Glycosaminoglycans Bind to Homologous Cardiotoxins with Different Specificity†

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ABSTRACT: We have reported that some cardiotoxins (CTXs), homologous basic polypeptides of cobra venom, bind strongly to heparin. Herein we show that CTXs from spitting cobra venom bind avidly to chondroitin-6-sulfate (CS6) and dermatan sulfate (DS), the glycosaminoglycans (GAGs) abounding in the cornea and elsewhere. We compared the binding strength of Tγ, a major component of spitting cobra, *Naja nigricollis*, venom with that of CTX A3, a major component of *Naja atra* venom to various GAGs including CS6, chondroitin-4-sulfate (CS4), DS, keratan sulfate (KS), hyaluronan (HYA), and heparin. The binding strength of Tγ followed the order CS6 > KS > HYA > DS > CS4 > heparin, whereas that of CTX A3 was heparin > KS > CS4 > DS > CS6 > HYA. The binding specificity displayed by different CTXs toward GAGs is impressive, given the high homology among CTXs and among GAGs. Strong binding of Tγ to CS6, rather than to the highly anionic and versatile cousin, heparin, implies specific interaction with CS6. Heparin, at high concentration, displaced CS6 from CS6−Tγ and CS6−A3 complexes. We also show that corneal CS/DS likely allow Tγ to bind to corneal epithelium. CTXs of spitting cobra venom are known to cause corneal opacity and/or blindness. Taken together with these observations, our results suggest that corneal CS/DS play a role in the action of CTX in the eye. Most importantly, the present results establish CTXs as cationic, readily available, avidly binding ligands of CS/DS.

Glycosaminoglycans (GAGs)[1] represent the saccharide moieties of proteoglycans (PGs) which occur abundantly in all tissues, on cell surfaces, and in extracellular matrix and perform a myriad of physiological functions (*1*-*3*). GAGs are extensively sulfated, repeating copolymers of hexosamine and hexuronic acid (*4*, *5*). Heparin and heparan sulfate (HS) are classified as glucosaminoglycans, while chondroitin-6-sulfate (CS6), chondroitin-4-sulfate (CS4), and dermatan sulfate (DS) are classified as galactosaminoglycans, according to their hexosamine residues (*4*, *6*, *7*). Keratan sulfate (KS) contains galactose residues instead of hexuronic acid, and hyaluronan (HYA) is an atypical GAG in that it exists free of core proteins and lacks sulfates (*4*, *6*). Heparin/HS bind, for example, to antithrombin III and to fibroblast growth factor (FGF) and allow the proteins to exercise their physiological function (reviewed in refs *8*, *9*), whereas CS/DS regulate blood coagulation through their association with thrombomodulin and heparin cofactor II (reviewed in refs *2*, *3*, *10*). Human placental CS allows *Plasmodium falciparum* to adhere to this tissue and thereby renders pregnant women susceptible to malaria (*11*). HYA binds to CD44 proteins (*12*) in addition to others with high specificity and participates in cell−cell and cell−matrix interaction, as well as in cell migration during wound healing and embryogenesis (*13*, *14*). Many aspects of GAG−protein interaction pertaining mostly to heparin, HS, and DS have been uncovered (*15*, *16*). To date, few proteins are known to bind to CS, particularly to CS6.

Proteoglycans mediate cell−cell and cell-extracellular matrix interaction (*1*, *17*−*22*). PGs of simian and avian corneal tissues consisting of CS/DS and KS (*18*, *19*) hold collagen in a regular fashion, an arrangement that maintains corneal transparency (*19*, *20*). Certain cobra species spit venom (some are loathe to bite) into eyes of the offender. The animal then injects potent venom into the bloodstream of victim wherefrom the toxins rush to their targets to paralyze the motion and cause systolic heart arrest. Immediate and extensive washing with water removes venom, which, if remains in the eye, causes corneal opacity and/or blindness (*23*). That CTX is the venom component causing corneal opacity was proved for the African black-necked spitting cobra, *N. nigricollis* (*24*).

Cardiotoxins (CTXs) are highly homologous, basic proteins conserved throughout the genus (*25*−*27*), but they display remarkable tissue specificity (*28*−*30*). CTXs fold into an all β-sheet structure with three fingerlike loops emanating from a core, which is held by four disulfide bonds. Structures of about a dozen CTXs are established by X-ray crystallography and NMR spectroscopy (*31*, and refs cited therein). Shown in Figure 1 are structures of Toxin γ (Tγ) and CTXs A3 and A5; the overall structure of the toxins shown resemble one another, as do structures of other CTXs known. Small differences in amino acids are found mostly in the loop regions; in the structure these differences translate into variation in flexibility of each loop, especially loop 2 of the molecule (*26*). Specific properties of CTXs are hence

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[1] Abbreviations: CTX, cardiotoxin; CS, chondroitin sulfate; DS, dermatan sulfate; GAG, glycosaminoglycan; HS, heparan sulfate; HYA, hyaluronan; KS, keratan sulfate; PG, proteoglycan; Tγ, toxin γ; K_d, dissociation constant; ELISA, enzyme-linked immunoadsorbent assay.

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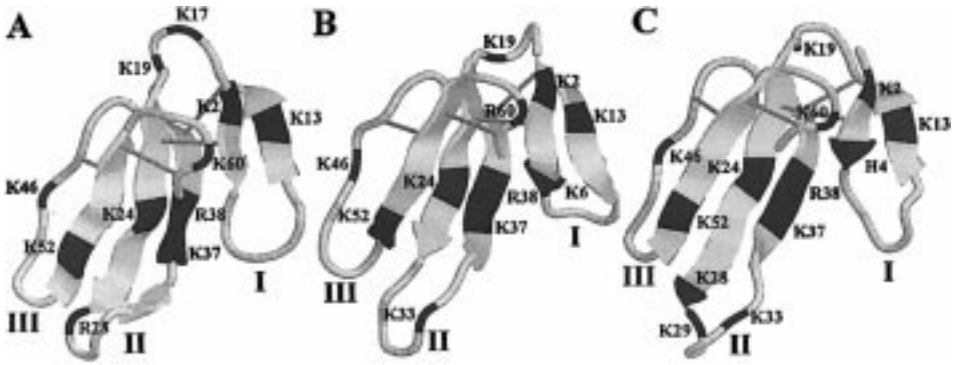

FIGURE 1: Ribbon representation of 3-dimensional structures of CTXs. The 3-D structure of all CTXs analyzed (by X-ray and/or NMR spectroscopy) are highly similar as exemplified by Tγ (A), A3 (B), and A5 (C). CTXs adopt a three-fingered loop structure formed by antiparallel β-sheets. The three loops (numbered) emanate from a core formed by four disulfide bonds (represented by rods at the upper half of the structure). The basic residues are distributed as a regular equatorial band (shaded dark). Small variations are found in the loop and β-sheet regions. The figures were generated using program Rasmol V2.6 and the coordinates from the Brookhaven Protein Data Bank.

generally attributed to this loop or variations therein (*27*). Small differences in the β-sheets are also observed (*26*, *31*).

We have reported that CTX of Taiwan cobra venom binds to heparin/HS and that the binding potentiates penetration of toxin into the membrane monolayer (*32*). We have also proposed that the loop regions of CTXs may be involved in heparin binding (*33*). However, binding affinity of CTXs varied by 3−4 orders of magnitude (*33*). The marked variation in binding affinity of CTXs toward heparin and the different tissue distribution of GAGs prompted us to believe that CTXs might react specifically with different GAGs. We reasoned that toxins of spitting cobra venom first encounter the eye of the victim where CS, DS, KS, and HYA abound.

We report herein that CTXs of spitting cobra venom, especially Tγ, bind avidly to CS, DS, KS, and HYA, whereas toxins from venom of *N. atra*, a cobra that rarely spits, bind weakly to these GAGs but strongly to heparin. The specificity in binding toward different GAGs displayed by CTXs is impressive, given the homology in nature and structure of the polypeptides and GAGs. The highly homologous toxins present themselves as a series of readily available model proteins to study protein−carbohydrate interaction, especially protein−CS/DS interaction because few proteins are known to bind to CS6 and DS.

MATERIALS AND METHODS

Crude venom from *Naja mossambica*, *N. atra*, and *N. nigricollis*, as well as chondroitin sulfate C (CS6, shark cartilage), chondroitin sulfate A (CS4), hyaluronan (both from bovine trachea), chondroitin sulfate B (dermatan sulfate), heparin (porcine intestinal mucosa), and keratan sulfate (bovine cornea) were from Sigma Chemicals. Molecular weights of the above GAGs determined by using low angle laser light scattering were 50,000, 45500, 80000, 14000, 15000, and 8900 respectively (determined by Sigma). Chondroitin sulfate C used herein contained 90% CS6 and 10% CS4; chondroitin sulfate A contained 70% CS4 and 30% CS6, while dermatan sulfate contained 90% DS and 10% of CS4 and CS6. The sulfur content of CS6, CS4, and DS was 6.4, 5.1, and 5.6%, respectively (information supplied by Sigma). Chondroitinase ABC (*Proteus vulgaris*), heparinase III (*Flavobacterium heparinum*), fluorescein isothiocyanate (FITC), FITC-labeled anti rabbit antibodies, and *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester were also from Sigma. Aprotinin and leupeptin were purchased from Boehringer Mannheim. Chromatographic media and columns were obtained from Pharmacia Biotech, and all other chemicals were from Merck. All chemicals were reagent grade. CTXs were purified as described previously (*27*, *33)*. The purity of CTXs was analyzed by SDS−PAGE, and HPLC was found to be higher than 99%. Protein concentration was determined by the Lowry method.

Fluorescence Measurements. Change in intrinsic fluorescence intensity and anisotropy of Tyr or Trp residue of CTXs were monitored during titration of CTXs with GAGs as reported (*32*, *33*). Briefly, excitation and emission wavelengths were set at 285 and 318 nm for Tyr and at 295 and 347 nm for Trp, respectively, by using a SLM 4800 fluorescence spectrometer. GAG was titrated against the protein (constant, 2.5 μM for Trp-containing, and 20 μM for Tyr-containing CTX). In competition experiments, excitation and emission wavelengths were set at 492 and 520 nm, respectively, for fluorescein−CS6.

Determination of Dissociation Constants. Dissociation constants were determined by nonlinear least-squares fitting of fluorescence data (*32*) and by assuming that *n* independent, but equivalent, binding sites are present in heparin as described in detail (*32*, *33*). Complete binding was assumed to occur when the fluorescence intensity reached a plateau.

GAG Affinity Chromatography. GAG−Sepharose columns were prepared as reported (*34*) and fitted to a BioRad FPLC system. The columns were washed and equilibrated in phosphate-buffered saline (PBS) of the following composition: 8 mM Na_2HPO_4, 2.7 mM KCl, 1.8 mM KH_2PO_4, 138 mM NaCl, pH 7.4. Crude *N. nigricollis* venom (3 mg) dissolved in PBS was loaded onto the column, after which the column was washed extensively with PBS. A linear gradient of 0−1 M NaCl in PBS was then applied, and fractions were collected. Identity of toxin peaks was confirmed by comparing HPLC retention times of the eluted fractions with those of pure toxins.

Fluorescent Labeling of CS6 and Tγ. Fluorescent labeling of Tγ was carried out as reported for other proteins (*35*). Briefly, 2.8 mg of Tγ and fluorescein isothiocyanate (FITC, 0.165 mg) were dissolved in 0.1 M sodium bicarbonate buffer; Celite (2 mg) was added to the solution, and the resulting suspension was stirred for 8−10 h in the dark at 4

Table 1: Sequence of CTXs and Comparison of Their Dissociation Constants (K_d) with CS6, DS, and Heparin and Number of Binding Sites (n)[a]

CTX	Amino acid sequences (10 20 30 40 50 60)	CS6		DS		Heparin
		K_d(μM)	n	K_d(μM)	n	K_d(μM)[b]
Tg	LKC-NQLIPP FWKTCPKGKN LCYKMTMRAA P-MVPVKRGC IDVCPKSSLL IKYMCCNTDK CN	**0.06**	13	4.3	7	140
M1	LKC-NQLIPP FWKTCPKGKN LCYKMTMRAA P-MVPVKRGC IDVCPKSSLL IKYMCCNTNK CN	**0.29**	12	4.1	7	135
M2	LKC-NQLIPP FWKTCPKGKN LCYKMTMRGA -SKVPVKRGC IDVCPKSSLL IKYMCCNTDK CN	**0.35**	12	0.9	8	0.120
M4	LKC-NRLIPP FWKTCPEGKN LCYKMTMRLA P-KVPVKRGC IDVCPKSSLL IKYMCCNTNK CN	**0.85**	13	0.06	8	0.130
M3	LKC-NKLIPI AYKTCPEGKN LCYKMMLASK -KMVPVKRGC INVCPKNSAL VKYVCCSTDR CN	**0.90**	10	0.07	8	0.02
A5	LKCHNTQLPF IYKTCPEGKN LCFKATLKKF PLKFPVKRGC ADNCPKNSAL LKYVCCSTDK CN	20	9	3	7	0.25
A3	LKC-NKLVPL FYKTCPAGKN LCYKMFMVAT P-KVPVKRGC IDVCPKSSLL VKYVCCNTDR CN	28	7	5	7	1.42
A4	RKC-NKLVPL FYKTCPAGKN LCYKMFMVSN -LTVPVKRGC IDVCPKNSAL VKYVCCNTDR CN	95	9	13	7	2.55
A2	LKC-NKLVPL FYKTCPAGKN LCYKMFMVSN -LTVPVKRGC IDVCPKNSAL VKYVCCNTDR CN	102	9	17	7	3.83
A1	LKC-NKLIPI ASKTCPAGKN LCYKMFMMSD -LTIPVKRGC IDVCPKNSLL VKYVCCNTDR CN	105	7	22	7	2.68

[a] CTXs are arranged in decreasing order of CS6 binding strength. R-28 (boxed) containing CTXs exhibit strong CS6 binding. CTX M3 with 2 contiguous Lys (boxed) binds strongly to all 3 GAGs. All CTXs containing K-33 (shaded) and K-32 (shaded) in CTX M3 in the loop 2 region (amino acid 27−34) bind stronger to heparin. [b] Data are taken from ref *33*.

°C. The supernatant was loaded onto a Sephadex G25 column to remove salt and excess FITC. The protein eluted out when distilled water was used as eluant. Fluorescent labeling of CS6 was performed as reported (*36*).

Competition Binding Assay. Competition binding assays were performed by titrating fluorescein−CS6 (100 nM, constant) against a CTX until near saturation (3.5 μM for Tγ and CTX M3, and 40 μM for CTX A3), and change in fluorescence intensity and anisotropy were monitored at each point. After that, heparin was added instead of the CTX to the same solution. The fluorescence intensity rose steadily until it reached the initial value. A binding isotherm was plotted using these data. Similarly, flourescein−CS6 (100 nM) was titrated against Tγ (3.5 μM), and unlabeled CS6 was then added until the fluorescence intensity of fluorescein−CS6 remained constant.

Generation of Specific Antibodies against CTX A5. Antibodies were generated (in New Zealand white rabbits) against a synthetic peptide corresponding to aa residues 25−34, unique to CTX A5 (see Table 1 for sequence) and located in a highly variable region at the tip of loop 2 of the toxin. The peptide was conjugated to BSA using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester as reported (*37*). Antiserum obtained after III and IV boosts was clarified by centrifugation. The supernatant was loaded onto an affinity matrix constructed by covalently coupling the synthetic peptide used for immunization to CNBr-activated sepharose 4B according to standard protocol (*38*). Bound antibodies eluted out when cycles of high and low pH were applied. Purity, specificity, and titer of the antibodies were determined by SDS−PAGE, western blotting, and ELISA, respectively.

Binding of CTXs to Corneal Epithelium. Intact eye balls (bovine) were obtained fresh from the slaughterhouse and used immediately. Corneal epithelium was isolated as reported (*39*) with minor modification. Briefly, the eyeball was rinsed using PBS and the epithelium was peeled off as a sheet from the underlying stroma using a surgical scalpel. Approximately 10 mg of corneal epithelial tissue was homogenized in a total volume of 5 mL of ice-cold PBS (supplemented with 1 mM EDTA, 150 μM PMSF, 1 μM leupeptin, and 0.1 μM aprotinin; PBSA) so as to maintain the integrity of the tissue. Aliquots (1 mL) were then centrifuged at 3000*g* for 10 min at 4 °C, and the resulting pellets were reconstituted to 0.5 mg/mL in PBSA. Aliquots (500 μL and 250 μL, respectively) were incubated with chondroitinase ABC (0.5 units) for 3.5 h at 37 °C and with heparinase III (2.5 units) for 5 h at 25 °C, essentially as reported (*4*, *40*). For double enzymatic digestion the chondroitinase-treated tissue was washed thoroughly with PBSA, resuspended in PBSA, and incubated with heparinase III as described above. The reactions were monitored spectrophotometrically at 232 nm for the release of saccharide moieties, as described (*41*). At the end of the incubation period the samples were centrifuged at 14 000 rpm for 10 min in an eppendorf centrifuge, the supernatant was discarded, and the pellet was washed three times and resuspended in PBSA. Protein concentration of the pellets was determined by BioRad dye binding assay using BSA as a standard. The protein concentration of the pellet was adjusted to 1 mg/mL using PBSA supplemented with 0.2% Tween 20 (PBSB) to prevent nonspecific binding during subsequent treatments. Aliquots, in triplicate, (200 μg/200μL) of untreated and glycanase-treated samples were incubated with 10 μM fluorescein−Tγ or unlabeled CTX A5 with end-over-end shaking for 30 min at room temperature. At the end of the incubation period, unbound toxin was removed by washing three times with PBSB followed by centrifuging the samples at 14 000 rpm for 10 min. The pellet of Tγ-treated tissue was treated with 1M NaCl containing PBSB (150 μL). Aliquots of supernatant (125 μL) of Tγ-treated samples were transferred to 96 well plates (Costar), and binding was quantitated directly by reading the intensity at 490 nm using an automated ELISA plate reader.

CTX A5-treated samples were washed three times with PBSB and centrifuged, and the pellet was resuspended in PBSB containing 1% BSA (200 μL). To this solution were

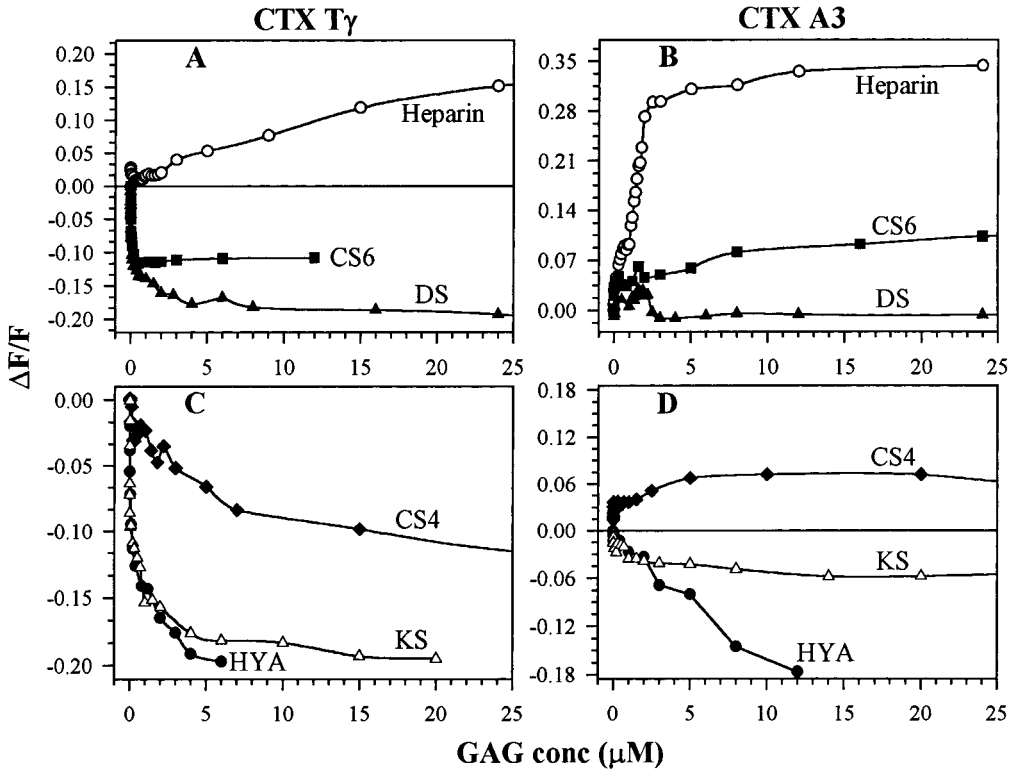

FIGURE 2: Change in fluorescence intensity of Trp of Tγ (left panel) and Tyr of CTX A3 (right panel) plotted against GAG concentration. Clearly evident is the higher avidity of binding of Tγ for CS6/DS (A) and for KS and HYA (C), than for heparin (A). With CTX A3 the opposite change prevails; it exhibits stronger binding for heparin than for C6S/DS (B) and HYA (D). The binding of the two toxins to CS4 is also different (C and D). A negative value of $\Delta F/F$ indicates quenching of fluorescence intensity upon GAG binding.

added CTX A5-specific antibodies at a dilution of 1:500, and the sample was allowed to shake in an end-over-end shaker for 1 h at room temperature. Addition of primary antibodies was omitted in simultaneous control. Following this incubation, the samples were washed three times with PBSB. The pellet was resuspended in PBSB containing 1% BSA (200 μL). The sample was then treated with FITC-labeled anti rabbit antibodies, diluted to 1:20 for 1 h at room temperature, and allowed to shake end-over-end. They were then washed and treated with 1M NaCl containing PBSB (150 μL). Binding of CTX A5 was quantitated as described above.

RESULTS

Specificity of Tγ from N. nigricollis Contrasts That of CTX A3 from N. atra. We performed titration of Tγ with CS6 and DS and monitored intrinsic fluorescence intensity of Trp of CTX. Change in fluorescence intensity in Tγ versus concentration of GAG is plotted in Figure 2A. Whereas fluorescence intensity increased in the presence of heparin, it decreased in the presence of CS6, suggesting that the mode of binding of these two GAGs to Tγ may be different. Also evident is the marked contrast in behavior of Tγ with the GAGs of two classes; that Tγ binds strongly to CS6 and DS but weakly to heparin is clearly discernible from traces shown in Figure 2A. CTX A3, on the other hand, exhibits the reverse specificity; it binds strongly to heparin but weakly to CS6/DS, as evidenced by an increase in fluorescence intensity of Tyr residue of toxins, plotted *vs* GAG concentration (Figure 2B).

Figure 3 depicts the binding isotherms of Tγ with CS6 (Figure 3A) and DS (Figure 3B) and the change in fluorescence anisotropy *vs* GAG concentration. Change in fluorescence intensity and anisotropy are similar, authenticating a decrease in fluorescence intensity as a reflection of GAG binding. We estimate, by nonlinear least-squares fitting of fluorescence data, that Tγ binds to CS6 with K_d = 0.06 μM and number of binding sites, n = 13 (Table 1). Tγ binds to DS with K_d = 4.3 μM, n = 7 (Table 1). Molecular weights of DS and heparin are comparable (*32*); therefore, binding strength may reflect specificity of CTX for a GAG. Tγ binds to CS6, four orders of magnitude stronger than to heparin, although CS6 is less sulfated. CTX A3, on the other hand, prefers to bind to heparin.

The salt concentration required to dissociate a ligand from immobilized GAGs reflects the relative strength of binding between the two. We performed GAG affinity chromatography of CTXs with immobilized CS6 and DS. Tγ eluted at ~500 mM NaCl concentration from CS6-Sepharose. No other component of *N. nigricollis* venom bound to CS−Sepharose to a detectable extent under these conditions.

Affinity chromatography of Tγ with immobilized DS was likewise performed, except that semipure Tγ was loaded onto the column in PBS. Tγ required ~460 mM NaCl to elute from DS−Sepharose. In contrast, Tγ elutes from heparin at 300 mM NaCl concentration (*33*). These results strengthen our observation that Tγ binds strongly to CS/DS, although heparin is more anionic. On the other hand, highly homologus CTX A3 binds to GAGs in reverse order: heparin > DS > CS6 (500, 310, and 180 mM NaCl respectively) (*32*). Most proteins, including basic fibroblast growth factor (bFGF), lipoprotein lipase, and hepatic lipase bind avidly to

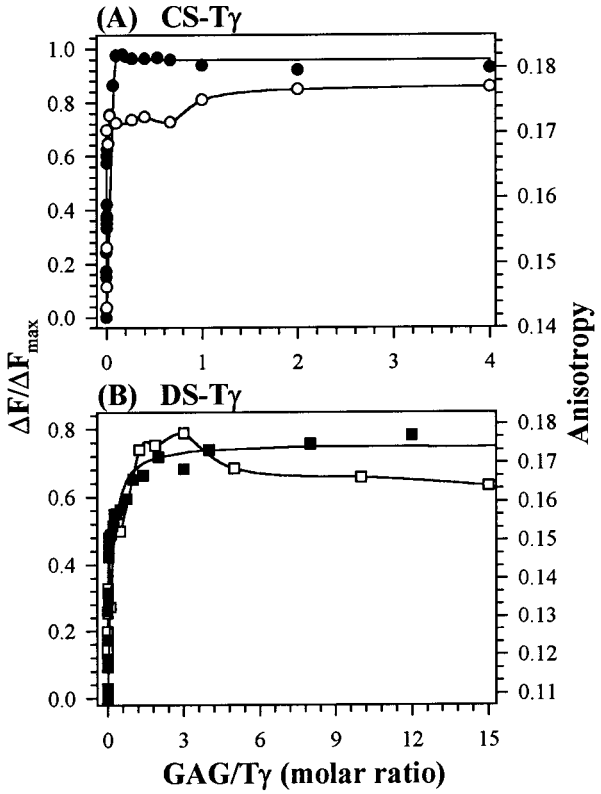

FIGURE 3: Binding isotherm of Tγ as monitored by relative change of fluorescence intensity (solid symbols) and anisotropy (open symbols) of Trp plotted against CS6 (A) and DS (B) concentration. Fluorescence intensity and anisotropy exhibit the same trend, rising and saturating around the same GAG concentration, confirming that fluorescence measurements truly reflect change in CTX upon binding to GAG. For DS, anisotropy decays somewhat preceding saturation. This trend is reminiscent of the CTX−heparin behavior (*32*).

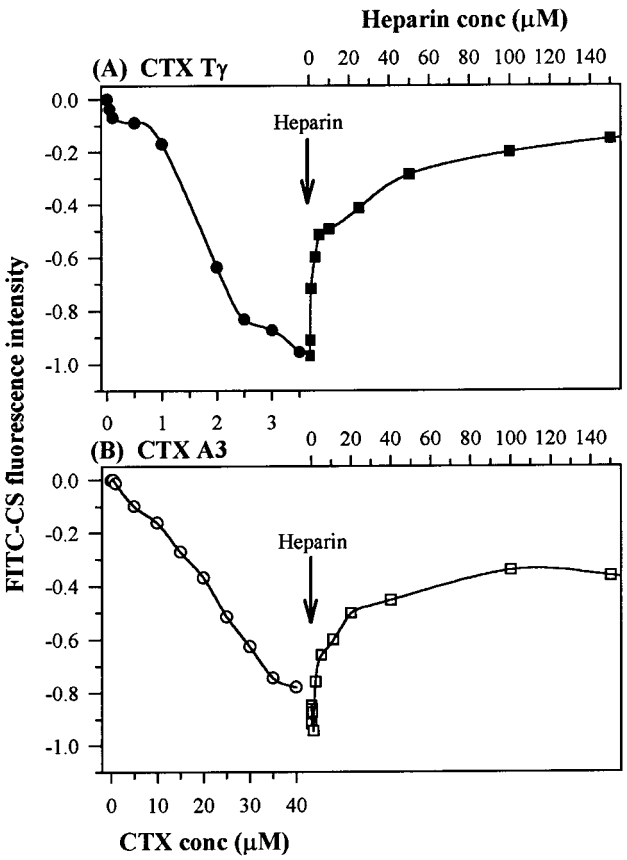

FIGURE 4: Competition binding assay for binding of CTXs with CS6 and heparin. To a constant amount of fluorescein−CS6 was added CTX. Relative fluorescence intensity of fluorescein fluorophore is plotted (representative traces) against the concentration of CTXs (circles). Addition of CTX was stopped near the saturation concentration (3.5 μM for Tγ, closed symbols; 40 μM for CTX A3, open symbols), and heparin was added instead to the same solution. Arrows indicate the point at which heparin was added. Change in fluorescence intensity upon addition of heparin is plotted against heparin concentration (squares) in the right side of same panel for Tγ (A) and CTX A3 (B). CTX M3 exhibited a similar trend (data not shown).

heparin, HS, and DS, but weakly or not at all to CS6 (see for example, refs 32, 42, 43). For example, bFGF requires 1.5−1.75 M NaCl to dissociate from heparin matrix but only 0.2−0.5 M NaCl for dissociation from CS/DS matrix (*33*). Tγ clearly distinguishes itself by its stronger binding for this less versatile GAG.

Affinity of Different CTXs for CS6/DS. CTXs are readily available and highly homologous; they can be regarded as natural mutants of Tγ, which binds to CS6. To search for features that equip a protein for GAG binding and to understand the interaction of CTXs with GAGs, we studied the interaction of 10 CTXs with CS6 and with DS. Structures of eight out of ten CTXs studied here have been established unequivocally (*31*, and refs cited therein) and are found to be highly similar in topology (Figure 1 shows representative structures) and amino acid sequences (Table 1). We have proposed that loop regions of CTXs are possibly involved in binding to heparin (*33*). We performed titration against CS6 and DS of ten toxins from the venom of three cobra species by monitoring changes in intrinsic fluorescence intensity and anisotropy of Trp or Tyr residues of the protein. Dissociation constants of ten CTXs with CS6 and DS determined from the binding isotherm are listed in Table 1 in decreasing order of binding strength to CS6. It should be pointed out that the heterogeneous nature of GAGs precludes determination of exact K_d values; accuracy of values listed lies in order of magnitude. Nevertheless, since identical procedures for data accumulation and treatment were applied for all toxins and GAGs, comparison among CTXs toward the same GAG should be meaningful and our conclusion of specificity of CTXs holds good. Loop 2 of CTXs is generally regarded as instrumental in many properties of the toxin (*28*, *30*). Alignment of primary sequence of CTXs reveals that toxins exhibiting high CS6/DS affinity contain Arg at position 28 (Table 1), near loop 2. CTXs M4 and M3, from venom of *N. mossambica*, also a spitting cobra, clearly stand out for stronger affinity toward DS. Tγ and CTX M1 display a somewhat weak affinity for DS, but stronger than that for heparin. The residues proposed to be important in binding to heparin (*33*) are also highlighted in Table 1.

To determine if heparin and CS6 associate with CTX in similar fashion, we performed competition experiments with the two GAGs. To a solution of fluorescein−CS6 was added Tγ, and the fluorescence intensity was measured at each point. The intensity decreased steeply (Figure 4A), reflecting the behavior observed when titration is performed in reverse order (previous experiments, Figure 2). Heparin was then added to the solution instead of Tγ, when intensity rose steadily and eventually assumed the initial value (Figure 4A). Heparin at high concentration can thus displace CS6 from

the Tγ−CS complex. Heparin likewise displaced CS6 from a CS−A3 complex (Figure 4B). Furthermore, unlabeled CS6 was found to displace FITC−CS6 from the Tγ−CS complex around 1 μM (data not shown). CTX M3 displays comparable affinity for heparin (K_d = 0.02 μM) as well as CS6 (K_d = 0.9 μM). Also, the presence of the contiguous lysine residues in the accessible loop 2 region of the toxin might contribute to GAG binding, so we performed a competition study on CTX M3. Heparin could, likewise, displace CS6 from the CTX M3−CS complex (data not shown).

It is difficult to dissociate the contribution of each or a pair of species or to draw a conclusion about the location of binding sites based on the competition experiment, because many processes may be operating in this tripartite interaction. The result obtained (Figure 4, panel A) is better interpreted in the light of a plausible model proposed for the binding between GAGs and toxins (*32*). According to this model, at 3 μM Tγ for example, just before heparin is added, the toxin is in excess and CS6 is scarce. Hence, Tγ may be forced to bind indiscriminately. Consequently, this binding may be weaker than that observed when proportionate quantities of both species are present in the solution or when the GAG is in excess. This behavior is especially typical of Tγ, a protein that is involved in numerous nonspecific associations when the GAG is scarce (*33*). The steep rise seen upon the initial addition of heparin (Panel A) may reflect such a phenomenon, not true association (this initial binding does not exhibit saturation). It may be the later, saturable phase of the curve, that reflects binding.

Specific Binding of Tγ and CTX A5 to Corneal Epithelium. To investigate if binding of CTXs observed in vitro occurs also *in vivo*, we performed binding studies of the toxins with corneal tissue. Cornea is made up of three different cellular layers (*17*−*20*), but we reasoned that epithelium should be directly susceptible to the effect of CTX for the following reasons: (1) this tissue would be the first to contact venom, and (2) opacity/blindness caused by CTX can be prevented if the eye is washed extensively with water or other bland solution; damage incurred in stroma is permanent and scarring, unlike the effect of venom.

Corneal epithelium from bovine eye was obtained, and the tissue was minced finely and homogenized in the presence of protease inhibitors so that tissue integrity may not be compromised. Homogenized tissue was then allowed to incubate with fluorescein−Tγ in PBSA. Fluorescence microscopy revealed that the tissue retained fluorescein fluorescence, indicating that Tγ binds to bovine corneal epithelium (data not shown). Tissue treated with FITC alone showed no significant fluorescence (data not shown). We also studied binding of the toxin to tissue treated with chondroitinase ABC. This enzyme cleaves CS4 and CS6, DS, and to a much less extent, HYA (*4*). Therefore enzyme-treated tissue should present less cell surface-associated GAGs than would intact tissue. Chondroitinase ABC-treated tissue exhibited lower binding, judging by change in fluorescence intensity (data not shown).

To determine if the toxins display similar specificity toward CS/DS and heparin/HS in binding to corneal epithelium, we quantitated the amount of toxin bound to intact tissue and to enzyme-treated tissue using a multiplate reader. We found that binding of fluorescein−Tγ to chondroitinase ABC-treated tissue was reduced by 22% that of intact tissue

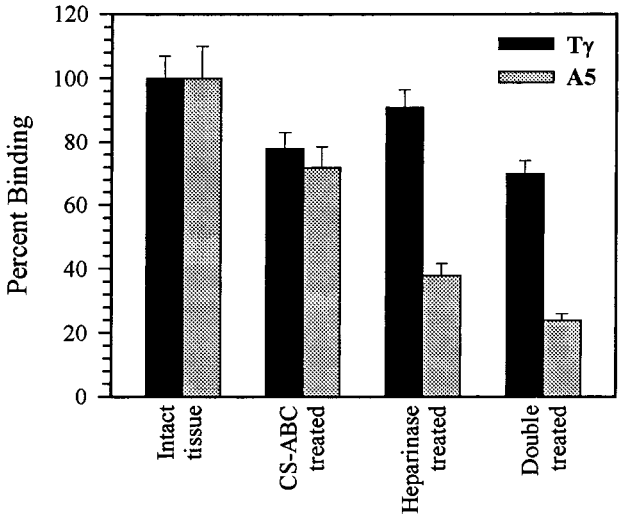

FIGURE 5: Binding specificity of CTXs to corneal epithelium. Binding of Tγ (dark bars) and CTX A5 (shaded bars) to corneal epithelium as determined by the intensity of fluorescein fluorophore read on automated ELISA plate reader. Tγ and CTX A5 bind to corneal epithelium (intact tissue). Tγ binds to chondroitinase ABC-treated tissue less strongly than to intact or to heparinase III-treated tissue. CTX A5, on the other hand, binds less strongly to heparinase III-treated tissue than to intact or to chondroitinase ABC-treated tissue. Binding of each toxin to tissue treated with both enzymes (double treated) is considerably reduced. Fluorescein−Tγ was used for visualization. For CTX A5, visualization was indirect; specific antibodies against CTX A5 and FITC-labeled secondary antibodies were used (please refer to text for details). Data are presented as the mean of two independent experiments with tissue obtained at different times.

(Figure 5), whereas binding to heparinase III-treated tissue was reduced only by 8% that of intact tissue (Figure 5). Heparinase III cleaves HS into small oligosaccharides (*4*). Tissue treated with this enzyme should present less HS. Binding to tissue treated with both enzymes was ~31% lower than that of intact tissue. This observation suggests that Tγ may bind to CS/DS of corneal epithelium but weakly to HS of this tissue. The decrease in binding to chondroitinase ABC-treated tissue is rather modest, indicating that other receptors, such as KS for example, may also be involved in binding of Tγ to corneal epithelium (see the following section).

Binding of CTX A5, the toxin exhibiting higher affinity for heparin than for CS/DS, was quantitated likewise, except that visualization of fluorescence was done using specific antibodies generated against CTX A5. To the primary antibody-treated sample was allowed to bind a fluorescein-labeled secondary antibody. In the absence of CTX A5, no fluorescence was observed in the tissue, confirming that this indirect mode of visualization presents no artifacts (data not shown). CTX A5 clearly exhibits the reverse preference of Tγ; CTX A5 bound very weakly (reduced to 63% of intact tissue) to heparinase III-treated tissue (Figure 5), while binding to chondroitinase-treated tissue was relatively more strong (reduced by 25% of intact tissue). Binding of CTX A5 to tissue treated with both enzymes was rather low, ~75% less than that for intact tissue. These observations corroborate those made using fluorescence binding measurements and suggest that while Tγ binds to CS/DS, CTX A5 binds to heparin/HS.

Binding Specificity of All GAGs for Tγ and CTX A3. To study what features of GAGs allow binding to a basic

Table 2: Comparison of Dissociation Constant K_d (μM) of CTX Tγ and CTX A3 with GAGs[a]

GAGs	Tγ	A3
CS6	0.06	28
KS	0.45	1.6
HYA	3	190
DS	4.3	5
CS4	29	4
heparin	140	1.42[b]

[a] GAGs are arranged in decreasing order of CTX Tγ binding strength. [b] Data are taken from ref *33*.

polypeptide specifically, we compared binding strength of individual toxins, Tγ and CTX A3 each, with all GAGs (Table 2). The specific and strong binding of Tγ to CS6 is unique because few known proteins bind to this GAG (*7*). Furthermore, since Tγ is a major component of spitting cobra venom (*44*), it is pertinent to study its interaction with the other GAGs, i.e., HYA, KS, and CS4, abundant in cornea.

HYA, the atypical GAG, lacks sulfates and abounds in cornea and elsewhere. HYA binds to CD44 and abounds in extracellular matrixes and mediates important functions (*12*−*14*). From fluorescence measurements, we estimated that Tγ binds to HYA with a K_d of 3 μM and the number of binding sites, $n = 23$ (HYA is a giant GAG, MW ~80 000) (Table 2). The binding isotherm is depicted in Figure 2C. Thus, the binding of Tγ to this GAG with appreciable strength points again to an element of specificity in the binding. We found that CTX A3 binds to HYA weakly, with a $K_d = 190$ μM, and $n = 20$ (Figure 2D, Table 2).

KS is another GAG whose properties are far less well understood than other GAGs, especially heparin. Also, the abundance of KS in the cornea (*19*) prompted us to quantitate the binding of CTX from the spitting cobra with this GAG. Both, Tγ and CTX A3 exhibited strong binding to KS, with respective K_d values of 0.45 and 1.6 μM, and $n = 3$ (Figure 2C,D; Table 2). The affinity of Tγ is about 3.5-fold higher than that of CTX A3.

CS4 contains the same monomeric residues as CS6, except that the galactosamine is sulfated at the 4-position rather than in the 6-position (*4*, *7*). The exocyclic 6-sulfate of CS6 should be more accessible than the 4-sulfate. To compare the effect of this sulfation on ligand binding of CS, we studied the binding of the two toxins to CS4. Tγ bound CS4 with a K_d of 29 μM and $n = 10$ (Figure 2C; Table 2). This drastic reduction in the binding affinity to CS4 compared to CS6 ($K_d = 0.06$ μM) lends further credence to the suggestion that Tγ is unique in its ability to bind CS6. Interestingly, an opposite trend was observed for the binding of CTX A3 to these two GAGs. CTX A3 bound more strongly to CS4 ($K_d = 4$ μM, and $n = 10$) (Figure 2D; Table 2) compared to CS6 ($K_d = 28$ μM). Thus Tγ and CTX A3 display the reverse specificity of each other for different GAGs. This contrast is striking in view of the high homology among the CTXs and suggests that binding to GAGs may be mediated not just by clusters of basic residues but rather by their specific location.

DISCUSSION

We show herein that Tγ, a major component of spitting cobra venom, binds strongly to CS6, DS, HYA, and KS. CTXs M4, M3, and M2, also from the venom of spitting cobra *Naja mossambica*, display high affinity for DS. Heparin and CS possibly compete for similar binding sites in the toxin, but the binding mode of the two GAGs may be different. Our results establish basic proteins that bind to CS6, DS, and heparin with specificity. The preference of Tγ for CS/DS and of CTX A5 for heparin/HS was also observed in binding of the toxins to corneal epithelium. These results may explain the tissue specificity of CTXs and reinforce our suggestion that sulfated oligosaccharides may act as primary target of CTXs (*32*).

To explain their versatile binding ability it is proposed that the presence in heparin, HS, and DS of the conformationally more flexible iduronic residue renders these GAGs amenable to protein (*7*). Extensive sulfation in heparin, less often in DS and HS, further may strengthen the binding by ionic interaction. CS6 contains, on the other hand, glucuronic acid, which is conformationally restrained. Also, CS6 is more sparsely sulfated than heparin. Nonetheless, Tγ binds strongly to CS/DS, which is remarkable.

The 3-D structures of about a dozen CTXs are established (*31*) and are found to be homologous, as can be seen from Figure 1 and Table 1. Despite their high homology, Tγ and CTX A3 display remarkable specificity for CS6 and heparin, respectively. Furthermore, CTXs are readily available in large amounts. These features make CTXs ideal tools with which to probe details of protein−carbohydrate interaction. For this purpose, we studied herein binding of 10 CTXs with CS/DS. The marked variation in affinity they display, especially toward CS6 and CS4, suggests that the specificity may lie with the toxin.

Small variations in the largely homologous structures of the toxins are found in the loop and β-sheet regions, producing subtle variations in the length and freedom of loops (*26*, *31*). The most flexible, and therefore versatile, loop 2 invariably carries a basic residue. A characteristic feature of the CTX molecule is that the cationic charges lie along the structure as a regular, equatorial band (Figure 1, dark band). Maximum overlap with the polyanionic polymers is imaginable if the saccharide is thought to bind along this cationic belt. While this belt may well mediate binding (*33*), it cannot explain the specificity toward different GAGs. Therefore, the specificity may be decided by the subtle variations in the flexibility, charge distribution, and length of the loop and β-sheet of CTXs.

The effect of snake venom is manifested promptly and extends over a long period. To allow for rapidity and efficiency, each component of venom ought to act independently, yet in concert with other proteins. For this purpose, venom has evolved to retain a variety of active proteins. Low affinity of Tγ for heparin eliminates the possibility that Tγ targets the heparinlike molecules of the cell surface. Incidentally, Tγ from spitting cobra venom binds strongly to CS6 and HYA, the GAGs present in cornea. Corneal collagen fibrils are held together by CS-, DS-, and KS-containing PGs (*18*, *19*); disruption in the regular spacing causes collapse of the assembly (*20*). It is shown that CTXs, including Tγ, from *N. nigricollis* venom cause opacity when injected into rabbit cornea (*23*, *24*). The concentration of CS6 and HYA in rabbit tears was determined recently (*45*). It was found that the concentration of CS was 7.6 times higher than HYA; among the CS isomers, concentration of CS6 was highest, followed by CS4. Taking these results together with our present results, it is conceivable that Tγ

may bind to GAGs abundant in the cornea and elsewhere. Binding of the foreign toxin to corneal GAGs, then, may affect assembly of collagen fibrils, leading eventually, to opacity or temporary blindness. The mechanism of toxicity remains to be established.

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