Lysine residues of annexin VI are responsible for glycosaminoglycan- and phospholipid-binding activities

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Abstract

Annexin VI is known to bind to glycosaminoglycans as well as phospholipids. We investigated the effects of chemical modification of annexin VI lysine residues on glycosaminoglycan-binding by solid phase assays and on phospholipid-binding by an optical biosensor system. As the concentration of the modifying reagent, pyridoxal 5'-phosphate, was increased, the extent of modification increased and the bindings of modified annexin VI to the ligands decreased, suggesting that lysine residues are important for the interactions of annexin VI with both glycosaminoglycans and phospholipids. Quantitatively however, the chemical modification affected the binding to glycosaminoglycans more than to phospholipids.

1. Introduction

Annexin VI is a member of a widely distributed family of proteins that bind phospholipid membranes in a calcium-dependent manner. Annexin VI differs from the other members of the family in its unique structure: eight repetitive 70 amino acid domains instead of the four such repeats found in other annexins. The biological functions of annexin VI have not been elucidated yet, although calcium-dependent associations with phospholipid membranes suggest its involvement in various processes such as membrane-microfilament interaction, sarcoplasmic reticulum calcium channeling, and membrane or vesicular trafficking (e.g., exocytosis, endocytosis) [1]. In molecular terms, annexin VI seems to interact directly with the headgroups of phospholipids through calcium ions. The major mechanism may be ionic interaction of positively charged residues of annexin molecules with negatively charged headgroups of phospholipid [2], but the precise phospholipid-binding sites of annexins remain to be identified.

In addition to these intracellular functions, many extracellular events have been linked to annexins [3-6]. We previously demonstrated that annexin VI binds to glycosaminoglycans (GAGs [1]) such as heparin, heparan

sulfate, and chondroitin sulfate [7]. Several annexins, such as annexin II, II tetramer, IV, and V have also been reported to bind GAG [7-11], suggesting close physiological roles in extracellular spaces via GAG-binding. The interaction of the proteins with GAG chains occurs by positively charged basic amino acids of the proteins and negatively charged sulfate or carboxylate groups of GAGs. Some of these critical basic residues required for GAG-binding have been identified by chemical modification, site-directed mutagenesis, crystallographic analysis, or molecular modeling [12,13]. In another field, the blood coagulation cascade, chemical modification [14,15] and site-directed mutagenesis [16] have identified lysine and arginine residues of antithrombin as essential amino acids in the binding to heparin.

Because the elucidation of the unique GAG- and phospholipid-binding sites for annexin VI will provide a basis for understanding the mechanism of the interactions and thier physiological roles, we investigated, in the present study, the effects of chemical modification of annexin VI lysine residues on bindings to ligands. Further, we discuss differences found between GAG- and phospholipid-binding regions.

2. Materials and methods

2. 1. Materials

Heparin (porcine intestinal mucosa) and pyridoxal 5'-phosphate (PLP) were purchased from Wako Pure Chemicals (Osaka, Japan). Chondroitin sulfate A (shark cartilage, super-special grade) was purchased from Seikagaku Kogyo (Tokyo, Japan). Phospholipids, phosphatidylcholine (PC, bovine brain), phosphatidylethanolamine (PE, egg yolk), phosphatidylinositol (PI, bovine liver) and phosphatidylserine (PS, bovine brain) were purchased from Sigma Chemical (St. Louis, MO). Bovine serum albumin (BSA)-conjugated GAGs were prepared using *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline as described previously [17]. Human annexin VI cDNA was inserted into plasmid pGEX-3X (Amersham Pharmacia Biotech, Uppsala, Sweden), and recombinant proteins were produced as glutathione *S*-transferase-fusion proteins and digested with factor Xa (Boehringer Mannheim, Mannheim, Germany). Anti-human annexin VI monoclonal antibodies were purchased from Transduction Lab. (Lexington, KY).

2. 2. Chemical modification of annexin VI with PLP

Chemical modification of lysine residues of annexin VI was performed by a method modified from Tsubaki *et al.* (1989) [18]. Annexin VI (0.3 mg/ml) was incubated in the dark in a reaction mixture containing 20 mM potassium phosphate (pH 7.4), 0.1 mM EDTA, and the desired amounts of PLP. After 30 min at 25°C, a freshly prepared chilled solution of $NaBH_4$ was added to give a final concentration of fivefold excess of $NaBH_4$ to PLP. The reaction mixture was kept at room temprature for an additional 10 min and then dialyzed at 4°C in

[1]Abbreviations: GAG, glycosaminoglycan; PLP, pyridoxal 5'-phosphate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; BSA, bovine serum albumin; TBS, Tris-buffered saline; PAGE, polyacrylamide gel electrophoresis

the dark against 10 mM Tris-HCl (pH 7.4), and 0.15 M NaCl (TBS). For a control, annexin VI was treated similarly but without PLP. Absorption spectra of annexin VI and modified molecules were recorded in TBS with a spectrophotometer (Ubest-55, JASCO Inc., Tokyo, Japan). After modification with PLP, annexin VI was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) [19]. Protein concentrations were determined with the Micro BCA reagent (Pierce Chemical Co., Rockford, IL) according to the manufacturer's protocol.

2. 3. BSA-conjugated GAG-binding Assay

A microtiter plate was coated with BSA-conjugated heparin or chondroitin sulfate A in TBS at 4°C overnight. The plate was blocked with 3% BSA in TBS for 2 h. A volume of 100 μl of annexin VI or modified annexin VI at various concentrations was added to each well and incubated for 1 h in the presence of 5 mM $CaCl_2$. The amounts of annexin VI bound to BSA-GAG were monitored using anti-human annexin VI monoclonal antibodies, which have nearly identical reactivities to annexin VI and modified annexin VI.

2. 4. Measurement of phospholipid-binding activity by surface plasmon resonance

Interaction of annexin VI with phospholipids was measured by surface plasmon resonance using the Biacore system (BIACORE™ 2000, Biacore AB, Uppsala, Sweden). Phospholipid vesicles composed of PI/PC, PE/PC, and PS/PC in the ratio of 1:1 or PC were prepared by sonication. A hydrophobic chip HPA (Biacore AB) was initially washed with 0.1 mM ß-octyl glucoside. Vesicles of phospholipids in TBS were injected for 30 min at a flow rate of 1 μl/min. The flow was increased to 5 μl/min, and 10 mM NaOH was injected to wash off the unbound phospholipids. The sensor chip had the following phospholipid mixtures immobilized on it: PC (1320 RU), 1:1 ratio of PI/PC (690 RU), PE/PC (1280 RU), or PS/PC (1170 RU). A volume of 40 μl of a 6.25 μg/ml solution of annexin VI in TBS was then injected in the presence of 1 mM $CaCl_2$. The sensor chip was regenerated by injection of 10 μl of 0.5 M EDTA after each measurement cycle.

3. Results and Discussion

3. 1. Chemical modification of annexin VI lysine residues with PLP

One of the initial steps in the identification of substrate- and ligand-binding sites is to selectively chemically modify amino acids. To examine whether or not annexin VI lysine residues are responsible for the GAG- and phospholipid-bindings, annexin VI was chemically modified with PLP, a selective lysine residue reagent. After treatment with PLP followed by $NaBH_4$ reduction, absorption spectra of annexin VI were modified with a new maximum at 325 nm (Fig. 1A), the characteristic peak of PLP lysine substitution. The number of lysine residues substituted was calculated from an extinction coefficient of pyridoxal 5'-phosphate-*N*-lysine residues (9.72 mM^{-1} cm^{-1}) at 325 nm. At the concentration of 1 mM, the PLP treatment modified about 4.4 lysine residues out of 52 available in the annexin VI molecule. When the concentration was 10 mM, about 6.2 residues were modified. On SDS-PAGE analysis, modified annexin VI appeared as a band with a slightly

higher molecular weight than intact annexin VI (Fig. 1B). As the concentration of PLP was increased, the apparent molecular weight on SDS-PAGE of modified annexin VI increased (Fig. 1B).

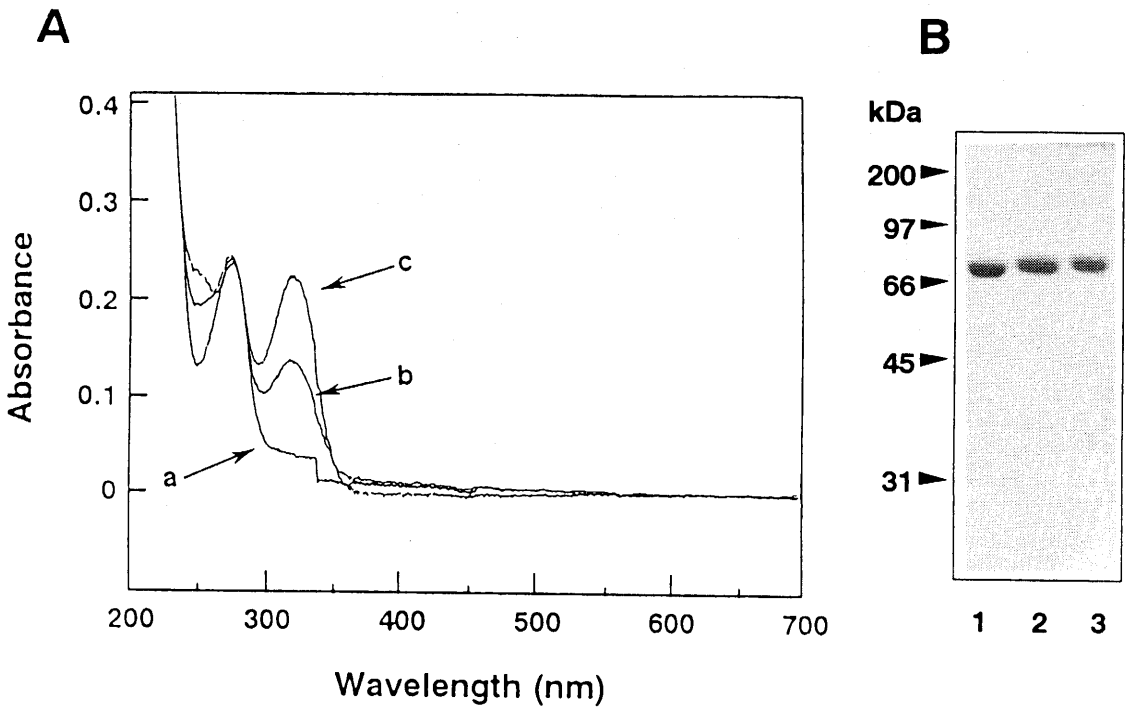

Fig. 1. Absorption spectra and SDS-PAGE analysis of annexin VI modified with PLP. A, Absorption spectra of intact annexin VI (a) and annexin VI modified with PLP at a concentration of 1 mM (b) or 10 mM PLP (c) were recorded in TBS. B, Intact annexin VI (lane 1) and annexin VI treated with PLP at a concentration of 1 mM (lane 2) or 10 mM PLP (lane 3) were analysed on 9.5% SDS-PAGE and stained with Coomassie Brilliant Blue.

3. 2. Effects of the modification of annexin VI lysine residues on the GAG-binding activity

The effects of the modification of annexin VI lysine residues on the GAG-binding activity were estimated by solid phase binding assay using BSA-GAG [7]. Treatment of annexin VI with PLP decreased its heparin-binding activity (Fig. 2A). After treatment with 10 mM PLP solution, the binding of annexin VI to heparin was only about 10% of that observed with intact annexin VI at a concentration of annexin VI where the binding to heparin was saturated (0.39 μg/ml). Even the 1 mM PLP treatment resulted in a reduction of 50% of the activity of intact annexin VI at the same concentration of annexin VI. When chondroitin sulfate was used as a ligand, the modification resulted in an almost complete loss of binding of annexin VI (Fig. 2B). These results indicate that annexin VI lysine residues are responsible for the binding to both heparin and chondroitin sulfate. However, the lysine modification had a more drastic effect on the binding to chondroitin sulfate than to heparin, suggesting a heterogeneity in the role of lysine residues (Fig. 2). When we observed inhibitory effects of soluble GAGs on binding of annexin VI to immobilized GAG-BSA, heparin was found more effective than

chondroitin sulfate (unpublished data). Taken together, these results indicate that annexin VI may have several heparin-binding sites, of which some were modified with PLP under these conditions, but others not.

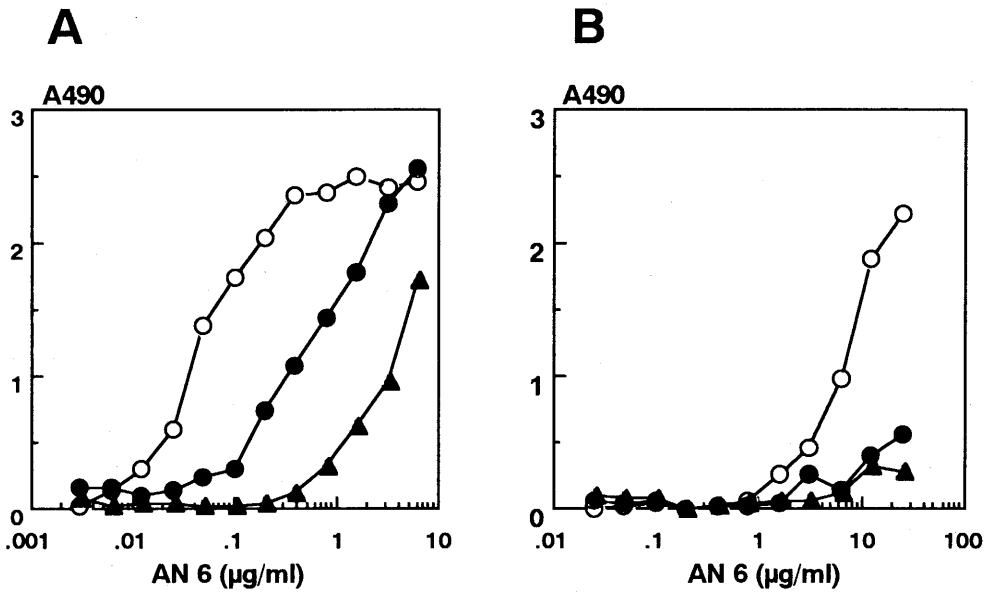

Fig. 2. Effects of the modification of annexin VI on the GAG-binding activiies. Intact annexin VI (open circles) and annexin VI modified with PLP at a concentration of 1 mM (solid circles) or 10 mM PLP (solid triangles) were incubated with (A) BSA-heparin and (B) BSA-chondroitin sulfate A immobilized on plates in the presence of 5 mM $CaCl_2$. Detection of intact annexin VI and PLP-modified annexin VI adsorbed on BSA-GAG was performed by anti-annexin VI antibodies.

Many heparin-binding proteins share consensus sequences rich in basic amino acids, XBBXBX and XBBBXXBX (where B is a basic and X, any other amino acids) [20,21]. Kassam *et al.* showed that annexin II contains a heparin-binding consensus sequence in the repeat 4: ^{306}FKKKYGKS^{314} and actually binds to heparin [10]. Recently, Capila *et al.* (1999) reported kinetics data of the interaction between annexin V and heparin, and speculated that annexin V might have two potential heparin-binding sites in repeat 3 and 4 [11]. Annexin VI does not have these complete consensus motifs but only some basic amino acids in the corresponding regions (repeat 4 and 8), which are located on concave sides facing the cytosol or extracellular milieu (See 3. 2.). Although our results show that annexin VI lysine residues are important for the GAG-binding, the identification of their locations remains to be done. As described later, lysine modification reduced the GAG-binding more than the phospholipid binding (Fig. 3), suggesting that some lysine residues required for the GAG-binding were selectively modified under our conditions. When we preliminarily modified annexin VI both in the absence and presence of heparin, we found that heparin partially protected lysine residues from PLP-modification. More proteolytic peptides, including lysine residues, modified with PLP were obtained from annexin VI modified in

the absence of heparin than in the presence (data not shown), suggesting that selectively modified lysine residues can be identified by amino acid sequence analysis of these peptides. Besides lysine residues, other amino acid residues may also participate in the binding process. The next step should be the identification of the critical specific sequences of annexin VI with the lysine residues required for the GAG-binding.

3. 3. Effects of the modification of annexin VI lysine residues on the phospholipid-binding activity

Annexin VI has been reported to bind to phospholipid vesicles containing PS, PE, or PI, but not to those containing only PC, in a calcium-dependent manner [1]. In this study, we investigated the effects of the modification of annexin VI lysine residues on the binding activity to PS, PE, or PI by measurement of surface plasmon resonance using the Biacore system. We did not observe any binding of annexin VI to single-immobilized PC on the sensor chip (data not shown) so we assumed that the bindings of annexin VI to each vesicle came from vesicle components other than PC. As shown in Fig. 3, annexin VI was found to bind clearly to the three kinds of phospholipids, and the modification of lysine residues decreased the bindings in relation to the concentrations of PLP used. The extent of the decrease of the binding was related to the kind of phospholipid, although the factors involved could not be identified. Residual phospholipid-binding activity of each PLP-modified annexin VI was calculated from the RU values corresponding to the amounts of annexin VI bound to phospoholipids, with 100% for the intact annexin VI (Table I). The bindings of modified annexin VI to PE and PS were estimated to be 80 ~ 90% those of intact annexin VI. In the case of binding to PI, annexin VI modified by 1 mM PLP and 10 mM PLP had residual binding activity of 46% and 73%, respectively. However, the effects of the modification on phospholipid-binding were lower than on GAG-binding (Fig. 2). These results indicate that annexin VI lysine residues partially contribute to the phospholipid binding and that other amino acids may contribute more to the binding.

Annexins are composed of four (or eight in the case of annexin VI) homologous repeats containing a region of increasing homology called the "endonexin fold" with a characteristic GXGTDE motif. Each repeating unit consists of five α-helices, and the four units are arranged in an almost planar cyclic array. The annexin molecules have an overall flat, slightly curved shape with a convex and a concave side. The convex side faces the biological membrane and contains the calcium ion- and phospholipid-binding sites [22]. Crystallographic analysis of annexin VI reveals that the molecule consists of two four-repeating lobes, closely resembling those of other annexin molecules [23,24]. Considering that annexin VI seems to interact, via positively charged sites, with the headgroups of the phospholipids [2], the lysine residues modified with PLP should exist in the consensus sequences of the repeating units. A recent study has however revealed that the binding of annexin I to phospholipids induces the formation of a second phospholipid binding site, which may exist on the concave side of the protein [25]. Like annexin I, annexin VI may also have phospholipid binding regions on the concave side, and the identification of the modified lysine residues may give information on phospholipid-binding sites of annexin VI.

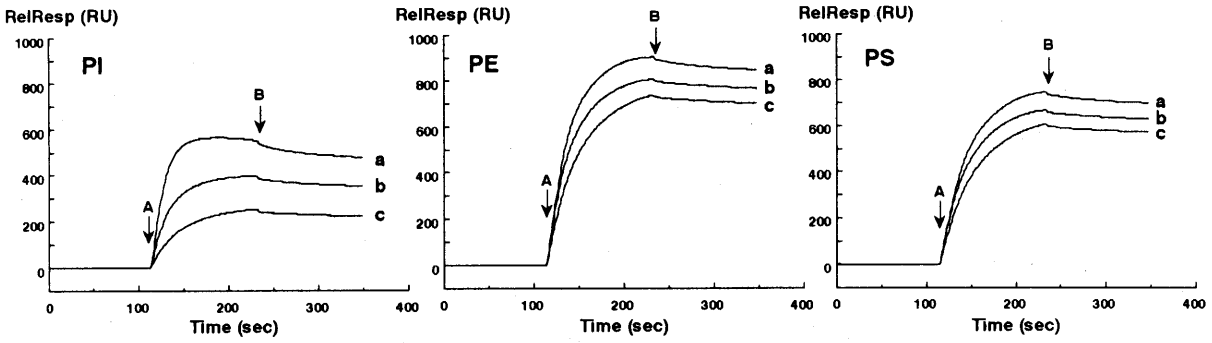

Fig. 3. Effects of the modification of annexin VI on the phospholipid-binding activities. PI/PC, PE/PC, and PS/PC were immobilized on the HPA chip. Intact annexin VI (a) and annexin VI modified with PLP of a concentration of either 1 mM (b) or 10 mM PLP (c) were used as the soluble ligand at a concentration of 6.25 μg/ml. Arrows indicate the beginning (A) and the end (B) of the application of the soluble ligand.

Table I Phospholipid-binding activities of annexin VI and PLP-modified annexin VI

Concentration of PLP	% residual phospholipid-binding activity *		
	PI	PE	PS
0 (intact annexin VI)	100	100	100
1 mM	73	90	90
10 mM	46	82	81

*Phospholipid-binding activity of each PLP-modified annexin VI was calculated from RU values 20 seconds after the end of the application of the soluble ligand, with 100% for intact annexin VI.

3. 4. Relationship between two kinds of ligand-binding sites on annexin VI

The results of the experiments presented here show that annexin VI lysine residues are responsible, to different degrees, for the binding to both GAGs and phospholipids. However, it is not clear whether lysine residues essential to GAG binding are identical to those essential to phospholipid binding. It has been previously shown that binding of annexin IV [9] and annexin II tetramer [10] to phospholipid vesicles is not

affected by the presence of heparin, suggesting that these annexins have different binding sites for these two kinds of ligands, namely GAGs and phospholipids. Similarly, binding of annexin VI to phospholipids was not blocked by GAGs (data not shown). However, it is not clear whether the same molecule of annexin VI can simultaneously bind to GAG and phospholipid because annexin VI has been described as self-associating on the membrane surface [26]. Further studies, including a protected chemical modification by each ligand, are required to determine whether GAG- and phospholipid-binding regions are different from each other.

The crystallographic analysis of annexin VI shows that both half molecules are arranged perpendicular to each other connected by an α-helical segment and reorient flexibly relative to each other by about 90° upon membrane binding [23]. Furthermore, other conformational changes of annexin VI may also be induced upon its binding to phospholipid membranes, as shown for other annexins. For example, because of conformational changes, calcium-dependent binding of annexin VI to phospholipids (PS) partially protects the protein against proteolysis [27]. It is known that upon binding to phospholipid membranes annexins undergo conformational changes that alter their phospholipid-binding properties [26] and proteolysis sensibilities [27]. Similarly, the binding of annexin VI to phospholipids may influence GAG-binding activities and may result in the formation of new GAG-binding sites. Therefore, all these factors must be considered in the discussion on the relationship between the GAG- and phospholipid-binding regions of annexin VI.

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