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# Characterization of Sugar Binding by the Mannose Receptor Family Member, Endo180*

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**Members of the mannose receptor family, the mannose receptor, the phospholipase $A_2$ receptor, DEC-205, and Endo180, contain multiple C-type lectin-like domains (CTLDs) within a single polypeptide. In addition, at their N termini, all four family members contain a cysteine-rich domain similar to the R-type carbohydrate recognition domains of ricin. However, despite the common presence of multiple lectin-like domains, these four endocytic receptors have divergent ligand binding activities, and it is clear that the majority of these domains do not bind sugars. Here the functions of the lectin-like domains of the most recently discovered family member, Endo180, have been investigated. Endo180 is shown to bind in a $Ca^{2+}$-dependent manner to mannose, fucose, and *N*-acetylglucosamine but not to galactose. This activity is mediated by one of the eight CTLDs, CTLD2. Competition assays indicate that the monosaccharide binding specificity of Endo180 CTLD2 is similar to that of mannose receptor CTLD4. However, additional experiments indicate that, unlike the cysteine-rich domain of the mannose receptor, the cysteine-rich domain of Endo180 does not bind sulfated sugars. Thus, although Endo180 and the mannose receptor are now both known to be mannose binding lectins, each receptor is likely to have a distinct set of glycoprotein ligands *in vivo*.**

The mannose receptor family comprises the mannose receptor, the M-type phospholipase $A_2$ receptor, the dendritic cell receptor DEC-205, and Endo180 (also known as uPARAP) (1–3). The four members of the family share a common structural organization. Each receptor is a type I transmembrane receptor with an extracellular region containing an N-terminal cysteine-rich domain similar to the galactose-binding domains of ricin, a fibronectin type II (FNII)[1] domain, and either 8 (mannose receptor, phospholipase $A_2$ receptor, and Endo180) or 10 (DEC-205) C-type lectin-like domains (CTLDs). The short cytoplasmic domain of each receptor mediates internalization of the receptor into the cell and recycling back to the plasma membrane (4–8). These common features and the fact that the mannose receptor functions in the clearance of glycoproteins, initially suggested that a main function of each of these receptors would be to mediate cellular uptake of glycosylated ligands. However, although each receptor contains multiple lectin-like domains, the majority of these domains do not bind sugars.

CTLDs are found in a wide variety of proteins and are characterized by a sequence motif that specifies a conserved fold (9). They were initially described in C-type lectins such as serum mannose-binding protein (MBP-A) and the asialoglycoprotein receptor, which bind sugars in a $Ca^{2+}$-dependent manner. However, it has become clear that many of these domains lack the residues required for $Ca^{2+}$-dependent sugar binding and are thus predicted to have other functions. The majority of CTLDs within proteins of the mannose receptor family have not conserved the key amino acids required for coordination of $Ca^{2+}$ ions and sugar residues, and on this basis none of the CTLDs within the phospholipase $A_2$ receptor and DEC-205 are predicted to bind sugar (2, 10) Indeed, sugar binding activity of DEC-205 has never been demonstrated, and the phospholipase $A_2$ receptor binds a nonglycosylated protein ligand, phospholipase $A_2$. The mannose receptor does mediate $Ca^{2+}$-dependent sugar binding, but this activity appears to be is restricted to only two of the eight CTLDs (11).

In addition to the CTLDs, all four receptors in the mannose receptor family contain an N-terminal cysteine-rich domain with homology to the R-type carbohydrate-recognition domains found in ricin, some glycosyltransferases, and bacterial hydrolases (12). The cysteine-rich domain of the mannose receptor binds oligosaccharides terminating in GalNAc-4-$SO_4$, such as those found on the pituitary hormones lutropin and thyrotropin (13, 14). However, again this activity is unlikely to be shared with other members of the family. DEC-205 does not bind lutropin (15), and sequence analysis predicts that the phospholipase $A_2$ receptor and Endo180 will also not bind sulfated sugars (3, 16).

Endo180 was originally identified in a monoclonal antibody screen for novel fibroblast cell surface receptors and demonstrated to be an endocytic recycling glycoprotein (17). Isolation of murine and human cDNAs revealed it to be the fourth member of the mannose receptor family (1, 18, 19), and examination of the human genome sequence suggests that this is the final family member. In contrast to the phospholipase $A_2$ receptor and DEC-205, but in common with the mannose receptor, preliminary studies have demonstrated that Endo180 from cultured cell lysates binds in a $Ca^{2+}$-dependent manner to immobilized GlcNAc (18). In this study, the roles of the multiple lectin-like domains in sugar binding activity of Endo180 have been investigated using a combination of mutational analysis and expression of isolated domains.

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[1] The abbreviations used are: FNII, fibronectin type II; CTLD, C-type lectin like domain; MBP-A, rat serum mannose-binding protein; BSA, bovine serum albumin.

## EXPERIMENTAL PROCEDURES

*Materials*—Restriction enzymes and other DNA modifying enzymes were obtained from New England Biolabs. Nitriloacetic acid-agarose, monosaccharides, and bovine serum albumin were from Sigma-Aldrich. $Na^{125}I$ and isopropyl-β-D-thiogalactoside were from Amersham Biosciences. $Mannose_{30}$-BSA was purchased from E.-Y. Laboratories and iodinated by the chloramine-T method (20). Immulon 4 96-well microtitre plates were obtained from Dynex Technologies. GlcNAc-, galactose-, mannose-, and fucose-Sepharose were prepared by the divinyl sulfone method (21). Lutropin was purified from bovine pituitaries following the method of Papkoff and Gan (22) and coupled to Affi-Gel 15 (Bio-Rad). The generation of the human Endo180-specific monoclonal antibody A5/158 is described elsewhere (17, 18). Horseradish peroxidase-conjugated second layer antibodies were purchased from Jackson Immunoresearch.

*Generation and Expression of Endo180-Fc Constructs*—The cloning of Endo180 and generation of the pcDNA3-Endo180 expression construct has been previously described (18). To generate Endo180-Fc fusion proteins in which the Endo180 was truncated after CTLD4 or CTLD2 (ΔCTLD5–8-Fc and ΔCTLD3–8-Fc, respectively; see Fig. 1), PCR was performed with Expand™ long polymerase (Roche Molecular Biochemicals) and pcDNA3-Endo180 as a template with a 5′ modified T7 primer (5′-CTGGCTTATCGAAATTAATACGACTCACTATAGGGA-3′) and the following 3′ primers, 5′-GTCTCGAGTTGAGGGCTGTCGTCGGGCTCC-3′ for ΔCTLD5–8-Fc and 5′-GTCTCGAGCCGGCTGCCATGGTCCTCCTCG-3′ for ΔCTLD3–8-Fc, where the underlined bases indicate an *Xho*I restriction site. Amplified DNA was digested with *Hin*dIII and *Xho*I and ligated into the pIgplus vector (R & D Systems) digested with the same enzymes. Mutation of $Asn^{472}$ to an aspartic acid residue within ΔCTLD5–8-Fc was generated using the QuikChange™ mutagenesis method (Stratagene) with oligonucleotides 5′-GGCACCCCTTTGAGCCCGACAAACTTCCGGG-3′ and 5′-CCCGGAAGTTGTCGGGCTCAAAGGGGTGCC-3′, where the underlined bases indicate changes from the wild type sequence. pIgplus-Endo180-Fc constructs (100 μg) were transfected into 50–75% confluent COS-1 cells with 400 μg/ml DEAE/dextran, 100 μM chloroquine diphosphate in serum-free Dulbecco's modified Eagle's medium. The cells were incubated for 4 h at 37 °C. The medium was aspirated and replaced with 15 ml of phosphate-buffered saline with 10% $Me_2SO$ osmotic shock medium for 2 min. The osmotic shock medium was replaced with 25 ml of Dulbecco's modified Eagle's medium with 10% fetal calf serum, and the cells were incubated overnight at 37 °C. 24 h later the medium was replaced with serum-free Dulbecco's modified Eagle's medium. The supernatant was harvested after a 7-day period.

*Expression of Endo180 CTLD2 in Bacteria*—The portion of the human Endo180 cDNA (18) coding for CTLD2 (nucleotides 1259–1651) was cloned into the expression vector pIN-IIIompA-2 (23) using standard recombinant DNA techniques. Synthetic oligonucleotides were used to fuse the 5′ end of the cDNA to the codons specifying the *omp*A signal sequence and to add a stop codon at the 3′ end. The integrity of the final expression plasmids was checked by DNA sequencing using an ABI prism 310 Genetic Analyzer. Luria-Bertani medium (1 liter) containing 50 μg/ml ampicillin was inoculated with 30 ml of an overnight culture of *Escherichia coli* strain JA221 containing the CTLD2 expression plasmid. The culture was grown with shaking at 25 °C to an $A_{550}$ of ~1. Isopropyl-β-D-thiogalactoside and $CaCl_2$ were then added to final concentrations of 50 μM and 100 mM, respectively. After growth for a further 18 h at 25 °C, the cells were harvested by centrifugation at 4,000 rpm for 15 min in a Beckman JS-4.2 rotor. Bacterial pellets were resuspended in cold 10 mM Tris-HCl, pH 7.8, followed by centrifugation at 12,000 rpm for 15 min at 4 °C in a Beckman JA14 rotor. The bacteria were sonicated in 30 ml of 25 mM Tris-HCl, pH 7.8, 0.5 M NaCl, 20 mM $CaCl_2$ (loading buffer). Lysed bacteria were centrifuged at 10,000 × *g* for 15 min, and the supernatant was recentrifuged at 100,000 × *g* for 1 h at 4 °C. The supernatant was passed over a 10-ml GlcNAc-Sepharose column equilibrated in loading buffer. The column was washed with 30 ml of loading buffer and eluted with 10 × 2 ml of elution buffer (25 mM Tris-HCl, pH 7.8, 0.5 M NaCl, 2 mM EDTA). Elution fractions were analyzed by SDS-polyacrylamide gel electrophoresis, and CTLD2 was identified by N-terminal sequencing on a Beckman LF3000 protein sequencer following transfer to polyvinylidene difluoride membranes. Fractions containing pure CTLD2 were pooled, and protein was assayed using the Bio-Rad protein assay reagent with BSA as standard. The yield of pure CTLD2 ranged from about 0.5 to 1 mg/liter.

*Expression of Endo180 Cysteine-rich Domain in Bacteria*—The portion of the human Endo180 cDNA (18) coding for the cysteine-rich domain (nucleotides 236–643) was cloned into the expression vector pIN-IIIompA-2. Synthetic oligonucleotides were used to fuse the 5′ end of the cDNA to the codons specifying the *omp*A signal sequence and to add codons specifying six histidine residues and a stop codon at the 3′ end. For protein expression, growth and induction of *E. coli* strain JA221 containing the cysteine-rich domain plasmid was as described above for production of CTLD2, except that no $CaCl_2$ was added at the time of induction. The bacteria were harvested as described above, resuspended in 100 ml of N1 buffer (25 mM Tris-HCl, pH 7.8, 0.5 M NaCl) containing 20 mM imidazole, and lysed by sonication. Lysed bacteria were centrifuged at 10,000 × *g* for 15 min, and the supernatant was recentrifuged at 100,000 × *g* for 1 h at 4 °C. The supernatant was passed down a 1-ml column of nitriloacetic acid-agarose that was preloaded with 5 ml of 50 mM $NiSO_4$ and equilibrated in N1 buffer containing 20 mM imidazole. The column was washed with 10 ml of N1 buffer containing 50 mM imidazole and eluted with N1 buffer containing 150 mM imidazole. Elution fractions were analyzed by SDS-polyacrylamide gel electrophoresis, and the cysteine-rich domain was identified by N-terminal sequencing.

*Sugar Binding Assays*—For Endo180-Fc chimeras, 100 μl of COS-1 tissue culture supernatant in 900 μl of loading buffer (150 mM NaCl, 25 mM Tris-HCl, pH 8.0, plus 25 or 10 mM $CaCl_2$) was loaded onto 2-ml columns of mannose-, GlcNAc-, fucose-, or galactose-Sepharose. The flow through was collected, and the columns were washed with 7 × 1 ml of loading buffer followed by 7 × 1 ml of elution buffer (150 mM NaCl, 25 mM Tris-HCl, pH 8.0, and 10 mM EDTA). For assay of Endo180 from Flow2000 fibroblasts, the cells were lysed in 150 mM NaCl, 10 mM Tris-HCl, pH 8.0, 0.15% Triton X-100, 1 mM $MgCl_2$, 10 mM $CaCl_2$. Lysate (containing ~20 μg of protein) was added to 1 ml of loading buffer containing 0.15% Triton X-100 and applied to the columns. The columns were washed in loading buffer plus 0.15% Triton X-100 and eluted in elution buffer plus 0.15% Triton X-100. The fractions were precipitated by incubation with 40 μg of BSA and 0.5 ml of 30% trichloroacetic acid for 30 min on ice and then centrifuged for 10 min at 4 °C at 15,000 × *g*. The pellets were washed twice in 1:1 ethanol ether, air dried for 10 min, and resuspended in 40 μl of nonreducing sample buffer. The samples (10 μl) were resolved by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose, and Endo180 was detected using monoclonal antibody A5/158 followed by horseradish peroxidase-conjugated anti-mouse immunoglobulin. The blots were developed using ECL reagent (Amersham Biosciences).

*Sugar Competition Assays*—Plastic microtitre plates with removable wells (Immulon 4) were coated with CTLD2 (50 μl/well of a 100 μg/ml solution of CTLD2 in 25 mM Tris-HCl, pH 7.8, 0.1 M NaCl, 25 mM $CaCl_2$). Following incubation overnight at 4 °C, the wells were washed three times with cold washing buffer (25 mM Tris-HCl, pH 7.8, 0.1 M NaCl, 25 mM $CaCl_2$). Nonspecific binding sites were blocked by filling the wells with 5% (w/v) BSA in washing buffer and incubating for 2 h at 4 °C. After washing the wells three times with cold washing buffer, aliquots (100 μl) of a range of concentrations of monosaccharide in washing buffer containing $^{125}I$-Man-BSA (1 μg/ml) and 5% BSA were added to the wells in duplicate. Following incubation at 4 °C for 2 h, the wells were washed three times with cold washing buffer and counted on a γ counter. The values for $K_i$ (the inhibitor concentration that gives 50% inhibition of $^{125}I$-Man-BSA binding) for each inhibitor were determined by fitting the data to the following equation for simple competitive inhibition: fraction of maximal binding = $K_I/(K_I + [\text{Inhibitor}])$.

## RESULTS

*Localization of $Ca^{2+}$-dependent Sugar Binding Activity of Endo180 to CTLD2*—Previous studies have demonstrated that Endo180 solubilized from cultured cells displays $Ca^{2+}$-dependent binding to immobilized GlcNAc (18). Sequence analysis predicts that this activity is likely to be mediated by CTLD2 because it is the only Endo180 CTLD that contains all of the five residues that ligate two hydroxyl groups of a monosaccharide and a $Ca^{2+}$ in other sugar-binding CTLDs (Fig. 1). In addition, the presence of the sequence EPN (amino acids 470–472) at the position equivalent to the principal $Ca^{2+}$-binding site of MBP-A and other mannose-specific C-type lectins predicts that Endo180 CTLD2 will ligate sugars with equatorial three and four hydroxyl groups, including GlcNAc and mannose (24). To define the roles of the different domains of Endo180 and to investigate further the specificity of the receptor, the sugar binding activity of several different Endo180 expression constructs has been assessed.

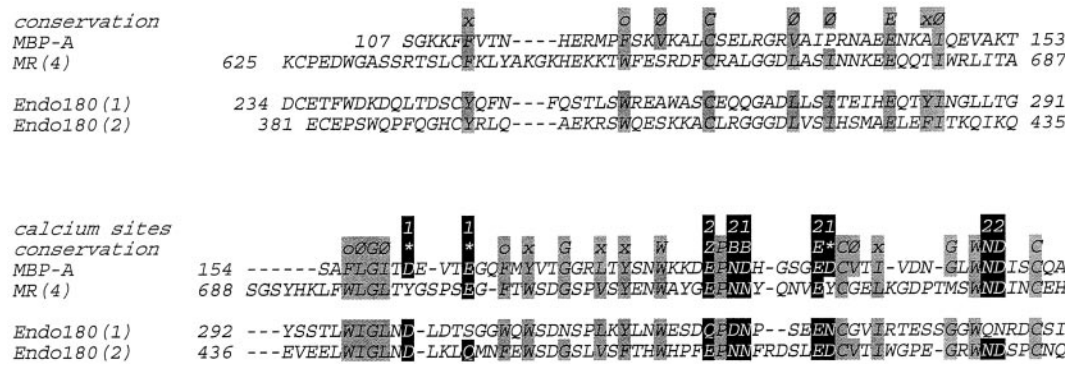


FIG. 1. **Sequence comparisons of C-type lectin-like domains.** The CTLD of MBP-A is aligned with human Endo180 CTLD1 and CTLD2 (*Endo180 (1)* and *Endo180 (2)*, respectively) and mannose receptor CTLD4 (*MR (4)*). Conserved residues that define the CTLD fold and the $Ca^{2+}$ sites are shaded in *gray* and *black*, respectively (46, 47). *1* and *2* denote residues involved in ligating two $Ca^{2+}$ ($Ca^{2+}$ 1 and $Ca^{2+}$ 2) to MBP-A. The binding site for $Ca^{2+}$ site 2, also known as the principal $Ca^{2+}$, is conserved in all sugar-binding CTLDs. The auxiliary $Ca^{2+}$ site 1 is conserved in some sugar binding CTLDs. *x*, aliphatic or aromatic; Φ, aliphatic; o, aromatic; *, side chain with carbonyl oxygen; *Z*, E or Q; *B*, D or N. Invariant amino acids are shown in single-letter codes.

Soluble Endo180 constructs were generated as chimeric proteins fused to the Fc portion of human Ig. The presence of the Fc tail enables detection using anti-human Fc antibodies and, if required, purification of constructs on protein G columns. Endo180-Fc constructs were expressed in COS-1 cells and tested for their ability to bind to immobilized monosaccharides. In these assays, monosaccharide-Sepharose columns were loaded with expressed Endo180-Fc protein in $Ca^{2+}$-containing buffer. After washing with the $Ca^{2+}$-containing buffer, the columns were eluted with buffer containing EDTA to detect proteins bound to the column in a $Ca^{2+}$-dependent manner. For initial studies, an Fc chimera containing Endo180 deleted after CTLD4 (Endo180ΔCTLD5–8-Fc; Fig. 2) was employed. A significant fraction of this Endo180-Fc construct is retained on columns of GlcNAc-, mannose-, and fucose-Sepharose but not on galactose-Sepharose and is eluted in the presence of EDTA (Fig. 3*A*). The results indicate that Endo180 has specificity for mannose and fucose as well as GlcNAc and that CTLDs 5–8 are not required for $Ca^{2+}$-dependent binding of these monosaccharides.

In a previous, preliminary study of sugar binding by Endo180, only binding to immobilized GlcNAc and not to immobilized mannose and fucose was detected (18). This apparent selectivity for GlcNAc was somewhat surprising because C-type lectins that bind GlcNAc, including the mannose receptor and MBP-A, generally bind mannose and fucose as well (24). The discrepancy in these data does not result from the use in this current study of chimeric constructs that are dimerized via their Fc tails because native Endo180 from Flow2000 cell lysates was also demonstrated to bind to mannose-Sepharose (Fig. 3*B*), and identical binding profiles were obtained with a soluble Endo180 construct generated without an Fc tail (data not shown). Differences in the monosaccharide resins used are the most likely explanation for the difference between the results obtained here and those reported previously (18). In this study, monosaccharides were conjugated to Sepharose in the laboratory using a procedure that has been used to produce resins for study of sugar binding by other C-type lectins. It is likely that these resins contain a higher ratio of attached sugar compared with the commercial monosaccharide-agarose matrices used previously and consequently provide more effective substrates for lectin binding.

Analysis of an Endo180-Fc chimera deleted after CTLD2 (Endo180ΔCTLD3–8-Fc; Fig. 2) indicates that CTLDs 3 and 4 are also not required for $Ca^{2+}$-dependent sugar binding. Like the construct deleted after CTLD4, this truncated construct

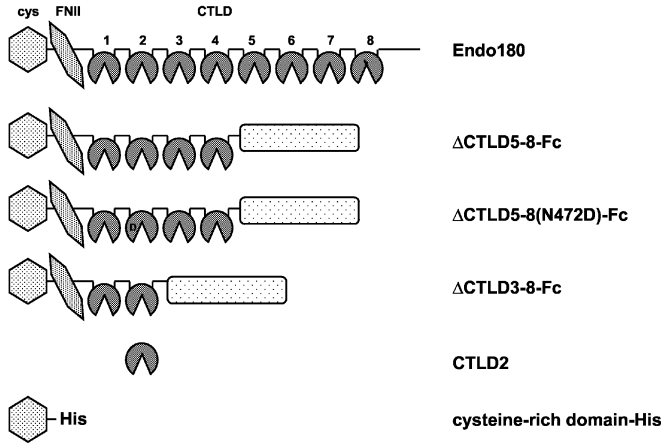


FIG. 2. **Diagram of Endo180 constructs.** Wild type Endo180 protein has an N-terminal cysteine-rich domain (*cys*) followed by a FNII domain and eight CTLDs. Soluble constructs were generated in which Endo180 truncated after CTLD4 (Endo180ΔCTLD5–8-Fc) or after CTLD2 (Endo180ΔCTLD3–8) was fused in frame with the Fc portion of human IgG. These constructs were expressed in COS-1 cells. CTLD2 and the cysteine-rich domain with a C-terminal His tag were expressed in bacteria. The N-terminal signal sequences are not shown.

binds to mannose-Sepharose (Fig. 4*A*) and GlcNAc-Sepharose (data not shown).

The involvement of Endo180 CTLD2 in sugar binding was assessed further by mutation of key residues in $Ca^{2+}$ site 2. In the first construct, $Glu^{470}$ and $Asn^{492}$ (Fig. 1) were each changed to alanine. However, this protein could not be expressed, suggesting that these two residues are required for the correct folding and stability of CTLD2. Because this unstable protein could not be assessed for sugar binding, a second construct was generated in which $Asn^{472}$, another of the residues predicted to be involved in ligation of sugar and $Ca^{2+}$ to CTLD2, was mutated. In MBP-A and E-selectin, the amide nitrogen of the equivalent asparagine residue forms a hydrogen bond to a sugar hydroxyl group, whereas the carbonyl oxygen forms a coordination bond with the $Ca^{2+}$ ion that is also ligated to the sugar. Mutation of this Asn residue to Asp in the CTLDs of MBP-A (25) and E-selectin (26) abolishes sugar binding activity because of a loss of the hydrogen bond to the sugar hydroxyl group. $Asn^{472}$ in CTLD2 was mutated to aspartic acid in the context of the Endo180-Fc chimera containing Endo180 deleted after CTLD4 (Endo180ΔCTLD5–8(N472D)-Fc; Fig. 2).

Binding of the mutated Endo80 construct to mannose-Sepharose is observed in the presence of 25 mM $Ca^{2+}$, but this binding

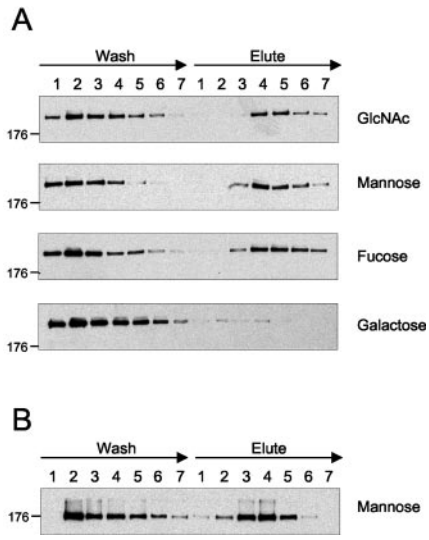


FIG. 3. **Specificity of Endo180 binding to immobilized monosaccharides.** Tissue culture supernatant containing Endo180ΔCTLD5–8-Fc construct (*A*) or Flow2000 cell lysate (*B*) was loaded onto GlcNAc-, mannose-, fucose-, or galactose-Sepharose columns. The columns were washed with loading buffer (containing 25 mM $Ca^{2+}$) and then elution buffer. The fractions (*Wash 1–7* and *Elute 1–7*) were analyzed by Western blotting as described under "Experimental Procedures." Note that the increased size of the Fc construct results from Fc-mediated dimerization on nonreducing SDS-polyacrylamide gel electrophoresis.

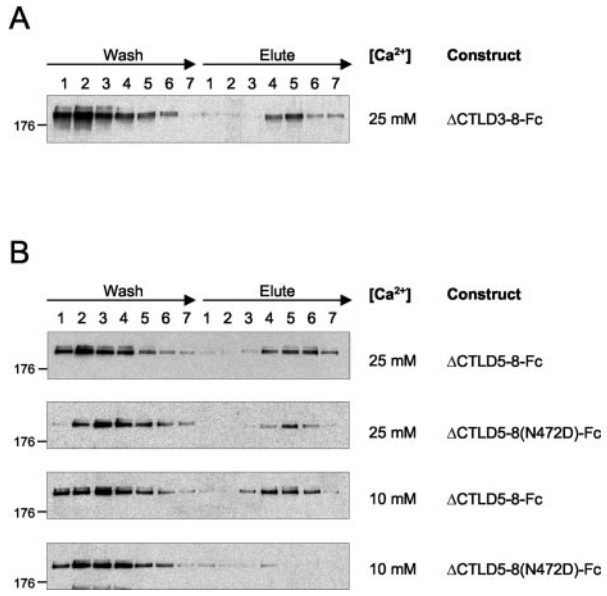


FIG. 4. **Mutation of Asn$^{472}$ in Endo180 CTLD2 impairs binding to mannose.** Tissue culture supernatant containing Endo180ΔCTLD3–8-Fc (*A*) or containing Endo180ΔCTLD5–8-Fc or Endo180ΔCTLD5–8(N472D)-Fc (*B*) were loaded onto mannose-Sepharose columns and analyzed as described in the legend to Fig. 2 except that loading buffers contained either 25 or 10 mM $Ca^{2+}$.

is reduced compared with the wild type construct (Fig. 4*B*). However, at 10 mM $Ca^{2+}$, the mutated construct does not bind to mannose-Sepharose, whereas binding of the wild type construct is not affected by the reduction in $Ca^{2+}$ concentration (Fig. 4*B*). Similar results were obtained when the constructs were tested on GlcNAc-Sepharose (data not shown). The assays were initially carried out at 25 mM $Ca^{2+}$, because this concentration has typically been used for assaying other C-type lectins and C-type carbohydrate recognition domains (25, 27–29). Measured affinities of C-type carbohydrate recognition domains for $Ca^{2+}$ are in the range 0.2–1.0 mM, and as long as $Ca^{2+}$ is saturating, varying $Ca^{2+}$ concentration does not affect

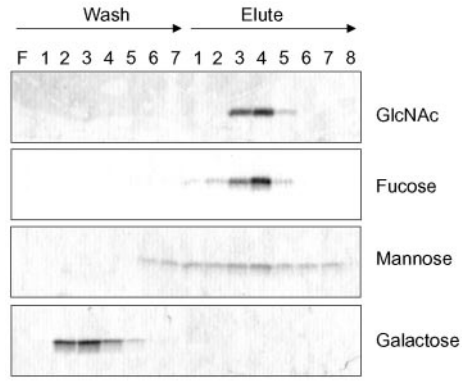


FIG. 5. **Binding of isolated Endo180 CTLD2 to monosaccharide columns.** Purified CTLD2 in 1 ml of loading buffer was passed over 2-ml columns of GlcNAc-, fucose-, mannose-, and galactose-Sepharose. The columns were washed with 7 × 1 ml of loading buffer and eluted with 8 × 1 ml of elution buffer. The flow through (*F*) and all fractions (*Wash 1–7* and *Elute 1–8*) were analyzed by SDS-polyacrylamide gel electrophoresis on 17.5% gels that were stained with Coomassie Blue.

sugar binding (29–31). Thus, the fact that the mutated Endo180(N472D) construct shows no sugar binding activity at 10 mM $Ca^{2+}$ indicates that the $Ca^{2+}$ binding affinity of CTLD2 has been substantially reduced by the mutation and that the mutated construct would not have sugar binding activity at physiological $Ca^{2+}$ concentration. These results indicate that Asn$^{472}$ of CTLD2 is likely to be involved in ligation of sugar and $Ca^{2+}$ and that CTLD2 is responsible for the sugar binding activity of the Endo180 construct deleted after CTLD4.

*Monosaccharide Binding Activity of Endo180 CTLD2*—The experiments with deletion constructs give a strong indication that CTLD2 is the domain responsible for mediating $Ca^{2+}$-dependent binding of mannose, GlcNAc, and fucose to Endo180. To allow direct assessment of sugar binding activity by CTLD2, this domain was expressed in bacteria using an expression system that has been successful for producing other C-type domains (27, 29). The CTLD is expressed as a fusion protein with the *omp*A signal sequence, which directs the protein into the periplasm where conditions are favorable for folding. Expressed CTLD2 was purified from the bacterial lysate by affinity chromatography on GlcNAc-Sepharose. Like the Endo180-Fc construct truncated after CTLD4, isolated CTLD2 binds to GlcNAc-, mannose-, and fucose-Sepharose in a $Ca^{2+}$-dependent manner but does not bind to galactose-Sepharose (Fig. 5).

The specificity of CTLD2 was further investigated using a competition assay in which monosaccharides compete for binding of $^{125}$I-$Man_{30}$-BSA to immobilized CTLD2. Unlike the column binding assays, which can only give a qualitative indication of specificity, this assay allows quantitative assessment of binding of different monosaccharides to CTLD2. Representative inhibition curves are shown in Fig. 6, with inhibition constants given in Table I. In agreement with the results obtained with monosaccharide resins, mannose, GlcNAc, and fucose are effective inhibitors of $^{125}$I-$Man_{30}$-BSA binding to CTLD2. The weak inhibition seen with galactose is likely to be due to interaction with the anomeric hydroxyl of the free sugar, because α-methylgalactoside does not inhibit binding of $^{125}$I-$Man_{30}$-BSA. Such nonphysiological binding of galactose has been seen with other mannose-specific C-type lectins (28, 32). The results suggest that Endo180 CTLD2 distinguishes between monosaccharides in a manner similar to that of other C-type lectins through recognition of the C-3 and C-4 hydroxyls and that like other mannose-specific C-type lectins, CTLD2 binds preferentially to sugars with equatorial C-3 and C-4 hydroxyl groups. Mannose, glucose, GlcNAc, and *N*-acetylman-

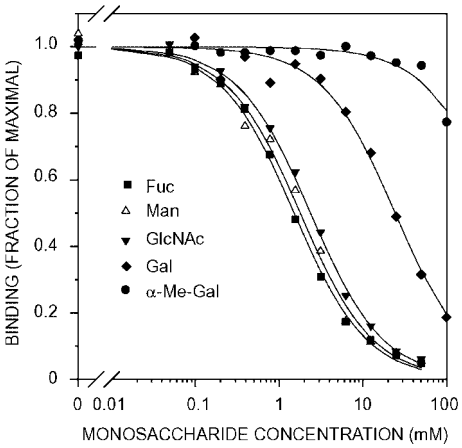


FIG. 6. **Inhibition of $^{125}$I-Man$_{30}$-BSA binding to Endo180 CTLD2 by monosaccharides.** The data were obtained using the competition assay. The experimental values (*symbols*) are shown together with the theoretical curves (*lines*) fitted to the data.

TABLE I
*Monosaccharide binding by Endo180 CTLD2*

Inhibition constants for each monosaccharide were determined using the competition assay. The results are presented as the means ± S.D. of 3–5 assays done in duplicate.

| Monosaccharide | $K_i$ | $K_{i\ \mathrm{Sugar}}/K_{i\ \mathrm{Man}}$ |
|---|---|---|
| | *mM* | |
| Mannose | 1.8 ± 0.1 | 1 |
| *N*-Acetylglucosamine | 2.3 ± 0.1 | 1.28 |
| L-Fucose | 1.6 ± 0.1 | 0.89 |
| Glucose | 2.6 ± 0.7 | 1.44 |
| α-Methylmannoside | 2.0 ± 0.6 | 1.11 |
| *N*-Acetylmannosamine | 2.2 ± 0.9 | 1.22 |
| Galactose | 22.7 ± 2.3 | 12.6 |
| α-Methylgalactoside | >400 | >200 |

nosamine are approximately equal in effectiveness as inhibitors of $^{125}$I-Man$_{30}$-BSA binding, suggesting that substituents at the C-2 position do not interact significantly with Endo180 CTLD2. Fucose, which can bind to mannose-specific C-type lectins through equatorial hydroxyl groups on C-2 and C-3, interacts with CTLD2 as strongly as mannose. Thus, Endo180 CTLD2 binds the same range of monosaccharides as other mannose-specific C-type lectin domains.

*The Cysteine-rich Domain of Endo180 Does Not Bind Sulfated Sugars*—Like the other members of the mannose family, Endo180 has an N-terminal cysteine-rich domain homologous to the galactose-binding R-type carbohydrate recognition domains of ricin. However, the cysteine-rich domain of Endo180 does not contain the residues that interact with galactose in ricin, nor the residues that interact with GalNAc-4-SO$_4$ in the mannose receptor cysteine-rich domain and is thus not predicted to have sugar binding activity (3, 16). The Endo180 cysteine-rich domain was produced in bacteria so that its sugar binding activity could be assessed. As for CTLD2, the cysteine-rich domain was expressed as a fusion protein with the *omp*A signal sequence, so that it was directed into the bacterial periplasm. Mannose receptor cysteine-rich domain produced in the same way folds correctly and can be purified from the bacterial lysate by affinity chromatography on lutropin-agarose.[2]

Endo180 cysteine-rich domain with a C-terminal His tag was purified by nickel affinity chromatography and tested for its ability to bind to lutropin-agarose. Analysis of the lutropin-agarose column fractions demonstrates that none of the

[2] C. T. Heise and M. E. Taylor, unpublished observations.

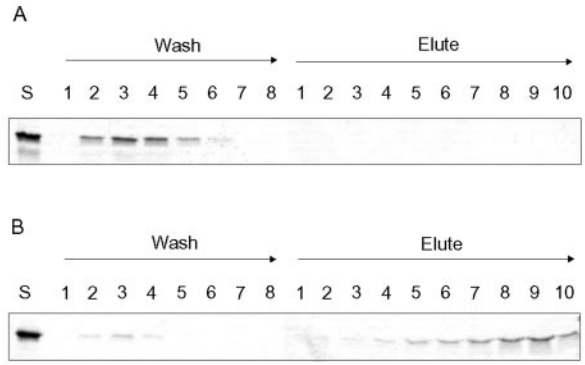


FIG. 7. **Endo180 cysteine-rich domain does not bind sulfated sugars.** Purified cysteine-rich domain in 1 ml of 20 mM Tris-HCl, pH 7.4, 100 mM NaCl (washing buffer) was passed over a 2-ml column of lutropin-agarose. The column was washed with 8 × 1 ml of washing buffer and eluted with 10 × 1 ml of 25 mM glycine, pH 2.5, 0.5 M NaCl. The fractions (*Wash 1–8* and *Elute 1–10*) and a sample of the starting material loaded onto the column (*S*) were analyzed by SDS-polyacrylamide gel electrophoresis on 17.5% gels that were stained with Coomassie Blue. *A*, Endo180 cysteine-rich domain. *B*, mannose receptor cysteine-rich domain.

Endo180 cysteine-rich domain is retained on the column (Fig. 7*A*). In contrast, although a small fraction of the mannose receptor cysteine-rich domain is detected in the wash fractions, most binds to the lutropin column and is slowly eluted with the low pH elution buffer (Fig. 7*B*). Thus, as predicted from the sequence analysis, the cysteine-rich domain of Endo180 does not contain a binding site for GalNAc-4-SO$_4$. The cysteine-rich domain of Endo180 also does not bind to Gal- or GlcNAc-Sepharose (data not shown), indicating that this domain does not contribute to the sugar binding activity of the receptor.

## DISCUSSION

A combination of deletion mutagenesis and expression of isolated domains of Endo180 has defined the roles of individual domains of this receptor in sugar binding. Like the mannose receptor, Endo180 binds mannose, GlcNAc, and fucose in a $Ca^{2+}$-dependent manner, and this activity is associated with a single CTLD. However, unlike the mannose receptor, Endo180 lacks a binding site for sulfated sugars in the cysteine-rich domain.

$Ca^{2+}$-dependent binding of mannose, GlcNAc, and fucose to Endo180 is mediated by CTLD2. It is likely that the mechanism of sugar binding by this domain is similar to other mannose-specific CTLDs and involves ligation of two equatorial hydroxyl groups of a monosaccharide by two pairs of asparagine and glutamic acid residues at the conserved principal $Ca^{2+}$ site (25). In the mannose receptor, a single CTLD is also mainly responsible for mediating $Ca^{2+}$-dependent binding to a similar range of monosaccharides, but in this case it is CTLD4 rather than CTLD2 (11). Interestingly, although the phospholipase $A_2$ receptor does not bind sugars, a single CTLD of this receptor, CTLD5, is also largely responsible for the $Ca^{2+}$-independent binding to nonglycosylated secretary phospholipases $A_2$ (33).

Although it is clear that CTLD2 is largely responsible for sugar binding by Endo180, the possibility that an additional CTLD may be involved in binding glycoprotein ligands, as is the case in the mannose receptor, should be considered. Only CTLD4 of the mannose receptor binds sugars when expressed in isolation, but the five residues that ligate the principal $Ca^{2+}$ are also absolutely conserved in CTLD5, and there is strong evidence that CTLD5 contributes to binding of natural glycoproteins to the receptor (11, 34). However, in Endo180, no other CTLD apart from CTLD2 contains all of the residues required

for ligating $Ca^{2+}$ and sugar. CTLD1 contains several of these residues, but Gln and Asn replace Asn and Asp of the conserved WND sequence ($Asn^{205}$ and $Asp^{206}$ in MBP-A) (Fig. 1). Thus, $Ca^{2+}$ and sugar could not be ligated at this site in exactly the same way as in known sugar-binding CTLDs. In addition, the presence of the sequence QPD (amino acids 326–328) rather than EPN predicts that CTLD1 would be more likely to bind galactose-like rather than mannose-like monosaccharides (24). The fact that neither intact Endo180 nor any of the deletion constructs containing CTLD1 bind to galactose-Sepharose suggests that there is unlikely to be any interaction of this domain with galactose. However, the possibility that CTLD1 contributes to binding of natural ligands to Endo180 in a manner similar to that of CTLD5 of the mannose receptor cannot be absolutely ruled out.

The finding that Endo180 exhibits $Ca^{2+}$-dependent binding to a similar spectrum of monosaccharides as the mannose receptor is of interest because it raises the possibility that these two receptors might have some overlap in function, particularly as they are both expressed on macrophages (18, 35). The main role of the mannose receptor appears to be in the clearance of proteins bearing high mannose oligosaccharides, such as lysosomal enzymes that are released as part of the inflammatory response (36). Endocytic activity of Endo180 has been well characterized (8, 17), and given the results presented here it is likely that this receptor will also mediate uptake of glycoproteins. However, several lines of evidence suggest that there will probably be only limited, if any, overlap in the ligands and functions of these two receptors *in vivo*.

The sugar binding CTLDs of Endo180 and the mannose receptor are located in different positions relative to the other domains in the protein, and this difference is likely to affect the interactions of the two proteins with glycoprotein ligands. Hydrodynamic analysis and protease resistance studies reveal that the extracellular region of the mannose receptor adopts a relatively rigid extended conformation with the cysteine-rich domain projected furthest from the membrane. In addition there are close interactions between the domains with the exception that the linker regions on either side of CTLD3 and CTLD6 are flexible and exposed (37). Thus, CTLD4 and CTLD5 are in close contact with each other, but are separated from the neighboring domains, and form a ligand-binding core in the middle of the polypeptide. This arrangement is likely to be important for binding multiple mannose residues on high mannose oligosaccharides. If, as is likely, the conformation of the extracellular region of Endo180 is similar to that of the mannose receptor, then Endo180 CTLD2 will be projected further from the membrane and may be accessible to glycoprotein ligands that cannot bind to the mannose receptor. In addition, the Endo180 cysteine-rich domain, the FNII domain, CTLD1, and CTLD2 will be closely associated and separated from CTLD3. Thus, sugar binding to CTLD2 may be modulated by the close proximity of CTLD1 and additionally by the FNII domain, especially if, like FNII domains found in several other proteins, this domain has a role in collagen binding (3).

Endo180 and the mannose receptor also have distinct patterns of expression. Although both are expressed on macrophages (18, 35), Endo180 is also found on fibroblasts and chondrocytes, chondrocytes, a subset of endothelial cells, and in tissues undergoing ossification (1, 17, 18, 38–40), whereas the mannose receptor has been detected on lymphatic and hepatic endothelium, smooth muscle cells, and some epithelia (41–44). These differences in distribution may reflect accessibility to distinct sets of ligands *in vivo*.

Finally, evidence is presented here that the Endo180 cysteine-rich domain does not bind sugars. In contrast, the equivalent domain of the mannose receptor binds sulfated GalNAc found on the oligosaccharides of soluble glycoproteins such as lutropin (13), as well as sulfated Lewis blood group antigens and chondroitin 4-sulfate groups of proteoglycans (15). The mannose receptor cysteine-rich domain can also mediate association with transmembrane glycoproteins bearing sulfated oligosaccharides, including sialoadhesin and CD45 (45). Consequently Endo180, unlike the mannose receptor, will not function in the uptake of soluble glycoproteins or extracellular matrix components bearing sulfated sugars nor act as a counter-receptor for transmembrane proteins with sulfated oligosaccharides.

Further understanding of the function of Endo180 will require the identification of natural glycoprotein ligands. Type V collagen and a complex of the pro form of urokinase-type plasminogen activator and its receptor have been identified as potential ligands for Endo180 (19). Little is yet known about the molecular basis for these interactions, but each of these molecules is glycosylated, raising the possibility that recognition of sugars could be involved.

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REFERENCES

1. Wu, K., Yuan, J., and Lasky, L. A. (1996) *J. Biol. Chem.* **271,** 21323–21330
2. Taylor, M. E. (1997) *Glycobiology* **7,** v–viii
3. East, L., and Isacke, C. M. (2002) *Biochim. Biophys. Acta* **1572,** 364–386
4. Kruskal, B. A., Sastry, K., Warner, A. B., Mathieu, C. E., and Ezekowitz, R. A. (1992) *J. Exp. Med.* **176,** 1673–1680
5. Mahnke, K., Guo, M., Lee, S., Sepulveda, H., Swain, S. L., Nussenzweig, M., and Steinman, R. M. (2000) *J. Cell Biol.* **151,** 673–684
6. Zvaritch, E., Lambeau, G., and Lazdunski, M. (1996) *J. Biol. Chem.* **271,** 250–257
7. Schweizer, A., Stahl, P. D., and Rohrer, J. (2000) *J. Biol. Chem.* **275,** 29694–29700
8. Howard, M. J., and Isacke, C. M. (2002) *J. Biol. Chem.* **277,** 32320–32331
9. Weis, W. I., Taylor, M. E., and Drickamer, K. (1998) *Immunol. Rev.* **163,** 19–34
10. Taylor, M. E., Conary, J. T., Lennartz, M. R., Stahl, P. D., and Drickamer, K. (1990) *J. Biol. Chem.* **265,** 12156–12162
11. Taylor, M. E., Bezouska, K., and Drickamer, K. (1992) *J. Biol. Chem.* **267,** 1719–1726
12. Dodd, R. B., and Drickamer, K. (2001) *Glycobiology* **11,** 71R–79R
13. Fiete, D. J., Beranek, M. C., and Baenziger, J. U. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 2089–2093
14. Simpson, D. Z., Hitchen, P. G., Elmhirst, E. L., and Taylor, M. E. (1999) *Biochem. J.* **2,** 403–411
15. Leteux, C., Chai, W., Loveless, R. W., Yuen, C. T., Uhlin-Hansen, L., Combarnous, Y., Jankovic, M., Maric, S. C., Misulovin, Z., Nussenzweig, M. C., and Ten, F. (2000) *J. Exp. Med.* **191,** 1117–1126
16. Liu, Y., Chirino, A. J., Misulovin, Z., Leteux, C., Feizi, T., Nussenzweig, M. C., and Bjorkman, P. J. (2000) *J. Exp. Med.* **191,** 1105–1116
17. Isacke, C. M., van der Geer, P., Hunter, T., and Trowbridge, I. S. (1990) *Mol. Cell. Biol.* **10,** 2606–2618
18. Sheikh, H., Yarwood, H., Ashworth, A., and Isacke, C. M. (2000) *J. Cell Sci.* **113,** 1021–1032
19. Behrendt, N., Jensen, O. N., Engelholm, L. H., Mortz, E., Mann, M., and Dano, K. (2000) *J. Biol. Chem.* **275,** 1993–2002
20. Greenwood, F. C., Hunter, W. M., and Glover, J. S. (1963) *Biochem. J.* **89,** 114–123
21. Fornstedt, N., and Porath, J. (1975) *FEBS Lett.* **57,** 187–191
22. Papkoff, H., and Gan, J. (1970) *Arch. Biochem. Biophys.* **136,** 522–528
23. Ghrayeb, J., Kimura, H., Takahara, M., Hsiung, H., Masui, Y., and Inouye, M. (1984) *EMBO J.* **3,** 2437–2442
24. Drickamer, K. (1992) *Nature* **360,** 183–186
25. Weis, W. I., Drickamer, K., and Hendrickson, W. A. (1992) *Nature* **360,** 127–134
26. Graves, B. J., Crowther, R. L., Chandran, C., Rumberger, J. M., Li, S., Huang, K. S., Presky, D. H., Familletti, P. C., Wolitzky, B. A., and Burns, D. K. (1994) *Nature* **367,** 532–538
27. Burrows, L., Iobst, S. T., and Drickamer, K. (1997) *Biochem. J.* **324,** 673–680
28. Mitchell, D. A., Fadden, A. J., and Drickamer, K. (2001) *J. Biol. Chem.* **276,** 28939–28945
29. Mullin, N. P., Hall, K. T., and Taylor, M. E. (1994) *J. Biol. Chem.* **269,** 28405–28413
30. Weis, W. I., Crichlow, G. V., Murthy, H. M., Hendrickson, W. A., and Drickamer, K. (1991) *J. Biol. Chem.* **266,** 20678–20686
31. Wragg, S., and Drickamer, K. (1999) *J. Biol. Chem.* **274,** 35400–35406
32. Ng, K. K., Drickamer, K., and Weis, W. I. (1996) *J. Biol. Chem.* **271,** 663–674
33. Nicolas, J. P., Lambeau, G., and Lazdunski, M. (1995) *J. Biol. Chem.* **270,** 28869–28873
34. Taylor, M. E., and Drickamer, K. (1993) *J. Biol. Chem.* **268,** 399–404
35. Wileman, T. E., Lennartz, M. R., and Stahl, P. D. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 2501–2505
36. Lee, S. J., Evers, S., Roeder, D., Parlow, A. F., Risteli, J., Risteli, L., Lee, Y. C.,

Feizi, T., Langen, H., and Nussenzweig, M. C. (2002) *Science* **295,** 1898–1901
37. Napper, C. E., Dyson, M. H., and Taylor, M. E. (2001) *J. Biol. Chem.* **276,** 14759–14766
38. Engelholm, L. H., Nielsen, B. S., Dano, K., and Behrendt, N. (2001) *Trends Cardiovasc. Med.* **11,** 7–13
39. Nielsen, B. S., Rank, F., Engelholm, L. H., Holm, A., Dano, K., and Behrendt, N. (2002) *Int. J. Cancer* **98,** 656–664
40. St Croix, B., Rago, C., Velculescu, V., Traverso, G., Romans, K. E., Montgomery, E., Lal, A., Riggins, G. J., Lengauer, C., Vogelstein, B., and Kinzler, K. W. (2000) *Science* **289,** 1197–1202
41. Irjala, H., Johansson, E. L., Grenman, R., Alanen, K., Salmi, M., and Jalkanen, S. (2001) *J. Exp. Med.* **194,** 1033–1042
42. Lew, D. B., Songu-Mize, E., Pontow, S. E., Stahl, P. D., and Rattazzi, M. C. (1994) *J. Clin. Invest.* **94,** 1855–1863
43. Linehan, S. A., Martinez-Pomares, L., Stahl, P. D., and Gordon, S. (1999) *J. Exp. Med.* **189,** 1961–1972
44. Shepherd, V. L., Tarnowski, B. I., and McLaughlin, B. J. (1991) *Invest. Ophthalmol. Vis. Sci.* **32,** 1779–1784
45. Martinez-Pomares, L., Crocker, P. R., Da Silva, R., Holmes, N., Colominas, C., Rudd, P., Dwek, R., and Gordon, S. (1999) *J. Biol. Chem.* **274,** 35211–35218
46. Weis, W. I., Kahn, R., Fourme, R., Drickamer, K., and Hendrickson, W. A. (1991) *Science* **254,** 1608–1615
47. Drickamer, K. (1993) *Curr. Opin. Struct. Biol.* **3,** 393–400

# Characterization of Sugar Binding by the Mannose Receptor Family Member, Endo180

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