Characterization of the Heparin Binding Properties of Annexin II Tetramer*

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In this report, we have characterized the interaction of heparin with the Ca\(^{2+}\)- and phospholipid-binding protein annexin II tetramer (AIIt). Analysis of the circular dichroism spectra demonstrated that the Ca\(^{2+}\)-dependent binding of AIIt to heparin caused a large decrease in the a-helical content of AIIt from 44 to 31%, a small decrease in the b-sheet content from 27 to 24%, and an increase in the unordered structure from 20 to 29%. The binding of heparin also decreased the Ca\(^{2+}\) concentration required for a half-maximal conformational change in AIIt from 360 to 84 mM. AIIt bound to heparin with an apparent K\(_d\) of 32 ± 6 mM (mean ± S.D., n = 3) and a stoichiometry of 11 ± 0.9 mol of AIIt/mol of heparin (mean ± S.D., n = 3). The binding of heparin to AIIt was specific as other sulfated polysaccharides did not elicit a conformational change in AIIt. A region of the p36 subunit of AIIt (Phe\(^{306}\)–Ser\(^{313}\)) was found to contain a Cardin-Weintraub consensus sequence for glycosaminoglycan recognition. A peptide to this region underwent a conformational change upon heparin binding. Other annexins contained the Cardin-Weintraub consensus sequence, but did not undergo a substantial conformational change upon heparin binding.

The annexins are a family of ~13 proteins that bind to acidic phospholipids and biological membranes in a Ca\(^{2+}\)-dependent manner (see Refs. 1–3 for reviews). These proteins are expressed in a wide range of organisms such as slime molds, higher plants, invertebrates, and vertebrates. Studies of the amino acid sequence of the annexins have established the homology of these proteins. All annexins contain four repeats (eight repeats in the case of annexin VI) of ~70 amino acids that are highly homologous. In contrast, the N terminus of each of the annexins is unique and displays the greatest variation in sequence and length. The crystal structure of several of the annexins has been reported (4–6) and has established that the annexins are composed of two distinct sides. The convex side faces the biological membrane and contains the Ca\(^{2+}\)- and phospholipid-binding sites. The concave side faces the cytosol and contains the N and C termini.

Annexin II (p36) contains three distinct functional regions, the N-terminal region, the C-terminal region, and the core region. The core region of p36 contains the Ca\(^{2+}\)- and phospholipid-binding sites, whereas the C-terminal region contains the 14-3-3 homology domain (7) and the plasminogen-binding domain (8). The N terminus of annexin II (p36) contains two important regulatory domains, the L and P domains. The L domain consists of the first 14 residues of the N terminus and contains a high affinity binding site for the p11 protein (reviewed in Ref. 9). The P domain of p36 contains the phosphorylation sites for protein kinase C (Ser\(^{25}\)) and pp60\(^{c-src}\) (Tyr\(^{324}\)). The N-terminal L and P domains play regulatory roles; activation of the phosphorylation sites of annexin II tetracer results in an increase in the A\(_{254}\)(Ca\(^{2+}\)) for chromaffin granule aggregation and F-actin binding, whereas binding of the p11 subunit decreases the A\(_{254}\)(Ca\(^{2+}\)) for these activities. The heterotetrameric complex (p36\(_2\)·p11\(_2\)) formed by the binding of p11 to p36, referred to as annexin II tetramer (AIIt), is the predominant form in most cells (reviewed in Ref. 9).

AIIt has been shown to be present at both the cytosolic and extracellular surfaces of the plasma membrane of many cells (9). Extracellular AIIt has been proposed to function as a cell adhesion factor (10, 11), a receptor for plasminogen and tissue plasminogen activator (8, 12), and a receptor for tenascin-C (13, 14). In a previous study (15), we reported that AIIt bound to a heparin affinity column and that the phosphorylation of AIIt on tyrosine residues blocked the heparin-binding activity of the protein. In this report, we have characterized the interaction of AIIt with heparin. Our results identify AIIt as a specific, high affinity heparin-binding protein. Furthermore, we show that the Ca\(^{2+}\)-dependent binding of heparin to AIIt causes a dramatic conformational change in the protein. Last, we show that the p36 subunit of AIIt contains a Cardin-Weintraub glycosaminoglycan recognition site (16) and that a peptide to this region of AIIt binds heparin.

EXPERIMENTAL PROCEDURES

Materials—Annexin II tetramer and annexin II monomer were prepared from bovine lung as described (17) and stored at −70 °C in 40 mM Tris·HCl, pH 7.5, 1.0 mM DTT, 0.1 mM EDTA, and 150 mM NaCl. Carbohydrates were obtained from Sigma and were the purest grade available. Heparin (bovine lung) was obtained from Calbiochem and had an average molecular mass of 17 kDa and an activity of 149 USP units/mg. Porcine intestinal mucosa heparin (6 kDa, 70 USP units/mg), bovine intestinal mucosa heparin (3 kDa, 50 USP units/mg), and bovine kidney heparan sulfates (7.5 kDa) were obtained from Sigma. Measurement of Protein Secondary Structure by Circular Dichroism—CD measurements were performed with a Jasco J-715 spectropolarimeter. The spectropolarimeter was calibrated with an aqueous solution of recrystallized ammonium camphor sulfonate·d\(_{16}\). At a concentration of 1 mg/ml in a 1-mm cell, this compound had a \(\Delta\epsilon\) of 2.36 at its CD maximum of 290.5 nm. 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 The abbreviations used are: AIIt, annexin II tetramer; DTT, dithiothreitol.

* This work was supported by a grant from the Medical Research Council of Canada. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1.0 mM CaCl₂ or buffer B (10 mM Tris-HCl, pH 7.5, 0.1 mM DTT, 150 mM NaCl, and 0.5 mM EGTA) in the presence or absence of ligand (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 ligand in buffer. The measurements at 222 nm were obtained using the time scan mode of the software, and each point was the average of a minimum of 20 × 1-s measurements (at a bandwidth of 1 nm). Results are expressed as Δε (m⁻¹ cm⁻¹).

Analysis of CD Spectra—CD spectra of the AIIt preparations were analyzed using the computer program SELCON, which was a generous gift from Dr. Robert W. Woody (Department of Biochemistry and Molecular Biology, Colorado State University, Fort Collins, CO). Typically, scans from six individual experiments were analyzed, and these values were averaged. The root mean square difference between the predicted and experimental CD spectra was always <0.15.

AIIt Affinity Chromatography—Approximately 1.4 g of CNBr-activated Sepharose 4B was washed with 400 ml of 1 mM HCl, followed by extensive washing in buffer C containing 25 mM NaCl, 0.1 mM EDTA, and DTT (1 mM), which was used as the couplng buffer (20). AIIt (10 mg) was extensively dialyzed against coupling buffer, followed by step elution with buffer C containing 150 and 500 mM NaCl. The heparin concentration of these fractions was determined by the uronic acid/carbazole reaction (19).

Heparin Binding Assays—Heparin binding experiments were performed in a total volume of 200 μl in a reaction mixture containing 25 mM Hepes, pH 7.5, 1 mM DTT, and 1 mM CaCl₂) or buffer B (10 mM Tris-HCl, pH 7.5, 0.1 mM DTT, 150 mM NaCl). AIIt (10 mg) was extensively dialyzed against coupling buffer, followed by step elution with buffer C containing 150 and 500 mM NaCl. The heparin concentration of these fractions was determined by the uronic acid/carbazole reaction (19).

RESULTS

CD Spectra of the AIIt-Heparin Complex—Consistent with a previous report (23), we also found that the binding of Ca²⁺ to AIIt caused a 10% reduction in intensity of the CD spectrum of AIIt (Fig. 1A). In contrast, the binding of heparin to AIIt in the presence of Ca²⁺ resulted in a decrease in the intensity of the CD spectrum at 222 nm of ~40%. Furthermore, the CD spectrum of AIIt lost intensity and changed shape as the protein bound heparin in the presence of Ca²⁺ (Fig. 1B). The mixture was sonicated (4 × 15-s bursts at 75 watts with a Braun probe sonicator), and the phospholipid liposomes were stored at room temperature prior to their use.

Quantitative assessment of the secondary structure components of the CD spectra of AIIt was made as described (18). The results are summarized in Table I. In the presence of Ca²⁺, the
binding of heparin to AIIt resulted in a decrease in the α-helical content of AIIt from ~44 to 31%, a decrease in the β-sheet content from ~27 to 24%, an increase in the content of β-turns from ~12 to 14%, and an increase in the unordered structure from ~20 to 29%.

In the absence of Ca²⁺, heparin induced a more moderate change in the conformation of AIIt. Of interest was the heparin-induced increase in the β-sheet from ~21 to 27% and decrease in unordered structure from 22 to 18%.

These results establish that in the presence of Ca²⁺, heparin induces a large conformational change in AIIt, resulting in a substantial change in the conformation of the protein. However, heparin can also interact with AIIt in the absence of Ca²⁺ and, to a much smaller degree, affect the conformation of the protein.

Dose Dependence of the Heparin-induced Conformational Change in AIIt—Fig. 2 presents the dose dependence of the heparin-induced conformational change in AIIt as determined by the changes in the CD spectrum at 222 nm. Increasing the heparin concentration decreased the intensity of the CD spectrum of AIIt until a maximum was reached at ~0.59 μM heparin. At the optimal concentration of heparin, the CD intensity decreased by ~36%. The heparin concentration required for a half-maximal decrease in the CD intensity of 2.2 μM AIIt was ~0.10 μM. Increasing the heparin concentration in excess of 0.59 μM resulted in an increase in the CD intensity.

Ca²⁺ Dependence of the Heparin-induced Conformational Change in AIIt—AIIt binds Ca²⁺ with a K_{Ca²⁺} of ~0.5 mM. When AIIt is bound to chromaffin granules, phospholipid liposomes, or F-actin, the K_{Ca²⁺} is reduced to μM values (9). The decrease in the K_{Ca²⁺} upon phospholipid binding is due to a conformational change in the protein resulting from the binding of the phospholipid to the protein (6). We therefore used CD to examine the Ca²⁺ requirement for the changes in the conformation of AIIt observed upon heparin binding. As shown in Fig. 3, when assayed in the presence of saturating heparin, the half-maximal change in the CD spectrum of AIIt at 222 nm required 0.084 ± 0.008 μM Ca²⁺ (mean ± S.D., n = 3). In contrast, the half-maximal change in the CD spectrum of AIIt at 222 nm in the absence of heparin required 0.361 ± 0.149 μM Ca²⁺ (mean ± S.D., n = 3). This result therefore establishes that the binding of heparin to AIIt increases the affinity of the protein for Ca²⁺.

Specificity of the Heparin-induced Conformational Change in AIIt—We have also examined the effect of a variety of charged polysaccharides on the CD spectra of AIIt at 222 nm. As shown in Table I, charged polysaccharides such as heparin sulfate, chondroitin sulfate, and dextran sulfate failed to induce a large conformational change in AIIt. Similarly, other amino monosaccharides such as N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, and N-acetylneuraminic acid or 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 heparin-binding site of AIIt without producing a conformational change. This possibility was ruled out by our observation that 50 μM heparan sulfate or 50 μM N-acetylgalactosamine failed to block the conformational change in AIIt induced by 0.5 μM heparin.

We have also observed that smaller molecular mass forms of heparin can induce a conformational change in AIIt. As shown in Table II, both 3- and 6-kDa forms of heparin could induce a conformational change in AIIt. However, we have been unable to detect a conformational change in AIIt in the presence of disaccharide heparin derivatives (data not shown). These results therefore suggest that the heparin-induced conformational change in AIIt requires the interaction of AIIt with a region of heparin larger than a disaccharide repeating unit.

Binding of Heparin to an AIIt Affinity Column—Heparin is

### Table I

| Structure     | Ca²⁺ | Ca²⁺, heparin | EGTA | EGTA, heparin |
|---------------|------|---------------|------|---------------|
| α-Helix       | 43.8 ± 1.63 | 31.4 ± 2.2    | 44.6 ± 1.0 | 40.1 ± 0.6    |
| β-Sheet       | 26.6 ± 4.2  | 24.3 ± 3.9    | 20.9 ± 3.8 | 26.5 ± 6.2    |
| β-Turns       | 11.5 ± 5.0  | 13.8 ± 0.8    | 11.9 ± 1.0 | 14.8 ± 1.5    |
| Unordered     | 20.0 ± 3.6  | 29.4 ± 4.4    | 22.3 ± 7.3 | 18.4 ± 4.0    |

FIG. 2. Concentration dependence of the heparin-induced conformational change in AIIt. AIIt (2.2 μM) was incubated for 20 min at 20 °C in buffer A with variable concentrations of heparin. The circular dichroism spectrum was then measured at 222 nm. Data shown are expressed as means ± S.D. (n = 3).

FIG. 3. Ca²⁺ dependence of the heparin-induced conformational change in AIIt. AIIt (2.2 μM) was incubated for 20 min at 20 °C in buffer (10 mM Tris-HCl, pH 7.5, 0.1 mM DTT, and 0.15 mM NaCl) containing variable concentrations of Ca²⁺ in the absence (○) or presence (■) of 0.56 μM heparin. The circular dichroism spectra were measured at 222 nm. Data shown are expressed as means ± S.D. (n = 3). The conformational change at 222 nm is plotted as a function of the maximal conformational change in AIIt at 222 nm, where the maximal conformational change (100% of maximum) was determined by averaging the Δε_{222 nm} value for AIIt in the presence of 2 mM Ca²⁺ and saturating heparin. 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") and four-fitting parameters, a (asymptotic maximum), b (slope parameter), c (value at inflection point, A_{0.5}), and d (asymptotic minimum), which were allowed to float during the computer iterations.
an acidic polysaccharide characterized by a disaccharide repeating unit of hexosamine and uronic acid (\(L\)-iduronic or \(D\)-glucuronic acid) connected through 1–4-linkages. Commercially prepared heparin is heterogeneous due to the varying degree of modification of the functional groups in the disaccharide unit and also to variations in the size of the polysaccharide chains (24). This presented the possibility that the heterogeneous heparin preparation might contain subpopulations of heparin that might not bind to AIIt. We therefore examined the binding of the heparin preparation to an AIIt affinity column. As shown in Fig. 4, ~3% of the heparin applied to the AIIt affinity column flowed through the column, whereas ~85% was recovered when the AIIt column was washed with 150 mM NaCl. Washing the column with 1 M NaCl failed to further elute any heparin.

**Characterization of the Binding of AIIt to Heparin**—We observed that in the presence of Ca\(^{2+}\), the AIIt-heparin complex could be pelleted by centrifugation at 400,000 \(\times g\). This simple pelleting assay allowed an alternative estimation of the affinity and capacity of the binding of AIIt to heparin. As shown in Fig. 5, half-maximal binding of AIIt to heparin required 0.115 \( \pm \) 0.021 \( \mu \)g heparin (mean \( \pm \) S.D., \( n = 3 \)). The asymptotic maximum of the plot was 1.904 \( \mu \)M AIIt, which suggested that ~97% of the total AIIt could be recovered in the pellet. Analysis of the binding data (Fig. 5, inset) estimated that AIIt bound to heparin with an apparent \( K_f \) of 32 \( \pm \) 6 nM (mean \( \pm \) S.D., \( n = 3 \)) and a stoichiometry of 1\( \times \)0.9 mol of AIIt/mol of heparin (mean \( \pm \) S.D., \( n = 3 \)).

We also examined the Ca\(^{2+}\) dependence of AIIt-heparin complex formation. Fig. 6 presents a typical result. Half-maximal formation of the AIIt-heparin complex required \( pCa^{2+} \) = 4.259 \( \pm \) 0.035. This value (55 \( \mu \)M Ca\(^{2+}\)) was similar to the value of 84 \( \mu \)M Ca\(^{2+}\) determined for the half-maximal Ca\(^{2+}\) dependence of the heparin-induced conformational change in AIIt.

As discussed under "Experimental Procedures," AIIt can be purified by NaCl-dependent elution from a heparin affinity column in the presence of Ca\(^{2+}\), a 500 mM NaCl wash was sufficient to elute the AIIt from the heparin affinity column. We therefore examined the dependence of the formation of the AIIt-heparin complex on ionic strength. As shown in Fig. 7, half-maximal dissociation of the AIIt-heparin complex required ~380 \( \pm \) 99 mM NaCl. This result is consistent with the formation of AIIt-heparin complexes under physiological conditions.

**Identification of a Heparin-binding Site in AIIt**—It has been suggested that many heparin-binding proteins contain consensus sequences for glycosaminoglycan recognition (16). Two consensus sequences, referred to as Cardin-Weintraub sequences, that have been identified are XBBBXXBX and XBBBXYB, where B has the probability of a basic residue and X is a hydrophatic residue. We have found that the p36 subunit of AIIt contains a Cardin-Weintraub consensus sequence (17 kDa).

**TABLE II**

| Polysaccharide         | Change at \( \Delta_{222} \) nm |
|------------------------|----------------------------------|
| Heparin                | 4.0 \( \pm \) 3.4                |
| Heparan sulfate        | 3.3 \( \pm \) 1.0                |
| Chondroitin sulfate    | 2.3 \( \pm \) 0.9                |
| Dextran sulfate        | 6.9 \( \pm \) 4.1                |
| N-Acetylgalactosamine  | 6.2 \( \pm \) 1.7                |
| N-Acetylneuraminic acid| 5.1 \( \pm \) 1.0                |
| Glucose                | 3.9 \( \pm \) 1.0                |
| Galactose              | 2.8 \( \pm \) 0.1                |
| Mannose                | 9.5 \( \pm \) 0.1                |
| Fucose                 | 6.1 \( \pm \) 0.2                |
| Heparin (3 kDa)        | 40.9 \( \pm \) 1.2               |
| Heparin (6 kDa)        | 58.0 \( \pm \) 2.3               |
| Heparin + hepan sulfate| 39.3 \( \pm \) 2.4               |
| Heparin + N-acetylgalactosamine | 35.3 \( \pm \) 2.2               |

**FIG. 4.** Binding of heparin to an AIIt affinity column. Heparin (0.5 mg) was dissolved in buffer C containing 25 mM NaCl and applied to an AIIt affinity column equilibrated against the same buffer. The column was washed with buffer C containing 50 mM NaCl and eluted with buffer C containing 150 mM NaCl. Fractions were analyzed for the presence of heparin with the uronic acid/carbazole reaction (see "Experimental Procedures").

**FIG. 5.** Binding isotherm for the titration of AIIt with heparin. AIIt (1.971 mg) was incubated with 25 mM Hepes, pH 7.5, 1 mM DTT, 150 mM NaCl, 1 mM CaCl\(_2\), and variable concentrations of heparin at room temperature for 20 min. The reaction mixture was centrifuged at 400,000 \( \times g \) for 30 min, and the amount of AIIt in the pellet was determined as described under "Experimental Procedures." 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: \( a = 1.904 \) \( \mu \)M (asymptotic maximum), \( b = 1.592 \) (slope parameter), \( c = 0.115 \) \( \mu \)M (asymptotic minimum). Inset, the fractional saturation (mol of AIIt in pellet/total mol of AIIt) is plotted against the total heparin 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 (Equation 1 under "Experimental Procedures") with the assumption of a single class of heparin-binding sites on AIIt and two fitting parameters, \( K_L \) and \( n \), which were allowed to float during the computer iterations. The convergent best fit for these experiments was determined for \( K_L = 32 \) nM and \( n = 0.091 \) mol of heparin/mol of AIIt.
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### Table III

Comparison of heparin-binding domains of heparin-binding proteins

| Protein              | Consensus Sequence |
|----------------------|--------------------|
| Annexin II           | KIRSEFKKGYKGLLY    |
| Vn                   | QFRFRHRNKGVYGRQRG  |
| ApoB                 | KFIIPSPKRPKVLSSG   |
| bFGF                 | GHKDPKRLYCKNGG     |
| NCAM                 | DGGSPIRHYLIYKAK    |
| PCI                  | GLSEKTLKWLKMKKKF   |
| AT-III               | KLNCRLYRKANKSSKL   |
| ApoE                 | SHRLKRKRRLLRADD    |
| β-TG                 | PDAPRIKIVQKKLAG    |
| IGFBP-3              | DKKGFYKQCPPRGK     |
| Fibrin               | GHRPLDKREEAPSLR    |
| FGFR-1               | AAVPVHLKKEM        |

*Bold type indicates position in the consensus sequence of an invariant residue.*

### DISCUSSION

Previous work from our laboratory established that AIIt is a Ca$^{2+}$-dependent heparin-binding protein (15). The interaction of AIIt with heparin was also shown to be inhibited by tyrosine phosphorylation of AIIt (15). Since the role that heparin binding plays in the structure or function of AIIt is unknown, the current study aimed at the characterization of the interaction of heparin with AIIt. Analysis of the CD spectra of AIIt showed that the binding of heparin to AIIt resulted in a pro-

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**Figure 6.** Ca$^{2+}$ dependence of AIIt-heparin binding. AIIt (1.9 μM) was incubated at room temperature for 20 min with 25 mM Hepes, pH 7.5, 1 mM DTT, 150 mM NaCl, 0.56 μM heparin, and 1 mM Ca$^{2+}$-nitrilotriacetic acid buffers. The reaction mixture was centrifuged at 400,000 × g for 30 min, and the amount of AIIt in the pellet was determined as described under “Experimental Procedures.” Data shown are means ± S.D. (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 parameters of which were allowed to float during the computer iterations.

**Figure 7.** Salt dependence of AIIt-heparin binding. AIIt (1.2 μM) was incubated at room temperature for 20 min with 25 mM Hepes, pH 7.5, 1 mM DTT, 0.56 μM heparin, and variable concentrations of NaCl. The reaction mixture was centrifuged at 400,000 × g for 30 min, and the amount of AIIt in the pellet was determined as described under “Experimental Procedures.” Data shown are means ± S.D. (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”) and four fitting parameters, a (asymptotic maximum), b (slope parameter), c (value at inflection point, A$_c$), and d (asymptotic minimum), which were allowed to float during the computer iterations.

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of the p36 subunit conforms exactly to the Cardin-Weintraub consensus sequence.

To determine if the Cardin-Weintraub consensus sequence present in the p36 subunit of AIIt participates in heparin binding, we synthesized a peptide to this region of the p36 subunit and examined the potential interaction of the peptide with heparin. As shown in Fig. 8A, the CD spectrum of the peptide comprising $^{399}$LKIRSEFKKGYKGLLY$^{416}$ demonstrated a significant heparin-dependent conformational change. In contrast, only a slight change in conformation was observed when the consensus sequence peptide was incubated with N-acetylglucosamine. We also examined the interaction of two other peptides with heparin. Although heparin produced a conformational change in a peptide modeled to the actin-binding region of the p36 subunit (GGTLIRIMVSR) (25), a similar conformational change was also induced by the interaction of this peptide with N-acetylglucosamine (Fig. 8B). Similarly, a peptide modeled to the phosphorylation sites of the p36 subunit (15), but containing an additional two lysines at the N terminus (KKKLSEGLDHHSTPPSAYGSVKAYT) (26), demonstrated conformational changes in the presence of both heparin and N-acetylglucosamine (Fig. 8C). Therefore, these results indicate that the region of the p36 subunit of AIIt that contains the Cardin-Weintraub consensus sequence probably participates in heparin binding.

**Heparin-dependent Conformational Changes in Other Annexins**—Since the p36 subunit of AIIt contains the heparin-binding domain, it was reasonable to suspect that both AIIt and the isolated p36 subunit (annexin II) would undergo conformational changes upon heparin binding. This was not the case, however; and the p36 subunit did not undergo a significant conformational change in the presence of heparin (Table IV). Furthermore, of the six monomeric annexins examined, none demonstrated a specific conformational change in the presence of heparin. This was surprising because all these annexins were purified by heparin affinity chromatography. This observation therefore suggested that the specific heparin-induced conformational change in AIIt did not occur in other annexins.

Affect of Heparin on Membrane-bound AIIt—AIIt is a phospholipid-binding protein that has been shown to be located on both the intracellular and extracellular surfaces of the plasma membrane (9). Since the binding of heparin to AIIt has such a profound effect on the conformation of the protein, it was important to investigate the possibility that the interaction of AIIt with heparin might disrupt the binding of AIIt with membranes. However, as shown in Fig. 9, heparin did not block the interaction of AIIt with phospholipid liposomes.
found change in the conformation of AII (Fig. 1 and Table I). We also found that in the absence of Ca2+, a small change in the conformation of AII occurred upon heparin binding.

Animal carbohydrate-binding proteins can be broadly classified into seven major groups. These include the C-type or Ca2+-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 (26). The C-type lectins bind several carbohydrates including mannose and galactose and require Ca2+ to form a coordination bond with the sugar ligand. The galectins bind only b-galactoside, whereas the P-type proteins bind only mannose 6-phosphate. The I-type lectins bind only sialic acid, whereas the pentraxins bind several carbohydrates such as heparin and sialic acid as well as phosphorylcholine. The hyaluronan-binding proteins bind only hyaluronan. The heparin-binding proteins bind only heparin.

### FIG. 8. Heparin-induced conformational change in a peptide to the heparin-binding domain of AII.

Wavelength scans were conducted at 20 °C in buffer A alone (solid line) or containing 0.56 μM heparin (dotted line) or 5 μM N-acetylglucosamine (dashed line). Peptides were added at a concentration of 100 μg/ml. A, heparin-binding site consensus peptide (309LKRSEFKKKYKSLY326); B, actin-binding site consensus peptide (286YLIRIMVSR294); C, phosphorylation site consensus peptide (KK9KLSLEGDHSTPPSAYGSVKAYT).

### FIG. 9. Effect of heparin on the binding of AII to phospholipid vesicles.

Phospholipid liposomes consisting of phosphatidylserine, phosphatidylethanolamine, and cholesterol were prepared as described under “Experimental Procedures” and then incubated at 20 °C with 30 mM Hepes, pH 7.5, 50 mM KCl, 2.0 mM MgCl2, 0.2 mM CaCl2, 1.0 μM heparin, and 0.56 μM AII. After 30 min, the reaction was centrifuged at 14,000 × g for 10 min, and the pellet was analyzed by SDS-polyacrylamide gel electrophoresis. Lane 1, AII standard; lane 2, liposomes and heparin incubated for 10 min before addition of Ca2+ and AII; lane 3, AII incubated with heparin and Ca2+ before addition to the liposomes; lane MWM, molecular weight markers.

### TABLE IV

Heparin-induced conformational changes in several annexins

Results are expressed as means ± S.D. (n = 5). Percent change at \( \Delta \varepsilon_{222} \) nm = \( \Delta \varepsilon_{222} \) nm(AII) - \( \Delta \varepsilon_{222} \) nm(AII + ligand)/\( \Delta \varepsilon_{222} \) nm(AII) × 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 CaCl2, and 5.0 or 0.5 μM heparin (AII). Annexins used in this study were isolated as described (17) and further purified by Ca2+-dependent binding to a heparin affinity column as described (15).

| Annexin  | Heparin binding | Change at \( \Delta \varepsilon_{222} \) nm |
|----------|----------------|-----------------------------------|
| N-Acetylglucosamine | Heparin | |
| Annexin I | 6.2 ± 1.7 | 1.0 ± 1.0 |
| Annexin II | 6.2 ± 1.7 | 6.4 ± 3.8 |
| Annexin II tetramer | 6.2 ± 1.7 | 41.0 ± 3.4 |
| Annexin III | 4.5 ± 0.7 | 5.3 ± 2.1 |
| Annexin IV | 10.0 ± 1.4 | 3.7 ± 2.6 |
| Annexin V | 5.0 ± 0.5 | 3.6 ± 2.1 |
| Annexin VI | 21.5 ± 9.1 | 17.8 ± 4.3 |
| p11 | ND* | 1.0 ± 0.1 |

*ND, not determined.
binding proteins generally demonstrate Ca$^{2+}$-independent binding of both heparin and heparan sulfate. Since AIIt binds heparin (Table II) but not phosphocholine (15), AIIt is most likely a member of the heparin-binding family of proteins. However, AIIt appears to be unique among heparin-binding protein members in that the binding of AIIt to heparin is stimulated by Ca$^{2+}$. Furthermore, AIIt appears to be a unique member of the heparin-binding proteins because AIIt can discriminate between heparin and heparan sulfate ligands.

Several consensus sequences have been identified among members of the heparin-binding family of proteins. For example, the heparin-binding sequence of the C-terminal region of fibronectin has been identified as WQPPRARI (27). In contrast, a region of thrombospondin containing the sequence WSPW has been identified as the heparin-binding region of the protein (28, 29). Analysis of several heparin-binding proteins has suggested the potential existence of two consensus sequences referred to as Cardin-Weintraub heparin-binding consensus sequences (16, 30). Site-directed mutagenesis and binding studies with synthetic or isolated peptides from several of these proteins have confirmed that this consensus region is often involved in binding heparin (30–34, 36). Other studies have shown that the orientation of the Cardin-Weintraub consensus sequence within the protein is critical and may determine if the consensus sequence participates in heparin binding (33, 37). As shown in Table III, the p36 subunit of AIIt contains a Cardin-Weintraub heparin-binding consensus sequence. Furthermore, a peptide to this region of the p36 subunit of AIIt (307-LKRISEFKKYKGLS) and undergoes a conformational change upon heparin binding (Fig. 8). These results therefore suggest that residues 300–316 of the p36 subunit of AIIt are involved in heparin binding.

Although the monomeric annexins I–VI bind to a heparin affinity column in the presence of Ca$^{2+}$, a heparin-dependent conformational change was not observed for these proteins (Table IV). The p36 subunit of AIIt can exist as a monomer or as a heterotetramer. Heterotetrameric AIIt is composed of two p36 subunits and two p11 subunits. Considering that the p36 subunit (annexin II) binds to a heparin affinity column and contains the Cardin-Weintraub consensus sequence, it was surprising that the p36 subunit did not undergo a conformational change upon heparin binding. This suggests that the heparin-binding site of the p36 subunit and other monomeric annexins is preformed and does not require the recruitment of residues from other regions of the protein. This is consistent with the observation that carbohydrate-binding proteins undergo few if any changes in conformation upon carbohydrate binding (26). The p11 subunit of AIIt does not bind heparin and does not contain any heparin-binding consensus sequences. It is therefore unlikely that the heparin-dependent conformational change in AIIt was due to the coordinated binding of heparin by both the p36 and p11 subunits of AIIt. We cannot, however, rule out the possibility that the binding of the p36 subunit to the p11 subunit induces a conformational change in the p11 subunit that results in exposure of a novel heparin-binding domain. The simplest explanation for the large conformational change in AIIt upon heparin binding is that the orientation of the p36 subunits in AIIt is not optimal for heparin binding. Therefore, the binding of heparin to AIIt results in the realignment of the p36 subunits.

Of particular interest was our observation that the Ca$^{2+}$-dependent conformational change in AIIt was induced by heparin, but not by other negatively charged glycosaminoglycans such as heparan sulfate, chondroitin sulfate, and dextran sulfate. Heparan sulfates are structurally related glycosaminoglycans that are found on cell surfaces and in the extracellular matrix, where they form the chains of heparan sulfate proteoglycans and bear only short stretches of dense sulfation. In contrast, heparin is the glycosaminoglycan that is secreted by mast cells and other hematopoietic cells and therefore may serve as a signaling molecule (38, 39). To date, a heparin-binding protein capable of distinguishing between heparin and heparan sulfate has not been described. Recently, annexin IV was shown to bind heparin, but the binding of heparin to this protein was inhibited by a variety of carbohydrates including glucose, N-acetylgalactosamine, heparan sulfate, and chondroitin sulfate (40). In contrast, we have found that heparan sulfate or other glycosaminoglycans do not induce a conformational change in AIIt (Table II). Furthermore, high concentrations of heparan sulfate (50 μM) do not inhibit the conformational change in AIIt elicited by 0.5 μM heparin (Table II), therefore suggesting that heparan sulfate does not bind to AIIt. However, considering the heterogeneity of the cell-surface heparan sulfate proteoglycan (38), it is possible that AIIt may interact with other heparan sulfate proteoglycans.

We also observed that AIIt formed a large complex with heparin and that this complex was pelleted by centrifugation at 400,000 × g. Analysis of the binding isotherm suggested that AIIt bound heparin with an apparent Kd of 32 ± 6 nM (mean ± S.D., n = 3) and a stoichiometry of 11 ± 0.9 mol of AIIt/mol of heparin (mean ± S.D., n = 3). This Kd for the binding of heparin to AIIt is slightly lower than the Kd reported for the binding of heparin to heparinase (60 nM), acidic fibroblast growth factor (50–140 nM), or fibroblast growth factor (2.2 nM), or antithrombin III (11 nM) (32, 41, 42). AIIt does not bind to disaccharides of heparin, but does bind to 3-kDa heparan sulfate that contains ~10 monosaccharides (Table II). The binding of ~11 molecules of AIIt to a single 17-kDa heparin strand that contains ~50 monosaccharide units (Fig. 5) suggests that AIIt requires ~4–5 monosaccharide units for binding.

The physiological significance of the binding of heparin to AIIt is unclear. Heparin has been shown to interact with enzymes of the clotting and fibrinolysis systems (24), protect proteins from inactivation, play an essential role in the interaction of growth factors with their receptors, directly activate growth factor receptors, and serve as an essential cofactor in cell-cell recognition and cell-matrix adhesion processes (27, 35, 43–47). AIIt is the major cellular receptor for tenascin-C and plasminogen (8, 14). It is therefore possible that heparin might be involved in the regulation of the interaction of AIIt with these ligands.

Acknowledgments—We thank Dr. Robert W. Woody for the generous gift of the SELCON computer program and Dr. Narasimha Sreerama (Colorado State University) for helpful discussions concerning interpretation of CD data using the SELCON computer program.

REFERENCES

1. Kaetzel, M. A., and Dedman, J. R. (1995) News Physiol. Sci. 10, 171–176
2. Raynal, P., and Pollard, H. B. (1994) Biochim. Biophys. Acta 1197, 63–93
3. Swairjo, M. A., and Seaton, B. A. (1994) Annu. Rev. Biophys. Biomol. Struct. 23, 193–213
4. Favier-Perron, B., Lewit-Bentley, A., and Russo-Marie, F. (1996) Biochemistry 35, 1740–1744
5. Loecke, H., Chang, B. T., Maillard, W. S., Schlaepfer, D. D., and Haigler, H. T. (1995) Nature 378, 512–515
6. Swairjo, M. A., Concha, N. O., Kaetzel, M. A., Dedman, J. R., and Seaton, B. A. (1995) Nat. Struct. Biol. 2, 968–974
7. Roth, D., Morgan, A., and Burgoyne, R. D. (1993) FEBS Lett. 320, 207–210
8. Hajjar, K. A., Jacovina, A. T., and Chacko, J. (1994) J. Biol. Chem. 269, 21191–21197
9. Waisman, D. M. (1995) Mol. Cell. Biochem. 149/150, 301–322
10. Tressler, R. J., Updyke, T. V., Yeatman, T., and Nicolson, G. L. (1993) J. Cell. Biol. 123, 23–35
11. Loecke, H., Chang, B. T., Maillard, W. S., Schlaepfer, D. D., and Haigler, H. T. (1995) Nature 378, 512–515
12. Tressler, R. J., Updyke, T. V., Yeatman, T., and Nicolson, G. L. (1993) J. Cell. Biol. 123, 23–35
13. Chung, C. Y., Murphy-Ullrich, J. E., and Erickson, H. P. (1996) Mol. Biol. Cell 7, 21198–21203
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14. Chung, C. Y., and Erickson, H. P. (1994) *J. Cell Biol.* **126**, 539–548
15. Hubaisby, I., Jones, P. G., Bjorge, J., Bellagamba, C., Fitzpatrick, S., Fujita, D. J., and Waisman, D. M. (1995) *Biochemistry* **34**, 14527–14534
16. Cardin, A. D., and Weintraub, H. J. (1998) *Arterioscler Thromb Vasc Biol* **18**, 21–32
17. Khanna, N. C., Helwig, E. D., Iebuzhi, N. W., Fitzpatrick, S., Bajwa, R., and Waisman, D. M. (1996) *Biochemistry* **35**, 4852–4862
18. Sreerama, N., and Woody, R. W. (1993) *Anal. Biochem.* **209**, 32–44
19. Bitter, T., and Muir, H. M. (1962) *Anal. Biochem.* **4**, 330–334
20. Laemmli, U. K. (1970) *Nature* **227**, 680–685
21. Jones, P. G., Fitzpatrick, S., and Waisman, D. M. (1994) *Biochemistry* **33**, 13751–13760
22. Reed, K. C., and Bygrave, F. L. (1975) *Annu. Rev. Biochem.* **44**, 44–54
23. Shadle, P. J., Gerke, V., and Weber, K. (1985) *J. Biol. Chem.* **260**, 16354–16360
24. Aigner, G. (1996) *Hemostasis* **26**, Suppl. 2, 2–9
25. Jones, P. G., Moore, G. J., and Waisman, D. M. (1992) *J. Biol. Chem.* **267**, 13993–13997
26. Weis, W. I., and Drickamer, K. (1996)*Annu. Rev. Biochem.* **65**, 441–473
27. Woods, A., McCarthy, J. B., Furcht, L. T., and Couchman, J. R. (1993)*Mol. Biol. Cell* **4**, 605–613
28. Guo, N. H., Krutzsch, H. C., Negre, E., Vogel, T., Blake, D. A., and Roberts, D. D. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 3040–3044
29. Guo, N., Krutzsch, H. C., Negre, E., Zabrenetsky, V. S., and Roberts, D. D. (1992) *J. Biol. Chem.* **267**, 19349–19355
30. Cardin, A. D., Demeter, D. A., Weintraub, H. J., and Jackson, R. L. (1991)*Methods Enzymol.* **203**, 556–583
31. Bae, J., Desai, U. R., Pervin, A., Caldwell, E. E., Weiler, J. M., and Linhardt, R. J. (1994) *Biochem. J.* **301**, 121–129
32. Sasekharan, R., Venkataraman, G., Godavarti, R., Ernst, S., Conney, C. L., and Langer, R. (1996) *J. Biol. Chem.* **271**, 3124–3131
33. Ma, Y., Henderson, H. E., Liu, M. S., Zhang, H., Forsythe, I. J., Clarke-Lewis, I., Hayden, M. R., and Brunzell, J. D. (1994) *J. Lipid Res.* **35**, 2049–2059
34. Barkalow, F. A., and Schwarzauer, J. K. (1994) *J. Biol. Chem.* **269**, 3957–3962
35. Persson, B., Bengtsson-Olivecrona, G., Enorback, S., Olivecrona, T., and Jornvall, H. (1989) *Eur. J. Biochem.* **179**, 39–45
36. Booth, B. A., Borse, M., and Drennec, D. L., Dake, B. L., Kiefer, M. C., Maack, C., Linhardt, R. J., Bar, K., Caldwell, E. E., Weiler, J., and Bar, R. S. (1995) *Growth Regul.* **5**, 1–17
37. Wong, P., Hampton, B., Stuhlbruck, E., Gallagher, A. M., Jaye, M., and Burgess, W. H. (1995) *J. Biol. Chem.* **270**, 25865–25871
38. Lindblom, A., Bengtsson-Olivecrona, G., and Fransson, L. A. (1991) *Biochem. J.* **279**, 821–829
39. Matsuzato, K., Sali, A., Ghildyal, N., Karplus, M., and Stevens, R. L. (1995) *J. Biol. Chem.* **270**, 19524–19531
40. Kojima, K., Yamamoto, K., Irimura, T., Osawa, T., Ogawa, H., and Matsumoto, I. (1996) *J. Biol. Chem.* **271**, 7679–7685
41. Mach, H., Volkin, D. B., Burke, C. J., Middaugh, C. R., Linhardt, R. J., Fromm, J. R., Loganathan, D., and Mattson, L. (1990) *Biochemistry* **29**, 5480–5489
42. Lee, M. K., and Lander, A. D. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 2768–2772
43. Kan, M., Wang, F., Xu, J., Crabb, J. W., Hou, J., and McKeenan, W. L. (1993) *Science* **259**, 1918–1921
44. Gitay-Goren, H., Soker, S., Vladavsky, I., and Neufeld, G. (1992) *J. Biol. Chem.* **267**, 6093–6098
45. Gleizes, P. E., Pouiller-Delpre, J., Amalric, F., and Gas, N. (1995) *Eur. J. Cell Biol.* **66**, 47–59
46. Murphy-Ullrich, J. E., Gurussiddappa, S., Frazier, W. A., and Hook, M. (1993) *J. Biol. Chem.* **268**, 26784–26789
47. Gas, G., and Goldfarb, M. (1995) *EMBO J.* **14**, 2183–2190