Immunochemical and Electrophoretic Characterization of the Major Pertussis Toxin Substrate of the RAW264 Macrophage Cell Line

Peter S. Backlund, Jr.,*,‡ Robert R. Aksamit,‡ Cecilia G. Unson,§ Paul Goldsmith,∥ Allen M. Spiegel,∥ and Graeme Milligan⊥

Laboratory of General and Comparative Biochemistry, National Institute of Mental Health, Bethesda, Maryland 20892, Department of Biochemistry, The Rockefeller University, New York, New York 10021, Metabolic Diseases Branch, National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892, and Department of Biochemistry and Pharmacology, University of Glasgow, Glasgow, Scotland

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ABSTRACT: The pertussis toxin substrate from RAW264 macrophage cell membranes was characterized by two-dimensional gel electrophoresis and by immunoblots using antibodies directed against different guanine nucleotide binding proteins. RAW264 membranes were found to contain one major pertussis toxin substrate, which was recognized by both antibodies AS/6 and LE/3. The AS/6 antibody was made against a synthetic peptide corresponding to the carboxyl-terminal decapeptide of the α-subunit of transducin, and the LE/3 antibody was made against the peptide corresponding to amino acids 160–169 of a guanine nucleotide binding protein (G_i-2-α) cloned from a mouse macrophage cell line. The RAW264 pertussis toxin substrate was not recognized by either antibody CW/6 or antibody RV/3, which recognize the 41-kilodalton α-subunit of brain G_i (G_i-1-α) and G_o-α, respectively. Pertussis toxin substrates from bovine brain were resolved into four major α-subunits by two-dimensional gel electrophoresis, and the LE/3 antibody recognized only one of the four proteins. The brain LE/3 reactive protein also reacted with the AS/6 antibody, migrated with a 40K molecular weight, and had an isoelectric point slightly more basic than the RAW264 pertussis toxin substrate. Therefore, the major pertussis toxin substrate in RAW264 cells appears to be G_i-2, and bovine brain contains a relatively minor amount of a closely related guanine nucleotide binding protein.

Guanine nucleotide binding proteins (G proteins)[1] are a family of related membrane proteins that are involved in the coupling of receptors to various effector proteins [see reviews by Stryer and Bourne (1986) and Spiegel (1987)]. Several lines of evidence have indicated a role for G proteins in the signal transduction of receptors for chemoattractants such as fMet-Leu-Phe, leukotriene B_4, and endotoxin-activated mouse serum (Backlund et al., 1985; Bokoch & Gilman, 1984; Goldman et al., 1985; Koo et al., 1983; Lad et al., 1985; Okajima & Ui, 1984). Chemoattractants elicit several cellular responses, including chemotaxis, degranulation, and stimulation of superoxide production. Biochemical changes induced by chemoattractants include the stimulation of phosphoinositide turnover (Bennett et al., 1980, 1982), increase in intracellular calcium (Naccache et al., 1977), and release of arachidonic acid (Hirata et al., 1979). Pertussis toxin, which inactivates certain G proteins, inhibits chemotaxis of macrophages (Backlund et al., 1985; Meade et al., 1984) and neutrophils (Lad et al., 1985; Goldman et al., 1985). Pertussis toxin also inhibits chemoattractant-stimulated phosphoinositide turnover (Brandt et al., 1985), calcium increase (Molski et al., 1984; Goldman et al., 1985), and arachidonic acid release (Okajima & Ui, 1984; Bokoch & Gilman, 1984). The association of a G protein with fMet-Leu-Phe receptors is also suggested by the inhibition of high-affinity binding of fMet-Leu-Phe to neutrophil membranes by analogues of GTP (Koo et al., 1983; Okajima et al., 1985).

Pertussis toxin can ADP-ribosylate transducin (Van Dop et al., 1984), the inhibitory subunit of adenylate cyclase (G_i) (Katada & Ui, 1982), and an abundant brain G protein of unknown function (G_o) (Sternweis & Robishaw, 1984; Neer et al., 1984), while the stimulatory subunit of adenylate cyclase (G_s) is not modified by the toxin (Bokoch et al., 1983). The inhibition of chemotaxis by pertussis toxin was correlated with the ADP-ribosylation of an approximately 40–41-kDa membrane protein (Backlund et al., 1985; Lad et al., 1985), which seemed to implicate the involvement of G_i or a G_i-like protein in the chemotactic response. However, several distinct pertussis toxin substrates migrate with similar mobility, so it was not clear which G protein is involved in the chemotactic response. In addition, changes in adenylate cyclase activity do not seem to be directly involved with the chemotactic response, since chemoattractants do not alter the activity of adenylate cyclase in membranes (Backlund et al., 1985; Verghese et al., 1985) and increasing cAMP levels with either isoproterenol or forskolin have no effect on chemotaxis of RAW264 cells (Aksamit et al., 1985).

All members of the G protein family have similar heterotrimer structure, consisting of α-, β-, and γ-subunits, with the α-subunit being unique for each member and containing the ADP-ribosylation site for pertussis toxin (Stryer & Bourne,

* Correspondence should be addressed to this author at the National Institute of Mental Health, Building 36, Room 3D-06, Bethesda, MD.
‡ National Institute of Mental Health.
§ The Rockefeller University.
∥ National Institute of Diabetes, Digestive and Kidney Diseases.
⊥ University of Glasgow.

[1] Abbreviations: G protein, guanine nucleotide binding protein; G_s, stimulatory subunit of adenylate cyclase; G_i, inhibitory subunit of adenylate cyclase; G_o, guanine nucleotide binding protein of unknown function isolated from brain; G_i-1, G_i-2, and G_i-3, G proteins corresponding to arbitrarily designated cloned cDNA sequences; G_i-1-α, G_i-2-α, G_i-3-α, and G_o-α, α subunits of the corresponding G protein; fMet-Leu-Phe, *N*-formylmethionylleucylphenylalanine; SDS, sodium dodecyl sulfate; kDa, kilodalton(s); Tris, tris(hydroxymethyl)aminomethane.

1986). The α-subunits of several different pertussis toxin substrates have been cloned and sequenced using cDNA libraries from a variety of tissues and species. The first G_i sequence isolated, G_i-1-α, has been cloned from bovine brain (Nukada et al., 1986), bovine pituitary (Michel et al., 1986), and human brain (Bray et al., 1987). A second G_i-like sequence, G_i-2-α, has been cloned from mouse macrophages (Sullivan et al., 1986), human monocytes (Didsbury et al., 1987), and rat glioma cells (Itoh et al., 1986). In addition, a third G_i-like sequence has been isolated from brain tissue (Jones & Reed, 1987), human liver (Suki et al., 1987), and HL-60 cells (Didsbury & Snyderman, 1987). The major pertussis toxin substrate in brain, G_o, has also been cloned and sequenced from both rat glioma cells (Itoh et al., 1986) and bovine brain (Van Meurs et al., 1987). Comparison of the cDNA sequences of the same G protein type in different species indicates that these proteins are highly conserved.

In order to differentiate between the different G proteins, specific antibodies have been recently developed. The antibody CW/6 was made against holotransducin and was found to cross-react with the 41-kDa α-subunit of G_i in brain (Pines et al., 1985). The antibody RV/3 was made against a purified mixture of bovine brain G_i and G_o and was found to recognize G_o, but does not recognize the 41-kDa G_i-1-α and has little reactivity with the major pertussis toxin substrate in neutrophils (Gierschik et al., 1986a,b, 1987). Since the proteins used for production of CW/6 and RV/3 both contained β-subunits, these two antibodies also cross-react with the β-subunits. More recently, antibodies have been made using specific peptides based on the translated sequences of the cDNA clones of specific G proteins (Goldsmith et al., 1987). The antibody AS/6 is directed against the carboxyl-terminal amino acid sequence of the α-subunit of tranducin, which contains the cysteine that is the site for ADP-ribosylation of pertussis toxin (Medynski et al., 1985). The AS/6 antibody recognizes G_i-1-α, but not G_o-α, which has a substantially different carboxyl-terminal sequence (Goldsmith et al., 1987). The AS/6 antibody does not differentiate between G_i-1-α and G_i-2-α, since they both have the same carboxyl-terminal decapeptide sequence. A peptide sequence specific to G_i-2-α (Table I) was used to generate the antibody LE/3, which does not recognize the 41-kDa G_i-1-α in brain but does recognize a pertussis toxin substrate in neutrophils (Goldsmith et al., 1987).

Here we combine the use of some of these specific antibodies with the high resolution of two-dimensional gel electrophoresis to characterize the pertussis toxin substrate in membranes of the macrophage cell line RAW264. On the basis of these results, RAW264 cells contain one major pertussis toxin substrate, which appears to be closely related to or identical with G_i-2. Bovine brain contains a G protein, present at a low level relative to the other pertussis toxin substrates in brain, that is closely related to the RAW264 macrophage pertussis toxin substrate.

Experimental Procedures

RAW264 cells were grown (Aksamit et al., 1981), and plasma membranes were isolated (Backlund et al., 1985) as previously described. Bovine brain plasma membranes were prepared, and the pertussis toxin substrates were purified as previously described (Milligan & Klee, 1985). RAW264 membranes (200 μg) or purified bovine brain G proteins (0.8 μg) were ADP-ribosylated using pertussis toxin in the presence of [^{32}P]NAD and 0.1 mM GTP (Backlund et al., 1985).

Electrophoresis. Proteins were separated by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970) with 10% acrylamide for the separating gel, or by two-dimensional gel electrophoresis according to the procedure of O'Farrell (1975) with modifications similar to Woolkalis et al. (1986) in order to improve the recovery from the two-dimensional gels of G proteins in membrane preparations. The membranes were solubilized in 5 mM potassium carbonate, 20 mM dithiothreitol, and 1% Lubrol-PX for 10 min on ice, followed by centrifugation at 15000*g* for 10 min. The supernatant was then added to 2 volumes of a solution containing 9.5 M urea, 2% ampholines (LKB, 1.6% 5/7 and 0.4% 3/10), 5% 2-mercaptoethanol, and 8% NP-40. The solubilized proteins were applied to the basic end of the isoelectric focusing gels, which had not been prefocused, and the isoelectric focusing was performed at 500 V for 18 h. The α-subunits of the G proteins migrated as distinct spots, using this procedure, with little streaking or aggregation of either immunoreactive or radiolabeled material at the basic end of the isoelectric focusing gel. The pH gradient was determined from gels run in parallel by measuring the pH of gel slices incubated with degassed 2 mM KCl for 2 h. Proteins were detected by silver staining (Merril et al., 1982).

Enzyme-Linked Immunoblots. The CW/6 antibody was made against holotransducin and recognizes both the α- and β-subunits of brain G_i (Pines et al., 1985). The RV/3 antibody was made against a mixture of purified brain G_o and G_i and recognizes both the α- and β-subunits of G_o (Gierschik et al., 1986a). The antibody AS/6 was made against the peptide KENLKDCGLF, corresponding to the C-terminus of the α-subunit of transducin, while the LE/3 antibody was made against the peptide LERIAQSDYI, a sequence specific for G_i-2-α (Goldsmith et al., 1987). After electrophoretic separation, the proteins were transferred from the gels to nitrocellulose filters, and excess protein binding sites were blocked by incubation in 150 mM NaCl, 50 mM Tris (pH 7.9), and 0.05% Tween-20 (TBST buffer) with 1% gelatin. The antisera were diluted to the indicated levels in TBST buffer with 1% bovine serum albumin and incubated with the filters for 2–3 h at room temperature. After being washed 3 times with TBST buffer, the filters were incubated with a 1:2000 dilution of horseradish peroxidase conjugated goat anti-rabbit IgG (Calbiochem) for 2 h. The filters were then washed, and the stain was developed by incubation with 125 mM NaCl, 42 mM Tris (pH 7.9), 83 mM imidazole, 20% methanol, 0.015% H_2O_2, and 0.5 mg of 4-chloro-1-naphthol/mL.

Results

Characterization of RAW264 Pertussis Toxin Substrates. Pertussis toxin will ADP-ribosylate a 40–41-kDa protein in RAW264 cell membranes (Backlund et al., 1985; Aksamit et al., 1985). In order to further characterize the RAW264 pertussis toxin substrate, antibodies directed against different pertussis toxin substrates were tested for reactivity against RAW264 membrane proteins separated by SDS–polyacrylamide gel electrophoresis (Figure 1). The pertussis toxin substrate in RAW264 cells was also compared with those present in the membranes of bovine brain. The antibodies AS/6 and LE/3 both recognize proteins migrating with a 40K–41K molecular weight in brain and RAW264 membranes (Figure 1). In RAW264 membranes, the intensity of staining with AS/6 and LE/3 antibodies was approximately equal, and the reactive protein migrated with the same mobility. In contrast, with brain membranes, the AS/6-reactive material was much greater than the LE/3-reactive material. In addition, the AS/6-reactive material in brain membranes migrated with a slightly higher molecular weight than the LE/3-reactive material, suggesting that AS/6 recognized more

Table I: Homology of Amino Acid Sequences in G Proteins to Peptides Used in Production of Antibodies[a]

G protein	LE/3 antibody	AS/6 antibody	ref
synthetic peptide[b]	LERIAQSDYI	KENLKDCGLF	
G_i-1-α			
bovine pituitary	-D----PN--	-N--------	Nichel et al. (1986)
bovine brain	-D----PN--	-N--------	Nukada et al. (1986)
human brain	-D----PN--	-N--------	Bray et al. (1987)
G_i-2-α			
mouse macrophage	----------	-N--------	Sullivan et al. (1986)
rat C6 glioma	----------	-N--------	Itoh et al. (1986)
human monocyte	----------	-N--------	Didsbury et al. (1987)
G_i-3-α			
human liver	-D--S--N--	-N---E---Y	Suki et al. (1987)
human HL-60	-D--S--N--	-N---E---Y	Didsbury & Snyderman (1987)
G_o-α			
rat C6 glioma	-D--GAA--Q	AN--RG---Y	Itoh et al. (1986)
bovine brain	-D--GAA--Q	AN--RG---Y	Van Meurs et al. (1987)

[a] Amino acids are indicated by the one-letter abbreviation. A dash indicates the amino acid residue is the same as in the synthetic peptide used in making the antibody. [b] Amino acid sequences of the synthetic peptides used in eliciting the antibodies. The LE/3 sequence corresponds to amino acids 160–169 of the mouse macrophage G_i-α (Sullivan et al., 1986), and the AS/6 sequence corresponds to the carboxyl terminal of transducin-α and contains the pertussis toxin ribosylation site (Medynski et al., 1985).

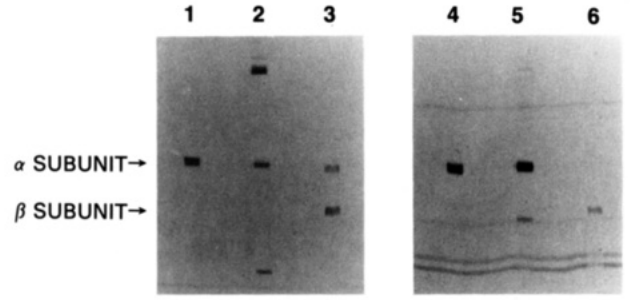

FIGURE 1: Immunoblot detection of G proteins in bovine brain and RAW264 macrophage membranes. Bovine brain membranes (300 μg, lanes 1–3) and RAW264 membranes (300 μg, lanes 4–6) were separated by SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose. Immunoblotting was then performed using the following antibodies: 1:300 dilution of AS/6 (lanes 1 and 4); 1:100 dilution of LE/3 (lanes 2 and 5); 1:5 dilution of affinity-purified RV/3 (lanes 3 and 6). The positions of the α- and β-subunits of the G proteins are indicated. The additional bands using the LE/3 antibody were due to the use of crude serum.

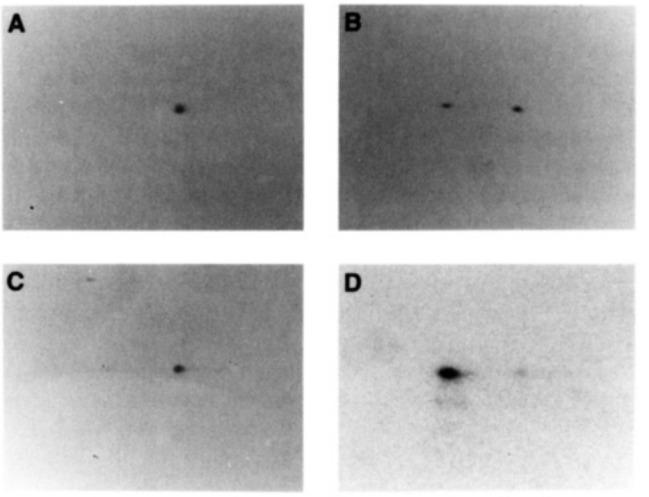

FIGURE 2: Characterization of RAW264 pertussis toxin substrate on two-dimensional gels. RAW264 membrane proteins (80 μg/gel) from untreated (panels A and C) or from membranes incubated with pertussis toxin and [^{32}P]NAD (panels B and D) were separated by two-dimensional gel electrophoresis. The proteins were transferred to nitrocellulose, and immunoblots were performed with the following antibodies: (A and B) 1:300 dilution of AS/6; (C) 1:100 dilution of LE/3; (D), autoradiography of immunoblot B. The two-dimensional gels are oriented with the isoelectric focusing dimension horizontal with the acidic end to the left and the basic end to the right. The portion of the gel containing the G proteins is shown and covers a pH range from pH 5.0 to pH 6.1.

than one protein in brain. The AS/6 antibody recognizes both G_i-1-α and G_i-2-α, since both G proteins share the identical C-terminus (Table I). In contrast, LE/3 should be specific for G_i-2-α, since the peptide sequence used to make LE/3 differs by three amino acids from the corresponding region of G_i-1-α and G_i-3-α, and by five amino acids from G_o-α (Table I). The antibody RV/3, which recognizes the α- and β-subunits of G_o, showed reactivity with a 39-kDa protein in brain membranes, but no reactive material was detected in the RAW264 membranes, indicating that G_o was not present in the RAW264 cells (Figure 1). In contrast, the reactivity of RV/3 with the common β-subunit was similar in both the brain and RAW264 membranes (Figure 1), indicating the presence of a similar amount of β-subunit in both brain and RAW264 membranes.

The α-subunits of different pertussis toxin substrates are not well resolved by one-dimensional SDS–polyacrylamide gel electrophoresis. However, it has previously been shown that two-dimensional gel electrophoresis, combining isoelectric focusing and SDS–polyacrylamide gel electrophoresis, can resolve multiple α-subunits in the isoelectric focusing dimension (Woolkalis et al., 1986). When the RAW264 membrane proteins were separated by two-dimensional gel electrophoresis and immunoblots performed with either the AS/6 or the LE/3 antibody, the same protein was recognized by both antibodies (Figure 2A,C). The protein recognized by the antibodies migrated with an apparent molecular weight of 40K and an isoelectric point of approximately 5.7. If the membrane proteins were separated after incubation with pertussis toxin and [^{32}P]NAD, a single major radiolabeled protein was observed migrating with a molecular weight of 40K and a pI of approximately 5.4 (Figure 2D). The shift to the more acidic pI is due to the negative charge of the ADP-ribose. A low level of radioactivity was also observed migrating with a 40K molecular weight and an isoelectric point of approximately 5.7, suggesting that a second pertussis toxin substrate may be present at very low levels.

Immunoblots of pertussis toxin treated RAW264 membranes contained two proteins recognized by the AS/6 antibody, instead of the single protein recognized in the untreated membranes (Figure 2A,B). The more acidic protein comigrated with the ^{32}P radioactivity (Figure 2D) and represented the ADP-ribosylated G protein, while the more basic protein represented the remaining unmodified G protein. The clear separation of these two proteins on two-dimensional gels

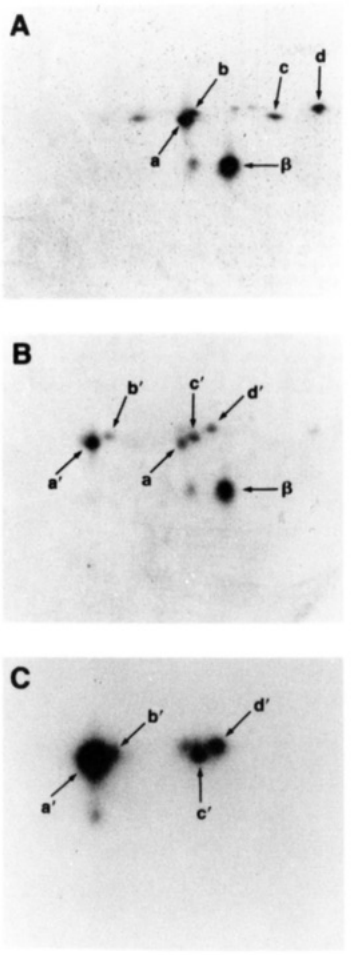

FIGURE 3: Two-dimensional gel electrophoresis of purified bovine brain pertussis toxin substrates. Pertussis toxin substrates from bovine brain (0.8 μg) were incubated with [^{32}P]NAD in the absence or presence of pertussis toxin. The proteins were separated by two-dimensional gel electrophoresis and detected by silver staining. (A) Minus pertussis toxin; (B) plus pertussis toxin; (C) autoradiogram of (B). The four major α-subunits and the β-subunit which were detected by silver staining are indicated (protein spots a–d). The ADP-ribosylated proteins corresponding to each of the α-subunits are indicated (protein spots a′–d′). The portion of the gel shown and the orientation are as indicated in Figure 2.

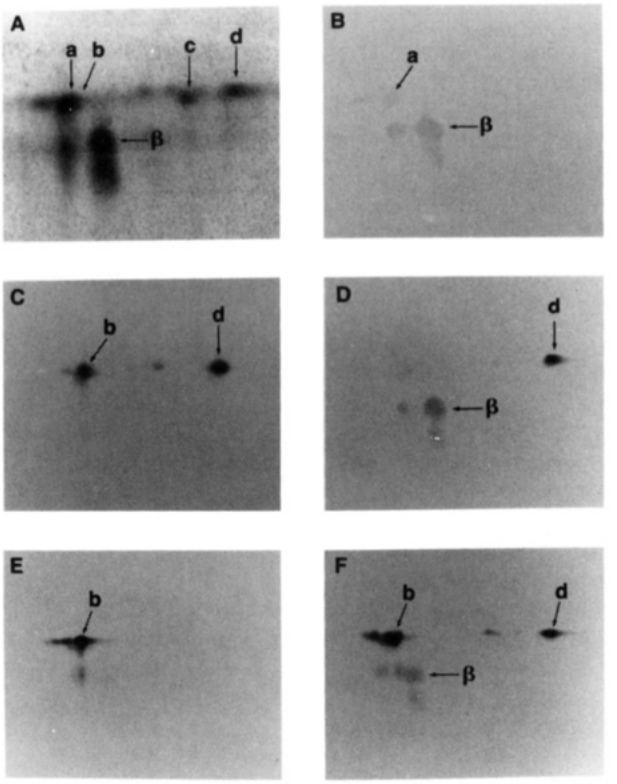

FIGURE 4: Antibody specificity of bovine brain pertussis toxin substrates. Pertussis toxin substrates (0.5 μg/gel from bovine brain were separated by two-dimensional gel electrophoresis and transferred to nitrocellulose for immunoblots. (A) Proteins detected by silver staining; (B–F) immunoblots using the following antibodies: (B) 1:5 dilution of affinity-purified RV/3; (C) 1:300 dilution of AS/6; (D) 1:50 dilution of CW/6; (E) 1:100 dilution of LE/3; (F) 1:50 dilution of CS/6 and 1:100 dilution of LE/3. The major α-subunits and the β-subunit detected by silver staining and the corresponding proteins detected by the antibodies on the immunoblots are indicated (protein spots a–d). The gels are oriented with the acidic end to the left, and the basic end to the right, and includes the pH range from pH 5.3 to pH 6.2.

demonstrated that the AS/6 antibody recognized both the unmodified and the ADP-ribosylated form of this G protein. The ^{32}P labeling with pertussis toxin combined with the antibody specificity indicates that RAW264 cells contain one major pertussis toxin substrate that is similar to or identical with G_i-2.

Characterization of Bovine Brain Pertussis Toxin Substrates. Brain tissue is known to contain at least three pertussis toxin substrates with α-subunits migrating in the 39–41-kDa range (Sternweis & Robishaw, 1984; Neer et al., 1984; Katada et al., 1987). Since a low level of LE/3-reactive material was observed in bovine brain membranes (Figure 1), two-dimensional gel electrophoresis was used to resolve the LE/3-reactive protein from the other G proteins and to compare the LE/3-reactive protein in brain with that in RAW264 cells. When purified bovine brain pertussis toxin substrates were separated by two-dimensional gel electrophoresis, four major protein spots were resolved in the 39–41-kDa range (Figure 3, protein spots a–d), and three less abundant proteins were also observed. The four major proteins were shown to be pertussis toxin substrates by ADP-ribosylation in the presence of [^{32}P]NAD. Treatment with pertussis toxin resulted in the incorporation of radioactivity (Figure 3C) and in a shift in the migration of the four major proteins to more acidic positions in the isoelectric focusing dimension (Figure 3B, spots a′–d′), reflecting the negative charge of the ADP-ribose.

After separation by two-dimensional gel electrophoresis, the brain pertussis toxin substrates were also tested for reactivity with the antibodies directed against different G proteins (Figure 4). The protein corresponding to the α-subunit of G_o was identified on the two-dimensional gels by reactivity with the RV/3 antibody (Figure 4B, protein spot a). In addition to being recognized by the RV/3 antibody, the α-unit of G_o was the most abundant protein both by silver staining and by the incorporation of [^{32}P]ADP-ribose (Figure 3, protein spots a and a′). In addition, the RV/3-reactive protein migrated with the lowest molecular weight of the brain G proteins, consistent with the 39K molecular weight reported for G_o-α. The protein corresponding to the brain form of G_i, G_i-1-α, was determined by reactivity with the antibody CW/6 (Figure 4D), which has previously been shown to recognize the 41-kDa α-subunit of G_i in brain (Pines et al., 1985). The protein recognized by the CW/6 antibody was second in abundance by silver staining and migrated with the highest molecular weight and most basic isoelectric point (approximately pI = 6.1) of the brain G proteins (Figure 4A,D). In addition to reacting with the CW/6 antibody, G_i-1-α was also recognized by the AS/6 antibody (Figure 4C). The third most abundant α-subunit in bovine brain (Figure 4, protein spot c) was poorly recognized by the antibodies tested. This protein

was a substrate for ADP-ribosylation by pertussis toxin (Figure 3, protein spots c and c'), and the protein appears to represent a distinct G protein in brain (unpublished results).

The antibody LE/3 primarily recognized a single protein in the brain pertussis toxin substrates that migrated just above and slightly more basic than the α-subunit of G_o (Figure 4E). This protein was easily detected with the LE/3 antibody, even though the protein was only fourth in abundance based on the intensity of the silver stain (Figures 3A and 4A, protein spot b), indicating that the LE/3 antibody reacted well with the protein. A slight reactivity of LE/3 toward the α-subunit of G_o resulted in a shadowlike spot just below the major LE/3-reactive protein on some of the immunoblots, due to the high level of G_o present in the sample (Figure 4E). In addition, a very low level of a second LE/3-reactive protein was observed migrating just to the acidic side of the major LE/3 positive protein (Figure 4E). Both the major and the minor LE/3-reactive proteins were also recognized by the AS/6 antibody (Figure 4C), and not by antibodies RV/3 or CW/6, indicating that the LE/3-reactive proteins are distinct from G_o or G_i-1. In order to demonstrate the migration of the LE/3-reactive protein relative to the other brain G proteins, a mixture of the LE/3 and CW/6 antibodies was used in an immunoblot of brain G proteins (Figure 4F). The LE/3-reactive protein migrated with an isoelectric point more acidic than the β-subunit and migrated with a molecular weight of 40K, instead of the 41K molecular weight of G_i-1-α.

It appeared that the LE/3-reactive proteins isolated from bovine brain and RAW264 cells migrated with very similar molecular weights and isoelectric points on the immunoblots of two-dimensional gels. In order to examine the migration of these two proteins more closely, RAW264 membranes were mixed with purified brain pertussis toxin substrates and then separated by two-dimensional gel electrophoresis. Immunoblots of the mixture of RAW264 membranes and bovine brain G proteins indicated that the LE/3-reactive protein in RAW264 cells migrated just to the acidic side of the major LE/3-reactive protein from bovine brain and comigrated exactly with the minor LE/3-reactive protein in brain (Figure 5). In addition to the similar migration on two-dimensional gels, both the RAW264 and bovine brain LE/3-reactive proteins were recognized by the AS/6 antibody, but not by the CW/6 or RV/3 antibodies. Since the LE/3 proteins compared here are from two different species, small changes in the amino acid sequences may occur and may result in slightly different mobilities. Therefore, even though the LE/3-reactive protein from bovine brain and mouse RAW264 cells do not exactly comigrate on the two-dimensional gels, they may be functionally the same protein.

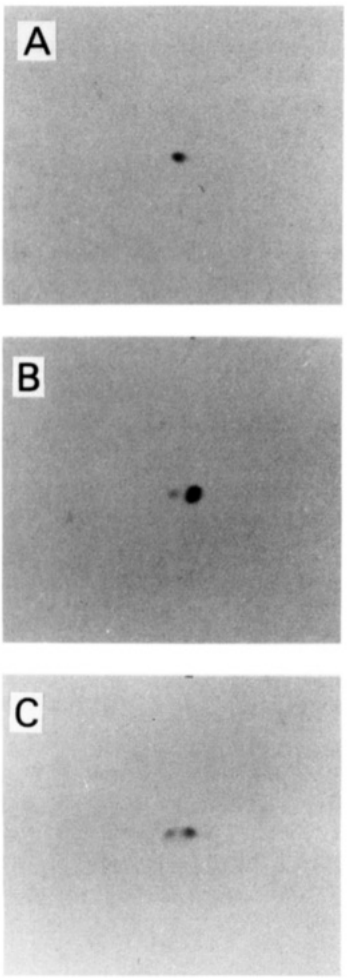

FIGURE 5: Migration of RAW264 and bovine brain LE/3-reactive proteins on two-dimensional gels. RAW264 membranes and purified bovine brain pertussis toxin substrates were separated by two-dimensional gel electrophoresis, and the proteins were transferred to nitrocellulose. Immunoblots were done using 1:100 dilution of the LE/3 antibody. (A) RAW264 membranes (50 μg); (B) bovine brain pertussis toxin substrates (0.5 μg); (C) mixture of RAW264 membranes (25 μg) and bovine brain pertussis toxin substrates (0.25 μg). The gels are oriented with the acidic end to the left and the basic end to the right, and includes the pH range 5.3–6.0.

Discussion

Previous data have demonstrated a role for pertussis toxin sensitive G proteins in signal transduction of chemoattractant receptors, and the inhibition of various responses to chemoattractants has been correlated with the ADP-ribosylation of a 40–41-kDa subunit by pertussis toxin. We have extended this characterization of the RAW264 macrophage G protein to provide evidence that the major pertussis toxin substrate in these cells is similar to or identical with G_i-2. The sequence for G_i-2-α has been determined from cDNA sequences cloned from cDNA libraries from several sources (Sullivan et al., 1986; Didsbury et al., 1987; Itoh et al., 1986); however, a protein corresponding to G_i-2-α has yet to be identified by peptide sequencing. The LE/3 antibody raised against a G_i-2-α-specific sequence (Table I) recognizes the major pertussis toxin substrate in RAW264 membranes (Figure 2). The antibody LE/3 appears to be very specific, since it reacted well with only one of the four major pertussis toxin substrates in brain tissue (Figure 4E), and does not react either with brain G_i-1-α or with G_o-α. Since a protein encoded by G_i-3-α has not yet been isolated, we cannot rule out the possibility that LE/3 will cross-react with this protein; however, this seems unlikely due to the differences in the corresponding amino acid sequences (Table I). Further evidence to indicate that the macrophage G protein is G_i-2 was the recognition of the protein by the AS/6 antibody, directed against the carboxyl-terminal sequence (Table I), and the failure of the protein to be recognized by either CW/6 or RV/3, which recognize G_i-1 and G_o, respectively.

Since the cDNA sequence of both G_i-1-α and G_i-2-α is known, it is possible to estimate the relative isoelectric points of the proteins based on the amino acid sequence of the translated cDNA sequence, assuming that there are no posttranslational modifications which would alter the net charge of the protein. On the basis of the translated sequences, G_i-1-α should have a more basic isoelectric point than G_i-2-α. Consistent with this prediction, G_i-1-α (Figure 4, protein spot d) migrated with a more basic pI than the LE/3-reactive protein (Figure 4, protein spot b). At this point, it is not

possible to rule out that a G protein other than G_i-2 may contain an amino acid sequence recognized by the LE/3 antibody. However, a G_i-2-α cDNA clone has been isolated from the PU-5 mouse macrophage cell line (Sullivan et al., 1986), indicating that this sequence is at least expressed at the mRNA level in a macrophage cell line. An LE/3-reactive protein was also detected in bovine brain, and the antibody reactivity and migration on two-dimensional gels demonstrated that the brain protein is very similar to the macrophage LE/3-reactive protein. The sequence for bovine G_i-2-α has not been determined, but few changes from the mouse sequence would be expected, since only small species differences have been observed with G proteins. For example, there are only seven amino acid changes between the mouse and human sequences for G_i-2-α (Sullivan et al., 1986; Didsbury et al., 1987), and only two of those changes, Arg_{87}-mouse to Ala_{87}-human and Gln_{281}-mouse to His_{281}-human, alter the charge of the protein.

The different pertussis toxin substrates have distinct mRNAs, and the amino acid sequences are highly conserved between species, suggesting that these proteins are also functionally distinct. These differences may be reflected in both the receptor and the effector proteins that interact with the different pertussis toxin substrates. Since the chemoattractant fMet-Leu-Phe does not alter adenylate cyclase activity in membranes of macrophages (Backlund et al., 1985) or neutrophils (Verghese et al., 1985), it seems that chemoattractant receptors are not directly coupled to adenylate cyclase, and it has been suggested that the chemoattractant receptors are coupled by a G protein to phospholipase C or calcium channels. However, these activities have not been ascribed to a specific G protein. It has been reported that both G_i and G_o purified from brain can reconstitute chemoattractant receptor-linked stimulation of phospholipase C (Kikuchi et al., 1986). The presence of multiple G proteins in brain, including a low level of an LE/3-reactive protein, suggests that these experiments should be reevaluated to determine if a minor G protein could be responsible for the activity.

It has been reported that neutrophils (Gierschik et al., 1986b, 1987) and differentiated HL-60 cells (Oinuma et al., 1987) contain a pertussis toxin sensitive G protein that is distinct from G_i or G_o. This G protein may be similar to or identical with the LE/3-reactive protein in RAW264 cells. In RAW264 cells, the LE/3-reactive G protein was the only pertussis toxin substrate identified, suggesting that this G protein may couple chemoattractant receptors to the effector proteins. An LE/3-reactive protein is also present in brain tissue, but the protein is quantitatively a minor pertussis toxin substrate, with three distinct G proteins present at higher levels. The experiments reported here indicate that RAW264 cells have G_i-2 as the major pertussis toxin substrate and therefore the cell line should be useful for further studies on the interaction of chemoattractant receptors and effector proteins with this G protein.

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Thermal Stability and Intersubunit Interactions of Cholera Toxin in Solution and in Association with Its Cell-Surface Receptor Ganglioside G_{M_1}†

Beth Goins and Ernesto Freire*
Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218
Received July 23, 1987; Revised Manuscript Received November 16, 1987

ABSTRACT: The thermal stability of cholera toxin free in solution and in association with its cell-surface receptor ganglioside G_{M_1} has been studied by using high-sensitivity differential scanning calorimetry and differential solubility thermal gel analysis. In the absence of ganglioside G_{M_1}, cholera toxin undergoes two distinct thermally induced transitions centered at 51 and 74 °C, respectively. The low-temperature transition has been assigned to the irreversible thermal denaturation of the active A subunit. The second transition has been assigned to the reversible unfolding of the B subunit pentamer. The isolated B subunit pentamer exhibits a single transition also centered at 74 °C, suggesting that the attachment of the A subunit does not contribute to the stability of the pentamer. In the intact toxin, the A subunit dissociates from the B subunit pentamer at a temperature that coincides with the onset of the B subunit thermal unfolding. In aqueous solution, the denatured A subunit precipitates after dissociation from the B subunit pentamer. This phenomenon can be detected calorimetrically by the appearance of an exothermic heat effect. In the presence of ganglioside G_{M_1}, the B subunit is greatly stabilized as indicated by an increase of 20 °C in the transition temperature. In addition, ganglioside G_{M_1} greatly enhances the cooperative interactions between B subunits. In the absence of ganglioside, each monomer within the B pentamer unfolds in an independent fashion whereas the fully ganglioside-bound pentamer behaves as a single cooperative unit. On the contrary, the thermotropic behavior of the A subunit is only slightly affected by the presence of increasing concentrations of ganglioside G_{M_1}. The exothermic process at the onset of the B subunit pentamer unfolding is not present when the toxin molecule is bound to ganglioside G_{M_1}. This effect is observed with both micellar and membrane-bound gangliosides but not with pure phospholipid vesicles. Differential solubility thermal gel analysis indicates that under these conditions the A subunit remains associated with the micellar ganglioside G_{M_1} or ganglioside G_{M_1}-containing membranes.

Cholera toxin is a multisubunit protein consisting of five identical binding (B) subunits (M_r 11 500) forming a pentameric ring which supports the A subunit (Gill, 1976). Treatment of the A subunit with reducing reagents produces two subunits: an ADP-ribosylating protein termed A_1 (M_r 21 000) which activates adenylate cyclase and a small protein, A_2 (M_r 6000), which plays a structural role in holding the A_1 and B subunits together.

The interaction of cholera toxin with the cell surface occurs when the B subunit recognizes and binds, with high affinity and specificity, to ganglioside G_{M_1}. This association is believed to cause a conformational change in the protein that facilitates the exposure and subsequent penetration of the A subunit into the membrane. Evidence for this conformational change has been derived from fluorescence studies using cholera toxin incubated either with ganglioside G_{M_1} (Mullin et al., 1976) or with the oligosaccharide moiety of G_{M_1} (Fishman et al., 1978) and NMR studies (Sillerud et al., 1981). Various techniques including photoaffinity labeling (Wisnieski & Bramhall, 1981; Tomasi & Montecucco, 1981; Tomasi et al., 1982), hydrodynamic studies (Dwyer & Bloomfield, 1982), and differential scanning calorimetry (Goins & Freire, 1985) have provided indirect evidence that upon association of cholera

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