Isolation of a Protein Fraction from *Bordetella pertussis* That Facilitates Entry of the Calmodulin-Sensitive Adenylate Cyclase into Animal Cells†

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ABSTRACT: *Bordetella pertussis*, the pathogen responsible for whooping cough, releases a soluble calmodulin-sensitive adenylate cyclase into its culture medium. Several investigators have shown that the partially purified adenylate cyclase is capable of entering animal cells and elevating intracellular cAMP levels [Confer, D. L., & Eaton, J. W. (1982) *Science 217*, 948-950; Shattuck, R. L., & Storm, D. R. (1985) *Biochemistry 24*, 6323-6328]. However, the mechanism for entry of the catalytic subunit of the adenylate cyclase into animal cells is unknown. Recently, it was determined that the purified catalytic subunit of the enzyme is unable to enter animal cells [Masure, H. R., Oldenburg, D. J., Donovan, M. G., Shattuck, R. L., & Storm, D. R. (1988) *J. Biol. Chem. 263*, 6933-6940]. On the basis of these data and other observations, we hypothesized that the culture medium of *B. pertussis* contains one or more additional polypeptides which facilitate entry of the adenylate cyclase catalytic subunit into animal cells. In this study, we report that a cell-invasive preparation of *B. pertussis* adenylate cyclase was rendered noninvasive after passage through a wheat germ lectin-agarose column. A fraction was eluted from the wheat germ lectin-agarose column with *N*-acetyl-D-glucosamine. This fraction, when combined with the noninvasive adenylate cyclase, was able to restore the ability of the adenylate cyclase preparation to enter neuroblastoma cells and increase intracellular cAMP levels. Furthermore, the fraction eluted from the wheat germ lectin-agarose column was found to be trypsin and chymotrypsin sensitive, suggesting that this material was proteinaceous. SDS gel electrophoresis of the eluate from the wheat germ lectin-agarose column revealed the presence of three polypeptides with apparent molecular mass values of 26, 28, and 30 kDa. These data provide the first direct evidence for the existence of an additional, separable protein component produced by *B. pertussis* that is required for entry of the catalytic subunit into animal cells.

Bordetella pertussis is a small, Gram-negative bacillus that is the pathogen responsible for whooping cough (Olson, 1975; Wardlaw & Parton, 1988). The culture medium of growing *B. pertussis* contains a number of biologically active components which are thought to play a role in the pathogenesis of the disease. One of these, islet activating protein (IAP),[1] has been purified and shown to attenuate receptor-mediated inhibition of adenylate cyclase in a variety of mammalian cell types (Katada & Ui, 1981; Yajima et al., 1978). Another factor which may play a role in the pathogenesis of whooping cough is a soluble adenylate cyclase [for a review, see Masure et al. (1987)]. An unusual property of this enzyme is that it is stimulated by calmodulin (CaM), although *B. pertussis* does not contain CaM (Wolff et al., 1980). In addition, the activation of the adenylate cyclase by CaM is unique in that it is stimulated both in the presence and in the absence of free Ca^{2+} (Greenlee et al., 1982; Killhoffer et al., 1983). The relatively high affinity of the enzyme for CaM, even at low Ca^{2+} concentrations, supports the proposed role of the adenylate cyclase as an intracellular toxin.

Confer and Eaton (1982) as well as Hanski and Farfel (1985) have shown that incubation of crude preparations of the *B. pertussis* adenylate cyclase with animal cells causes an increase in intracellular cAMP levels. Furthermore, an adenylate cyclase preparation, that had been purified to remove contaminating IAP, was found to elevate intracellular cAMP levels in human erythrocytes and mouse neuroblastoma cells (Shattuck & Storm, 1985). Invasion of animal cells by this enzyme preparation was inhibited by addition of exogenous CaM. Since the increase in intracellular cAMP occurred in mature red blood cells, which contain no endogenous adenylate cyclase activity, the elevated cAMP was not due to stimulation of a mammalian adenylate cyclase by a bacterial factor. These data indicated that the catalytic subunit of the *B. pertussis* adenylate cyclase is capable of entering animal cells. However, the mechanism for translocation of the adenylate cyclase catalytic subunit across the target cell membrane is unknown.

Recently, we reported that the *B. pertussis* adenylate cyclase is synthesized as a large precursor molecule (215 kDa). This precursor is transported to the outer membrane of the bacteria where it is proteolytically processed to a smaller form (45 kDa) that is released into the culture supernatant (Masure & Storm, 1989). The catalytic subunit of the adenylate cyclase that is isolated from bacterial culture supernatant has been purified to homogeneity. The purified catalytic subunit did not elevate intracellular levels of cAMP when incubated with N1E-115 mouse neuroblastoma cells. Although this suggests that there may be additional protein(s) released into the culture medium required for entry of the catalytic subunit into animal cells,

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[1] Abbreviations: IAP, islet activating protein; CaM, calmodulin; DMEM, Dulbecco's modified essential medium; GlcNAc, *N*-acetyl-D-glucosamine; WGA, wheat germ agglutinin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; QAE, diethyl(2-hydroxypropyl)aminoethyl; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride.

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no direct evidence for such a factor has been presented. In this paper, we report that there is a separable protein fraction that facilitates entry of the *B. pertussis* adenylate cyclase into neuroblastoma cells.

Materials and Methods

Materials. QAE-Sephadex, wheat germ agglutinin–agarose, and CNBr-activated Sepharose 4B were purchased from Pharmacia. Protein kinase, cAMP, and wheat germ agglutinin were from Sigma. [α-^{32}P]ATP was purchased from ICN, and [^{3}H]cAMP was purchased from International Chemical Nuclear. Molecular weight standards for SDS gel electrophoresis were from Bio-Rad. Dulbecco's modified essential medium (DMEM) with high glucose content (4.5 g/L) and fetal calf serum were purchased from Grand Island Biological Co. All other reagents were of the finest available grade from commercial sources.

Purification of an Invasive Calmodulin-Sensitive Adenylate Cyclase Preparation. B. pertussis (Tohama phase I strain) was grown from a 5% inoculum in supplemented Stainer–Scholte medium (Stainer & Scholte, 1971). Invasive preparations of the adenylate cyclase were purified from culture supernatants by QAE-Sephadex chromatography as described by Shattuck et al. (1985). Preparations of the enzyme collected from QAE-Sephadex chromatography were concentrated by ultrafiltration using Amicon PM-10 membranes and stored at −80 °C. The specific activity of these preparations was 22.8 μmol of cAMP min^{-1} mg^{-1} (±2.3, n = 3).

Wheat Germ Lectin–Agarose Chromatography. An invasive adenylate cyclase preparation (1.0 mL) was loaded at 30 mL/h onto a WGA–agarose column (1.5 mL) preequilibrated in a buffer which contained 20 mL Tris-HCl (pH 7.5), 40 mM NaCl, and 2 mM $MgCl_2$ (equilibration buffer). The column was washed with 4 column volumes of equilibration buffer and then eluted with 4 column volumes of the equilibration buffer containing 0.1 M GlcNAc. Fractions (0.5 mL) were collected from the column and assayed both for adenylate cyclase activity and for protein concentration. Peak fractions of adenylate cyclase activity were pooled and stored at −80 °C.

Adenylate Cyclase Assay. Adenylate cyclase was assayed at 30 °C by the method of Salomon et al. (1974) using [α-^{32}P]ATP as a substrate and [^{3}H]cAMP to monitor product recovery. Each assay contained 20 mM Tris-HCl (pH 7.5), 1 mM [α-^{32}P]ATP (10 cpm/pmol), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM β-mercaptoethanol, 0.1% (w/v) BSA, and 2.4 μM CaM in a final volume of 250 μL. Results are presented as the mean of duplicate assays.

Neuroblastoma Cells. N1E-115 mouse neuroblastoma cells (passages 16–28) were grown at 37 °C in DMEM supplemented with 5% fetal calf serum, without antibiotics, in an atmosphere of 10% CO_2/90% humidified air. The cells were grown to 80–90% confluency in plastic tissue culture dishes (60 mm) prior to the start of each experiment. The cells were subcultured weekly, and the culture medium was changed on days 3 and 5 and daily thereafter.

Cell Entry Experiments. On the day of the experiment, cultures were washed with serum-free DMEM and then preincubated for 20 min at 37 °C in 3 mL of DMEM supplemented with 5 mM theophylline and buffered with 10 mM Hepes–HCl (pH 7.4). After preincubation, cells were treated with various preparations of the adenylate cyclase for 20 min at 37 °C. Typically, a minimum of 100 nmol/min of enzyme activity was applied per plate of cells. The enzyme-containing solution was removed; the cells were washed twice with PBS and assayed for intracellular cAMP. In addition, each cell entry experiment included a determination of basal adenylate cyclase activity since we have observed this activity to vary, dependent upon the passage of neuroblastoma cells being used.

Intracellular cAMP Determination. Intracellular cAMP was determined by the method of Gilman (1970). Cells, which had been treated with various preparations of adenylate cyclase, were lysed with 4 mL of 5% trichloroacetic acid. The protein precipitate was removed by centrifugation, and cAMP was isolated from the supernatant by chromatography on 2-mL AG50W-X4 (200–400 mesh) Dowex columns. [^{3}H]cAMP was included for determination of column recovery. The concentration of cAMP was measured by a competition binding assay using the regulatory subunit of cAMP-dependent protein kinase. Results represent the mean and range of triplicate samples.

SDS–Polyacrylamide Gel Electrophoresis. SDS–polyacrylamide gel electrophoresis was performed as described by Laemmli (1970). Proteins were visualized with silver as described by Ansorge (1985).

Determination of Protein. Protein concentrations for the Gilman assay were determined by the method of Lowry et al. (1951). Samples were prepared by incubating the precipitated protein from the tissue culture plates with 2 mL of 1 N NaOH until they dissolved. Samples were then aliquoted for protein determination. Protein concentrations for WGA–agarose chromatography were determined by the method of Bradford (1976).

Iodo-WGA Gel Overlay. Adenylate cyclase (invasive preparation isolated by QAE-Sephadex chromatography) was subjected to SDS–PAGE [7.5% acrylamide/0.17% bis-(acrylamide)]. The gel was fixed with 40% methanol/10% acetic acid and washed with 10% ethanol overnight. The gel was washed with distilled water and equilibrated with buffer A (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 1 mg/mL defatted bovine serum albumin, and 1 mM $CaCl_2$) for 30 min. The gel was incubated in buffer A containing ^{125}I-WGA for 16 h. The gel was then washed 6 times with buffer A to remove nonspecifically bound ^{125}I-WGA. The gel was stained with Coomassie blue, dried, and autoradiographed.

Purification of the Catalytic Subunit. The 45-kDa catalytic subunit of the adenylate cyclase was purified to homogeneity by the procedure of Haiech et al. (1988). This procedure utilizes a genetically engineered CaM (VU-8) that has decreased affinity for the adenylate cyclase to construct an affinity matrix. VU-8 CaM was prepared as described by Craig et al. (1987) and coupled to CNBr-activated Sepharose 4B, yielding a final concentration of 2.5 mg of CaM/mL of resin. The resin was equilibrated in buffer B (50 mM Tris-HCl, pH 8, 6 mM $MgCl_2$, 20 mM NaCl, and 0.1 mM $CaCl_2$). Concentrated invasive adenylate cyclase (3 mL) was adjusted to 2 mM $CaCl_2$ and batched with VU-8 CaM–Sepharose (5 mL) for 1 h at 4 °C. The column was washed with 4 column volumes of buffer B followed by 8 column volumes of buffer B adjusted to 0.5 M NaCl. The column was eluted with buffer B containing 0.5 M NaCl and 3 mM EGTA. The column eluate was adjusted to 0.1 mM $CaCl_2$ prior to assaying for CaM-stimulated adenylate cyclase activity. Fractions containing peak enzyme activity were pooled and used the same day for cell entry experiments. The specific activity of the CaM–Sepharose-purified adenylate cyclase was 32 μmol min^{-1} mg^{-1} (±5.2, n = 3).

Results

Effect of Wheat Germ Agglutinin on the Invasion of Neuroblastoma Cells by B. pertussis Adenylate Cyclase. During an examination of possible inhibitors of adenylate cyclase entry into aminal cells, it was discovered that WGA

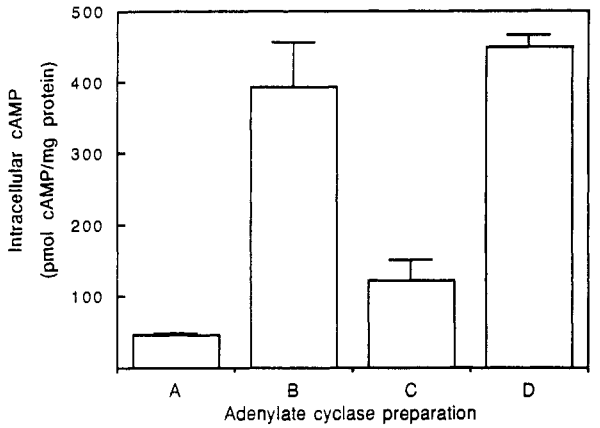

FIGURE 1: Wheat germ lectin inhibition of adenylate cyclase entry into N1E-115 cells. Neuroblastoma cell cultures were treated as described under Materials and Methods. During preincubation, the cells were treated with DMEM supplemented with 5 mM theophylline and buffered with 10 mM Hepes–HCl (pH 7.4) plus (A) no addition, (B) no addition, (C) 50 μg/mL WGA, and (D) 50 mM GlcNA and 50 μg/mL WAG. After preincubation, each plate of cells was incubated with 100 nmol/min of an invasive adenylate cyclase preparation (B–D) or with no addition (A) for 20 min at 37 °C. Intracellular cAMP was measured as described under Materials and Methods.

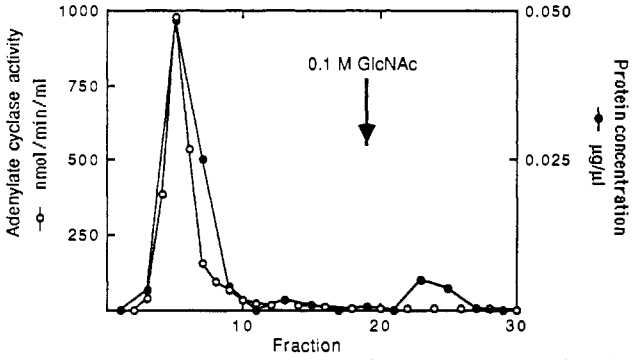

FIGURE 2: Wheat germ lectin–agarose chromatography. An invasive adenylate cyclase preparation (1202 nmol/min) was isolated from culture supernatant by the method of Shattuck et al. (1985) and applied to a WGA–agarose column (1.5 mL). The column was washed and eluted with 0.1 M GlcNAc as described under Materials and Methods. Fractions (0.5 mL) collected from the column were assayed for both adenylate cyclase activity and for protein concentration.

inhibited entry of *B. pertussis* adenylate cyclase into neuroblastoma cells (Figure 1). The invasive adenylate cyclase preparation, isolated from bacterial culture supernatant by QAE-Sephadex chromatography, increased intracellular cAMP levels in neuroblastoma cells approximately 8-fold. Pretreatment of the neuroblastoma cells with 50 μg/mL WGA almost completely inhibited this elevation in intracellular cAMP levels. Furthermore, WGA inhibition of cell entry was blocked by GlcNAc, a sugar specific for this lectin.

Wheat Germ Agglutinin–Agarose Chromatography. The effect of WGA on entry of the adenylate cyclase into neuroblastoma cells may have resulted from a direct interaction of the lectin with the catalytic subunit of the adenylate cyclase or with some other protein present in the adenylate cyclase preparation. WGA–agarose chromatography was utilized to determine whether any component of the invasive adenylate cyclase preparation interacted with the lectin. All of the adenylate cyclase activity that was applied to the WGA–agarose column appeared in the flow-through and showed no interaction with WGA (Figure 2). A small percentage of the total protein did absorb to WGA–agarose and was specifically eluted from the column with 0.1 M GlcNAc. This latter

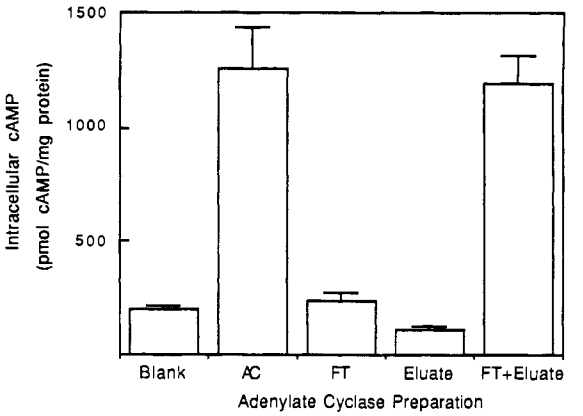

FIGURE 3: Reconstitution of the cell-invasive properties of the adenylate cyclase. Neuroblastoma cells were grown and treated as described under Materials and Methods. Following preincubation, cells were incubated for 20 min at 37 °C with DMEM (supplemented with 5 mM theophylline/10 mM Hepes–HCl, pH 7.4) that contained (blank) no addition, (AC) invasive adenylate cyclase preparation (150 nmol min^{-1} $plate^{-1}$), (FT) flow-through material from WGA–agarose chromatography (150 nmol min^{-1} $plate^{-1}$), (eluate) protein eluted from the WGA–agarose column using GlcNAc (4.2 μg/plate), and (FT + eluate) flow-through (150 nmol min^{-1} $plate^{-1}$) and eluate (4.2 μg/plate) precombined and then applied to the cells. Intracellular cAMP was measured as described under Materials and Methods.

fraction contained no detectable adenylate cyclase activity.

Reconstitution of the Cell-Invasive Properties of the Adenylate Cyclase. The two protein fractions isolated by WGA–agarose chromatography were examined for their effect on intracellular cAMP levels in neuroblastoma cells (Figure 3). Although the flow-through (FT) exhibited adenylate cyclase activity, there was no increase in intracellular levels of cAMP when this material was incubated with neuroblastoma cells. Similarly, the protein fraction that absorbed to the WGA–agarose column (eluate) did not increase the intracellular levels of cAMP. However, combination of the adenylate cyclase containing fraction with the fraction eluted by GlcNAc restored the ability of the enzyme preparation to invade neuroblastoma cells and elevate intracellular cAMP concentrations.

Characterization of the WGA Column Eluate. Pretreatment of the eluate from WGA–agarose chromatography with either trypsin or chymotrypsin destroyed the ability of this fraction to restore cells invasiveness to the adenylate cyclase and confirmed the proteinaceous nature of the reconstituting activity (Table I). SDS gel electrophoresis of the eluate from the WGA column revealed the presence of three polypeptides with apparent molecular weights of 26000, 28000, and 30000 (Figure 4). The presence of only three major polypeptides in this fraction and the ease with which they were separated by SDS gel electrophoresis should permit future sequencing analysis to determine whether they are distinct proteins or, instead, are related as a result of proteolytic processing.

^{125}I-WGA Binding to an Invasive Preparation of the Adenylate Cyclase. The association of these bacterial peptides with WGA–agarose implied that they may be glycoproteins. This was surprising since glycoproteins are rarely found in bacteria. However, binding of these peptides to WGA–agarose could have been due to indirect associations with a glycolipid contaminant. Therefore, direct interaction between WGA and components of the invasive adenylate cyclase preparation was examined by using ^{125}I-WGA and the SDS gel overlay technique described under Materials and Methods (Figure 5). This experiment revealed the presence of several ^{125}I-WGA binding polypeptides in the partially purified adenylate cyclase preparation that migrated with apparent molecular weights

Table I: Proteolytic Degradation of WGA Eluate Prevents Reconstitution of Cell-Invasive Activity

sample[a]	elevation in intracellular cAMP levels (pmol of cAMP/mg of protein)
flow-through + eluate[b]	174 ± 11
flow-through + chymotrypsin-treated eluate[c]	72 ± 6
invasive adenylate cyclase preparation[d]	180 ● 20
chymotrypsin-treated adenylate cyclase preparation[e]	73 ± 2
chymotrypsin- and PMSF-treated adenylate cyclase preparation[f]	123 ± 4
no addition	75 ± 9

[a] Samples were incubated with N1E-115 neuroblastoma cells as described under Materials and Methods. [b] An invasive adenylate cyclase preparation was applied to a WGA affinity column. Adenylate cyclase activity (flow-through) and a protein peak (eluate) were collected as described under Results. These samples (100 nmol/min of flow-through and 1.5 μg of eluate) were combined and applied to each plate of cells. [c] The eluate was pretreated with 10 μg/mL chymotrypsin for 10 min at 37 °C and then quenched with 0.2 mM PMSF. [d] Adenylate cyclase preparation (100 nmol min^{-1} $plate^{-1}$) before application to the WGA column. [e] The adenylate cyclase preparation was treated as described in *c*. [f] Chymotrypsin (10 μg/mL) and PMSF (0.2 mM) were precombined and then incubated with the adenylate cyclase preparation as described in *c*.

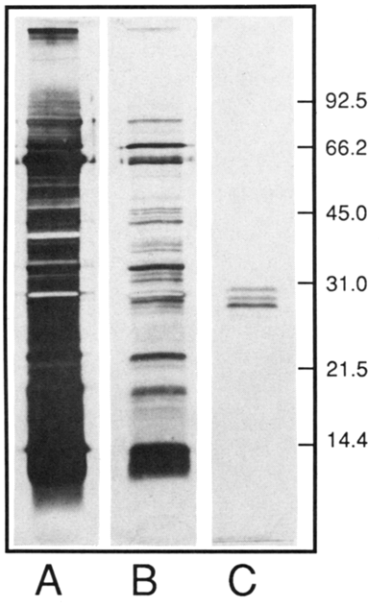

FIGURE 4: SDS–PAGE of wheat germ lectin–agarose chromatography fractions. Samples were precipitated with 72% trichloroacetic acid at 4 °C. The precipitates were pelleted by microcentrifugation, and the pellets were washed twice with 1 mL of cold acetone and recentrifuged. The acetone was removed, and the pellets were dissolved in 50 μL of Laemmli sample buffer. The samples were subjected to SDS–PAGE on a 12.5%, 0.75-mm slab gel. The gel was silver-stained as described under Materials and Methods. (A) Invasive adenylate cyclase preparation purified by QAE-Sephadex chromatography (5 μg); (B) column flow-through from WGA–agarose chromatography (3.5 μg); (C) protein eluted from the WGA–agarose column with 0.15 M GlcNAc (3 μg). The molecular weight standards that were used are phosphorylase *b* (92 500), bovine serum albumin (66 000), ovalbumin (45 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500), and lysozyme (14 400).

of approximately 30 000. The interaction with ^{125}I-WGA was specific since it was blocked by the addition of GlcNAc.

Reconstitution of Invasive Adenylate Cyclase Using the Purified Catalytic Subunit. The results of the reconstitution experiment described above suggested that the WGA eluate contained a protein fraction that was required for entry of the catalytic subunit into animal cells. However, the adenylate cyclase preparation used in those experiments was quite heterogeneous (Figure 4; lane B). Therefore, the 45 000-Da catalytic subunit of the enzyme was purified to homogeneity using CaM affinity chromatography (Haiche et al., 1988) and examined in reconstitution experiments. Incubation of the homogeneous catalytic subunit with neuroblastoma cells did not affect intracellular cAMP levels (Table II). When the protein fraction isolated by WGA–agarose chromatography was added to cells in combination with the catalytic subunit, a 5-fold increase in intracellular cAMP levels was observed.

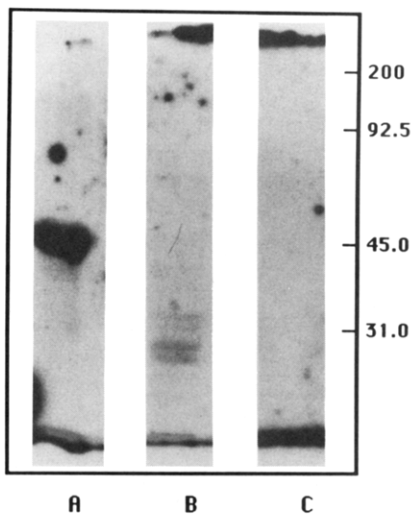

FIGURE 5: ^{125}I-WGA binding to a preparation of the invasive adenylate cyclase. A CaM-sensitive, invasive preparation of adenylate cyclase (9 μg, isolated by QAE-Sephadex chromatography) was subjected to SDS–PAGE. The gel was fixed, washed, and incubated with ^{125}I-WGA as described under Materials and Methods. For control, 0.2 M *N*-acetyl-D-glucosamine was added to buffer A (lane C). The gel was stained with Coomassie blue, dried, and autoradiographed. Lane A, molecular weight markers (phosphorylase *b*, 95 000; bovine serum albumin, 66 000; ovalbumin, 45 000; carbonic anhydrase, 31 000; soybean trypsin inhibitor, 21 500; and lysozyme, 14 400). Lanes B and C, adenylate cyclase isolated by QAE-Sephadex chromatography.

Table II: Entry of Purified Adenylate Cyclase Catalytic Subunit into N1E-115 Mouse Neuroblastoma Cells

sample[a]	elevation in intracellular cAMP levels (pmol of cAMP/mg of protein)
45 kDa + WGA eluate[b]	224 ± 11
45 kDa[c]	41 ± 3
no addition	36 ● 2

[a] Samples were incubated with N1E-115 neuroblastoma cells as described under Materials and Methods. [b] The catalytic subunit of the invasive adenylate cyclase was purified by VU8 CaM–Sepharose chromatography as described under Materials and Methods. The 45-kDa catalytic subunit [100 nmol min^{-1} (plate of cells)$^{-1}$] was precombined with eluate from WGA–agarose chromatography (3 μg/plate of cells) and then applied to the cells. [c] 100 nmol/min of the purified adenylate cyclase catalytic subunit was applied per plate of cells.

These experiments confirmed that the 45 000-Da catalytic subunit, by itself, is noninvasive (Masure et al., 1988). Furthermore, the data illustrated that reconstitution of the purified catalytic subunit with the protein fraction isolated by WGA–agarose chromatography resulted in an invasive adenylate cyclase preparation.

Discussion

Recently, Glaser et al. (1988) cloned the gene encoding the *B. pertussis* adenylate cyclase and determined its nucleotide sequence. Analysis of the nucleotide sequence revealed an open-reading frame that would code for a polypeptide of approximately 177 000 daltons. The catalytic domain was localized at the N-terminal end of the polypeptide. Biochemical studies have demonstrated that the *B. pertussis* adenylate cyclase is synthesized as a large precursor that is proteolytically processed to the 45 000-dalton catalytic subunit which is released into the culture supernatant (Masure & Storm, 1989). The catalytic subunit of the adenylate cyclase has been purified by SDS gel electrophoresis and shown to be noninvasive when incubated with mouse neuroblastoma cells (Masure et al., 1988). Data reproted in this study indicated that the catalytic subunit purified by the method of Haiche et al. (1988) was also noninvasive. On the basis of these observations, we hypothesized that the culture medium of *B. pertussis* contains one or more additional polypeptides which facilitate entry of the adenylate cyclase catalytic subunit into animal cells.

In the present study, data have been presented which prove that there is a separable protein factor that facilitates entry of the *B. pertussis* adenylate cyclase into neuroblastoma cells. Specifically, it has been determined that an adenylate cyclase preparation can be rendered noninvasive by passage over a WGA–agarose column. A protein fraction was eluted from the lectin column with GlcNAc. Reconstitution of the purified catalytic subunit with this protein fraction resulted in an invasive adenylate cyclase preparation. The eluate from WGA–agarose chromatography contained three polypeptides with apparent molecular weights of 26 000, 28 000, and 30 000. The chemical basis for absorption of these polypeptides to WGA–agarose is not established, although they were eluted from the column with GlcNAc and interacted directly with ^{125}I-WGA, suggesting that they may be glycopeptides.

We have published evidence that the catalytic subunit of the *B. pertussis* adenylate cyclase binds calcium. It was observed that in the presence of excess EGTA the Stokes radius of the invasive adenylate cyclase was 33.8 Å. This corresponds to an apparent molecular weight of 61 000. In the presence of 2 mM $CaCl_2$, the Stokes radius of the adenylate cyclase increased to 43.5 Å. This Stokes radius is consistent with an apparent molecular weight of 104 000 (Masure et al., 1988). In addition, optimal invasion of neuroblastoma cells by the enzyme required Ca^{2+} at approximately 2 mM, which is characteristic of extracellular Ca^{2+} concentrations. We hypothesized that calcium binding to the catalytic subunit promotes the association of one or more additional polypeptides which facilitate entry of the enzyme into animal cells (Masure et al., 1988). The data reported in this study are consistent with this hypothesis. There is, indeed, a separable protein factor present in the invasive adenylate cyclase preparation which facilitates entry of the catalytic subunit into animal cells. Considering the function of this protein component, it seems appropriate to refer to it as invasive factor.

The invasive factor apparently interacts directly with the catalytic subunit to facilitate cell entry, rather than with some other protein present in the invasive adenylate cyclase preparation, since an invasive adenylate cyclase preparation was obtained by reconstitution of the homogeneous catalytic subunit with the protein isolated from WGA–agarose. The Ca^{2+}-dependent increase in apparent molecular weight of the invasive adenylate cyclase from 61 000 to 104 000 together with results of the cell entry studies presented in this paper suggests a possible stoichiometry of 1:1 for interaction of the catalytic subunit with the invasive factor. However, the exact stoichiometry remains to be determined, and may be complicated by our observation that the catalytic activity of the CaM–Sepharose-purified adenylate cyclase is very unstable.

Although the mechanism for entry of the catalytic subunit into animal cells is not established, the discovery of a protein required for cell entry provides further insight into this process. It is interesting that the catalytic subunit of the adenylate cyclase (edema factor) from *Bacillus anthracis* requires the presence of an additional polypeptide (protective antigen) for entry into animal cells (Leppla, 1982). However, the mechanism for entry utilized by these two bacterial adenylate cyclases is apparently quite different (Gordon et al., 1988). The structure of the *B. pertussis* adenylate cyclase may be similar to several other bacterial toxins which are known to be composed of multiple subunits, one or more of which are required to facilitate entry of a catalytic subunit into animal cells (Tamura et al., 1982; Neville & Hudson, 1986; Gill, 1978).

Acknowledgments

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Registry No. Adenylate cyclase, 9012-42-4.

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Cloning, Analysis, and Bacterial Expression of Human Farnesyl Pyrophosphate Synthetase and Its Regulation in Hep G2 Cells

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ABSTRACT: A partial length cDNA encoding farnesyl pyrophosphate synthetase (hpt807) has been isolated from a human fetal liver cDNA library in λgt11. DNA sequence analysis reveals hpt807 is 1115 bp in length and contains an open reading frame coding for 346 amino acids before reaching a stop codon, a polyadenylation addition sequence, and the first 14 residues of a poly(A+) tail. Considerable nucleotide and deduced amino acid sequence homology is observed between hpt807 and previously isolated rat liver cDNAs for farnesyl pyrophosphate synthetase. Comparison with rat cDNAs suggests that hpt807 is about 20 bp short of encoding the initiator methionine of farnesyl pyrophosphate synthetase. The human cDNA was cloned into a prokaryotic expression vector and *Escherichia coli* strain DH5αF'IQ was transformed. Clones were isolated that express an active fusion protein which can be readily observed on protein gels and specifically stained on immunoblots with an antibody raised against purified chicken farnesyl pyrophosphate synthetase. These data confirm the identity of hpt807 as encoding farnesyl pyrophosphate synthetase. Slot blot analyses of RNA isolated from Hep G2 cells show that the expression of farnesyl pyrophosphate synthetase mRNA is regulated. Lovastatin increases mRNA levels for farnesyl pyrophosphate synthetase 2.5-fold while mevalonic acid, low-density lipoprotein, and 25-hydroxycholesterol decrease mRNA levels to 40–50% of control values.

Farnesyl pyrophosphate synthetase catalyzes the sequential condensation of isopentenyl pyrophosphate with the allylic pyrophosphates, dimethylallyl pyrophosphate, and then with the resultant geranyl pyrophosphate as shown in Scheme I.

The ultimate product of these two reactions, farnesyl pyrophosphate, is utilized in the synthesis of squalene, cholesterol, and other sterols. Farnesyl pyrophosphate synthetase has been purified from many eukaryotic sources including yeast (Eberhardt & Rilling, 1975) and liver tissue of chickens (Reed & Rilling, 1975), rats (V. Ding, unpublished results), and humans (Barnard & Popjak, 1981). The enzyme has a cytosolic localization and is found as a dimer consisting of two indistinguishable subunits of molecular weight between 38 000 and 42 000.

Farnesyl pyrophosphate synthetase is but one of a yet unknown number of enzymes generically referred to as prenyltransferases that catalyze the addition of isoprene units to a pyrophosphate primer. The microsomal enzyme dolichol phosphate synthetase or *cis*-prenyltransferase adds isopentenyl units in a cis configuration to farnesyl pyrophosphate to synthesize dolichyl phosphate, the long-chain polyisoprenoid involved in asparagine-linked glycoprotein synthesis (Adair et al., 1984). Farnesyl pyrophosphate also may be the primer utilized by a mitochondrial *trans*-prenyltransferase in the synthesis of the polyisoprenoid side chain of ubiquinone, the electron carrier in the electron transport chain of mitochondria (Nambudiri et al., 1980). To date, the prenyltransferases involved in dolichol and ubiquinone synthesis have not been purified or characterized sufficiently to know what similarities, if any, exist between them and farnesyl pyrophosphate synthetase. Likewise, relatively few details are known about the synthesis of the farnesyl moiety of the heme *a* prosthetic group of cytochromes *a* and a_3 (Weinstein et al., 1986) or the prenylation of proteins (Sinensky & Logel, 1985; Breunger

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