Articles

Fucoidan-Dependent Conformational Changes in Annexin II Tetramer†

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ABSTRACT: Fucoidan, a sulfated fucopolysaccharide, mimics the fucosylated glycans of glycoproteins and has therefore been used as a probe for investigating the role of membrane polysaccharides in cell−cell adhesion. In the present report we have characterized the interaction of fucoidan with the Ca^{2+}- and phospholipid-binding protein annexin II tetramer (AIIt). AIIt bound to fucoidan with an apparent K_d of 1.24 ± 0.69 nM (mean ± SD, $n = 3$) with a stoichiometry of 0.010 ± 0.001 mol of fucoidan/mol of AIIt (mean ± SD, $n = 3$). The binding of fucoidan to AIIt was Ca^{2+}-independent. Furthermore, in the presence but not the absence of Ca^{2+}, the binding of fucoidan to AIIt caused a decrease in the α-helical content from 32% to 7%. A peptide corresponding to a region of the p36 subunit of AIIt, F(306)−S(313), which contains a Cardin−Weintraub consensus sequence for heparin binding, was shown to undergo a conformational change upon fucoidan binding. This suggests that heparin and fucoidan bound to this region of AIIt. The binding of fucoidan but not heparin by AIIt also inhibited the ability of AIIt to bind to and aggregate phospholipid liposomes. These results suggest that the binding of AIIt to the carbohydrate conjugates of certain membrane glycoproteins may have profound effects on the structure and biological activity of AIIt.

Fucoidan is a complex sulfated polysaccharide from the brown seaweed *Fucus vesiculosus* that consists of a homopolymer of sulfated L-fucose. The core of the molecule consists of a polymer of α-1−3-linked fucose with sulfate groups substituted at the 4 position on some of the fucose residues, resulting in about 0.3 mol of sulfate/mol of fucose. The attachment of fucose to the polymer also results in the formation of branch points. These branch points occur at every 2−3 fucose residues within the chain (*1*).

Fucoidan has been shown to be a useful probe for investigating the role of membrane polysaccharides in cell−cell adhesion since it mimics the biologically relevant fucosylated glycans of glycoproteins. For example, fucoidan significantly inhibits the tight binding of human sperm to human zona pellucida in vitro and the stimulation of the acrosome reaction by acid-solubilized human zona pellucida (*2*−*6*). Similarly, fucoidan has been shown to block cell binding mediated by P- or L-selectin (*7*−*9*). Fucoidan binds to L-selectin and inhibits L-selectin-mediated lymphocyte adhesion to lymph node high endothelial venules. Fucoidan also inhibits leukocyte rolling, which is an early and essential step in the process of leukocyte extravasation into inflamed sites. L-Selectin binds to charged phospholipids, such as cardiolipin and phosphatidylserine, and this binding activity is blocked by fucoidan (*10*). In contrast, the binding of fucoidan to the scavenger receptor, SR-A, does not affect the adhesion properties of the molecule (*11*−*15*).

Animal carbohydrate-binding proteins can be broadly classified into seven major groups. These include the *C*-type or Ca^{2+}-dependent lectins, the *S*-type or Gal-binding galectins, *P*-type mannose 6-phosphate receptors, the *I*-type lectins, the pentraxins, the hyaluronan-binding proteins, and the heparin-binding proteins (*16*). A heparin-binding consensus sequence, termed the Cardin−Weintraub heparin-binding consensus sequence, has been identified among members of the heparin-binding family of proteins (*17*, *18*). Recent studies from our laboratory have established the presence of a Cardin−Weintraub sequence in the p36 subunit of the annexin protein annexin II tetramer (AIIt)[1] (*19*). Furthermore, we showed that AIIt bound heparin with high affinity and specificity and that the binding of heparin to AIIt required Ca^{2+}.

AIIt consists of two copies of a p36 subunit and two copies of a p11 subunit (reviewed in ref *20*). The p36 subunit, a

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[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; AIIt, annexin II tetramer; p11, p11 light chain of annexin II tetramer; p36, p36 heavy chain of annexin II tetramer; DTT, dithiothreitol; buffer A, 10 mM Tris-HCl, pH 7.5, 0.1 mM DTT, 0.15 M NaCl, and 1.0 mM $CaCl_2$; buffer B, 10 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.15 M NaCl, and 0.5 mM EGTA; buffer C, 20 mM Tris-HCl, pH 7.5, 1 mM DTT, and 1 mM $CaCl_2$; $\Delta\epsilon$, circular dichroic absorption coefficient ($\epsilon_L - \epsilon_R$).

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member of the annexin family of proteins, binds Ca^{2+}, acidic phospholipid, F-actin, and heparin. The p11 subunit, a member of the calmodulin family of Ca^{2+}-binding proteins, binds plasminogen and cytosolic phospholipase A_2 (*21*, *22*).

AIIt was originally identified as an intracellular Ca^{2+}-binding protein that could stimulate the Ca^{2+}-dependent adhesion of phospholipid liposomes (*23*) or biological membranes such as chromaffin granules (*24*). Subsequently, several laboratories have provided experimental evidence consistent with a role for AIIt in such membrane trafficking events as endocytosis or exocytosis (reviewed in refs *20*, *25*). More recently, AIIt has been show to be present at the extracellular surface of the plasma membrane of endothelial cells, where it has been shown to act as a receptor for plasminogen (reviewed in ref *26*). Recent studies have suggested that AIIt mediates the adhesion of RAW117 lymphoma cells to endothelial cells (*27*), indicating that AIIt may play a role in cell−cell adhesion.

In a previous study we demonstrated that AIIt bound to heparin with high affinity and specificity and that the binding of heparin to AIIt resulted in a large conformational change in the protein (*19*). Furthermore, we showed that the p36 subunit of AIIt contains a Cardin−Weintraub glycosaminoglycan recognition site that was involved in heparin binding.

In the present report we demonstrate that AIIt binds fucoidan with high affinity and specificity. Since a peptide copied from the heparin-binding site of AIIt also binds fucoidan, we propose that heparin and fucoidan bind to a similar region of AIIt. Thus, this is the first identification of a putative fucoidan-binding site in a protein. We also show that the interaction of fucoidan with AIIt inhibits the ability of AIIt to aggregate liposomes. We, therefore, conclude that fucoidan and heparin induce distinct changes in the structure of AIIt.

EXPERIMENTAL PROCEDURES

Materials. Carbohydrates were obtained from Sigma and were the purest grade available. The fucoidan used in these studies was unfractionated and consisted an average M_r of 180 000 (determined by low-angle laser light scattering). The sulfate content ranged from 7.6% to 10.2%. Heparin (bovine lung M_r 17 000) obtained from Calbiochem had a specific activity of 149 USP units/mg. Annexin II tetramer and annexin II monomer were prepared from bovine lung according to ref *28* and stored at −70 °C in 40 mM Tris-HCl, pH 7.5, 1.0 mM DTT, 0.1 mM EGTA, and 150 mM NaCl.

Measurement of Protein Secondary Structure by Circular Dichroism. A Jasco J-715 spectropolarimeter was used to obtain circular dichroism (CD) spectra by the methodology described in ref *19*. AIIt (2.2 μM) was incubated in either buffer A (10 mM Tris-HCl, pH 7.5, 0.1 mM DTT, 0.15 M NaCl, and 1.0 mM $CaCl_2$) or buffer B (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1 mM DTT, and 0.5 mM EGTA) in the presence or absence of carbohydrate (0.5−50 μM) for 20 min at room temperature. Samples (0.1 mL) were scanned in a quartz cuvette (0.5 mm path length) from 178 to 260 nm at a rate of 10 nm/s, using a bandwidth of 1 nm and a response time of 4 s. CD spectra of proteins were obtained by averaging four wavelength scans and were corrected by subtracting buffer scans or, where appropriate, scans of carbohydrate in buffer. The measurements at 222 nm were obtained at a bandwidth of 1 nm, using the time scan mode of the software and by averaging a minimum of 20 × 1 s measurements. Results are expressed as circular dichroic absorption coefficient $\Delta\epsilon$ ($\epsilon_L - \epsilon_R$). The α-helical content was calculated from circular dichroism data according to the equation % α-helix $= -100(\theta_{222} + 3000)/33\,000$ (*29*).

Data Analysis. Nonlinear least-squares fitting was performed with the computer program SigmaPlot (SPSS Scientific) as detailed in the SigmaPlot reference manual. Data were analyzed with the four-parameter logistic equation $f = (a - d)/[1 + (x/c)^b] + d$, where a = asymptotic maximum, b = slope parameter, c = value at inflection point ($A_{0.5}$) and d = asymptotic minimum. The nonlinear least-squares curve fitting was then iterated by allowing the four fitting parameters to *float* while the Marquardt method was utilized for the minimization of the sum of the squared residuals. The K_d and n values for the binding of fucoidan to AIIt were derived from plots of the fraction of AIIt bound vs the total fucoidan concentration (*Fuc*) as described in ref *19*, using the quadratic equilibrium binding equation

$$\alpha = [(n[\mathrm{AIIt}]_T + [\mathrm{Fuc}]_T + k_d) - \{(n[\mathrm{AIIt}]_T + [\mathrm{Fuc}]_T + k_d)^2 - 4n[\mathrm{AIIt}_T][\mathrm{Fuc}]_T\}^{1/2}]/(2n[\mathrm{AIIt}_T])$$

where α = fractional binding (AIIt pelleted/total AIIt), n = moles of fucoidan/moles of AIIt, and K_d = dissociation constant. The nonlinear least squares curve fitting was iterated, during which the K_d and n parameters were allowed to *float*. For the binding analysis it was assumed that there was only a single class of fucoidan-binding sites on AIIt.

Liposome Aggregation. Phospholipid liposomes were prepared as described in ref *30*. Essentially, 50 μL of 20 mg/mL phosphatidylserine, phosphatidylethanolamine, and cholesterol in chloroform were dried in a N_2 stream and hydrated with 1 mL of 30 mM Hepes, pH 7.5, and 2 mM $MgCl_2$. The mixture was sonicated (4 × 15 s bursts at 75W with a Braun probe sonicator) and the phospholipid liposomes were stored at room temperature prior to their use in aggregation assays. Liposomal aggregation was measured at 20 °C and at a wavelength of 450 nm, in a final volume of 0.6 mL. The reaction mixture contained 30 mM Hepes, pH 7.5, 2 mM $MgCl_2$, 50 mM KCl, 0.2 mM Ca^{2+}, and sufficient liposomes such that the initial A_{450nm} was 0.3. The A_{450nm} of the reaction mixture was determined prior to protein addition and a final reading was taken at 15 min.

Miscellaneous Techniques. AIIt concentration was determined spectrophotometrically with an extinction coefficient $A_{280nm} = 0.68$ for 1 mg/mL AIIt. The Ca^{2+} concentration of $CaCl_2$ stock solutions was determined by atomic absorption. Ca^{2+}−EGTA buffers were prepared according to ref *31*. Peptides were synthesized by the University of Calgary Peptide Facility and were purified by HPLC.

RESULTS

Characterization of the Binding of Fucoidan to AIIt. We previously reported that the binding of heparin to AIIt resulted in a large conformational change in the protein and

that, in the presence of Ca^{2+}, the heparin−AIIt complex could be pelleted by centrifugation (*19*). Other sulfated sugars such as chondroitin sulfate and dextran sulfate did not form a complex with AIIt. However, we have observed that, in the presence of Ca^{2+}, the binding of the sulfated polysaccharide fucoidan to AIIt resulted in the formation of a large complex that could also be pelleted by centrifugation at 400000*g*. However, since the fucoidan−AIIt complex was not pelleted at 10000*g*, it was unlikely that the interaction of fucoidan and AIIt resulted in the denaturation and precipitation of AIIt. Furthermore, gel-permeation chromatography of AIIt in the presence of 1 mM $CaCl_2$ and 5.0 μM fucoidan confirmed that fucoidan did not dissociate the subunits of AIIt (data not shown).

The pelleting assay was used to estimate the affinity and capacity of the binding of fucoidan to AIIt. As shown in Figure 1A, half-maximal binding of fucoidan to 2.2 μM AIIt required 0.0107 ± 0.005 μM (mean ± SD, $n = 3$) fucoidan. The asymptotic maximum of the plot was 2.065 μM AIIt, which suggested about 94% of the total AIIt could be recovered in the pellet. Analysis of the binding data (Figure 1A, inset) indicated that AIIt bound to fucoidan (M_r 180 000) with an apparent K_d of 1.24 ± 0.69 nM (mean ± SD, $n = 3$) and a stoichiometry of 0.010 ± 0.001 mol of fucoidan/mol of AIIt (mean ± SD, $n = 3$).

Previously, we had shown that the binding of AIIt to heparin required Ca^{2+}. We therefore examined the Ca^{2+} dependency of the AIIt−fucoidan complex formation. In contrast to the Ca^{2+} requirement for AIIt−heparin complex formation, the formation of the AIIt−fucoidan complex did not require Ca^{2+} (Figure 1B).

Ca^{2+} Dependency of the Fucoidan-Induced Conformational Change in AIIt. Previous work from our laboratory established that the binding of heparin to AIIt resulted in a large decrease in the α-helical content of AIIt (*19*). We therefore used circular dichroism to examine the changes in the conformation of AIIt that occurred in upon the binding of fucoidan. As shown in Figure 2, an approximate 10% reduction in the intensity of the CD spectra of AIIt was observed upon the binding of Ca^{2+} to AIIt. The binding of fucoidan to AIIt in the presence of Ca^{2+} resulted in a 65% decrease in the intensity of the CD at 222 nm. Furthermore, the CD spectra of AIIt lost intensity and changed in shape as the protein bound fucoidan in the presence of Ca^{2+}. We also observed that, in the absence of Ca^{2+}, the CD spectra of the fucoidan−AIIt complex were slightly less intense than the CD spectra of AIIt alone. These results are summarized in Table 1.

Quantitative assessment of the secondary structure components of the CD spectra of the AIIt−fucoidan complex was attempted according to the method of ref *32*. Previously, this method had successfully calculated the secondary structure parameters for heparin−AIIt complex from the CD spectra (*19*). However, the root-mean-square difference between the predicted CD data and the actual CD data for the AIIt−fucoidan complex was significantly greater than 0.15. This indicated that the analysis was unacceptable. Therefore, the method of McLean et al. (*29*) was used to calculate the α-helical content of AIIt. In the presence of Ca^{2+}, fucoidan decreased the α-helical content of AIIt from 32% to about 7%. In the absence of Ca^{2+}, the α-helical content of AIIt was increased by from 35% to 37% by fucoidan.

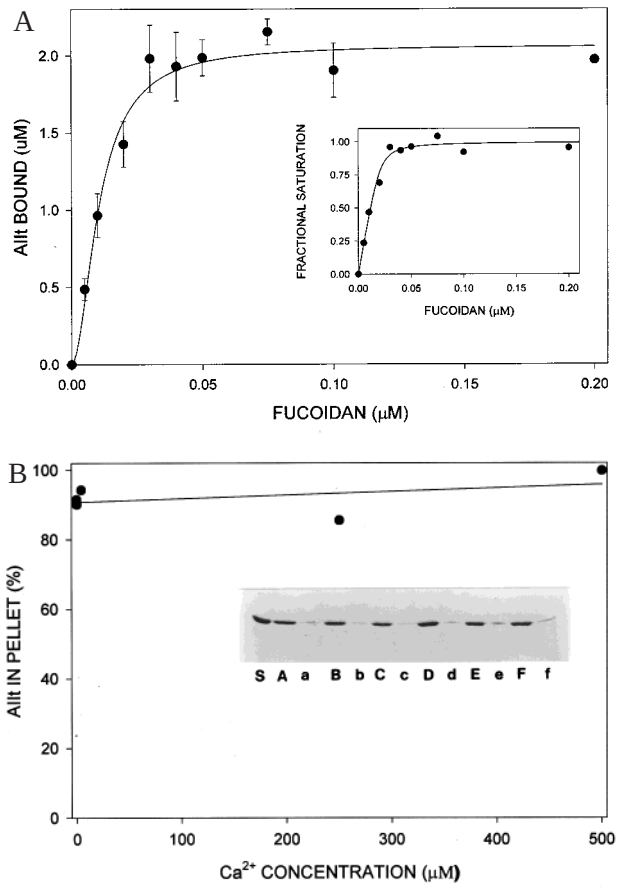

FIGURE 1: Binding isotherm for the titration of AIIt with fucoidan. (A) AIIt (2.2 μM) was incubated with 25 mM Hepes (pH 7.5), 1 mM DTT, 150 mM NaCl, 1 mM $CaCl_2$, and variable concentrations of fucoidan at room temperature for 20 min. The reaction mixture was centrifuged at 400000*g* for 30 min and the supernatants and pellets were analyzed by SDS−PAGE. The concentration of AIIt was determined by densiometric analysis of Coomassie-stained gels using a Storm 860 (Molecular Dynamics) phosphoimager and Image Quant software. The line through the points is a nonlinear least-squares curve fit of the data points calculated from computer modeling of the data using the logistic equation and the following values: $a = 2.065$ μM (asymptotic maximum), $b = 1.876$ (slope parameter), $c = 0.0107$ μM [value at inflection point ($A_{0.5}$)], and $d = 0$ (asymptotic minimum). (Inset) Fractional saturation (moles of AIIt in pellet/total moles of AIIt) is plotted against the total fucoidan concentration. The line through the points is a nonlinear least-squares curve fit of the data points calculated from computer modeling of data to the quadratic binding equation (see Experimental Procedures) with the assumption of a single class of fucoidan-binding sites on AIIt. The two fitting parameters, K_d and n, were allowed to float during the computer iterations. The convergent best fit for these experiments was determined for a K_d of 1.24 nM and an n of 0.010 mol of fucoidan/mol of AIIt. (B) AIIt (2.2 μM) was incubated at room temperature for 20 min with 25 mM Hepes (pH 7.5), 1 mM DTT, 150 mM NaCl, 5 μM fucoidan, 1 mM EGTA (lanes A−D only), and variable concentrations of Ca^{2+}. The reaction mixture was centrifuged at 400000*g* for 30 min and the supernatant (lower-case letters) and pellet (upper-case letters) were subjected to SDS−PAGE and stained with Coomassie blue. The concentrations of Ca^{2+} used were as follows: lane A, no added Ca^{2+} (2 mM EGTA); lane B, 0.014 μM; lane C, 0.12 μM; lane D, 4.3 μM; lane E, 0.25 mM; and lane F, 0.5 mM. S refers to the AIIt standard.

Dose-Dependency of the Fucoidan-Induced Conformational Change in AIIt. Figure 3 presents the dose-dependency

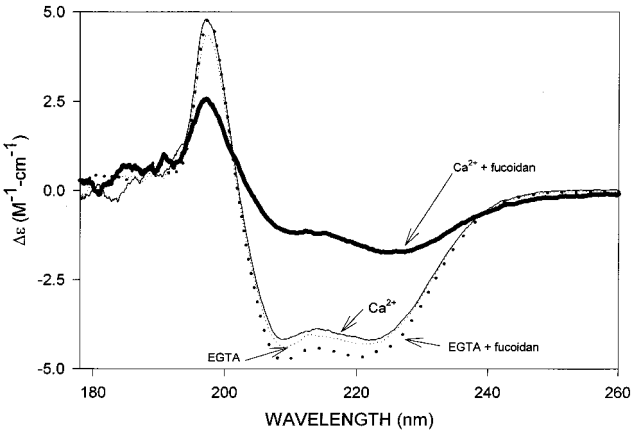

FIGURE 2: Fucoidan-dependent conformational changes in AIIt. Wavelength scans were conducted at 20 °C in the presence of 2.2 μM AIIt and in the presence (bold traces) or absence (thin traces) of 5.0 μM fucoidan. Experiments were performed with buffer A (10 mM Tris-HCl, pH 7.5, 0.1 mM DTT, 0.15 M NaCl and 1.0 mM $CaCl_2$) (solid traces) or buffer B (10 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.15 M NaCl and 0.5 mM EGTA) (dotted traces).

Table 1: Effect of Fucoidan on the CD Spectra of Annexin II Tetramer[a]

measurement	Ca^{2+}, fucoidan	Ca^{2+}	EGTA	EGTA, fucoidan
$\Delta\epsilon_{222nm}$	-1.64 ± 0.03	-4.18 ± 0.05	-4.39 ± 0.04	-4.65 ± 0.08
$\Delta\epsilon_{208nm}$	-1.03 ± 0.03	-4.11 ± 0.18	-4.27 ± 0.17	-4.68 ± 0.07
$\Delta\epsilon_{197nm}$	2.54 ± 0.06	4.77 ± 0.63	4.32 ± 0.54	4.59 ± 0.24

[a] Data are presented as mean ± SD ($n = 3$). CD scans were conduced at 20 °C in a buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2.2 μM AIIt, 0.2 mM DTT, 5 μM fucoidan, and either 1 mM $CaCl_2$ or 0.5 mM EGTA.

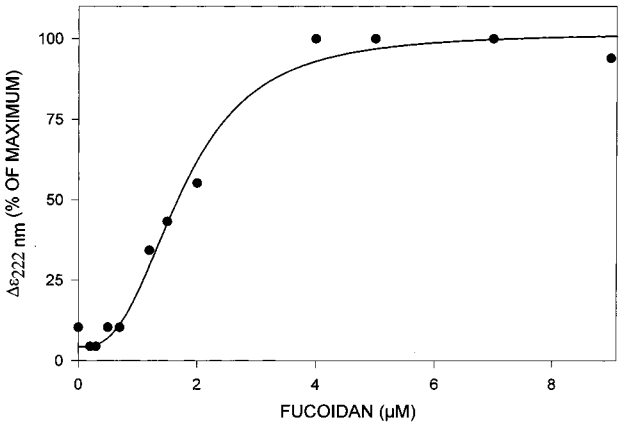

FIGURE 3: Fucoidan-induced conformational change in AIIt. AIIt (2.2 μM) was incubated for 20 min at 20 °C in buffer A (10 mM Tris-HCl, pH 7.5, 0.1 mM DTT, 0.15 M NaCl, and 1.0 mM $CaCl_2$) with variable concentrations of fucoidan. The circular dichroism was then determined at 222 nm. Data shown are expressed as mean ± SD ($n = 3$). The line through the points is a nonlinear least-squares curve fit of the data points calculated from computer modeling of data to the four-parameter logistic equation (see Experimental Procedures). The following values were derived from the curve fit: $a = 101.7\%$ (asymptotic maximum), $b = 2.805$ (slope parameter), $c = 1.748$ μM [value at inflection point ($A_{0.5}$)], and $d = 2.805\%$ (asymptotic minimum).

of the fucoidan-induced conformational change in AIIt as determined by the changes in CD at 222 nm. Increasing the fucoidan concentration decreased the intensity of the CD of AIIt until a maximum was reached at about 4 μM fucoidan.

Table 2: Specificity of the Fucoidan-Induced Conformational Change in Annexin II Tetramer[a]

polysaccharide	change at $\Delta\epsilon_{222nm}$
fucoidan	60.5 ± 2.3
heparin	41.0 ± 3.4
heparan sulfate	3.3 ± 1.0
chondroitin sulfate	2.3 ± 0.9
dextran sulfate	6.9 ± 4.1
N-acetylglucosamine	6.2 ± 1.7
N-acetygalactosamine	5.1 ± 1.0
N-acetylneuraminic acid	3.9 ± 1.0
glucose	2.8 ± 0.1
galactose	9.5 ± 0.1
mannose	6.1 ± 0.2
fucose	5.1 ± 1.0
fucoidan + dextran sulfate	54.0 ± 1.5
fucoidan + heparin	55.0 ± 3.7

[a] Results are expressed as mean ± SD ($n = 5$). Percent change at $\Delta\epsilon_{222nm} = |\Delta\epsilon_{222nm}(AIIt)| - |\Delta\epsilon_{222nm}(AIIt + ligand)|/|\Delta\epsilon_{222nm}(AIIt)| \times 100$. Annexin II tetramer (2.2 μM) was incubated in buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.2 mM DTT, and 1 mM $CaCl_2$ plus 50 μM carbohydrate, 5.0 μM fucoidan, or 0.56 μM heparin.

The fucoidan concentration required for a half-maximal decrease in the CD intensity of 2.2 μM AIIt was about 1.7 μM. In contrast, we observed that the Ca^{2+}-dependent conformational change in AIIt was optimal at 0.56 μM heparin and decreased slightly at higher heparin concentrations. Considering that on a molar basis fucoidan binds about 10-fold more AIIt than heparin, these data suggest that the binding site concentration of fucoidan required for the half-maximal conformational change in AIIt is much higher than that of heparin.

Specificity of the Fucoidan-Induced Conformational Change in AIIt. Fucoidan is a negatively charged polyelectrolyte consisting of a polymer composed primarily of α-1−3-linked fucose with sulfate groups substituted at the 4-position on some of the fucose groups. Theoretically, any negatively charged carbohydrate residue could interact with the positively charged domains of proteins, and therefore the demonstration of the binding of fucoidan to AIIt and a fucoidan-dependent conformational change in AIIt does not necessarily imply that a specific site exists on AIIt for fucoidan. We, therefore, examined the effect of a variety of charged polysaccharides on the CD spectra of AIIt at 222 nm. As shown in Table 2, sulfated polysaccharides such as chondroitin sulfate and dextran sulfate, amino monosaccharides such as *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, and *N*-acetylneuraminic acid, and other monosaccharides such as glucose, mannose, fucose, and galactose failed to induce a substantial change in the conformation of AIIt. We also examined the possibility that other carbohydrates could bind to the fucoidan-binding site of AIIt without producing a conformational change. This possibility was deemed unlikely because 50 μM heparin sulfate failed to block the conformational change in AIIt induced by 5 μM fucoidan. Furthermore, we also observed that the addition of both heparin and fucoidan to AIIt failed to increase the fucoidan-dependent conformational change. This observation presented the possibility that a common site existed on AIIt for both carbohydrates and that this site participated in the carbohydrate-induced conformational change in AIIt.

Identification of a Fucoidan-Binding Site in AIIt. We have reported that AIIt contains a Cardin−Weintraub sequence

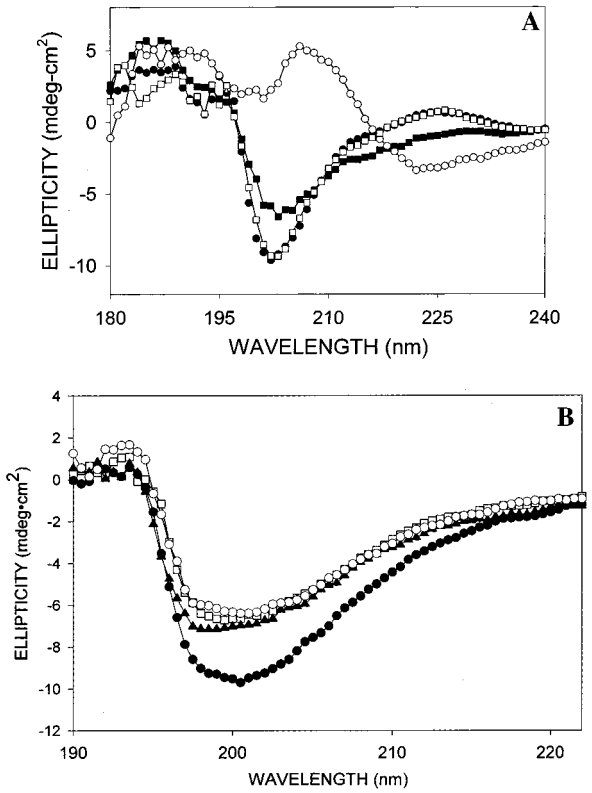

FIGURE 4: Fucoidan-dependent conformational change in a peptide to the heparin-binding domain of AIIt. Wavelength scans were conducted at 20 °C in buffer A (10 mM Tris-HCl, pH 7.5, 0.1 mM DTT, 0.15 M NaCl, and 1.0 mM $CaCl_2$) (●) or in buffer A containing 5 μM *N*-acetylglucosamine (□), 5 μM heparin (▲), 5.0 μM fucoidan (○) or 5.0 μM dextran sulfate (■). Scans were performed with either 200 μM glycosaminoglycan binding site consensus peptide ([300]-L-K-I-R-S-E-F-K-K-K-Y-G-K-S-L-Y-Y-[316]) (A) or 100 μg/mL of a peptide to the phosphorylation site of the p36 subunit (K-K-[9]-K-L-S-L-E-G-D-H-S-T-P-P-S-A-Y-G-S-V-K-A-Y-T-[30]) (B).

of the [XBBBXXBX] type (*19*) and that a peptide to this region of AIIt comprising [306]-FKKKYGKS-[314] undergoes a conformational change upon heparin binding.

To determine if the Cardin−Weintraub consensus sequence present in the p36 subunit of AIIt participates in fucoidan binding, we synthesized a peptide corresponding to this region of the p36 subunit and examined the potential interaction of the peptide with fucoidan. As shown in Figure 4A, the CD spectra of the peptide comprising [300]-L-K-I-R-S-E-F-K-K-K-Y-G-K-S-L-Y-Y-[316] demonstrate significant heparin- and fucoidan-dependent conformational changes. The concentrations of heparin and fucoidan that induced the conformational change in the peptide were similar to the concentrations of these carbohydrates required for a maximal change in the conformation of AIIt. In contrast, only a slight change in conformation was observed when the consensus sequence peptide was incubated with *N*-acetylglucosamine or dextran sulfate.

We also examined the interaction of two other peptides with fucoidan. Although fucoidan produced a conformational change in a peptide modeled on the actin bundling region of the p36 subunit (*33*) ([286]-V-L-I-R-I-M-V-S-R-[294]), a similar conformational change was also induced by the interaction of this peptide with *N*-acetylglucosamine (data not shown). Similarly, a peptide modeled on the phosphorylation sites of the p36 subunit (*34*), but containing an additional two lysines at the N-terminus (K-K-[9]-K-L-S-L-E-G-D-H-S-T-P-P-S-A-Y-G-S-V-K-A-Y-T-[30]), demonstrated conformational changes in the presence of fucoidan, heparin, and *N*-acetylglucosamine (Figure 4B). These results indicate that the region of the p36 subunit of AIIt that contains the Cardin−Weintraub consensus sequence probably participates in heparin and fucoidan binding.

Table 3: Fucoidan-Induced Conformational Changes in Several Annexins[a]

annexin	% change at $\Delta\epsilon_{222nm}$	
	N-acetylglucosamine	fucoidan
annexin I	6.2 ± 1.7	11.2 ± 3.3
annexin II	6.2 ± 1.7	3.0 ± 1.9
annexin II tetramer	6.2 ± 1.7	60.5 ± 2.3
annexin III	4.5 ± 0.7	51.9 ± 13.2
annexin IV	10.0 ± 1.4	45.7 ± 2.9
annexin V	5.0 ± 0.5	−26.7 ± 4.4
annexin VI	21.5 ± 9.1	−9.7 ± 6.4
p11	ND	1.0 ± 0.1

[a] Results are expressed as mean ± SD (n = 5). Percent change at $\Delta\epsilon_{222nm} = |\Delta\epsilon_{222nm}(AIIt)| - |\Delta\epsilon_{222nm}(AIIt + ligand)|/|\Delta\epsilon_{222nm}(AIIt)| \times 100$. Annexins (0.2 mg/mL) were incubated in buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.2 mM DTT, 1 mM $CaCl_2$, and 50.0 μM *N*-acetylglucosamine or 5.0 μM fucoidan. Annexins used in this study were isolated according to ref *28* and further purified by Ca^{2+}-dependent binding to a heparin affinity column as described in ref *34*. ND, not determined.

Fucoidan-Dependent Conformational Changes in Other Annexins. We had previously demonstrated that although many annexins bound to a heparin affinity column, only AIIt demonstrated a large conformational change upon heparin binding. AIIt also undergoes a dramatic conformational change in the presence of fucoidan (Figure 2), and since the p36 subunit of AIIt (annexin II) contains the lectin domain, it was reasonable to suspect that AIIt and the isolated p36 subunit would both undergo conformational changes in the presence of fucoidan. However, this was not the case, and neither the p36 subunit nor the p11 subunit of AIIt demonstrated a significant conformational change in the presence of fucoidan (Table 3). Furthermore, as shown in Table 3, all of the annexins tested except the p36 subunit of AIIt showed a significant conformational change in response to fucoidan.

Inhibition of AIIt-Dependent Liposomal Aggregation by Fucoidan. L-Selectin and AIIt are examples of proteins that bind Ca^{2+}, acidic phospholipids, and sulfated polysaccharides. Since the binding of fucoidan to L-selectin inhibits the ability of L-selectin to interact with acidic phospholipids (*10*), we were interested in exploring the possibility that fucoidan might also bind to AIIt and influence the ability of AIIt to aggregate phospholipid liposomes. As shown in Figure 5, fucoidan totally inhibited AIIt-dependent phospholipid aggregation. Furthermore, neither heparin, which binds to AIIt, nor *N*-acetylglucosamine, which does not interact with AIIt (*19*), affected AIIt-dependent phospholipid aggregation.

To determine if the disruption of AIIt-dependent liposomal aggregation by fucoidan was due to a loss in the binding of AIIt to the liposomes, AIIt was incubated with liposomes in the presence or absence of carbohydrate and the AIIt content of the pelleted liposomes was examined. As shown in Figure 5 (inset), fucoidan blocked the binding of AIIt to liposomes.

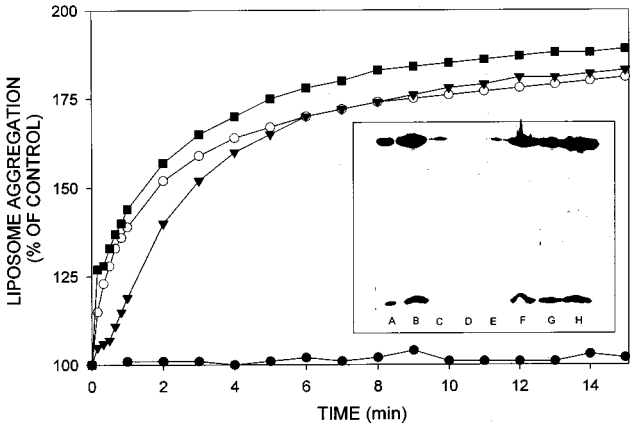

FIGURE 5: Inhibition of AIIt-dependent liposomal aggregation by fucoidan. Phospholipid liposomes consisting of phosphatidylserine, phosphatidylethanolamine, and cholesterol were prepared as per Experimental Procedures and then incubated at 20 °C with 30 mM Hepes, pH 7.5, 50 mM KCl, 2.0 mM $MgCl_2$, and 0.02 mM $CaCl_2$ (○) plus 50.0 μM heparin (▼), 50.0 μM *N*-acetylglucosamine (■), or 5.0 μM fucoidan (●). Immediately after the first reading of optical density was taken, AIIt (0.56 μM) was added to the reaction mixture and the A_{450nm} was continuously measured. Results are expressed as a percentage of starting A_{450nm} (no added AIIt). Data shown are expressed as mean ± SD ($n = 3$). (Inset) After 30 min the liposomal aggregation reaction mixtures were centrifuged at 14000*g* for 15 min and the pellet was analyzed by SDS−PAGE. AIIt-dependent liposomal aggregation was conducted in the absence of carbohydrate (lane B) or in the presence of 2 μM (lane C), 5 μM (lane D), or 10 μM (lane E) fucoidan or 50 μM heparin (lane F), 50 μM *N*-acetylglucosamine (lane G) or 50 μM fucose (lane H). AIIt standard is shown in lane A.

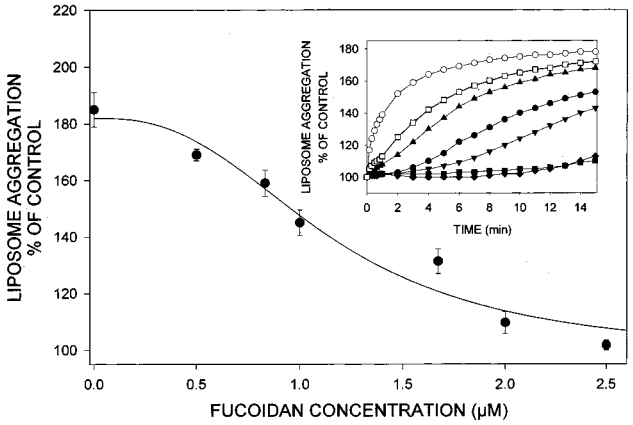

FIGURE 6: Concentration dependence of the inhibition of AIIt-dependent liposomal aggregation by fucoidan. Phospholipid liposomes (Experimental Procedures) were incubated at 20 °C with 30 mM Hepes, pH 7.5, 50 mM KCl, 2.0 mM $MgCl_2$, and 0.02 mM $CaCl_2$. Immediately after the first reading of optical density was taken, AIIt (0.56 μM) was added to the reaction mixture and the A_{450nm} was determined after 10 min. Results are expressed as a percentage of starting A_{450nm} (no added AIIt). Data shown are expressed as mean ± SD ($n = 3$). The line through the points is a nonlinear least-squares curve fit of the data points calculated from computer modeling of data using the logistic equation and the following values: $a = 182\%$ (asymptotic maximum), $b = 2.67$ (slope parameter), $c = 1.14$ M [value at inflection point ($A_{0.5}$)], and $d = 98.5\%$ (asymptotic minimum). The inset presents the time course data used for the analysis. The concentrations of fucoidan used were 2.5 μM (▲), 2.0 μM (●), 1.6 μM (◆), 1.0 μM (■), 0.75 μM (▼), 0.5 μM (□) and none (○).

In contrast, heparin or *N*-acetylglucosamine did not affect the binding of AIIt to the liposomes.

Figure 6 presents the concentration dependence of the inhibition of AIIt-dependent phospholipid aggregation by fucoidan. A concentration of fucoidan as low as 0.5 μM was found to inhibit the rate of AIIt-dependent liposomal aggregation (Figure 6, inset) and maximal inhibition was observed at about 2.0 μM fucoidan. Half-maximal inhibition of AIIt-dependent liposomal aggregation occurred at 1.14 ± 0.08 μM (mean ± SD, $n = 3$) fucoidan.

The data in Figure 5 established that the binding of fucoidan but not heparin to AIIt inhibited the liposomal aggregating activity of AIIt. If fucoidan and heparin bind to the same site on AIIt, then we would expect that heparin would block the inhibition of AIIt-stimulated liposomal aggregation by fucoidan by displacing fucoidan from the binding site on AIIt. As shown in Figure 7, at high concentration heparin but not *N*-acetylglucosamine reversed the inhibition of AIIt-stimulated liposomal aggregation by fucoidan.

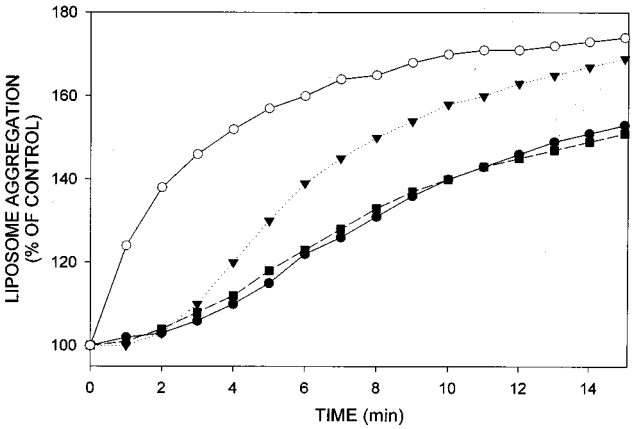

FIGURE 7: Reversal of fucoidan-dependent inhibition of AIIt-dependent liposomal aggregation by heparin. Phospholipid liposomes (Experimental Procedures) were incubated at 20 °C with 30 mM Hepes, pH 7.5, 50 mM KCl, 2.0 mM $MgCl_2$, and 0.02 mM $CaCl_2$ in the absence (○) or presence (●) of 1.0 μM fucoidan. Immediately after the first reading of optical density was taken, AIIt (0.56 μM) and either 50.0 μM heparin (▼) or 50.0 *N*-acetylglucosamine (■) were added to the reaction mixture and the A_{450nm} was continuously monitored. Results are expressed as a percentage of starting A_{450nm} (no added AIIt). Data shown are representative of three experiments.

DISCUSSION

Previous work from our laboratory established that AIIt was capable of binding heparin with high affinity and specificity. In the present report we have detailed the interaction of AIIt with fucoidan. The binding of fucoidan to AIIt was Ca^{2+}-independent and occurred at nanomolar concentrations of fucoidan. In contrast, the binding of fucoidan to AIIt caused a large conformational change in AIIt that required Ca^{2+} and occurred at micromolar concentrations of fucoidan. By comparison, both the binding of heparin to AIIt and the heparin-dependent conformational change required Ca^{2+}.

Although explication of the crystal structure of AIIt will be required before a detailed structural analysis of the interaction of fucoidan with AIIt can be formulated, it is possible to predict the mechanism of the binding of carbohydrate to AIIt. The X-ray crystallographic analysis of annexin II has revealed that the protein is composed of two distinct sides: one is a convex side that faces the biological

membrane and contains the Ca^{2+} sites. These Ca^{2+} sites also bind phospholipid and are therefore referred to as Ca^{2+}- and phospholipid-binding sites. The binding of phospholipid increases the affinity of Ca^{2+} binding to these sites. The concave side of annexin II faces the cytosol and contains the N- and C-termini and the Cardin−Weintraub consensus sequence (*35*−*37*). However, the alignment of the p36 subunits within AIIt is not known so the exact mechanism of lectin binding to AIIt is at present unclear. If a single lectin site is present on AIIt, then it is possible that fucoidan can, at low concentration, bind to this site in the absence of Ca^{2+}. At higher concentrations of fucoidan and in the presence of Ca^{2+}, fucoidan may form intramolecular cross-links between the lectin site of each of the two p36 subunits. The putative cross-linking of the lectin domain of the p36 subunits could cause a realignment of the subunits, resulting in a large conformational change in AIIt. Ca^{2+} binding to the Ca^{2+}- and phospholipid-binding sites may be necessary to alter the geometry of the p36 subunits such that cross-linking of the lectin domains by fucoidan could occur. This would explain the Ca^{2+} dependence of the fucoidan-induced conformational change in AIIt. Alternatively, AIIt may have two lectin sites, and one of the sites requires Ca^{2+} to activate fucoidan binding. The interaction of Ca^{2+} and fucoidan with this site would be responsible for producing a large conformational change in AIIt. The second putative fucoidan-binding site would be responsible for high-affinity Ca^{2+} independent fucoidan binding.

Since the CD analysis established that the conformation of the Ca^{2+}−AIIt−fucoidan complex is distinct from that of the Ca^{2+}−AIIt−heparin complex, the one-site model predicts that the heparin-bridged AIIt is not sufficiently contorted as to disrupt the Ca^{2+} and phospholipid sites. Therefore, fucoidan but not heparin blocks AIIt-dependent liposomal binding. Conversely, the two-sites model predicts that since the half-maximal fucoidan concentration required for either the conformational change or inhibition of liposomal aggregation is similar, then the interaction of Ca^{2+} and fucoidan with the Ca^{2+}- and fucoidan-binding site results in both the loss of the conformational change and the inhibition of liposomal binding. It is possible that the putative Ca^{2+}- and fucoidan-binding site and the Ca^{2+}- and phospholipid-binding sites are identical, i.e., fucoidan binds to the Ca^{2+}- and phospholipid-binding sites. The binding of fucoidan to the Ca^{2+}- and phospholipid-binding sites of AIIt could directly block the interaction of AIIt with phospholipid. Alternatively, the binding of fucoidan to a distinct Ca^{2+}- and fucoidan-binding site may change the conformation of AIIt so as to disrupt the Ca^{2+}- and phospholipid-binding sites, therefore resulting in inhibition of liposomal binding.

The large conformational change observed upon the binding of fucoidan to AIIt suggested that fucoidan might dissociate the AIIt subunits. However, gel-permeation chromatography of AIIt in the presence of Ca^{2+} and fucoidan did not result in the separation of the subunits, establishing that fucoidan did not disrupt the heterotetramer.

Cardin and Weintraub first reported the existence of heparin-binding consensus sequences in proteins (*17*). Two consensus sequences consisting of the sequences XBBBXX-BX or XBBXBX, where B is a basic amino acid and X is a hydropathic residue, were identified and shown to exist in several heparin-binding proteins. Modeling studies suggested that if the XBBXBX pattern was in a β-strand conformation, then the basic amino acids would be aligned on one face of the β-strand and the hydropathic amino acids would point back into the protein core. Similarly, if the XBBBXXBX pattern were aligned in an α-helix, then the basic amino acids would be arranged on one side of the helix with the hydropathic residues pointing back into the protein core. Other consensus sequences that have been identified include the XBBBXXBBBXXBBX sequence of von Willebrand factor (*38*, *39*) and the sequence TXXBXXTBXXXTBB, where T defines a turn (*40*). Recently we demonstrated that AIIt bound to heparin with an apparent K_d of 32 ± 6 nM (mean ± SD, $n = 3$) and a stoichiometry of 11 ± 0.9 mol of AIIt/mol of heparin (mean ± SD, $n = 3$). The binding of heparin to AIIt was Ca^{2+}-dependent and specific since other sulfated polysaccharides did not elicit a conformational change in AIIt. We also reported that although the isolated p36 subunit of AIIt (annexin II) did not show a conformational change upon fucoidan binding, a region of the p36 subunit of AIIt, F(306)−S(313) contained a Cardin−Weintraub consensus sequence of XBBBXXBX. A peptide corresponding to this region of the p36 subunit, [300]-LKIRSEFKKKYGKSLYY-[316], underwent a conformational change upon heparin binding (*19*). Therefore, our data suggested the presence of a C-terminal lectin domain in AIIt. Although speculative, our observation that a peptide corresponding to the lectin domain of AIIt also undergoes a conformational change upon binding fucoidan suggests but does not prove that fucoidan also binds to this region of AIIt. As discussed above, the binding of fucoidan with AIIt is complex and could involve multiple binding sites. However, our data are the first report that the Cardin−Weintraub consensus sequence can interact with fucoidan. Moreover, our data provide the first demonstration that an annexin can bind to sulfated fucopolysaccharides such as fucoidan.

The fucoidan-binding properties of AIIt are unique in certain aspects as compared to other fucoidan-binding proteins. First, AIIt has higher affinity (K_d 1 nM) than other fucoidan-binding proteins such as antistatin [K_d 50 nM (*41*)] or bindin [K_d 55 nM (*42*)], and the interaction of AIIt with fucoidan is more specific. For example, dextran sulfate competes with the fucoidan-binding site of GMP-740 (*43*), antistasin (*41*), proacrosin (*44*), selectin (*10*, *45*), and bindin (*42*).

Second, the specificity of the binding of fucoidan to AIIt cannot be explained on the basis of ionic interactions due to the density of charged sulfate groups. The charge density of fucoidan is less than 1 sulfate/saccharide residue, whereas the charge density of heparin is between 1 and 2 and that of dextran sulfate is about 2. However, dextran sulfate does not induce a conformational change in AIIt, nor does it produce a dramatic conformational change in the peptide modeled to the putative lectin domain of AIIt.

We also observed that AIIt formed a large complex with fucoidan and that this complex was pelleted by centrifugation at 400000*g*. Analysis of the binding isotherm suggested that AIIt bound fucoidan with an apparent K_d of 1.24 ± 0.69 nM (mean ± SD, $n = 3$) and a stoichiometry of 0.010 ± 0.001 mol of fucoidan/mol of AIIt (mean ± SD, $n = 3$). If it is assumed that the core region of fucoidan comprises a polymer of α1−3-linked fucose with α1−4 branch points every 2−3 fucose residues and sulfate groups substituted at

the 4-position of every three fucose residues, then the smallest average structural repeat would be about 1 kDa. Assuming a M_r of 180000 for fucoidan, this provides an estimate of about 180 repeats per molecule. If a repeat unit is the minimum fucoidan structure capable of interacting with AIIt, then a stoichiometry of 100 AIIt per fucoidan molecule is reasonable. How AIIt can stack along the polysaccharide chain is unclear and will require crystallographic analysis of the structure of the AIIt−fucoidan complex. By comparison, AIIt does not bind to disaccharides of heparin but does bind to 3 kDa heparin that contains about 10 monosaccharides. The binding of about 11 molecules of AIIt to a single 17 kDa heparin strand that contains about 50 monosaccharide units suggests that AIIt binds to about 4−5 monosaccharide units of heparin.

Fucoidan is a complex sulfated polysaccharide that has been shown to be an inhibitor of several biological processes. For example, fucoidan inhibits sperm−egg binding by blocking the interaction of the sperm inner acrosomal membrane protein proacrosin with glycoproteins associated with the zona pellucida (*44*, *46*−*49*). Fucoidan also inhibits sperm−zona pellucida binding (*3*, *4*). In addition, fucoidan has been shown to inhibit cell−cell binding mediated by P- or L-selectins (reviewed in refs *50* and *51*). The biological activity of fucoidan is thought to be due to the similarity in structure between fucoidan and fucosylated glycans of certain extracellular membrane glycoproteins. Specifically, fucoidan is thought to act by antagonizing the lectin−glycoprotein interaction involved in cell−cell binding by interacting with the lectin domain of extracellular membrane proteins. For example, the tetrasaccharide sialyl-Lewis^x is a binding determinant for the selectins and is present on P-selectin glycoprotein 1 (PSGL-1). PSGL-1 is a major ligand for the selectins, and loss of fucose from PSGL-1 results in a loss in binding activity (reviewed in ref *52*). The ability of fucoidan to efficiently block P- or L-selectin-mediated binding is probably due to regions within the fucoidan polysaccharide that have sulfate groups and fucose branches in the same configuration as the selectin ligand (*1*). In support of this suggestion are the recent reports that inhibition of the fucosylation and sulfation of Lewis^X blocks the interaction of selectins with their extracellular binding partners (*53*, *54*).

Although the physiological significance of the fucoidan-binding property of AIIt is unclear, it is reasonable to predict that AIIt may bind to the carbohydrate conjugates of certain membrane glycoproteins. The composition of the carbohydrate sequences (glycosaminoglycan or sulfated fucopolysaccharide) that interact with the lectin site of AIIt could have a profound effect on the biological activity of AIIt in vivo.

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