera might not be specific for the two calmodulin-sensitive adenylyl cyclases but might simply recognize the ATP-binding sites of these and unrelated enzymes, or domains shared by other bacterial adenylyl cyclases. To eliminate these possibilities, we tested for possible cross-reaction in Western blots two *E. coli* enzymes: adenylate kinase and the adenylyl cyclase–β-galactosidase fusion protein, the latter exhibiting both adenylyl cyclase and β-galactosidase activities (Danchin et al., 1984). As shown in Figure 1B–E (lanes 3), none of the sera reacted with the latter enzymes. The antibodies raised against the brain 155-kDa C catalytic component thus specifically recognized *B. pertussis* adenylyl cyclase.

Conversely, when antibodies raised against *B. pertussis* adenylyl cyclase were tested for cross-reaction with the brain adenylyl cyclase catalytic C subunit and its putative cleavage products, they gave positive reactions (Figure 2C,D) comparable to those obtained with the anti rat brain adenylyl cyclase antibodies (Figure 2B,F,G). The preimmune sera gave no signals. As mentioned above, the rat brain 155-kDa and 64-kDa proteins seem to be structurally related, since antibodies raised against either of them recognized both polypeptides. The finding that both antisera recognized *B. pertussis* adenylyl cyclase reinforces the hypothesis of the structural relationship between the two proteins, the 64-kDa protein probably being a cleaved fragment of the 155-kDa catalytic component.

Homologous and Heterologous Antibodies Inhibit B. pertussis Adenylyl Cyclase Activity and Immunoprecipitate the Enzyme. In order to substantiate further the specificity of the immunological cross-reactivity, we studied the effects of the different antisera on inhibition of adenylyl cyclase enzymatic activity. Figure 3A shows that *B. pertussis* adenylyl cyclase activity was inhibited by the two homologous immune sera, albeit not to the same extent. Most significantly, the serum raised against the rat brain 64-kDa protein also inhibited the activity of the bacterial enzyme. Immunoprecipitation of *B. pertussis* adenylyl cyclase with the two homologous immune sera and one heterologous immune serum was also tested in a parallel experiment conducted with ^{125}I-labeled purified *B.*

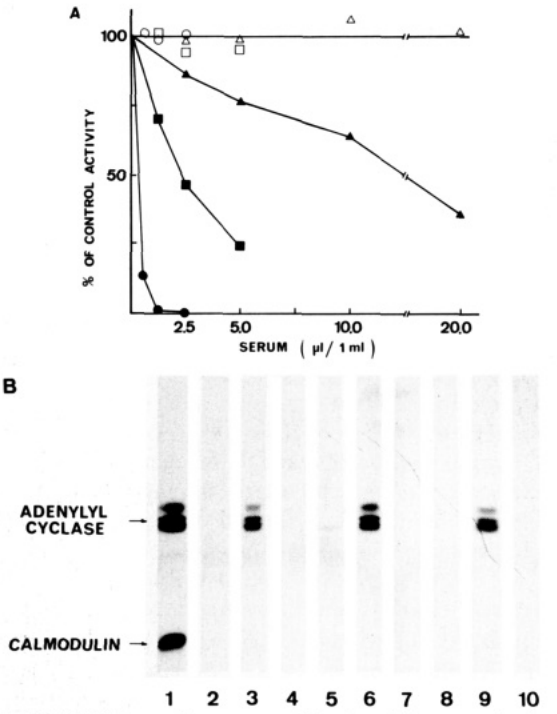

FIGURE 3: Inhibition and immunoprecipitation of *B. pertussis* adenylyl cyclase by homologous and heterologous immune sera. (A) Purified adenylyl cyclase was incubated with increasing amounts of preimmune or immune sera at 4 °C for 15 h. Adenylyl cyclase was then assayed. Open symbols, preimmune sera; closed symbols, immune sera. (●) and (■) Anti *B. pertussis* adenylyl cyclase sera obtained in guinea pigs (●) or rabbit (■); (▲) anti rat brain C subunit serum 27. (B) Purified fractions of iodinated *B. pertussis* adenylyl cyclase (3×10^{-3} units, 6×10^4 cpm of ^{125}I per tube) were incubated with preimmune or immune sera and processed as described under Experimental Procedures. In lane 1, a typical adenylyl cyclase fraction not subjected to addition of serum and protein A–Sepharose is shown. Lanes 2, 3, and 4 correspond to fractions immunoprecipitated with 1:2000 dilutions of guinea pig serum (lane 2, preimmune serum; lanes 3 and 4, immune serum raised against *B. pertussis* adenylyl cyclase). Lanes 5, 6, and 7 correspond to fractions immunoprecipitated with 1:2000 dilutions of rabbit serum (lane 5, preimmune serum; lanes 6 and 7, immune serum raised against *B. pertussis* adenylyl cyclase). Lanes 8, 9, and 10 correspond to fractions immunoprecipitated with 1:50 dilutions of serum 27 (lane 8, preimmune serum; lanes 9 and 10, immune serum raised against the rat brain 64-kDa polypeptide). Lanes 4, 7, and 10 correspond to ^{125}I-labeled fractions to which a 100-fold excess of unlabeled *B. pertussis* adenylyl cyclase was added prior to the addition of immune sera.

pertussis adenylyl cyclase. As shown in Figure 3B the three immune sera specifically immunoprecipitated the enzyme; in the presence of an excess of unlabeled adenylyl cyclase or in the presence of preimmune sera the iodinated enzyme was not immunoprecipitated. The *E. coli* adenylyl cyclase–β-galactosidase fusion protein was neither inhibited nor precipitated by these sera (not shown). However, antisera raised against the 155-kDa brain catalytic subunit did not inhibit or immunoprecipitate the *B. pertussis* enzyme.

Taken as a whole, these results are again in favor of an immunological relatedness between the bacterial and the mammalian adenylyl cyclases. It could be argued, however, that the immune sera had detected a common calmodulin-binding site shared by these and other calmodulin-binding proteins. This interpretation is unlikely since immunoprecipitation of the bacterial enzyme was not modified by a large excess of calmodulin over antibodies and since the adenylyl cyclase/calmodulin complex is very stable (Ladant et al., 1986).

Parallel experiments have been conducted with the brain adenylyl cyclase, but neither homologous nor heterologous

antibodies inhibited enzymatic activity in Lubrol extracts, suggesting either the absence of active-site-directed antibodies or a nonaccessibility of the active site to antibodies.

Discussion

Our experiments establish the existence of an immunological relatedness between *B. pertussis* and brain adenylyl cyclases: antibodies specific for either enzyme reacted with the heterologous counterpart. The specificity of the recognition seems demonstrated since none of the preimmune sera gave any positive reaction and none of the immune sera reacted with an adenylyl cyclase that is not regulated by calmodulin.

Furthermore, homologous immune sera as well as a heterologous one inhibited *B. pertussis* adenylyl cyclase activity and precipitated the enzyme. The extent of enzyme inhibition was, however, dependent upon the type of antisera. This is not surprising since the occurrence of active-site-directed antibodies can vary from one immune serum to another. Besides, the immune sera raised against the brain catalytic subunit were obtained with a denatured enzyme preparation.

Such an immunological relatedness between the bacterial and brain adenylyl cyclases is suggestive of a common evolutionary origin of the two enzymes. Besides catalyzing the same reaction, they share a striking functional property, namely, calmodulin regulation. So far, only two bacterial adenylyl cyclases are known to be stimulated by calmodulin: those of *B. pertussis* and *Bacillus anthracis* (Leppla, 1982), both being toxins secreted in culture supernatants and able to enter animals cells.

On the basis of the evolutionary distance between bacteria and mammals, a convergent evolution of the two adenylyl cyclases seems unlikely. The immunological relatedness could indicate that the two enzymes originate from a common ancestor to bacteria and mammals and that a functional selection maintained some common properties such as calmodulin regulation. However, since calmodulin is not known to occur in bacteria, we would rather suggest that the bacterial enzyme arose by gene transfer from the host eukaryote to the bacterium.

Although natural gene transfer from prokaryotes to eukaryotes is well known, examples of the reverse transfer are scarce. So far, the best documented example for the eukaryotic origin of a bacterial enzyme is the case of glutamine synthetase II from *Bradyrhizobium japonicum* (Carlson & Chelm, 1986). The structural similarity between a bacterial and eukaryotic copper/zinc superoxide dismutase has been accounted for by a gene transfer between the host ponyfish and the symbiotic bacterium (Bannister & Parker, 1985). This proposal has been, however, challenged recently (Leunissen & de Jong, 1986). Finally, the existence of an extensive amino acid sequence homology between a membrane glycoprotein, encoded by a mammalian multidrug resistance gene, and several bacterial periplasmic transport proteins (Chen et al., 1986; Gros et al., 1986) argues in favor of a common evolutionary origin of these proteins.

From immunological tests alone, no definite conclusion can be reached as to the degree of homology between *B. pertussis* and mammalian adenylyl cyclases. They provide, however, for the first time an experimental basis for their relatedness. Comparisons of macromolecular sequences, as soon as they are available, will permit the quantitative assessment of the evolutionary relationship between the two adenylyl cyclases.

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