Vol. 92, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 29, 1980 Pages 638-646

THE AMPHIPATHIC MEMBRANE PROTEINS ASSOCIATED WITH GANGLIOSIDES: THE PAUL-BUNNELL ANTIGEN IS ONE OF THE GANGLIOPHILIC PROTEINS*

K. Watanabe†, S. Hakomori‡, M.E. Powell, and M. Yokota

Program of Biochemical Oncology, Fred Hutchinson Cancer Research Center, and Departments of Pathobiology, Microbiology and Immunology, University of Washington, Seattle, Washington 98104

Received December 10, 1979

Summary-Two amphipathic protein fractions soluble in organic solvents as well as in water have been isolated from the ganglioside fraction of bovine erythrocyte membranes by successive chromatography in chloroform-methanol mixture on DEAE-Sephadex, silicic acid, and α-hydroxypropylated Sephadex G50 (LH60) columns. These two fractions contained a similar low molecular weight protein but with distinctively different amino acid composition. One of these proteins has been characterized by having a strong Paul-Bunnell antigen activity and had a binding affinity to ganglioside. A similar protein without Paul-Bunnell antigen activity was isolated as the major ganglioside-associated protein.

The presence of a chemically ill-defined polypeptide in ganglioside fraction has been known for many years since Folch *et al* (1), Rosenberg and Chargaff (2) and one of the authors (3) described "bound" polypeptides in "strandin" or in "mucolipid". Later studies on purification of gangliosides with silica gel chromatography eliminated such polypeptides from gangliosides, thus the proteins or polypeptides have been regarded to be mere contaminants.

During the course of the studies on erythrocyte gangliosides (4,5) we have noticed the presence of organic solvent soluble proteins associated with gangliosides. These proteins have been characterized by amphipathic property, being soluble in organic solvents as well as in water, having a characteristic amino acid composition, and display some affinity to ganglioside (gangliophilic property). A minor protein fraction (Fr. 2) has been identified as having a strong activity with Paul-Bunnell (P-B) antigen specificity (6) *i.e.* inhibited sheep and bovine

*This investigation has been supported by research grants from the National Institutes of Health, CA20026 and CA19224.
†Present address: The Shigei Medical Research Institute, 2117 Yamada, Okayama, Japan. ‡To whom communication should be addressed. Abbreviations: Salt/Pi, 0.8% NaCl, 0.2% KCl containing 9.5 mM phosphate, pH 7.0; P-B antigen, Paul-Bunnell antigen; TLC, thin-layer chromatography. Solvent composition is indicated by volume ratio. Ganglioside was abbreviated according to the assignment by Svennerholm (21). Globoside (GalNAcβ1→3Galα1→4Galβ1→4Glc→Cer). Sialylparagloboside (NeuAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer).

0006-291X/80/020638-09$01.00/0

erythrocyte hemagglutination caused by the P-B antibodies present in sera of patients with infectious mononucleosis. This is a preliminary note for the isolation and partial characterization of two types of ganglioside-associated proteins, the major protein co-purified with GM_3-ganglioside, and the minor one with P-B antigen activity.

MATERIALS AND METHODS

Separation and Fractionation of Ganglioside-Associated Proteins: The major protein associated with ganglioside fraction was prepared by the same procedure for preparing gangliosides from erythrocyte stroma (4,5). Cell membranes were homogenized with ethanol followed by brief heating; the total ethanol extract, after being dried *in vacuo*, was dissolved in chloroform-methanol (2:1) and partitioned according to the method of Folch *et al* (1). The total ganglioside fraction was isolated from the Folch's upper phase fraction by chromatography on DEAE-Sephadex (7). The ganglioside fraction (1.5 g) was subjected to chromatography on Biosil A (Biorad Chem. Co., Richmond, CA) by the same procedure as described previously (4). The distribution of polypeptides or proteins was monitored by fluorescamine assay in test tube (8) or by fluorescamine-triethylamine spray (9) on TLC. The major protein was eluted with GM_3-ganglioside by solvent 1 (Fig. 1), whereas the minor fraction with P-B antigen activity was eluted with the polar solvents (Solv. 3 and 4) (Fig. 1).

Purification of the Major Ganglioside-Associated Protein (Pooled Fraction No. 2-4, Fig. 1): The major protein was further purified by gel filtration through LH60 column (1.6 x 120 cm) in chloroform-methanol (2:1) and was eluted together with GM_3-ganglioside (Fr. No. 33-42, each Fr. 4 ml). The protein was separated from GM_3-ganglioside by chromatography on magnesium oxide-silicic acid ("Florisil", Floridin, Co., Tallahasse, FL), namely fraction eluted from LH60 column was loaded on Florisil column (1 x 15 cm) in ethylene dichloride-methanol (2:1); GM_3 was eluted with 5 column volumes of ethyelene dichloride-methanol-water (10:90:1), whereas the proteins were eluted with chloroform-methanol-water (10:90:5). The protein was further purified by TLC on Silica gel H; developed with chloroform-methanol-water (60:30:8) and the fluorescamine positive area, indicated by the guide spray, was scraped, extracted in 10 volumes of 1% tetraethylammonium chloride in chloroform-methanol-water (1:8:2) and sonicated for 2 minutes. The extract was evaporated in a rotary evaporator to a small volume (0.1-1 ml) and dialyzed against distilled water. Details of the preparation procedure and biological property will be described elsewhere.

Purification of the Protein with P-B Antigen Activity: Two step procedures with Biosil A chromatography and gel filtration through an LH60 column are described in the legends of Fig. 1 and 2.

Determination of the Paul-Bunnell Antigen Activity: Antisera were obtained from patients with infectious mononucleosis through the courtesy of Dr. Bruce C. Gilliland, Director of Clinical Immunology Laboratory, University of Washington. The titer of the antisera was determined after adsorption with guinea pig kidney acetone powder with microtiter plates using intact sheep erythrocytes or trypsin-treated bovine erythrocytes (1% Difco trypsin, 37°C for 30 minutes). Inhibition of bovine or sheep erythrocyte hemagglutination was performed with 3 hemagglutination doses of antiserum. The method is essentially the same as previously described by Fletcher *et al* (10,11) and by Merrick *et al* (12). The activity was expressed by the minimum quantity of peptide in μg per one microtiter well that inhibit hemagglutination caused by 3 hemagglutination doses of antiserum (footnote 1), or by the dilution factor that inhibits the same amount of antibodies (Fig. 1 and 2). The activity was further determined by precipitation

Vol. 92, No. 2, 1980
January 29, 1980

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 647-654

MECHANISM OF INHIBITION OF TRANSLOCATION BY KANAMYCIN AND VIOMYCIN: A COMPARATIVE STUDY WITH FUSIDIC ACID

Masarou Misumi and Nobuo Tanaka
Institute of Applied Microbiology, University of Tokyo, Tokyo 113

Received December 10, 1979

SUMMARY

Kanamycin and viomycin were found to block a single cycle of translocation on the poly[U]-ribosome, carrying N-acetyl-diPhe-tRNA on the acceptor site and deacylated tRNA at the donor site. The inhibition of translocation was demonstrated by enhanced puromycin-reactivity of N-acetyl-diPhe-tRNA and by release of deacylated tRNA. The GTPase reaction, catalyzed by EF-G and ribosomes, was not significantly affected by the antibiotics. The results with kanamycin and viomycin differed from those obtained with fusidic acid, indicating that the mechanism of translocation inhibition may be different. Kanamycin and viomycin seemed to interfere with the translocation by fixing N-acetyl-Phe-tRNA to the acceptor site, but not to the donor site.

Kanamycin, viomycin, and related antibiotics have been recently observed to interact with both large and small subunits of ribosomes, and to inhibit translocation of peptidyl-tRNA from the acceptor site to the donor site (1-6). The results of a more detailed study of the mechanism of translocation inhibition are presented in this communication.

Since fusidic acid is known to block the overall translocation without affecting the first cycle of translocation (7-9)[cf. a review (20)], we have investigated the effects of kanamycin and viomycin on a single cycle of translocation on the ribosome, carrying N-acetyl-diPhe-tRNA on the acceptor site and deacylated tRNA on the donor site. In addition, the effects of the antibiotics on the interaction of EF-G, GTP and ribosomes with or without fusidic acid, and stabilization of peptidyl-tRNA at the acceptor and donor sites have been examined.

MATERIALS AND METHODS

[^{14}C]Phenylalanine (513 mCi/mmole) and [5,6-^{3}H]uracil (43.4 Ci/mmole) were purchased from New England Nuclear, Boston, Mass., and [^{14}C]GTP (495 mCi/mmole) from the Radiochemical Centre, Amersham, England. Puromycin, poly[U], *E. coli* tRNA, GTP and GMPP(NH)P were products of Boehringer-Mannheim, Germany.

EF-G and 1 M NH_4Cl-washed ribosomes were prepared from *E. coli* Q13, as described previously (3). N-Acetyl-[^{14}C]phenylalanyl-tRNA was synthesized by the method of Haenni and Chapeville (10). The preparation of [^{3}H]tRNA and the assay for the release of [^{3}H]

0006-291X/80/020647-08$01.00/0

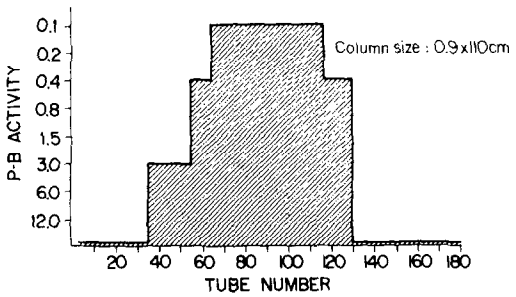

Fig. 2. Elution pattern of P-B activity through hydroxypropylated Sephadex G50 (LH60) in chloroform-methanol (2:1).

The pooled fraction No. 22-23 in Fig. 1 were combined, evaporated and the dried residue was re-dissolved in 2 ml of C-M (2:1) and applied onto an LH60 column (0.9 x110 cm). Each fraction 1 ml, ten fractions were pooled, evaporated and the residue dissolved in 1 ml salt/Pi, from this 25 µl was taken for determination of P-B activity. The ordinate indicates the amount of original solutions to be diluted which inhibit P-B hemagglutination. Fractions with P-B activity were named as follows: S1 (Tube #31-40), S2 (#41-50), S3 (#51-60), S4 (#61-70), S5 (#71-80), S6 (#81-90), S7 (#91-100), S8 (#101-110), S9 (#111-120), S10 (#121-130). Essentially the same elution pattern was obtained from other pooled fractions (pooled fraction No. 20-21; 24-25 in Fig. 1).

from a Biosil A column by solvent #1 (the pooled Fr. No. 2-4 in Fig. 1), and was consequently eluted from an LH60 column (1.6 x 120 cm) together with GM_3 (Fr. No. 38-42, each Fr. 4 ml). The protein fraction was finally separated from GM_3 through chromatography on a magnesium oxide-silicic acid column. GM_3 was eluted with ethylene dichloride-methanol-water (10:90:1), whereas, proteins were eluted with chloroform-methanol-water (10:90:5).

A similar chloroform-methanol soluble protein was isolated from the pooled fractions NO. 15-25 (Fig. 1), and showed a strong P-B antigen activity.[1] The P-B active fraction separated on Biosil A chromatography was further purified through gel filtration on an LH60 column (0.9 x 110 cm) in chloroform-methanol (see Fig. 2). Both Fr. 1 protein and Fr. 2 (P-B active) protein were non-dialyzable, soluble in chloroform-methanol in various ratios as well as in water, and gave a characteristic amino acid composition (Table I). On electrophoresis

[1]Activities of the purified samples, S4: 0.3 µg, S5: 0.15 µg, S6: 0.075 µg, S7: 0.075 µg per well (25 µl) that inhibit the hemagglutination caused by 25 µl of 3 agglutination doses of P-B antibodies. On agarose gel plate, these antigens showed a strong percipitin line with the P-B antibodies. Some fractions (S4, S5) showed two or three lines, possibly due to the presence of ganglioside-protein complex.

TABLE I. Amino acid composition of ganglioside associated proteins and that with Paul-Bunnell antigen activity (in mole %).*

	Fr. 1 Protein	S6 (from Fr. 2) (P-B antigen)	S7 (from Fr. 2) (P-B antigen)
Cys O_3	ND	(1.4)	(1.4)
Asp	4.5	10.3	10.4
Thr	4.3	7.5	8.1
Ser	22.9	11.2	11.3
Glu	11.3	7.4	7.5
Pro	3.4	11.6	11.5
Gly	17.4	6.1	5.7
Ala	7.7	10.5	10.7
Val	3.6	5.2	4.8
Met	0	(1.1)	(0.8)
Ileu	3.7	(1.8)	(1.9)
Leu	3.1	9.8	10.6
Tyr	2.0	(0.7)	(0.1)
Ph-al	2.1	4.9	5.3
Lys	9.2	3.7	3.3
His	3.4	5.2	5.3
Arg	(1.4)	(1.9)	(1.6)

*Analysis was kindly performed by Dr. Kazuo Fujikawa (Dept. of Biochemistry, Univ. of Wash.) and by Dr. Kirk Chen (Dept. of Pathobiology, Public Health Service Hospital). Samples were hydrolyzed with 6 N NCl at 110°C for 24 hrs in a sealed vacuum tube; serine increased by 10% and threonine increased by 5% to compensate for destruction during hydrolysis. Amino acid less than 2 mole % (shown in parenthesis) could be a contaminant for molecular weight of these proteins is less than 5000. About 2 mole % of hexosamine was detected in S6 and S7, but not in S4, nor in Fr. 1.
ND: not determined.

in 0.1% sodium dodecyl sulfate-15% polyacrylamide, the major Fr. 1 protein (Gel 7, Fig. 3) and P-B active fraction (Gel 3 to 6, Fig. 3) contained a low molecular weight component migrated to the same mobility as glucagon, but slightly behind the dye front region. However, the early eluate from an LH60 column (S1 to S4) with P-B activity contained an additional higher molecular weight component (M.W. about 25,000) (Gel 2, Fig. 3). Thus P-B active fractions contained a common component which had the same electrophoretic mobility as Fr. 1 protein and as glucagon (Fig. 3). These low molecular weight proteins were difficult to detect by normal staining procedures. They were stained by Coomassie blue, but de-stained with the background stain during

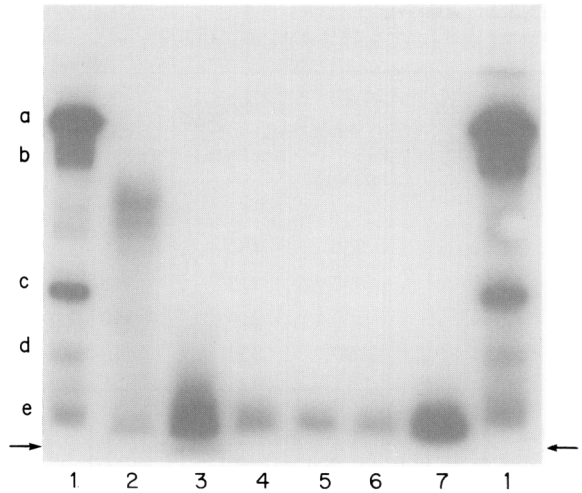

Fig. 3. Sodium dodecyl sulfate electrophoresis pattern of amphipathic protein fractions with P-B antigen activity and ganglioside binding proteins.

The condition of electrophoresis, see the text.
Gel 1 reference run, a. bovine serum albumin (M.W. 52,000); b. ovalbumin (M.W. 43,000); c. trypsin inhibitor (M.W. 15,000); d. hemoglobin (M.W. 12,000); e.glucagon (M.W. 3,500). Gel 2, S4 (tube #61-70 see legend to Fig. 2); Gel 3, S7 (tube #91-100 see legend to Fig. 2); gel 4, 2nd preparation of S4; Gel 5, 2nd preparation of S5; Gel 6, 2nd preparation of S7; Gel 7, fraction 1 protein of Fig. 1 purified by Florisil column. The arrow indicates the position of the dye front. Fractions S1 to S3 showed a similar pattern as S4, *i.e.* contained a higher molecular weight component (M.W. about 25,000) in addition to a low molecular weight component (Gel 2). S5 to S10 showed a similar pattern as S7, *i.e.* contained only a low molecular weight component (Gel 3). Second S4 did not exactly match with the 1st preparation and did not contain higher molecular weight component. The higher molecular weight component might be eluted earlier.

the de-staining procedure. The gel pattern was therefore obtained by iodination with ^{125}I followed by autoradiography. The precipitin line formed between [^{125}I]-P-B and its antibodies was strongly radioactive as evidenced by autoradiogram of the precipitin line (data not shown). However, these components have not yet been completely purified and therefore, definitive chemical composition and molecular weight is yet to be determined. The protein with P-B antigen activity did bind with GM_3-ganglioside as revealed by co-precipitation with anti-P-B antibodies, *i.e.* GM_3 but not globoside and sialylparagloboside were precipitated with anti-P-B and its second antibodies, except for S4 ("Exp 4") which contained a higher molecular component, and which co-precipitated with both GM_3 and globoside (see Table II). The interaction of P-B antigen with GM_3 was also demonstrated by the change of micellar aggregation of GM_3 in the

TABLE II. Interaction of P-B antigen and GM_3-ganglioside demonstrated by co-precipitation of P-B antigen and GM_3 with anti-P-B antibodies*

		GM_3 with P-B antigen	GM_3 without P-B antigen	Globoside with P-B antigen	Globoside without P-B antigen	Sialyl paragloboside with P-B antigen	Sialyl paragloboside without P-B antigen
Radioactivity (cpm) found in the precipitate formed by anti-P-B and anti-human IgM antibodies	Exp 1†	7800	750	450	365	540	750
	Exp 2†	7600	430	315	310	ND	ND
	Exp 3‡	3500	150	250	150	130	120
	Exp 4§	3250	1100	6500	1560	ND	ND

*Each 1 µg of [^{3}H]-labeled glycolipid, GM_3-ganglioside (15,000 cpm/µg), globoside (16,000 cpm/µg) and sialylparagloboside (25,000 cpm/µg) were dissolved in each 30 µl of chloroform-methanol (2:1), and was, respectively, mixed with P-B antigen polypeptide (1-2 µg) in the same solvent. The mixture was evaporated to a dryness under nitrogen stream and the dried residue was dissolved in 30 µl of salt/Pi and placed, respectively, in a "Microfuge tube" (Beckman Instr. Co.). Twenty µl of an anti-P-B antiserum (1:5 diluted with salt/Pi; the titer of the undiluted serum, 1:3000 with sheep erythrocytes) added and incubated at 37°C for 30 minutes and at 4°C for 1 hour, followed by the addition of 10 µl of anti-human IgM rabbit antibody (1.8 mg Ig/ml, Miles-Yeda, Ltd., Israel). The whole mixture was incubated for overnight at 4°C, and centrifuged in a Microfuge. The precipitate was washed twice with salt/Pi, and dissolved in "NC-solubilizer" (Nuclear Chicago) and the radio-activity was determined in a Scintillation counter. †Exp 1 and 2 were carried out with the 2nd preparation of S5 (Gel 5, Fig. 3); ‡Exp 3 was carried out with S7 (Gel 3, Fig. 5); §Exp 4 was carried out with S4 (Gel 2, Fig. 3). ND: not determined.

presence of P-B antigen polypeptide on ultracentrifugation. The major protein from Fr. 1 (Fig. 1) showed a similar binding affinity to glycolipid (data not shown; details will be described elsewhere).

Paul and Bunnell (6) reported that sera of patients with infectious mononucleosis agglutinated sheep erythrocytes and trypsinized bovine erythrocytes. Although the sera of patients with infectious mononucleosis contain antibodies with various specificities (16), the P-B antibody is of major clinical importance for diagnosis and pathogenesis of this disease (16,17,18). Recently, the appearance of P-B antigen in malignant lymphoma and some other tumors has been reported (18). Fletcher *et al* (10,11) and Merrick *et al* (12) have extracted and purified the P-B antigen from bovine erythrocyte membranes. A glycoprotein with relative molecular weight of 25,000 with 50% carbohydrate content was isolated and the activity was significantly reduced by treating with sialidase (11). On the other hand, a glycoprotein with a similar molecular weight but with about 10% carbo-

hydrate content was isolated by Merrick *et al* (12) and the P-B activity was destroyed by pronase and was reduced by sialidase treatment (12).[2] The latter study suggested that the antigen is an amphipathic protein associated with lipid components. The active protein reported in this paper is distinctively different from the glycoprotein antigens previously reported (10-12). It is possible, however, that the low molecular weight active polypeptide could be tightly associated with the reported sialoglycoprotein because the active polypeptide has a gangliophilic property and may have an affinity to sialosyl residue of glycoprotein as well. "Ganglioproteins", the cross reacting protein components to anti-ganglioside antibodies (20) may include the gangliophilic protein as described in this paper.

A possible role of gangliophilic proteins in regulating the mobility and the organization of membrane ganglioside is an important subject for future study.

REFERENCES

1. Folch-Pi, J. Arsove, S., and Meath, J.A. (1951) J. Biol. Chem. 191, 819-830.
2. Rosenberg, A., and Chargaff, E. (1958) J. Biol. Chem. 232, 1031-1049.
3. Hakomori, S., Ishimoda, T., Kawauchi, H., and Nakamura, K. (1963) J. Biochem. (Tokyo) 54, 458-461.
4. Watanabe, K., Hakomori, S., Childs, R.A., and Feizi, T. (1979) J. Biol. Chem. 254, 3221-3228.
5. Watanabe, K., Powell, M.E., and Hakomori, S. (1978) J. Biol. Chem. 253, 8962-8967.
6. Paul, J.R., and Bunnell, W.W. (1932) Amer. J. Med. Sci. 183, 90-104.
7. Yu, R.K., and Ledeen, R.W. (1972) J. Lipid Res. 13, 680-686.
8. Udenfriend, S., Stein, S., Böhlen, P., Dairman, W., Leingruber, W., and Wiegle, M. (1972) Science 178, 871-872.
9. Sherman, J., and Touchstone, J.C. (1974) Analytical Letters 7, 279-287.
10. Fletcher, M.A., and Woolfolk, B.F. (1971) J. Immunology 107, 842-853.
11. Fletcher, M.A., Lo, T.M., and Graves, W.R. (1976) J. Immunol. 117, 722-729.
12. Merrick, J.M., Schifferle, R., Zadarlik, K., Kano, K., and Milgrom, F. (1977) J. Supramolecular Structure 6, 275-290.
13. Laemmli, U.K. (1970) Nature 227, 680-685.
14. Freeman, T. (1967) in Handbook of Experimental Immunology, ed. D.M. Weir, F.A. Davis Co. (Philadelphia, PA) pp. 597-607.
15. Schwarzmann, G. (1978) Biochim. Biophys. Acta 529, 106-144.
16. Langhorne, J., and Feizi, T. (1977) Clin. exp. Immunol. 30, 354-363.

[2]Although sialidase susceptibility of P-B antigen *in situ* has been reported by four independent laboratories (10,12,17,19), the purified protein with P-B activity was negative in resorcinol reaction for sialic acid, and P-B activity was not altered by treatment with *Vibrio cholerae* sialidase. It is possible that P-B activity carried by polypeptide is stabilized or enhanced by association with ganglioside or sialoglycoprotein due to its "gangliophilic" property.

17. Lowenthal, R.M., McLauchlan, S.L., and Tyrrell, D.A.J. (1973) Nature 244, 109-111.
18. Kano, K., and Milgrom, F. (1977) Current Topics in Microbiology and Immunology 77, 43-69.
19. Springer, G.F., and Rapport, M.J. (1957) Proc. Soc. Exp. Biol. Med. 96, 103-107.
20. Tonegawa, Y., and Hakomori, S. (1977) Biochem. Biophys. Res. Commun. 76, 9-17.
21. Svennerholm, L (1963) J. Neurochem. 10, 613-623.