On the structure of cytolipin R, a ceramide tetrahexoside hapten from rat lymphosarcoma

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Abstract Cytolipin R, a ceramide tetrahexoside isolated from rat lymphosarcoma, was studied by sequential hydrolysis with specific glycosidases which revealed the anomeric configurations of the glycosidic bonds. Sugar linkages were established by combined gas–liquid chromatography and mass spectrometry of the partially methylated alditol acetates prepared after permethylation and hydrolysis of the intact lipid. Results indicated the structure of cytolipin R to be *N*-acetylgalactosaminyl(β1→3)galactosyl(α1→3)galactosyl(β1→4)glucosyl ceramide. Cytolipin K (globoside I) differs in having a -galactosyl(α1→4)galactosyl- internal linkage, and this difference must account for the immunological differences between cytolipin K and cytolipin R.

Supplementary key words glycosphingolipid · hapten · cytolipin K · asialo-G_{M1}-ganglioside · glycosidases · alditol acetates · gas–liquid chromatography · mass spectrometry

Glycosphingolipids have important specific immunologic properties (1) and may be involved in other cell surface phenomena such as cell–cell recognition and contact inhibition (2–8). Three naturally occurring ceramide tetrasaccharides are known (1), and all have similar compositions: ceramide bound to one residue of glucose, two of galactose, and one of *N*-acylgalactosamine. These are asialo-G_{M1}-ganglioside from mammalian brain (9), cytolipin K (also called globoside I) from human kidney and human red cells (10), and cytolipin R, from rat lymphosarcoma (11). Asialo-G_{M1}-ganglioside has the structure galactosyl(β1→3)-*N*-acetylgalactosaminyl(β1→4)galactosyl(β1→4)glucosyl ceramide (12), and cytolipin K is *N*-acetylgalactosaminyl(β1→3)galactosyl(α1→4)galactosyl(β1→4)glucosyl ceramide (13). Until very recently, Forssman hapten was thought to be a member of this group of compounds, but evidence was recently presented indicating that Forssman hapten is a ceramide pentasaccharide: *N*-acetylgalactosaminyl-(α1→3)-*N*-acetylgalactosaminyl(β1→3)galactosyl(α1→4)galactosyl(β1→4)glucosyl ceramide (14).

In this report we present evidence for the linkages and anomeric configurations of the glycosidic bonds in cytolipin R.

MATERIALS AND METHODS

Isolation of cytolipin R

Cytolipin R was isolated from chloroform–methanol extracts of rat lymphosarcoma tissue after acetone dehydration (11). The method of purification was modified from that previously reported (11). Four sequential fractionations on columns were carried out. Fractions from chromatography on Unisil eluted with acetone–methanol 85:15 and 80:20 (v/v) were passed through a DEAE-cellulose column to remove acidic phospholipids. The glycolipid fractions eluted with chloroform–methanol 90:10 and 70:30 (v/v) were combined and refractionated on Unisil. The fractions eluted with chloroform–methanol 80:20 and 75:25 (v/v) were combined and reprocessed on DEAE-cellulose; the fractions eluted with chloroform–methanol 70:30 and 50:50 (v/v) were collected. The combined material was recrystallized from methanol, and it then migrated on thin-layer plates of silica gel G as a homogeneous material. Analysis showed a long-chain base content of 21.4%, and galactosamine, 13.8%. Calculated values for a molecular weight of 1300 (11) require 23.0% and 13.8%, respectively. The phosphorus content was less than 0.05%.

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; CMH, ceramide monohexoside; CDH, ceramide dihexoside; CTH, ceramide trihexoside.

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GLC analysis of trimethylsilyl methyl glycosides (15) indicated the molar ratio of galactosamine:galactose:glucose to be 1:2:1. From 1076 g of lymphosarcoma tissue, 41 mg of crystallized cytolipin R was isolated. An additional 60–70 mg could be obtained from the mother liquor. Complete details of the method of isolation will be described[1] at a later date.

Standard glycolipids and oligosaccharides

Fabry ceramide trihexoside was obtained from the kidney of a patient with Fabry's disease. The kidney was homogenized in chloroform–methanol 2:1 to obtain a crude lipid fraction. Addition of ether at room temperature produced a precipitate which was subjected to mild alkaline methanolysis (15). The crude glycolipid mixture (3 g) was chromatographed on a column (52 × 290 mm) containing 400 g of heat-activated Unisil prepared in 5% methanol in chloroform. The column was successively eluted with 1500 ml each of 12%, 14%, 16%, 20%, 30%, and 50% methanol in chloroform. Fabry ceramide trihexoside was eluted (1.5 g) as a pure compound in the 20% fraction. The 16% and 30% fractions also contained some (1 g) of the Fabry lipid mixed with other glycolipids. Porcine erythrocyte stroma globoside was prepared according to Vance and Sweeley (15). Neuraminyllactose and lactose were purchased from Sigma Chemical Co., St. Louis, Mo.

Estimation of long-chain base; sugars

Long-chain base was estimated colorimetrically according to Lauter and Trams (16), galactosamine was measured by the method of Levvy and McAllan (17), and galactose:glucose:galactosamine ratios were determined by GLC according to Sweeley and Walker (18) and Vance and Sweeley (15). The terminal sugar was identified after mild acid hydrolysis (19) of cytolipin R, and by mass spectrometry of the intact lipid as the trimethylsilyl derivative (20).

Glycosphingolipid hydrolysis with glycosidases

Cytolipin R was incubated with the following enzymes: β-hexosaminidase from jack bean (21), α-galactosidase from ficin (13), and β-galactosidase from jack bean (22). Reactions were carried out on 100 μg of lipid, in a volume of 0.1 ml containing 0.1 M sodium citrate buffer, pH 5.0, and 100 μg of crude sodium taurocholate. After 18 hr at 37°C, reaction mixtures were frozen and lyophilized. 1 ml of chloroform–methanol 2:1 was added and the mixture was sonicated for 5 min. After centrifugation, the supernatant fractions were dried and then taken up in a small amount of chloroform–methanol 2:1. The solution of lipid was spotted on 250-μm layers of silica gel HR and chromatographed in chloroform–methanol–water 100:42:6. Lipids were visualized with iodine or α-naphthol, or by charring.

Permethylation and hydrolysis of glycolipids; preparation and analysis of partially methylated alditol acetates

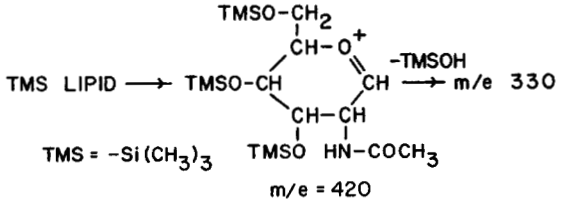
Permethylation was carried out by the method of Hakomori (23). Hydrolyses of the methylated glycolipids and oligosaccharides were then performed according to Jamieson, Jett, and DeBernardo (24). GLC of partially methylated alditol acetates was performed according to Björndal, Lindberg, and Svensson (25) on an F&M model 402 gas–liquid chromatograph (Hewlett-Packard Co., Avondale, Pa.) equipped with a 2 m × 3 mm column of 3% ECNSS-M on 100–120 mesh Supelcoport (Supelco, Inc., Bellefonte, Pa.), using helium as carrier gas. Mass spectra were recorded at 70 ev on an LKB 9000 fitted with a 2 m × 2 mm column of 3% ECNSS-M maintained isothermally at 155°C. Mass spectra were interpreted with reference to those published by Björndal et al. (26, 27) and Puro (28).

RESULTS

Identification of the terminal sugar in cytolipin R

Partial acid hydrolysis in 0.1 N HCl at 98°C for 45 min liberated *N*-acetylgalactosamine as the major monosaccharide product, and a lipid product with the properties of a ceramide trihexoside. The same result was obtained with cytolipin K, whereas with asialo-G_{M1}-ganglioside no hexosamine was released and no ceramide trihexoside was produced.

Mass spectrometry of the intact cytolipin R as the trimethylsilyl derivative, using a direct probe inlet system on the LKB 9000 at 70 ev, gave high values for *m/e* 420 and 330, indicating ions formed from electron impact-induced liberation of a terminal tristrimethylsilyl-*N*-acetylgalactosamine and its M − 90 (M − trimethylsilanol) product, respectively (20).



Enzymatic degradation of cytolipin R; configuration of anomeric linkages

A TLC separation of products resulting from incubation of cytolipin R with specific glycosidases is depicted in Fig. 1. The β-*N*-acetylhexosaminidase preparation

[1] Rapport, M. M., L. Graf, H. Schneider, and A. Kisic. Unpublished results.

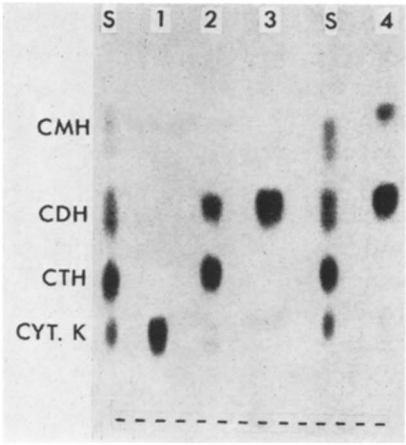


FIG. 1. TLC of cytolipin R incubated with glycosidases as follows: lane *1*, cytolipin R alone; lane *2*, cytolipin R plus jack bean β-*N*-acetylhexosaminidase with an α-galactosidase contaminant; lane *3*, cytolipin R, jack bean β-*N*-acetylhexosaminidase, and ficin α-galactosidase; lane *4*, same as lane *3* with the addition of jack bean β-galactosidase. Lanes marked *S* are standards of ceramide monohexoside (CMH), ceramide dihexoside (CDH), Fabry ceramide trihexoside (CTH), and porcine globoside (CYT. K). A 250-μm layer of silica gel HR (Brinkmann) was used with a solvent system of chloroform–methanol–water 100:42:6.

from jack bean contained some α-galactosidase activity toward Fabry ceramide trihexoside (CTH), known to have a terminal α-galactosyl bond (29). When pure ficin α-galactosidase was incubated with cytolipin R, no reaction occurred. Jack bean β-*N*-acetylhexosaminidase, however, cleaved the lipid to a product with TLC behavior similar to Fabry CTH. Further degradation to the ceramide dihexoside took place in this preparation, due to the presence of the α-galactosidase contaminant. Combined reaction of fig α-galactosidase and jack bean β-hexosaminidase with cytolipin R produced one component with TLC properties of a ceramide dihexoside. Removal of a third sugar resulted when specific jack bean β-galactosidase was added to the incubation mixture. The yield of this reaction was limited because of lower activity of this enzyme plus the reduced aqueous solubility of ceramide dihexosides when compared with higher oligomers.

Porcine erythrocyte stroma ceramide tetrahexoside having the serological activity of cytolipin K[2] gave the same chemical results as cytolipin R with these glycosidases.

These results show that the anomeric linkage between the *N*-acetylgalactosamine and galactose residues had a β-configuration, that the anomeric linkage between the galactose residues had an α-configuration, and that the anomeric linkage between the galactose and glucose residues had a β-configuration. No differences were discernible in configuration of anomeric linkages in the two molecules, cytolipin R and cytolipin K, which have different immunological specificities.

[2] Graf, L., and M. M. Rapport. Unpublished results.

GLC and mass spectral analysis of partially methylated alditol acetates from permethylation experiments; internal linkages

GLC analyses of the partially methylated alditol acetates from cytolipin K, lactose, and Fabry CTH are shown in Fig. 2. Two peaks would be expected for lactose, corresponding to 2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetyl galactitol (*A*) and 2,3,6-tri-*O*-methyl-1,4,5-tri-*O*-acetyl glucitol (*D*) (28). Comparison of mass spectra for these peaks with those published by Björndal et al. (26, 27) and Puro (28) confirmed these structures. A third peak (*C*) was present in the GLC tracing of Fabry CTH representing 2,3,6-tri-*O*-methyl-1,4,5-tri-*O*-acetyl galactitol (27, 30). Since the mass spectrometer cannot normally distinguish between epimers, peaks *C* and *D* have essentially identical mass spectra, reflecting the identical arrangement of *O*-methyl and *O*-acetyl functions. In molecules of the cytolipin K type, no peak *A* occurs (30), due to the lack of a terminal hexose. Instead, the penultimate residue (1→3 linkage) in this molecule provides a 4-*O*-methyl substitution in the alditol acetate species 2,4,6-tri-*O*-methyl-1,3,5-tri-*O*-acetyl galactitol (peak *B*), which has a shorter retention time than either *C* or *D*.

Fig. 3 shows analogous chromatograms for cytolipin R and authentic neuraminyllactose, neither of which has a peak *C* for 4-linked galactose. Integration[3] of the peaks *B* and *D* from cytolipin R gave a value of 2.15:1 for the ratio of the relative amounts of 3-linked galactose and 4-linked glucose, respectively. Neuraminyllactose gave a ratio of 1:1 for peaks *B* and *D*. Mass spectra of peaks *B* and *D* for both neuraminyllactose and cytolipin R are shown in Fig. 4. Spectrum *B* for neuraminyllactose is identical with spectrum *B* for cytolipin R; this comparison is also true for the spectra for peaks *D*. The ion fragment patterns in these spectra agree with those published by Björndal et al. (26, 27) and Puro (28) for 2,4,6-tri-*O*-methyl-1,3,5-tri-*O*-methyl hexose (*B*) and 2,3,6-tri-*O*-methyl-1,4,5-tri-*O*-acetyl hexose (*D*). These results show that the two galactose residues in cytolipin R are both linked through the 3 position.

Conclusion

On the basis of the experimental results presented, we propose that cytolipin R has the structure *N*-acetyl-

[3] The tetra-*O*-methyl hexitol acetates (*A*) do not give equivalent GLC peak areas when compared with the tri-*O*-methyl hexitol acetates (*B*, *C*, *D*). This may be due to losses during concentration because of high volatility of the tetra-*O*-methyl derivatives, as suggested by Björndal et al. (27).

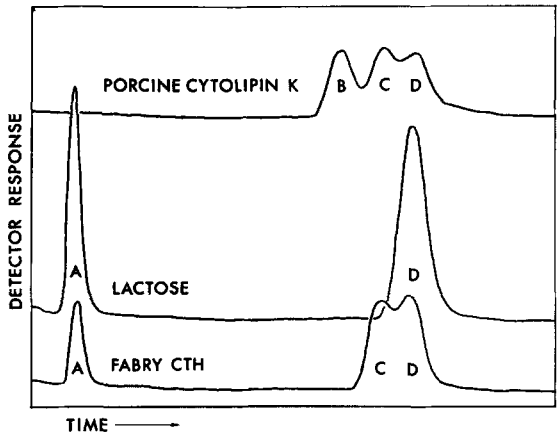


FIG. 2. GLC of partially methylated alditol acetates derived from porcine cytolipin K (erythrocyte stroma globoside), lactose, and Fabry CTH. Peak *A* corresponds to 2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetyl galactitol; peak *B* to 2,4,6-tri-*O*-methyl-1,3,5-tri-*O*-acetyl galactitol; and peaks *C* and *D* to 2,3,6-tri-*O*-methyl-1,4,5-tri-*O*-acetyl galactitol and -glucitol, respectively. Performed on a 2 m × 3 mm column of 3% ECNSS-M on 100–120 mesh Supelcoport maintained isothermally at 155°C using he'ium as carrier gas.

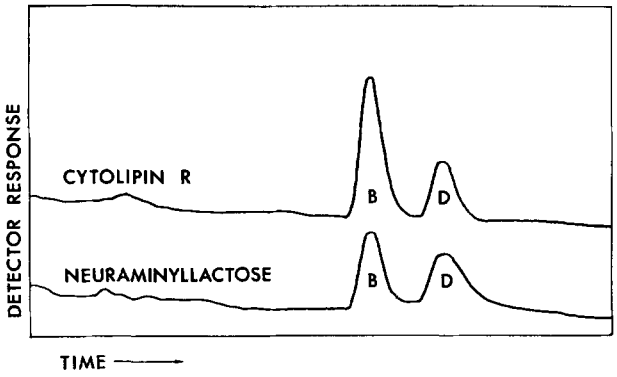
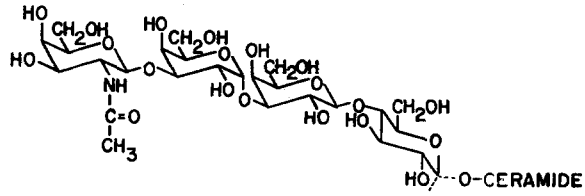


FIG. 3. GLC of partially methylated alditol acetates derived from neuraminyllactose and cytolipin R. Peaks *B* and *D* for both compounds correspond to those in Fig. 2 and were recorded under the same conditions.

galactosaminyl(β1→3)galactosyl(α1→3)galactosyl(β1→4)glucosyl ceramide, as shown below.



CYTOLIPIN R

DISCUSSION

Previous studies based on partial acid hydrolysis[1] provided evidence that the sequence of residues in cytolipin R was *N*-acetylgalactosamine-galactose-galactose-glucose ceramide. Mass spectral evidence[4] confirmed the presence of a terminal *N*-acetylgalactosamine residue and indicated that one of the internal linkages to galactose was in either the 2 or the 3 position. The present studies provide definitive evidence for both the position and the anomeric configurations of all linkages between the monosaccharide residues of cytolipin R. The structure is found to differ from that of cytolipin K in only one respect, namely, the position through which the two galactose residues are linked. In cytolipin R this linkage is galactose(α1→3)galactose, and in human cytolipin K (13) and porcine cytolipin K (31) it is galactose-(α1→4)galactose. This close structural relationship accounts for the cross reactions observed between the cytolipin K and cytolipin R systems (1), but even more importantly, it confirms the exquisite degree of immunological specificity that can be attained, since some antibodies to cytolipin R do not cross-react with cytolipin K despite the identical configurations of the terminal and penultimate residues.

This structural identification of cytolipin R raises several questions concerning the glycosphingolipids in rat tissues. A ceramide trihexoside precursor should also have a galactose(α1→3)galactose linkage, and rat tissues should be examined for the presence of this substance. Kawanami (32) has proposed that a ceramide trihexoside with a galactose(1→4)galactose linkage and a related ceramide tetrahexoside are present in rat kidney, basing the evidence on methylation analysis. His preparation of the ceramide tetrahexoside was less active serologically than cytolipin R,[5] suggesting that a mixture of ceramide tetrahexosides was present or that alkaline hydrolysis used in the isolation procedure produced some alteration. It would be difficult to resolve this problem with any of the techniques currently available.

Not all immunological techniques appear to be capable of discriminating between structures as similar as those of cytolipin R and cytolipin K. Naiki and Taketomi (33), using inhibition of the hemolysis of pig erythrocytes by antiserum to pig spleen, were unable to discriminate between ceramide tetrahexosides from human red cells and rat kidney. This led them to question the validity of the differentiation between cytolipin K and cytolipin R. The present study confirms previous chemical evidence indicating structural individuality of these compounds and suggests that antiserum to pig spleen is not a useful reagent for discriminating between them.

Although mixtures of cytolipin K and cytolipin R cannot yet be separated chromatographically, it is possible that such mixtures could be detected by GLC analysis of the partially methylated alditol acetates,

[4] Sweeley, C. C. Unpublished results.
[5] Graf, L. Unpublished results.

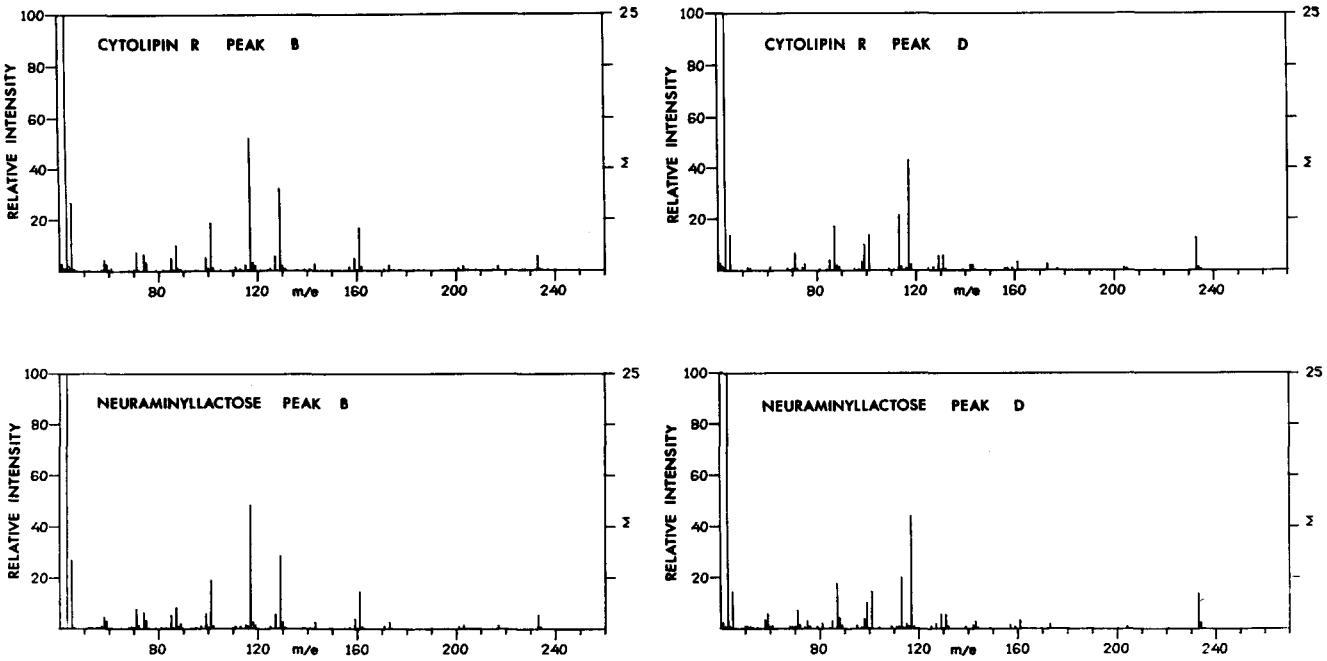


FIG. 4. Mass spectrometry of the partially methylated alditol acetates shown in Fig. 3. Mass spectra were recorded at 70 ev on an LKB 9000 combined GLC–mass spectrometer equipped with a computerized data handling system.

examining the proportion of peak *C* (Fig. 2) to peak *B* (Figs. 2 and 3). This method would be limited quantitatively by the incomplete resolution of these peaks.

A final question is concerned with the specific glycosyl transferases certain to be involved in the biosynthesis of cytolipin K and cytolipin R. Because of recent evidence for changes in glycosyl transferase activities in virus-transformed cells (8), comparison of these enzymes in rat lymphosarcoma and normal tissues would be of interest.

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