Carbohydrate-binding Protein 35 (Mac-2), a Laminin-binding Lectin, Forms Functional Dimers Using Cysteine 186*

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Carbohydrate-binding protein 35 (CBP35), also known as the macrophage surface antigen Mac-2, is a lactosamine-specific lectin whose extracellular properties include the ability to agglutinate cells and to bind avidly to the basement membrane glycoprotein laminin. Although these and other properties would be facilitated by dimerization of this lectin, previous studies have argued against multimeric forms of this protein. We report here that macrophage CBP35, purified by laminin affinity chromatography, exists as several distinct species (M, 35,000, 67,000, and 80,000) when analyzed under non-reducing conditions. This unexpected finding prompted us to study the biochemistry of multimerization using recombinant (rCBP35). rCBP35 expressed in Escherichia coli forms disulfide-linked homodimers (M, 67,000). The dimeric form of CBP35 binds to laminin with higher affinity than does monomer and by a lactosamine-dependent mechanism. Site-directed mutagenesis indicated that cysteine 186, the single cysteine residue in CBP35, is required for dimerization. These results raise the possibility that homo- and heterodimeric forms of CBP35 contribute to its postulated functions in cell-matrix interactions and growth regulation.

Carbohydrate-binding protein 35 (CBP35) is a lactosamine-specific animal lectin (1). Molecular cloning studies have revealed that CBP35 is identical to many previously characterized antigens including Mac-2, a cell surface macrophage differentiation antigen (2), RL-29, a developmentally characterized neuronal antigen (3), low affinity IgE-binding protein (4), and L-34, a tumor metastasis-associated antigen (5, 6). CBP35 is considered a thiol-dependent or S-type lectin because reducing conditions had been thought necessary to maintain carbohydrate binding activity (7). Divalent cations, however, are not required for binding, a property that distinguishes CBP35 from the Ca"-dependent or C-type lectins (7).

Recently, we reported that CBP35 is the major, non-integrin laminin-binding protein expressed on the surface of inflammatory mouse macrophages (8), a property that may be one of the primary extracellular functions of S-type lectins (9). In this context, the importance of the carbohydrate moieties of laminin in cell-laminin interactions has been suggested by several groups (10-13). Since CBP35 can agglutinate red blood cells, as well as other cell types, it may function in cell-matrix interactions by "bridging" cell surface oligosaccharides with oligosaccharidides on matrix proteins such as laminin (14, 15). The major inconsistency in both an agglutination and adhesion function for CBP35 is that all previous studies had indicated that CBP35 is monomeric, and no evidence for dimers or other multimers could be obtained (16, 17), findings consistent with the data that reducing conditions were necessary for S-lectin function.

In contrast to the prevailing hypotheses on CBP35 structure, we report here that macrophage CBP35, purified by laminin affinity chromatography, exists as several distinct, multimeric species when analyzed under non-reducing conditions. This unexpected finding prompted us to study the biochemistry of multimerization using rCBP35. rCBP35 expressed in Escherichia coli forms disulfide-linked homodimers (M, 67,000). The dimeric form of CBP35 was functional and bound to laminin by a lactosamine-dependent mechanism. Site-directed mutagenesis indicated that cysteine 186, the single cysteine residue in CBP35, is required for dimerization. These results raise the exciting possibility that homo- and heterodimeric forms of CBP35 contribute to its postulated function in cell-matrix interactions (15) and growth regulation (18).

EXPERIMENTAL PROCEDURES

Endogenous Macrophage CBP35—Thioglycollate-elicited peritoneal macrophages were obtained as described previously (8) from C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME). Macrophages were resuspended in RPMI medium without fetal bovine serum, and 10^6 cells were plated in 60-mm tissue culture dishes (Falcon Labware, Oxnard, CA). The cells were allowed to adhere for 1 h at 37°C, and non-adherent cells were then removed by vigorous washing with RPMI. After an additional 2-h incubation in RPMI at 37°C, adherent macrophages were washed with RPMI and then solubilized using a 0.01 M Tris buffer, pH 8.0, that contained 1% Triton X-100, 0.15 M NaCl, 2 mM CaCl_2 and MgCl_2, 2 mM phenylmethylsulfonyl fluoride, and 1 μM each of aprotinin, pepstatin, and leupeptin. Cell extracts were centrifuged at 13,000 x g for 5 min. to remove nuclei and other debris and used in the experiments described below.

Isolation of rCBP35—An expression construct was obtained from Dr. J. Wang (Michigan State University) in which the murine CBP35 cDNA was inserted into the pBluescript or pIB plasmid vectors. This secretion construct is designated pCBP35. The rCBP35 produced by this construct has the sequence NH_2-Ala-Glu-Phe-Arg-Asp-Ser. The 4th residue of this sequence, Arg, corresponds to the first amino acid of the cDNA sequence reported by Jia and Wang (1). The first 3 residues of rCBP35 represent extra amino acids inserted to generate the cloning site.

Secreted rCBP35 was purified from the periplasmic space following the recommended protocol of the Wang laboratory. Briefly, E. coli strain JA221 (American Type Tissue Collection 33875) was grown in an incubator shaker at 37°C to an A_600 of 0.5-1.0. An aliquot of this...
culture (10 ml) was expanded to a liter of medium, and this culture was grown for 2 h at 37 °C. The medium was then adjusted to 0.1 mM isopropyl-β-D-thiogalactopyranoside (Promega, WI). After an overnight incubation at 25 °C, cells were harvested by centrifugation at 3000 × g for 10 min. To isolate recombinant protein from the periplasmic space, the bacteria were hypotonically shocked by incubation in a 1 M Tris buffer, pH 7.0, containing protease inhibitors (2 mM phenylmethylsulfonyl fluoride and 1 μM each of aprotinin, pepstatin, and leupeptin) for 30 min at 4 °C. After centrifugation at 70,000 × g for 30 min, the supernatant was precipitated by 65% ammonium sulfate saturation. The precipitate was then dissolved and dialyzed against several changes of Dulbecco's phosphate-buffered saline containing protease inhibitors. Purified protein was stored in Dulbecco's phosphate-buffered saline at -80 °C at a concentration of ≤1 mg/ml. All of the above procedures were carried out in the absence of reducing agent.

Affinity Chromatography and Immunoblotting—Laminin purified from the Engelbreth-Holm-Swarm sarcoma (20) was conjugated to Sepharose CN 4B (Pharmacia LKB Biotechnology Inc.) at a ratio of 2 mg of protein to 1 ml of Sepharose according to the manufacturer's instructions. For affinity chromatography, approximately 0.4 mg of rCBP35 was incubated in the presence of 0.2 ml of laminin-Sepharose for 6-18 h at 4 °C with the gentle agitation. The supernatants were removed, and the gel was washed with high salt buffers as described previously (8). The bound proteins were eluted in Laemmli sample buffer and were subjected to SDS-PAGE. In some experiments, specific carbohydrates (Sigma) were added to the initial laminin-Sepharose incubation at the concentration noted in the figure legend. For analysis of macrophage extracts, aliquots (0.2-0.3 ml) containing the equivalent of 2-3 × 10⁶ macrophages were incubated in the presence of an equivalent volume of laminin-Sepharose as described for recombinant protein. All experiments were performed in the absence of reducing agents.

Proteins bound to laminin-Sepharose were resolved by SDS-PAGE and detected either by Coomassie staining or they were transferred to Immobilon-P membranes (Millipore, MA). The CBP35 (Mac-2) specific monoclonal antibody M3/38 (21) (American Type Tissue Collection) was used for immunoblotting. Bound antibodies were detected using a peroxidase-conjugated goat anti-rat IgG (Cappel, PA).

Site-directed Mutagenesis of rCBP35—Site-directed mutagenesis of the prCBP35s cDNA was carried out by overlap extension. This method requires introduction of nucleotide changes within oligonucleotide polymerase chain reaction (PCR) primers and a secondary PCR amplification to incorporate changes into an internal site within amplified DNA fragments (22, 23). A cysteine 186 to valine 186 mutation in the prCBP35s cDNA was generated by using pairs of complementary mutagenic oligonucleotide primers (5'-AGA AGA GTC ATT GTG AAC ACG AAG CAG GAC) representing nucleotides 541-573 (1). Underlined letters identify nucleotide changes that were introduced. The outer set of oligonucleotide primers from complementary strands representing the 5' and 3' ends of the prCBP35s gene-coding sequence were synthesized with an EcoRI recognition sequence at their 5' ends to facilitate cloning. Two PCR amplification products from the first round of PCR which shared overlapping sequences at the ends to be joined were purified from an agarose gel, restricted with EcoRI, and cloned into the EcoRI site of pBS KS+ (Stratagene, CA). PCR amplifications were performed with a DNA thermal cycler (Perkin-Elmer Cetus Instruments) using the following conditions: 30 cycles of 2 min at 55 °C for annealing, 2 min at 72 °C for polymerization, and 1 min at 94 °C for denaturation.

The entire nucleotide sequence of the recombinant inserts was confirmed by primer-directed dyeoxy chain termination sequencing. Finally, the EcoRI fragment containing the recombinant insert was transferred into the EcoRI cloning site of pBluescript II KS+ plasmid DNA (Stratagene, CA). PCR amplifications were performed with a DNA thermal cycler (Perkin-Elmer Cetus Instruments) using the following conditions: 30 cycles of 2 min at 55 °C for annealing, 2 min at 72 °C for polymerization, and 1 min at 94 °C for denaturation.

RESULTS

Mouse macrophage proteins that bound to laminin-Sepharose were resolved by SDS-PAGE and detected by immunoblotting using a Mac-2 specific monoclonal antibody. Under non-reducing conditions, several immunoreactive bands were observed (Fig. 1a). In addition to CBP35 monomer (32-35 kDa), prominent bands were seen at 67 and 80 kDa. Some of the minor bands that migrated below the 67- and 35-kDa bands probably represent degradation products of these two protein species based on similar observations made by other laboratories for the 35-kDa protein (e.g. Refs. 2, 5, and 21). Upon reduction of the sample with 2-mercaptoethanol (2-ME), only the 35-kDa monomeric form of CBP35 was observed (Fig. 1a). These data suggest that CBP35 forms disulfide-linked multimers.

In order to examine the biochemistry of CBP35 multimerization in more detail, we used a rCBP35 expression system. prCBP35s (obtained from Dr. J. Wang) is a construct in which the cDNA for CBP35 was inserted into the pLNI-III ompA secretion vector (19). Using this expression system, rCBP35 was isolated from the periplasmic space of E. coli using standard protocols with the exception that reducing agents were omitted from all buffers. After laminin-Sepharose chromatography and immunoblotting with the Mac-2 antibody, two major protein bands (M, 67,000 and 35,000) were evident when the recombinant protein was resolved under non-reducing conditions by SDS-PAGE (Fig. 1b). Upon reduction of the sample with 2-ME, only the 35-kDa protein band was seen (Fig. 1b). Both the 67- and 35-kDa proteins were detected by Coomassie staining (Fig. 2). Similar results were obtained when both non-reduced and reduced...
samples were resolved by non-denaturing gel electrophoresis (not shown). Taken together, these data indicate that rCBP35 forms disulfide-linked homodimers. CBP35 dimers are converted to monomer only by reducing reagents, and they are not dependent on the presence of SDS.

A priori, dimeric CBP35 should bind laminin more avidly than monomer. However, previous studies had indicated that reducing conditions were required to maintain the carbohydrate binding function of S-lectins (7) suggesting that this assumption may not be correct. We examined this discrepancy by analyzing the ratio of rCBP35 monomer to dimer in our initial preparation, in the material that did not bind laminin-Sepharose and in the material that bound specifically to laminin-Sepharose either in normal buffer (lane 3), in buffer containing 0.1 M lactose, or in buffer containing 0.1 M lactosamine. Bound proteins were analyzed by SDS-PAGE (12%) under non-reducing conditions and detected by immunoblotting using M3/38. Dimeric rCBP35 (molecular mass, 67 kDa) is seen only in the wild type under non-reducing conditions (lane 1), but not in the Val→Cys mutation (lane 2).

186. It is interesting to note that mutated rCBP35 retained the ability to bind laminin-Sepharose (not shown). However, considerably less mutated rCBP35 was secreted into the periplasmic space than was wild type rCBP35 suggesting that dimerization of CBP35 may facilitate its secretion in E. coli (24).

DISCUSSION

The data presented provide the first demonstration that CBP35 (Mac-2), a lactosamine-binding lectin, forms disulfide-linked homodimers that retain lectin activity. Most likely, dimers were not detected in previous studies because the protocols used to isolate this protein invariably employed reducing conditions (e.g. Refs. 5, 7, 8, 16, and 17). Such conditions were thought necessary to maintain the carbohydrate binding activity of this lectin. Our results, however, indicate that reducing conditions are not required for either the purification or lectin function of CBP35. In addition, other S-lectins including IgE binding protein, a rat homolog of CBP35, does not dimerize in prCBP35s was constructed by PCR in which a valine was substituted for cysteine 186. This mutant cDNA, as well as wild type CBP35 cDNA, was inserted into the pIN-III ompA2 secretion vector and expressed in E. coli. Results (Fig. 1a, b), proteins secreted into the periplasmic space were analyzed by SDS-PAGE (12%) under non-reducing conditions and detected by immunoblotting using M3/38. Dimeric prCBP35 (molecular mass, 67 kDa) is seen only in the wild type under non-reducing conditions (lane 1), but not in the Val→Cys mutation (lane 2).

Using site-directed mutagenesis of prCBP35s, we have established that cysteine 186, the single cysteine residue in this molecule, is required for dimerization. This cysteine is highly conserved in rat and human homologs of CBP35, as well as in the related 14-kDa family of galactose lectins often referred to as galaptins (27-33). Conservation of this cysteine may relate to the functional importance of dimerization of these lectins. Recently, however, it was reported that the low affinity IgE binding protein, a rat homolog of CBP35, does not form dimers when expressed in E. coli (17). This observation was substantiated by their finding that the single cysteine was resistant to alkylation under non-denaturing conditions and presumably not exposed to the extracellular environment. Although our mutagenesis data refute this conclusion, we also
note that in their study dithiothreitol was included in the initial purification of this protein from E. coli and that the recombinant cDNA was not expressed in a secretion vector.

Perhaps the most intriguing aspect of our observations is that CBP35 may form disulfide-linked heterodimers, as well as homodimers, in macrophages using cysteine 186. Determining the nature of such dimers could provide considerable insight into CBP35 function. CBP35 is localized in the cytoplasm (16), nucleus (34), as well as on the cell surface and in culture media (35). This pattern of localization, along with its ability to bind laminin through its lectin domain, has led to the hypothesis that CBP35 may function in such diverse processes as cell-matrix interactions (15) and growth regulation (18). It is tempting to speculate that these apparently disparate functions of CBP35 may be regulated by its ability to form intermolecular associations with nuclear, cytoplasmic, cell surface, and matrix proteins.

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