Structural Analysis of the Interaction between Shiga Toxin B Subunits and Linear Polymers Bearing Clustered Globotriose Residues

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Received 28 September 2005/Returned for modification 17 November 2005/Accepted 9 December 2005

We previously developed linear polymers bearing clustered trisaccharides of globotriaosylceramide (Gb3) as orally applicable Shiga toxin (Stx) neutralizers. Here, using a Gb3 polymer with a short spacer tethering the trisaccharide to the core, we found that shortening the spacer length markedly reduced the binding affinity for Stx2 but not Stx1. Moreover, mutational analysis revealed that the essential binding sites of the terminal trisaccharides were completely different between Stx1 and Stx2. These results provide the molecular basis for the interaction between Stx B subunits and Gb3 polymers.

Shiga toxin (Stx) is a major virulence factor in infections with Shiga toxin-producing *Escherichia coli* (STEC) in humans (6, 17, 19, 20). Stx is classified into two subgroups, Stx1 and Stx2, and Stx2 is more closely related to the severity of STEC infections than Stx1 (3, 18, 21, 24). Stx consists of a catalytic A subunit and a pentameric B subunit that is responsible for the binding of Stx to its functional cell surface receptor, globotriaosylceramide (Gb3) (Gal α [1-4]Gal β [1-4]Glc β 1-ceramide) (5, 10, 19). The crystal structure of the Stx1 B subunit in complex with a trisaccharide receptor analogue identified three trisaccharide-binding sites per B-subunit monomer, i.e., sites 1, 2, and 3 (8). A recent analysis of the crystal structure of Stx2 also predicted the presence of the corresponding trisaccharide-binding sites on its B subunit (4). Because multiple interactions of the B-subunit pentamer with the trisaccharide moiety of Gb3 are known to be essential for the high-affinity binding to its receptor, several synthetic Shiga toxin neutralizers that contain trisaccharide in multiple configurations have been developed (1, 2, 7, 13, 14, 25).

Recently, we developed linear polymers of acrylamide with clustered trisaccharides (Gb3 polymers) as oral therapeutic agents that function in the gut (25). Gb3 polymers with a high density of the trisaccharide bound to both Stx1 and Stx2 with high affinities, markedly inhibited their cytotoxic activities, and protected mice from a challenge with a fatal dose of *E. coli* O157:H7 when orally administered. Interestingly, reducing the trisaccharide density resulted in a decrease in the binding affinity for the Stx2 B subunit but not for the Stx1 B subunit, demonstrating that the interaction with a Gb3 polymer is different between Stx1 and Stx2. In the present study, we inves-

tigated the molecular basis of the interaction between Stx B subunits and Gb3 polymers.

First, to examine the effect of the spacer length of a Gb3 polymer on its binding affinities for Stx1 and Stx2, we synthesized a Gb3 polymer with a short spacer, referred to as Gb3 polymer 1:17s (Fig. 1). Gb3 polymer 1:17s was synthesized by polymerization of a trisaccharide derivative having an *n*-pentenyl group and a free acrylamide group at a 1:10 ratio, as described previously (12). Gb3 polymer 1:0, which contains the most dense clustering of the trisaccharides, and the other polymers, 2:17 and 1:12, were synthesized as described previously (12, 25). Polymers were indicated as X:Y, in which X and Y represent the average numbers of trisaccharide-assembled units and trisaccharide-free acrylamide units, respectively (Fig. 1). The X:Y ratio was determined by ¹H nuclear magnetic resonance spectroscopy. The spacer length of the Gb3 polymer 1:17s was about one-third of that of the other Gb3 polymers.

We determined the K_d value of Gb3 polymer 1:17s for the recombinant histidine-tagged Stx1 B subunit (1BH) and Stx2 B subunit (2BH), prepared as described previously (25), by using the BIAcore system (BIAcore, Uppsala, Sweden) (Fig. 2). These recombinant B subunits bound to Gb3 with binding affinities similar to those of native B-subunit pentamers (data not shown). The concentration of the polymer was given as the micromolar concentration of trisaccharide, which enables a direct comparison of the activity on a per-trisaccharide basis with the other Gb3 polymers. The K_d value and the maximum binding (RU_{max}) value of Gb3 polymer 1:17s for 1BH (Table 1) were similar to those of the other Gb3 polymers irrespective of the trisaccharide density (see the first line of Table 2 [cited from reference 25]). In contrast, not only the binding affinity but also the RU_{max} value of Gb3 polymer 1:17s for 2BH (Table 1) was markedly reduced compared with those of the other Gb3 polymers (see the first line of Table 3 [cited from reference 25]). These results clearly indicate the importance of the spacer length for high-affinity binding to Stx2 but not to Stx1.

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Gb3-polymers		X : Y	Molar content of trisaccharide (mmol/g)
Gb3-polymer 1:17s		1:17	0.56
Gb3-polymer 1:0 ^a		1:0	1.4
2:17 ^a		2:17	0.75
1:12 ^a		1:12	0.63

R : Gal α(1-4)-Galβ(1-4)-Glcβ1-

FIG. 1. Structures of Gb3 polymers. Linear polymers of acrylamide with trisaccharide of Gb3 are shown. X and Y represent the numbers of carbohydrate-assembled and carbohydrate-free acrylamide units, respectively. The X:Y ratio was determined by ^1H nuclear magnetic resonance spectroscopy.^a See reference 25.

FIG. 2. Kinetic analysis of the binding of Gb3 polymer 1:17s to histidine-tagged Stx B subunits using the BIAcore system. Recombinant 1BH and 2BH subunits were immobilized on a nitrilotriacetic acid sensor chip (BIAcore), and Gb3 polymer 1:17s was passed, starting at time zero, over the immobilized B subunits at a flow rate of 20 μ l/min for 3 min to reach the plateau. The resonance unit (RU) is an arbitrary unit used by the BIAcore system. The binding kinetics were analyzed by a Scatchard plot using the software BIAEVALUATION 3.0 (BIAcore).

Next, the inhibitory effect of Gb3 polymer 1:17s on the binding of Stx to Vero cells was examined. Vero cells were treated with 1 $\mu\text{g/ml}$ ^{125}I -labeled Stx1 (^{125}I -Stx1) or ^{125}I -Stx2 in the absence or presence of the desired amount of Gb3 polymer 1:0 or 1:17s for 1 h at 4°C. The 50% inhibitory concentration value of the Gb3 polymer 1:17s for ^{125}I -Stx1 binding was 0.17 μM , which was even lower than that of Gb3 polymer 1:0 (0.36 μM) (Fig. 3). In contrast, the 50% inhibitory concentration value for ^{125}I -Stx2 binding was 0.80 μM , which was 2.4 times higher than that of Gb3 polymer 1:0 (0.33 μM), indicating that shortening the spacer length substantially reduced the inhibitory activity of Gb3 polymers for the binding of Stx2, but not Stx1, to its functional cell surface receptor.

Finally, we determined the binding sites of various Gb3 polymers on the Stx1 and Stx2 B subunits by using a series of 1BH and 2BH with mutations at the trisaccharide-binding sites (15). Kinetic analysis was performed by using the BIAcore system. With regard to Stx1, Gb3 polymer 1:0 bound with high

TABLE 1. Kinetic analysis of the binding of Gb3 polymer 1:17s to histidine-tagged Stx B subunits

Subunit	Gb3 polymer 1:17s ($n = 3$)	
	Mean K_d ($\mu\text{mol/liter}$) $\pm$ SE	Mean RU_{max} $\pm$ SE
1B	0.4 ± 0.06	398 ± 102
2B	41 ± 5.4	372 ± 28

TABLE 2. Kinetic analysis of the binding of Gb3 polymers to the Stx1 B subunit mutants

Mutated site	Mutation	Gb3 polymer ^d							
		1:0		2:17		1:12		1:17s	
		<i>K_d</i> (μmol/liter) (SE)	RU _{max} (SE)	<i>K_d</i> (μmol/liter) (SE)	RU _{max} (SE)	<i>K_d</i> (μmol/liter) (SE)	RU _{max} (SE)	<i>K_d</i> (μmol/liter) (SE)	RU _{max} (SE)
Wild type		0.34 ^a	468 ^a	0.44 ^a	614 ^a	0.60 ^a	560 ^a	0.4	398
Site 1	D17E	2.1 (16)	334 (4.6)	0.75 (2.9)	1,091 (0.8)	0.50 (10)	1,100 (2.8)	0.80 (13)	760 (3.1)
	F30A	0.84 (16)	344 (3.8)	1.6 (7.1)	801 (2.6)	0.95 (4.1)	555 (1.3)	4.7 (29)	321 (11)
Site 2	A56Y	0.86 (9.8)	909 (3.4)	7.5 (2.8)	296 (1.6)	18 (1.9)	941 (0.9)	— ^c	— ^c
	G62A	1.4 (0.5)	162 (0.2)	13 (9.0)	416 (6.1)	4.4 (2.9)	649 (1)	— ^c	— ^c
Site 3	W34A	0.40 (15)	343 (3.9)	0.75 (11)	909 (3)	0.47 (4.5)	1,161 (1.2)	0.70 (12)	695 (2.6)
	D18E	0.61 (13)	1,036 (3.7)	0.58 (4.7)	764 (1.2)	0.23 (5.0)	404 (1)	0.56 (11)	724 (3.3)
Site 1+2	D17E/G62A	1.3 (14)	373 (5.4)	19 (4.2)	1,042 (2.2)	6.3 (8.1)	231 (4.6)	— ^c	— ^c
	F30A/G62A	1.7 (4.5)	376 (2)	— ^b	— ^b	20 (7.5)	318 (4.3)	— ^c	— ^c
Site 1+3	D17E/W34A	0.82 (20)	393 (6.7)	0.34 (17)	1,545 (2)	0.55 (8.1)	281 (2.3)	0.21 (7.1)	673 (1.9)
	F30A/W34A	2.3 (14)	357 (6.3)	— ^b	— ^b	— ^c	— ^c	— ^c	— ^c
Site 2+3	G62A/W34A	— ^c	— ^c	— ^c	— ^c	— ^c	— ^c	— ^c	— ^c
Site 1+2+3	D17E/G62A/W34A	— ^c	— ^c	— ^c	— ^c	— ^c	— ^c	— ^c	— ^c
	F30A/G62A/W34A	— ^c	— ^c	— ^c	— ^c	— ^c	— ^c	— ^c	— ^c

^a See reference 25.^b Did not saturate.^c Did not bind (RU_{max} <150).^d Numbers in parentheses are standard errors (percent) for linear fitting of equilibrium resonance unit (RU)/concentration versus equilibrium RU.

affinity to all of the single-point mutants and to two sets of double mutants with mutations at sites 1 and 2 or sites 1 and 3 but not to the double mutant with mutations at sites 2 and 3 or to the triple mutants (Table 2). This result indicates that site 2 or site 3 is sufficient for the high-affinity binding of Gb3 polymer 1:0 to the Stx1 B subunit. On the other hand, Gb3 polymers 2:17 and 1:12, both of which have a lower density of trisaccharides, did not efficiently bind to the single-point mutants with a mutation at site 2 (Table 2). Consistent with this result, these polymers bound to the double mutant with mutations at sites 1 and 3 (D17E/W34A), in which site 2 was intact, but not to the other double or triple mutants, demon-

strating that site 2 was sufficient for the high-affinity binding of these Gb3 polymers. Although having the intact site 2, the double mutant F30A/W34A did not bind to these Gb3 polymers, suggesting that Phe30 might affect the site 2-dependent binding of these polymers. Phe30 has been shown to be in a close configuration to Gly62, which is the residue involved in the binding with the terminal and the penultimate galactoses of the trisaccharide at site 2 (8), further supporting the above-mentioned contention. Interestingly, the same site selectivity was observed with Gb3 polymer 1:17s (Table 2).

In contrast, Gb3 polymer 1:0 bound with high affinity to all of the single-point mutants of 2BH and to its double mutants

TABLE 3. Kinetic analysis of the binding of Gb3 polymers to the Stx2 B subunit mutants

Mutated site	Mutation	Gb3 polymer ^d							
		1:0		2:17		1:12		1:17s	
		<i>K_d</i> (μmol/liter) (SE)	RU _{max} (SE)	<i>K_d</i> (μmol/liter) (SE)	RU _{max} (SE)	<i>K_d</i> (μmol/liter) (SE)	RU _{max} (SE)	<i>K_d</i> (μmol/liter) (SE)	RU _{max} (SE)
Wild type		0.68 ^a	1,340 ^a	1.4 ^a	803 ^a	7.1 ^a	961 ^a	41	372
Site 1	D16E	0.93 (22)	1,083 (5.7)	50 (3.3)	1,221 (1.6)	— ^b	— ^b	— ^c	— ^c
	W29A	0.93 (6.5)	1,784 (1.6)	47 (17)	1,481 (12)	— ^b	— ^b	— ^c	— ^c
Site 2	T55Y	0.73 (16)	1,610 (4.8)	0.60 (13)	1,216 (2.4)	0.60 (11)	451 (3.2)	18 (10)	387 (3.9)
	G61A	0.71 (17)	1,704 (3.8)	0.52 (6.4)	1,191 (1.6)	0.13 (1.3)	556 (0.3)	13 (8.7)	261 (3.1)
Site 3	W33A	1.8 (12)	2,693 (3.1)	87 (15)	2,244 (12)	— ^b	— ^b	— ^c	— ^c
	D17E	1.0 (19)	958 (6.1)	4.6 (21)	186 (10)	— ^c	— ^c	— ^c	— ^c
Site 1+2	W29A/G61A	1.5 (15)	1,268 (4.7)	84 (16)	2,565 (13)	— ^b	— ^b	— ^c	— ^c
	D16E/G61A	2.0 (7)	265 (2.6)	— ^b	— ^b	— ^c	— ^c	— ^c	— ^c
Site 1+3	W29A/W33A	— ^c	— ^c	— ^c	— ^c	— ^c	— ^c	— ^c	— ^c
Site 2+3	G61A/W33A	12 (11)	652 (5.8)	— ^b	— ^b	— ^c	— ^c	— ^c	— ^c
Site 1+2+3	W29A/G61A/W33A	69 (1.9)	218 (1.3)	141 (17)	241 (15)	— ^c	— ^c	— ^c	— ^c

^a See reference 25.^b Did not saturate.^c Did not bind (RU_{max} <150).^d Numbers in parentheses are standard errors (percent) for linear fitting of equilibrium resonance unit (RU)/concentration versus equilibrium RU.

FIG. 3. Inhibitory effects of Gb3 polymer 1:17s on ^{125}I -Stx binding to Vero cells. Vero cells maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum in a 24-well plastic microplate were treated with ^{125}I -Stx1 or ^{125}I -Stx2 (1 $\mu\text{g}/\text{ml}$; 3.8×10^6 cpm/ μg of protein) in the absence or presence of the desired amount of a given Gb3 polymer for 1 h at 4°C. After extensive washing, bound radioactivity was measured. The data are presented as the percentage of the activity in the absence of Gb3 polymer (mean $\pm$ standard error; $n = 3$). Filled circles, Gb3 polymer 1:17s; open diamonds, Gb3 polymer 1:0.

with mutations at sites 1 and 2 but not to the other double mutants or the triple mutant (Table 3). This result indicates that Gb3 polymer 1:0 bound to the Stx2 B subunit through sites 1 and 2 or site 3. Furthermore, Gb3 polymers 2:17 and 1:12 efficiently bound to only the single-point mutants with mutations at site 2 (T55Y and G61A), demonstrating that both sites 1 and 3 of the Stx2 B subunit were required for the high-affinity binding of these polymers. The same site selectivity of the binding was observed with Gb3 polymer 1:17s.

Previously, it was shown that the interaction with Gb3 was clearly distinguishable between Stx1 and Stx2 by analyzing their binding to a series of deoxy Gb3 analogues (16). Furthermore, a recent mutational analysis of Stx B subunits indicated that Stx1 required all the trisaccharide-binding sites for the high-affinity binding to Gb3 under physiological conditions (15, 22), whereas Stx2 required sites 1 and 3 but not site 2 (15). Based on these observations, our present results clearly demonstrate that Gb3 polymers inhibited the binding of Stx1 and Stx2 to target cells by competing with Gb3 for site 2 and sites 1 and 3, respectively.

A recent analysis of the crystal structure of Stx2 demonstrated that the conformation of Stx2 at site 2 distinctively differs from that of Stx1 (4). In particular, the presence of Ser54 in site 2 of the Stx2 B subunit is likely to hamper the accession of the penultimate galactose of the trisaccharide, suggesting that site 2 of Stx2 may barely contribute to trisaccharide binding. This observation may provide a rationale for the present result showing that site 2 of Stx2 was not involved in the interaction with the trisaccharide of Gb3 polymers.

In the present study, we found that a long spacer of a Gb3 polymer is required for the high-affinity binding with the Stx2 B subunit through sites 1 and 3. It is generally accepted that the fatty acid moiety of Gb3 can affect the binding of Stx to Gb3 (9). In addition, a recent analysis using trisaccharide analogues with alkyl chains of different lengths demonstrated that Stx2, but not Stx1, preferred a longer alkyl chain for high-affinity binding (11). Thus, the specific requirement of the long spacer may be explained by the structural differences between Stx1 and Stx2 at sites 1 and 3. In the crystal structure of the Stx2 B subunit, the five tryptophan rings present in site 3 (Trp33) were shown to adopt a common conformation and to form an irregular hydrophobic region on the receptor-binding surface (4),

whereas in the Stx1 B subunit, all five corresponding rings of Trp34 were packed in equal conformations (23). Such a bulky and irregular hydrophobic region of the Stx2 B subunit might be involved in the interaction with not only the trisaccharide but also the spacer moiety of a Gb3 polymer through hydrophobic interactions to ensure the high-affinity binding.

In conclusion, we found that the co-occupation of sites 1 and 3 of the Stx2 B subunit by a trisaccharide moiety with a long spacer is essential for the efficient binding of Gb3 polymers to Stx2. Considering the greater clinical significance of Stx2, our present results may be expected to further advance the development of practical therapeutic agents with more potential effectiveness against STEC infections.

This work was supported by a Health and Labor Sciences Research Grant on Advanced Medical Technology (14-N-9) and a Grant for International Health Cooperation Research (14-K-10) from the Ministry of Health, Labor, and Welfare, Japan.

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Editor: A. D. O'Brien