Characterization of Carbohydrate-binding Protein p33/41

RELATION WITH ANNEXIN IV, MOLECULAR BASIS OF THE DOUBLET FORMS (p33 AND p41), AND MODULATION OF THE CARBOHYDRATE BINDING ACTIVITY BY PHOSPHOLIPIDS

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Kyoko Kojima‡§, Kazuo Yamamoto¶, Tatsuro Irimura‡, Toshiaki Osawa‡, Haruko Ogawa‡, and Isamu Matsumoto‡

From the ‡Department of Chemistry, Faculty of Science, Ochanomizu University, Otsuka, Bunkyo-ku, Tokyo 112, Japan, and the ¶Division of Cancer Biology and Molecular Immunology, Faculty of Pharmaceutical Sciences, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan

A protein, p33/41, expressed in bovine kidney and many other tissues was identified as a lectin which binds to sialoglycoproteins and glycosaminoglycans in a calcium-dependent manner. Partial amino acid sequences of p33/41 are highly homologous to those of a calcium/phospholipid-binding annexin protein, annexin IV (endonexin). p33/41 exhibited similar calcium-dependent phospholipid binding activity (Kojima, K., Ogawa, H., Seno, N., Yamamoto, K., Irimura, T., Osawa, T., and Matsumoto, I. (1992) J. Biol. Chem. 267, 20536–20539). To further characterize p33/41, we cloned the p33/41 cDNA and characterized the recombinant protein encoded by this cDNA. Oligonucleotide probes were synthesized based on partial amino acid sequences of p33/41 and used for screening. A p33/41 cDNA clone was isolated encoding a protein of 319 amino acids with a calculated molecular mass of 35,769 Da. The deduced amino acid sequence was identical to that of bovine annexin IV except for one amino acid substitution. The recombinant protein gave two 33-kDa (p33) and 41-kDa (p41) bands on SDS-polyacrylamide gel electrophoresis under non-reducing conditions, and only one 33-kDa band under reducing conditions, as did the native protein. Mass spectrometric analysis combined with site-directed mutagenesis of each of the four cysteine residues of the recombinant protein revealed that p41 is a dimer of p33 cross-linked at Cys-198 via a disulfide bond. The recombinant protein bound to columns of heparin and fetuin cross-linked at Cys-198 via a disulfide bond. The recombinant protein revealed that p41 is a dimer of p33.

Various proteins have been found to interact with specific carbohydrate structures on selected glycoconjugates to perform their physiological functions. Many carbohydrate-binding proteins (lectins) have been identified in various contexts, both as a result of direct searches for proteins with selective carbohydrate binding activity, and in the course of investigations on biological recognition processes. In many cases, the physiological roles of lectins have been ascribed to biological recognition events. The list of lectin-mediated processes includes diverse biological phenomena, such as protein clearance from circulating blood, phagocytosis, intracellular routing of glycoproteins, and cell to cell adhesion (1–6).

We have purified a calcium-dependent carbohydrate-binding protein, p33/41, from bovine kidney extracts by successive affinity chromatography on fetuin- and heparin-Sepharose (7). Upon SDS-polyacrylamide gel electrophoresis (SDS-PAGE),1 the protein gave unique doublet bands corresponding to 33-kDa (p33) and 41-kDa (p41) proteins under non-reducing conditions. Under reducing conditions, the 41-kDa protein disappeared and only the 33-kDa protein was detected, suggesting that p41 is produced from p33. Therefore, the protein was termed, tentatively, p33/41. Immunoblot analysis of bovine tissue extracts revealed that p33/41 is expressed not only in the kidney but also in the liver, pancreas, and several other tissues (8). Immunohistochemical study of bovine kidney specimen showed that p33/41 is highly concentrated in the apical plasma membrane of the epithelial cells in the renal proximal tubules, and the results of sequential extraction with EDTA and Triton X-100 and phase transfer analysis with Triton X-114 suggested that p33/41 is integrated into the renal brush border membrane (9). To obtain information on its primary structure, we determined the amino acid sequence of the peptides derived by proteolysis of p33/41. The results demonstrate that partial amino acid sequences of p33/41 are strikingly homologous to those of annexin IV (8), one of the annexin family of proteins.

The annexins, which bind to phospholipids and membranes in the presence of calcium, comprise some 13 members expressed in diverse organisms such as higher plants, slime molds, metazoans, insects, birds, and mammals. Annexins are amphipathic and distinct from soluble and integral membrane proteins, but share features of both (9–11). These proteins consist of four or eight conserved repeating units of 70–80...
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amino acids, 17 of which are conserved in each unit. In contrast, the N-terminal segment of the annexin family of proteins exhibits the greatest variation in sequence and length. These proteins are claimed to have a wide range of biological functions related to their phospholipid/membrane-binding properties (12). They are anti-inflammatory proteins that inhibit phospholipase A2 activity in vitro by sequestering the substrate phospholipids from phospholipase A2 (13). Furthermore, the annexins exhibit anti-coagulant activity (14), calcium channel activity (15), and cyclic phosphate phosphohydrolase activity (16). They function in the membrane fusion process, exocytosis (17), endocytosis, membrane-cytoskeleton interactions (18), and regulation of calcium-dependent anion current activation (19).

In addition to its amino acid sequence homology to annexin IV, p33/41 exhibited calcium-dependent phospholipid binding activity. These two characteristics alone suggest that the two proteins p33/41 and annexin IV might be related or identical; however, their relationship has remained uncertain. For example, although most of the partial amino acid sequences obtained from p33/41 corresponded well to those of annexin IV, several did not. Annexin IV is a 32-kDa protein, and there has been no description of it as a doublet protein corresponding to 33 and 41 kDa. Annexin IV has not been identified as a lectin, and its biological functions have not been reported to be based on carbohydrate-binding properties. Although p33/41 can interact with two different types of ligands, carbohydrate and phospholipid, the influence of binding to one ligand on its affinity for the other ligand remains unclear. In the present study, we cloned a p33/41 cDNA by screening with oligonucleotide probes corresponding to partial amino acid sequences of p33/41. This cDNA was used to produce a recombinant protein in *Escherichia coli* that was characterized to obtain information on the carbohydrate and phospholipid binding properties of p33/41.

**EXPERIMENTAL PROCEDURES**

**Materials**—Phospholipids, phosphatidylcholine (PC, bovine brain), phosphatidylethanolamine (PE, egg yolk), and phosphatidylinositol (PI, bovine liver), and calf fetuin were purchased from Sigma. M13mp18, 5-bromo-4-chloro-3-indoyl-β-D-galactoside, the bacterial strains, *E. coli* JM109 and HB101, restriction enzymes, and T4 polynucleotide kinase were purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan). The expression vector pGEX-3X and isopropyl-β-D-thiogalactopyranoside (IPTG) were purchased from Pharmacia (Uppsala, Sweden); Glutathione and proteinase factor Xa (from *Bacillus subtilis*) were purchased from Boehringer Mannheim (Mannheim, Germany). Taq polymerase was from Perkin-Elmer. Horse radish peroxidase (HRP)-labeled heparin was prepared by the method described previously (7). Asialofetuin was prepared from fetuin by hydrolysis with 25 mM HSO₄ at 80 °C for 1 h. Affinity gels coupled with heparin, fetuin glycopeptide, asialofetuin glycopeptides, or glutathione were prepared by use of epoxy-activated Sepharose 4B (20–22). Bovine p33/41 was carried into the *E. coli* HB101. The recombinant fusion protein was induced by adding IPTG and purified on glutathione-Sepharose column. After digestion with proteinase factor Xa at a specific cleavage site, the recombinant protein was separated from glutathione-Sepharose column by use of epoxy-activated Sepharose 4B (20–22). Bovine p33/41 was used as the template for amplification of mutated partial cDNAs, and primer C was used as the downstream primer. Upstream primers for three mutants (C108S, C198S, and C242L) were designed to introduce the mutations.

**Cloning of Bovine p33/41 cDNA and Production of Recombinant Protein**—A bovine liver cDNA library in cloner vector Agt10 (Clontech, Palo Alto, CA) was screened with a 32P-labeled oligonucleotide probes (5'-CA/TG/CT/CA/CT/GA/AG/TG/TA-3', 5'-GG/CA/CA/CT/GA/CT/CG/CG/AG/CT-3', and 5'-GA/CT/GA/CT/TG/AG/CT/CG/AG/CT/CG/3'), which were synthesized based on partial amino acid sequences of p33/41. A cDNA clone that contained a 5'-truncated sequence of bovine p33/41 was isolated. This clone was used to synthesize mixed probes for rescreening with a multiprime DNA labeling system (Amersham Int. plc, Buckinghamshire, United Kingdom) involving α-32PdCTP. After plaque purification, the resulting positive clones were analyzed by Southern blotting. The cDNA inserts of five positive clones were subcloned into the M13mp18 vector at the EcoRI site for sequencing on both strands.

The coding region of the cDNA subcloned into M13mp18 was amplified by the polymerase chain reaction (PCR) using an upstream primer (primer N: 5'-GG/GA/AT/GC/GA/CC/GG/AG/AG/GG/3’) and a downstream primer (primer E: 5'-TC/CT/GA/GT/GA/AT/GG/TA/TA/CT-3’) containing *BanHI* and EcoRI restriction sites, respectively. The PCR-generated DNA was digested with EcoRI and *BanHI*, and the product was ligated into the *BanHI* and *EcoRI* sites of plasmid pGEX-3X and then used to transform *E. coli* HB101. The recombinant fusion protein produced in *E. coli* was induced by adding IPTG and purified on glutathione-Sepharose. After digestion with proteinase factor Xa at a specific cleavage site, the recombinant protein was separated from gluthathione-S-transferase and obtained in the flow-through fraction from the gluthathione-Sepharose column.

**Site-directed Mutagenesis**—The codons for Cys108, Cys198, Cys242, and Cys315 were altered to encode Ser, Ser, Leu, and Ser, respectively. The p33/41 cDNA subcloned into M13mp18 was used as the template for amplification of mutated partial cDNAs, and primer C was used as the downstream primer. Upstream primers for three mutants (C108S, C198S, and C242L) were designed to introduce the mutations.

Silent changes were introduced into each primer in order to generate or delete restriction sites to facilitate identification of the mutant introduced. The sense of individual primer pairs, 657-, 657-, and 256-base pair fragments corresponding to C108S, C198S, and C242L mutants, respectively, were amplified by PCR from the p33/41 cDNA. After chloroform-phenol extraction and ethanol precipitation, the reaction products were used as downstream primers for the next PCR. An amplification was performed from the bovine p33/41 cDNA subcloned into pGEX-3X using primer N as the upstream primer. Mutagenized partial cDNA for the C315S mutant was amplified from the p33/41 cDNA subcloned into M13mp18 using of primer N as the upstream primer and the following downstream primer.

**Effect on Heparin**—The recombinant proteins of p33/41, wild type and the C198S mutant, were dissolved in H₂O, 0.5% 2-mercaptoethanol or 10 mM dithiothreitol (approximately 100 μg/ml) and were mixed with a matrix solution (3.5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid, 0.1 M in methanol/acetonitrile = 1:1)) in the ratio of 1 to 9. The sample solution (1 μl) was applied onto the sample slide and dried in a vacuum. Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry was performed with a Kratos Compact MALDI-III mass spectrometer (Shimadzu, Co., Kyoto, Japan).

**Carbohydrate-binding Assay**—Calcium-dependent carbohydrate binding activity was assayed by affinity column chromatography. The recombinant p33/41 (rec-p33/41) was applied to a fetuin glycopeptide-, asialofetuin glycopeptidase-, or heparin-Sepharose column (inner diameter, 5 × 120 mm) in the presence of 5 mM CaCl₂, and the bound protein was eluted with 2 mM EDTA. The amount of rec-p33/41 in each fraction was monitored by ELISA using rabbit polyclonal anti-p33/41 antibodies, HRP-conjugated anti-rabbit IgG antibodies, and o-phenylene diamine (OPD).

**Effect of Heparin on the Binding of rec-p33/41 to Phospholipids**—PE and PC (1:1), PI and PC (1:1), or PS and PC (1:1) were suspended at 1
mg/ml in TBS containing 5 mM CaCl₂ and then sonicated for 15 min to prepare phospholipid vesicles. Solutions (50 μl) containing various amounts of p33/41 and 50 μl solutions containing phospholipid vesicles were incubated for 30 min in the presence or absence of heparin and then centrifuged at 15,000 × g for 5 min. The supernatants were discarded, and the pellets were resuspended in 100 μl of TBS containing 5 mM CaCl₂, mixed with added HRP-labeled heparin (0.2 μg/ml protein concentration) for 30 min, and then centrifuged. The pellets thus obtained were rinsed with TBS containing 5 mM CaCl₂ and the mixtures were centrifuged. Then, 200 μl of TBS containing 2.5 mM EDTA was added to each pellet followed by centrifugation. Each supernatant was transferred to the wells of a microtiter plate, and the amount of HRP-heparin released from p33/41 was monitored by ELISA.

RESULTS

cDNA Cloning of p33/41—In a previous study, we determined the amino acid sequences of 14 peptides derived from proteolysis of p33/41. Eleven of the 14 sequences corresponded to those of annexin IV, whereas three did not. Immunoblot analysis of bovine tissue extracts with polyclonal anti-p33/41 antibodies showed that p33/41 is abundantly expressed in the kidney, pancreas, and intestine (9). Therefore, we synthesized oligonucleotide probes based on partial amino acid sequences, some corresponding to, and others not corresponding to, those of annexin IV. These oligonucleotides were used to screen a bovine liver Agt10 cDNA library. After plaque purification, five positive clones were obtained, which were subcloned into the M13mp18 vector at the EcoRI site and sequenced on both strands. Three of the five positive clones were identical to each other and coded a full-length open reading frame, and the other two clones were 5'-truncated within the coding region. The nucleotide sequence and deduced amino acid sequence are shown in Fig. 1. The nucleotide sequence contains an open reading frame encoding a protein of 319 amino acids. The deduced amino acid sequence is identical to that deduced from the cDNA of bovine annexin IV (endonexin) (24) with the exception of one amino acid residue, i.e. residue 94 is Val in the cDNA sequence of p33/41, while it is Leu in that of bovine annexin IV. The N-terminal region does not appear to contain a hydrophobic signal sequence. The sequence includes two potential N-glycosylation sites (Asn-X-Ser/Thr). The mature polypeptide without the initial methionine has a calculated molecular mass of 35,769 Da, with a pI of 5.86.

The cDNA was subcloned into the E. coli expression vector pGEX-3X, and a recombinant fusion protein with glutathione S-transferase (26 kDa) was prepared. It was necessary to add EDTA to the buffer for sonication of E. coli cells to solubilize the recombinant fusion protein. Approximately 2 mg of the recombinant protein was purified from 1 liter of culture by column chromatography on glutathione-Sepharose. The fusion protein gave two protein bands, corresponding to 57- and 62-kDa proteins, on SDS-PAGE under non-reducing conditions and one band, corresponding to a 57-kDa protein, under reducing conditions (data not shown). After digestion of the fusion protein with proteinase factor Xa at a specific cleavage site, the recombinant protein was purified by a second chromatography on glutathione-Sepharose. Because of the location of the cleavage site for factor Xa and the introduction of a BamHI site, three extra amino acids (Gly-Ile-Pro) were present at the N terminus of the recombinant protein. Actually, the N-terminal amino acid sequence of the recombinant protein was determined to be Gly-Ile-Pro-Ala-Ala-Lys-Gly-Gly-Thr-Val-Lys-Ala-Ala-Ser-Gly-Phe-Asn-Ser/Thr. The mature polypeptide without the initial methionine has a calculated molecular mass of 35,769 Da, with a pI of 5.86.

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The results of SDS-PAGE analysis are shown in Fig. 2. Under non-reducing conditions, the recombinant protein also gave two protein bands with apparent molecular masses of 33 and 41 kDa, whereas only one protein band of 33 kDa was observed under reducing conditions. These results are in good agreement with those reported for native p33/41 and demonstrate that the 33- and 41-kDa proteins were both produced from a single cDNA of the isolated clone. When the recombinant protein was electrophoretically transferred to a PVDF membrane after SDS-PAGE and then probed with polyclonal anti-p33/41 antibodies, both the 33- and 41-kDa bands were observed under reducing conditions. These results are in good agreement with those reported for native p33/41 and demonstrate that the 33- and 41-kDa proteins were both produced from a single cDNA of the isolated clone. When the recombinant protein was electrophoretically transferred to a PVDF membrane after SDS-PAGE and then probed with polyclonal anti-p33/41 antibodies, both the 33- and 41-kDa bands were observed under reducing conditions. These results are in good agreement with those reported for native p33/41 and demonstrate that the 33- and 41-kDa proteins were both produced from a single cDNA of the isolated clone.
was, thus, also supported immunologically.

Native p33/41 was initially purified from a bovine kidney extract by calcium-dependent two-step affinity chromatography on fetuin-Sepharose and heparin-Sepharose. As shown in Fig. 3, the recombinant protein bound to fetuin glycopeptide-Sepharose as well as to heparin-Sepharose in the presence of 5 mM CaCl₂ and was eluted by chelating the calcium ions with 2 mM EDTA. When chromatography was performed in the presence of 5 mM EDTA, the recombinant protein did not bind either fetuin glycopeptide-Sepharose or heparin-Sepharose, indicating that the binding is calcium-dependent. When the recombinant protein was applied to an asialofetuin glycopeptide-Sepharose column, the protein was not retained on the column but was recovered in the flow-through fraction, suggesting that terminal sialic acids are required for the binding to fetuin glycopeptides. In addition, the recombinant protein was confirmed to be capable of binding to phospholipids in the presence of calcium (mentioned in detail below). Taken together, our data demonstrated that the recombinant protein produced from isolated cDNA possesses all the characteristics of p33/41; therefore, we concluded that the cDNA encodes p33/41.

The p41 Form Is a Dimer of p33 Cross-linked via a Disulfide Bond at Cys-198—To elucidate the molecular basis of the doublet forms of p33 and p41, mutational analyses of cysteine residues on p33/41 were performed. As shown in Fig. 1, p33/41 contains four cysteine residues, at positions 108, 198, 242, and 315. Therefore, four mutant proteins of rec-p33/41 were designed, in which each of the four cysteine residues was substituted with a serine or leucine residue. The mutant proteins thus prepared (C108S, C242L, C198S, and C315S) were subjected to SDS-PAGE and subsequent immunoblotting. As shown in Fig. 4, C108S, C242L, and C315S gave both the p33 and p41 forms, whereas C198S gave only the p33 form. Next, the wild type p33/41 and the cysteine mutant C198S were subjected to analysis by MALDI-TOF mass spectrometry. As shown in Fig. 5, two peaks corresponding to molecular ions of 36,074 ± 72 and 72,200 ± 144 Da were observed for the wild type p33/41 under non-reducing conditions and one peak corresponding to 36,104 ± 72 Da for the cysteine mutant C198S. The calculated molecular weight of the wild type p33/41 is 36,010, indicating that the p33 form is a monomer with a molecular mass of 36 kDa, and the p41 form is a homodimer with a molecular mass of 72 kDa. We also attempted MALDI-TOF mass analysis on the recombinant proteins under reducing conditions; however, no molecular ion derived from both of the recombinant proteins was observed in the presence of reducing agents (0.5% 2-mercaptoethanol or 10 mM dithiothreitol), suggesting that reducing agents inhibit ionization of the protein. Site-directed mutagenesis of the each of four cysteine residues of p33/41 combined with mass spectrometric analysis revealed that the p41 form is a homodimer having a single disulfide bond between the Cys-198 residues of two p33 monomers. The reason why the molecular weight of the homodimer estimated by SDS-PAGE is anomalously smaller (41 kDa) than the actual value (72 kDa), which was confirmed by MALDI-TOF mass spectrometry, may be that the disulfide bond is located close to the middle of the polypeptide chain of the monomer, making the overall length of the dimer molecule shorter than that of a single stretched polypeptide molecule with an equivalent molecular weight.

Heparin Does Not Compete with Phospholipids in the Binding to p33/41—In a previous study, we demonstrated that p33/41 exhibits binding activity toward two different types of ligands, i.e., p33/41 binds, on the one hand, to sialoglycoprotein...
and glycosaminoglycans, and on the other hand, to phospholipids. This suggests that p33/41 possesses recognition sites for both carbohydrates and phospholipids. To obtain information on the ligand binding of p33/41, we investigated the competitive inhibitory effect of heparin on the binding of rec-p33/41 to phospholipid vesicles.

Recombinant p33/41 was incubated with phospholipid vesicles and then centrifuged. Recombinant p33/41 co-precipitated with the phospholipid vesicles was released up on the addition of EDTA, and the released protein was immobilized on a plastic plate and detected by ELISA. As shown in Fig. 6, rec-p33/41 bound to phospholipid vesicles composed of PE/PC, PI/PC, and PS/PC in dose-dependent manner, with similar high-maximal concentrations of around 10 μg/ml. Even high concentrations of heparin (1 mg/ml) did not influence the binding, suggesting that heparin does not compete with phospholipid at its binding sites on rec-p33/41.

Phospholipid Modulates the Carbohydrate Binding Activity of p33/41—Next, the carbohydrate binding ability of rec-p33/41 associated with phospholipids was examined by means of binding assay using HRP-labeled heparin. Recombinant p33/41 associated with phospholipid vesicles was allowed to react with HRP-heparin in the presence of calcium. Then, HRP-heparin bound to rec-p33/41 was coprecipitated with phospholipid vesicles by centrifugation. The coprecipitated HRP-heparin was released up on the addition of EDTA. After centrifugation, the amount of HRP-heparin in the supernatant was monitored by a colorimetric reaction using OPD and H₂O₂. A control value obtained without the recombinant protein was used to subtract nonspecific binding. As shown in Fig. 7, the binding of rec-p33/41 to heparin was influenced by binding to phospholipid vesicles, and the degree varied with the phospholipid species. Among the vesicles tested, the strongest binding was observed with PE/PC vesicles, and the weaker with PI/PC or PS/PC vesicles. These results suggested that the carbohydrate binding ability of p33/41 is modulated by phospholipids.

DISCUSSION

In a previous study, we determined the amino acid sequence of 14 peptides derived by proteolysis of p33/41 (8). Eleven of the 14 sequences corresponded to those of annexin IV, whereas three peptide sequences did not. This striking sequence similarity between p33/41 and annexin IV suggested that the two proteins might be identical. Alternatively, p33/41 might consist of a segment of the annexin IV polypeptide and some additional peptide fragments. To prove the identity of p33/41, we isolated cDNA clones for p33/41 and characterized the resultant recombinant protein by biochemical analyses. Based on the results described herein, we concluded that p33/41 is identical to annexin IV. We screened a bovine liver cDNA library with oligonucleotide probes corresponding to the partial amino acid sequences of p33/41. Five positive cDNA clones, having different lengths but identical sequences, were obtained during the library screening. The amino acid sequence deduced from the cloned cDNA is identical to that deduced from bovine annexin IV cDNA, except for one amino acid substitution. As was observed for the p33/41 purified from bovine tissues, the recombinant protein produced from the cloned cDNA showed two forms, p33 and p41, on SDS-PAGE under non-reducing conditions. The recombinant protein exhibited both carbohydrate- and phospholipid binding activities in the presence of calcium, indicating that functional domains essential for the expression of binding activities toward two different types of ligands reside within the amino acid sequence deduced from the cloned cDNA. The inconsistency of the three non-corresponding sequences we reported before might be explained by the possibility that other proteins or peptides were present as contaminants during the protein or peptide purification steps.

Annexin IV (also called endonexin, protein II, chromobindin 4, placental anticoagulant protein II, and PP4-X) is one of a family of proteins that interact with phospholipids in the presence of calcium. In this study, we found that annexin IV has the additional unique property of recognizing carbohydrates. Calcium-dependent phospholipid binding activities are common and characteristic properties of the annexin family proteins. Phospholipids are suggested to bind via hydrophilic head groups to annexins, and the phospholipid-binding region is proposed to be localized on the convex surface side where calcium-binding sites are located in the crystal structure of annexin V (25). It is noteworthy that the carbohydrate binding...
activity of p33/41 is modulated by association with phospholipid vesicles; p33/41 associated with PE/PC vesicles is capable of responding to carbohydrate ligands whereas association with PI/PC or PS/PC vesicles decreased the carbohydrate binding activity. Proteins of the annexin family bind indiscriminately to every membrane fraction in cell-free systems, whereas, in intact cells, these proteins are localized along a specific domain of the plasma membrane and are associated with a restricted compartment. The limiting membrane may serve as a site at which p33/41 plays a functional role by expressing its carbohydrate binding activity.

Animal lectins have been classified into groups based on shared sequence characteristics. While the overall structure of lectins varies widely, their carbohydrate binding activity is ascribed to a limited portion of a given lectin. This active segment is designated as the carbohydrate recognition domain (CRD), and the CRDs in each group share a pattern of invariant and highly conserved amino acid residues. Three major groups of animal lectins, the C, S, and P-types, contain CRDs with a distinct CRD motif. The lectins in each group share certain functional properties beyond their amino acid sequence similarity. C-type lectins require calcium ions for expression of their carbohydrate binding activity and bind to a diversity of carbohydrates. S-type lectins (galectins) are all β-galactoside-specific and often depend on reducing agents for their full activity. P-type lectins recognize mannose 6-phosphate and function in intracellular targeting of lysosomal enzymes (26). p33/41 does not contain any of the consensus sequences conserved in the CRDs of any animal lectins known so far. Furthermore, consensus heparin-binding motifs (such as BXB-XBB) (27), previously identified glycosaminoglycan-binding sequences, or notably basic amino acid cluster regions capable of binding to acidic polysaccharides through simple ionic interaction were not observed within the primary sequence of p33/41, suggesting that p33/41 may contain a unique CRD. Mapping of the amino acid residues important for carbohydrate recognition is one of the ways to understand the molecular basis of the carbohydrate binding ability of p33/41.

In a series of experiments, the carbohydrate binding activity of p33/41 was examined with fetuin and heparin as carbohydrate ligands, because the detailed carbohydrate binding specificity remains uncertain. Our previous study showed that the binding of p33/41 to fetuin was inhibited by thyroglobulin, which has triantennary N-linked complex-type sialooligosaccharides, but not by transferrin, which only has biantennary N-linked complex-type sialooligosaccharides (7). Mucin-type glycoproteins having large amounts of terminal sialic acid residues such as bovine submaxillary mucin, however, did not inhibit the binding of p33/41 to fetuin (7). These results suggest that p33/41 preferentially recognizes the sialyl residues on triantennary N-linked complex-type sialooligosaccharides of glycoproteins. Since p33/41 exhibits significant binding activity toward heparin or heparan sulfate, it may function as a receptor for some proteoglycans. The next problems to be solved are to determine the biological processes in which p33/41 participates and the endogenous carbohydrate ligands for p33/41 under physiological conditions.

Because most carbohydrate chains on glycoconjugates are localized on the outer surface of plasma membrane or on the outside of cells rather than in the cytoplasm, p33/41, annexin IV, have to be expressed on the cell surface or secreted extracellularly to interact with carbohydrate ligands. Previously,
annexins had been considered to be primarily intracellular proteins and to be neither secreted from cells nor expressed on the cell surface, as predicted from the lack of hydrophobic N-terminal signal sequences in their cDNA- or amino acid sequences. Therefore, studies on their physiological functions have been mainly focused on intracellular events, such as endocytosis, exocytosis, and intracellular trafficking. However, recent studies have shown that annexins are actually exported from cells and expressed on the cell surface. Annexin I is selectively secreted by the ductal epithelium of the human prostate (28) and by inflammatory cells (29), and the export and expression of annexin II have been suggested in colon adenocarcinoma cells (30), large-cell lymphoma cells (31), chondrocytes (32), and endothelial cells (33). Annexin V, which was also identified as a collagen-binding protein, anchorin CII, was shown to be secreted and present in plasma, amniotic fluid, and post-culture medium (34). There is much emerging evidence that both annexin I and annexin V can bind to many types of cells (35–40), and the identification of annexins as new receptors of tissue plasminogen activator in human endothelial cells (41) and of influenza virus in cultured cells (42) implies functional expression of annexins on the cell surface. With regard to annexin IV, there is conflicting evidence with different cells. It was reported that annexin IV is synthesized in human prostate gland cells but not secreted by the cells (28), while it is expressed on the external surfaces of human and rodent tumor cells (30, 31). The specific extracellular transport and expression mechanisms of annexins have not yet been defined.

The results of this study revealed that p33 is a monomer of annexin IV, and p41 is a homodimer cross-linked via a single disulfide bond at Cys-198. Annexin II interacts with p11 protein via the short stretch of 12 amino acids situated at the N terminus and forms a heterotetramer composed of two annexin II and two p11 protein molecules (43). Annexin V is known to give doublet bands exhibiting an apparent difference of 4 kDa on SDS-PAGE. Unlike annexin IV, the annexin V isoforms are not sensitive to reducing agents and occur as a result of only two amino acid substitutions between the isoforms (44). This may be the first report of the formation of a covalently linked homodimer among annexins. Cysteine 198 is relatively conserved in annexins, and three of four cysteines (198, 242, and 315) in annexin IV are conserved in annexin III. Chen et al. (45) reported three-dimensional models of annexins (I, II, III, and VII) constructed by homology modeling using the crystal structure of annexin V as a template, and showed that cysteines 198, 242, and 315 in the annexin III model do not appear to be exposed on the protein surface. A conformational change to expose cysteine 198 to the outside and to allow it to form an intermolecular disulfide bridge appears to occur upon dimer formation of annexin IV.

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