Cell-specific Glycoforms of Sialoadhesin and CD45 Are Counter-receptors for the Cysteine-rich Domain of the Mannose Receptor*

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We previously reported that CR-Fc, an Fc chimeric protein containing the cysteine-rich (CR) domain of the mannos receptor, binds to marginal zone metallophilic macrophages (Mø) and B cell areas in the spleen and to subcapsular sinus Mø in lymph nodes of naive mice (CR-Fc+) cells. Several CR-Fc ligands were found in spleen and lymph node tissue lysates using ligand blots. In this paper we report the identification of two of these ligands as sialoadhesin (Sn), an Mø-restricted membrane molecule, and the leukocyte common antigen, CD45. CR-Fc bound selectively to Sn purified from spleen and lymph nodes and to two low molecular weight isoforms of CD45 in a sugar-dependent manner. CR-Fc binding and non-binding forms of Sn, probably derived from CR-Fc+ and CR-Fc− cells respectively, were selected from spleen lysates. Analysis of the glycan pool associated with the CR-Fc-binding form revealed the presence of charged structures resistant to sialidase, absent in the non-binding form, that could correspond to sulfated structures. These results confirm the identification of the CR region of the mannos receptor as a lectin. We also demonstrate that the same glycoprotein expressed in different cells of the same organ can display distinct sugar epitopes that determine its binding properties.

The mannos receptor (MR) is a type I membrane glycoprotein that mediates the uptake of mannansylated glycoconjugates by macrophages (Mø), dendritic cells (DCs), and hepatic endothelium (1–5). It is defined as a pattern recognition receptor due to its ability to bind sugar structures not normally found in the extracellular milieu but abundant in microorganisms (1). Carbohydrate recognition takes place through eight C-type lectin domains located in the extracellular region. These carbohydrate recognition domains are preceded by two poorly characterized domains, a cysteine-rich (CR) domain located at the amino terminus of the MR and a domain containing fibronectin type II repeats (6–9). A search for ligands for these two domains in mouse tissues led to the identification of several cell subpopulations able to bind a chimeric protein containing the CR domain of the murine form of the receptor fused to the Fc region of human IgG1 (CR-Fc) (10). These CR-Fc+ cells included marginal zone metallophilic Mø (MZMø) and subcapsular sinus Mø in spleen and lymph nodes (LN) of naive animals and, probably, follicular dendritic cells in immunized animals. During the course of an immune response CR-Fc+ cells with dendritic morphology appeared to migrate in draining LN through the follicles and interfollicular areas. Based on these data, an antigen transport pathway (highlighted by CR-Fc labeling) and an alternative role for the MR in antigen uptake and presentation were proposed (5, 10, 11).

A first step toward the interpretation of those results is the identification of the molecules that interact with CR-Fc. Preliminary data indicated the presence of several ligands in spleen and LN lysates with apparent molecular mass ranging from 360 to 100 kDa (10). This study presents the identification of two of these ligands as sialoadhesin (Sn), an Mø-restricted membrane molecule (12, 13), and CD45 (14, 15). Biochemical characterization of these interactions showed that CR-Fc binding to both molecules is sugar-dependent. These results indicate that the CR domain of the MR itself has lectin-like activity and that the different ligands recognized in tissue lysates could be different proteins expressed by CR-Fc+ cells sharing the same sugar structure. These findings provide additional evidence for the important role that tissue-specific post-translational modifications play in determining the function of a molecule and raise the possibility that Sn or CD45 may display different binding properties in different cells.

EXPERIMENTAL PROCEDURES

Animals

Balb/c mice were bred at the Sir William Dunn School of Pathology and were used at 8–10 weeks of age. CD45+/− mice (16) were bred at the Department of Pathology, University of Cambridge.

Antibodies, Cell Lines, and Chimeric Proteins

3D6 and SER-4 (anti-Sn) (17), YBM42.2.2 (pan anti-CD45) (18), and F4/80 (19) (specific for an Mø plasma membrane differentiation marker) were prepared in our laboratory. FDC-M2 (20) was kindly provided by Marie Kosco-Vilbois. 9B12 is a stable transfectant cell line expressing full-length sialoadhesin (21). CR-Fc (10), Sn 1-Fc (22), and MAG 1–5-Fc (23) were prepared as described.

Purification of CR-Fc Ligands

The procedure used was based on the method described by Williams and Barclay (24). Spleens from different mouse strains (100 g) were...
homogenized using a Polytron in 10 mM Tris-HCl, pH 8, 2.5% (v/v) Tween 40, 150 mM NaCl, 10 mM NaN₃, 2 mM EDTA and the following protease inhibitors: 5 mM iodoacetamide, 2 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin, and 1 μM leupeptin. Nuclei were removed by centrifugation at 500 × g for 15 min at 4 °C, and the supernatant was centrifuged at 35,000 rpm for 60 min at 4 °C using a 60Ti rotor. The membrane pellet was washed twice in 10 mM Tris-HCl, pH 8, 10 mM NaCl, 150 mM NaCl, 2 mM EDTA containing protease inhibitors and solubilized with stirring in 2 liters of 10 mM Tris-HCl, pH 8, 2% (v/v) Triton X-100, 10 mM NaN₃, 150 mM NaCl, 2 mM EDTA with protease inhibitors. The lysate was clarified by centrifugation at 35,000 rpm for 60 min at 4 °C in a 60Ti rotor and protein content was determined using bovine serum albumin coupled to protein A-Sepharose (10 ml, 2 mg/ml) (Amersham Pharmacia Biotech) prepared as described elsewhere (25). The lysate was then applied to a CR-Fc-protein A-Sepharose column (10 ml, 2 mg/ml). After extensive washing in 10 mM Tris-HCl, pH 8, 2% (v/v) Triton X-100, 150 mM NaCl (450 ml), bound material was eluted using 0.5% (v/v) diethylamine in 2% (v/v) Triton X-100 into tubes containing 1 mM Tris-HCl, pH 7 (1/10 of final volume). 2-ml fractions were collected and 10-μl aliquots were tested for CR-Fc binding activity by dot blot.

**Immunoprecipitation from Protein Lysates**

Tissue and cell lysates were prepared in lysis buffer (10 mM Tris-HCl, pH 8, 2% (v/v) Triton X-100, 10 mM NaCl, 2 mM EDTA) with protease inhibitors, clarified by centrifugation at 2,000 rpm for 5 min at 4 °C in a tabletop centrifuge to remove nuclei and at 55,000 rpm for 30 min at 4 °C in a Beckman TC100 centrifuge to remove insoluble material. Immunoprecipitation was performed using protein A-Sepharose for 2 h at 4 °C. After pre-clearance, lysates were incubated with 3D6-Sepharose or YMB422.2-Sepharose (5 mg Ab/ml, Amersham Pharmacia Biotech), prepared as described (24). Beads were washed in lysis buffer or in washing buffers A, B, and C (A: 10 mM Tris-HCl, pH 8, 500 mM NaCl, 0.5% (w/v) deoxycholate, 0.5% (v/v) Triton X-100, 0.05% (w/v) Tween 20 in 0.1% (v/v) Triton X-100; B: 10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.5% (w/v) deoxycholate, 0.5% (v/v) Triton X-100, 0.05% (w/v) SDS; C: 10 mM Tris-HCl, pH 8, 0.05% (w/v) SDS), and bound material was eluted with 0.5% (v/v) diethylamine in 1% (v/v) Triton X-100 or (w/v) octyl glucopyranoside, as described previously, or by boiling in 10 mM Tris-HCl, 0.05% (w/v) SDS.

**Western and Ligand Blot**

Analysis of CR-Fc binding and Sn and CD45 detection were performed following standard Western blot protocols. Briefly, samples (total tissue lysates or immunoprecipitated material) were resuspended in SDS-PAGE sample buffer (10 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 20% (v/v) glycerol, 0.001% (w/v) bromophenol blue), boiled, electroforesed in a 5 or 6% SDS-PAGE, as indicated, and transferred to nitrocellulose (Hybond C plus; Amersham Pharmacia Biotech). Filters were blocked in 5% (w/v) non-fat milk, 0.1% (v/v) Tween 20 in phosphate-buffered saline and incubated with primary antibodies conjugated to digoxigenin or a chemiluminescence kit (ECL, Amersham Pharmacia Biotech) prepared as described elsewhere (25). The lysate was then solubilized with stirring in 2 liters of 10 mM Tris-HCl, pH 8, 2.5% (v/v) Triton X-100, 10 mM NaN₃, 150 mM NaCl, 2 mM EDTA containing protease inhibitors and eluted using 0.5% (v/v) diethylamine in 0.5% (v/v) Triton X-100 as described previously.

**Sugar Analysis of the CR-Fc-binding and Non-binding Isoform of Sialoadhesin**

**Sugar Extraction—N-Glycans** were released from purified Sn by incubation with peptide N-glycosidase F (Oxford GlycoSystems Ltd.) as described (26). Briefly, purified Sn was electrophoresed under reducing conditions, visualized by Coomassie staining, excised from the gel, alkylated and treated with peptide N-glycosidase F to release the N-linked glycans.

**Fluorescent Labeling of the Oligosaccharide-reducing Terminus with 2-Aminoobenzamide (2-AB)—2-AB labeling was carried out using the Oxford GlycoSystems Signal labeling kit (27).**

**Weak Anion Exchange (WAX) Chromatography—** WAX was performed using a GlycoSep-C column (Oxford GlycoSystems Ltd.) (7 × 50 mm) attached to a Waters HPLC system as described previously (28). Simultaneous Oligosaccharide Sequencing of the Released Glycan Pool—2-AB-labeled glycan pools were aliquoted and dried using a vacuum centrifugal concentrator. Each aliquot was incubated with a different enzyme array. Conditions for the individual enzymes in the array were as follows: Arthrobacter ureafaciens sialidase (Oxford GlycoSystems Ltd.), 1–2 units/ml; bovine testes galactosidase (Oxford GlycoSystems Ltd.), 1–2 units/ml; bovine kidney fucosidase (Oxford GlycoSystems Ltd.) 1–2 units/ml. Enzyme digests were performed at 37 °C for 16–24 h in 100 mM citrate/phosphate buffer, pH 4.5, 0.2 mM zinc acetate, 150 mM NaCl. After incubation, enzymes were removed by filtration through nitrocellulose and analyzed by normal phase HPLC and described (29).

**Analysis of the Glycan Pool and Subsequent Digestion by Normal Phase Chromatography (NP-HPLC)—** NP-HPLC was performed using a Glycosep-N column as described (29) using 50 mM ammonium formate, pH 4.4, as solvent A and acetonitrile as solvent B. The column was calibrated in glucose units using 2-AB-labeled dextran hydrolysate. Structures were assigned from the NP-HPLC elution positions by comparison with previously determined sialidase (Oxford GlycoSystems Ltd.) converted glycans. The addition of monosaccharide residues to glycan cores (29) and the results of digests of each glycan pool using arrays of exoglycosidases (30).

**Ex Vivo Metabolic Labeling with ³⁵S Sodium Sulfate**

Spleens were cut into small pieces using a sterile surgical blade and incubated in RPMI media (ICN Biomedicals Ltd., Thame, UK) containing 2% dialyzed fetal calf serum (Life Technologies, Inc.), 10 mM l-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin ( RPMI, 2% fetal calf serum) for 30 min at 37 °C on a rotary shaker. Labeling was performed by incubating spleens fragments in RPMI, 2% fetal calf serum containing ³⁵S Sodium Sulfate (ICN Biomedicals, Ltd., 250 μCi/ml, 1 ml per spleen) at 37 °C for 4 h in the absence or presence of NaClO, as described in the presence of 0.5% (v/v) Triton X-100, pH 8, 10 mM NaCl, 150 mM NaCl, 2 mM EDTA containing protease inhibitors, transferred to a tissue grinder, and homogenized. Membrane proteins were extracted by adding 10% (v/v) Triton X-100, 10 mM Tris-HCl, pH 8, 10 mM NaCl, 150 mM NaCl, 2 mM EDTA with protease inhibitors (1/5 of total volume) and incubating the samples at 4 °C for 30 min. Lysates were processed for immunoprecipitation as described previously.

**RESULTS**

**Sn Is the Major Component of the CR-Fc Ligands**

Preparation Obtained by Affinity Chromatography

By using the chimeric protein CR-Fc, several putative ligands for the CR domain of the MR were detected in spleen and peripheral (p) LN lysates by ligand blot analysis (10). To characterize these molecules, affinity chromatography was performed using CR-Fc coupled to protein A-Sepharose and spleen lysates. By using the ligand(s) source (see “Experimental Procedures”) CR-Fc ligands were eluted at high pH and collected in 2-ml fractions. Most CR-Fc binding activity was found in fractions 4 and 5, but activity was also present in fractions 3 and 6. A major band of 180 kDa apparent mass on non-reducing SDS-PAGE was found in fractions 4 and 5 after Coomassie Blue staining (Fig. 1A, –DTT). Additional proteins could be detected by silver stain (data not shown). Ligand blot analysis of these fractions confirmed that the 180-kDa protein bound CR-Fc and...
The specificity of the purification procedure confirmed that Sn was the major component.

Coomassie staining of 30 μl of pooled fractions 4 and 5 electrophoresed under reducing (+ DTT) and non-reducing conditions (− DTT) and ligand blot analysis (CR-Fc binding) of CR-Fc ligands present in fractions 4 and 5, electrophoresed under non-reducing conditions. Digoxigenin-labeled CR-Fc and anti-digoxigenin antibody were used for detection. B, purified Sn binds CR-Fc. Sn selected from CR-Fc ligand preparation using 3D6-Sepharose (lane 1) and Sn (450 ng) purified from spleen using standard affinity chromatography (lane 2) were electrophoresed under non-reducing conditions, transferred to nitrocellulose, and probed with MAG1–5-Fc, Sn1-Fc, or CR-Fc as described under “Experimental Procedures.” CR-Fc bound to Sn from both sources in an Fc-independent manner.

The CR-Fc binding specific activity of Sn from pLN is higher than that from spleen. This result correlates with the data obtained by immunohistochemistry, since the percentage of Sn+, CR-Fc+ cells is higher in LN.

N-Glycosidase Treatment of Sn Abrogates CR-Fc Binding

Sn migrated as a broad band on SDS-PAGE indicating that it consisted of a range of glycoforms (data not shown). To determine the role that glycosylation might play in the interaction of Sn with CR-Fc, Sn immunoprecipitated from spleen, pLN, and thio-Mø + NMS protein lysates was treated with peptide N-glycanase F, to remove N-linked glycans and tested for the ability of the deglycosylated protein to bind CR-Fc. As shown in Fig. 2B, the removal of N-linked sugars completely abolished the ability of Sn to bind CR-Fc compared with control samples incubated in the same buffer but without the enzyme. Under these conditions the binding of 3D6 (an antibody that recognizes a conformation-dependent epitope) was reduced in the deglycosylated sample but was still detectable, indicating that N-glycans play a role in maintaining the structure of Sn. To test how CR-Fc binding was affected by protein conformation, Sn from pLN was reduced and alkylated and used in the binding assay. Under these conditions, the epitope recognized by 3D6 was eliminated, but CR-Fc binding, although reduced, could still be observed indicating that this interaction was maintained after the disruption of disulfide bonds (Fig. 2C).

Identification of CD45 as a Putative Ligand for CR-Fc

In addition to Sn, other CR-Fc ligands were present in the preparation obtained by affinity chromatography (Figs. 1 and 3). In an attempt to identify these molecules, the preparation of CR-Fc ligands was probed by Western blot with different mAbs. One of these Ab was YMB42.2.2, a rat IgG that recognizes all isoforms of CD45 (18). As shown in Fig. 3, YMB42.2.2 recognized a 100-kDa band that is strongly labeled by CR-Fc and two minor bands of about 180 kDa (see Fig. 4A for a better resolution). This result indicated that CD45 was present in this preparation but in a mostly degraded form that could have originated from two of the low molecular weight isoforms. Confocal analysis of splenic CR-Fc+ cells showed that they express CD45 (data not shown). To assess the contribution of CD45 to CR-Fc binding, spleen and LN from CD45+/− mice (16) were tested by immunohistochemistry as described (10). CR-Fc la-
Fig. 3. Identification of CD45 as a putative ligand for CR-Fc. A, CD45 is present in the preparation of CR-Fc ligands. 3 μl of fraction 3, pooled fractions 4 and 5 (4/5) and fraction 6, were probed with CR-Fc, 3D6 (α-Sn), and YBM42.2.2 (α-CD45) as described under “Experimental Procedures.” Most CR-Fc binding activity was detected in pooled fractions 4 and 5 which presented a complex binding pattern. In fractions 3 and 6 most binding activity was restricted to a 180-kDa band (probably Sn). Several forms of Sn (36D+ bands) were observed in sample 4/5. A major 100-kDa band and two minor 180-kDa bands were recognized by the anti-pan CD45 antibody in the same sample. The 100-kDa band could have originated by proteolytic cleavage of the two 180-kDa bands that correspond to two low molecular weight isoforms of CD45. B, pattern of CR-Fc binding to spleen lysates from CD45+/− and wild type (wt) mice. Total protein lysates from spleens of CD45+/− (2 mice, 132 μg, 165 μg) and wild type mice (2 mice, 120 μg, 105 μg) were tested for CR-Fc binding and for the presence of Sn and CD45 as described under “Experimental Procedures.” Two CR-Fc+ bands that co-migrated with low molecular weight isoforms of CD45 were absent in lysates of CD45−/− mice (indicated by arrows). Higher Sn expression was detected in the spleens from CD45−/− mice.

Selected Isoforms of CD45 Bind CR-Fc in a Sugar-dependent Manner

To test direct binding of CR-Fc to CD45, spleen and pLN lysates were immunoprecipitated using YBM42.2.2-Sepharose, as described under “Experimental Procedures,” and bound proteins were eluted using diethylamine or by boiling in 0.5% SDS. Early experiments showed that Sn co-precipitated with CD45 (data not shown) and that, for the satisfactory resolution of the different CR-Fc+ bands, samples had to be electrophoresed in a 5% SDS-PAGE gel. To establish the contribution that the presence of Sn could have on CR-Fc binding, an additional precleavage step was introduced in the immunoprecipitation protocol; after incubation with protein A-Sepharose, lysates were precleared with 3D6-Sepharose. CR-Fc binding was tested as described under “Experimental Procedures,” and the results are shown in Fig. 4A. Two low molecular weight isoforms of CD45 (~180 kDa) bound CR-Fc, and this interaction was independent of the presence of Sn. Peptide N-glycanase F treatment confirmed the sugar requirement for CR-Fc binding to CD45 (Fig. 4B).

Fig. 4. CR-Fc binds selected isoforms of CD45 in a sugar-dependent fashion. A, CR-Fc binding to CD45 immunoprecipitated from pLN lysates. pLN lysates were immunoprecipitated with 3D6-Sepharose (a) or YBM42.2.2-Sepharose (b and c), with (b) or without (c) a precleaving step using 3D6-Sepharose. Immunoprecipitated material was electrophoresed under non-reducing conditions, transferred to nitrocellulose, and probed with digoxigenin-labeled 3D6 (α-Sn) (10 μg/ml), CR-Fc (10 μg/ml), or YBM42.2.2 (5 μg/ml) (α-CD45). Binding was detected by incubation with peroxidase-conjugated anti-digoxigenin Fab, anti-human Fc Fab′/’, CR-Fc, or anti-rat IgG (α-CD45). In the sample immunoprecipitated with 3D6-Sepharose (a), CR-Fc recognized a single band corresponding to Sn, and no CD45 could be detected. In samples b and c, which contained undetectable (b) or trace (c) levels of Sn, CR-Fc recognized two bands (~180 kDa) that correspond to the low molecular weight isoforms of CD45 (indicated by arrows). Note that rat IgG is recognized in samples a–c by the anti-rat IgG secondary antibody (3rd panel). B, CR-Fc binding to CD45 is sugar-dependent. CD45 immunoprecipitated from pLN tissue lysates was incubated overnight in the absence (−) or in the presence (+) of PNGase F and probed for CR-Fc or YBM42.2.2 binding (α-CD45), as described under “Experimental Procedures.” Treatment of CD45 with N-glycosidase increased its mobility in SDS-PAGE and abrogated its ability to interact with CR-Fc.

CR-Fc Binding and Non-binding Forms of Sn and CD45 Can Be Selected from Spleen Lysates

To confirm the data obtained by immunoprecipitation, Sn and CD45 were purified from spleen lysates as described under “Experimental Procedures,” and CR-Fc binding and non-binding forms were separated using a CR-Fc-protein A-Sepharose column. Briefly, 0.5-ml fractions containing purified Sn (4–11) or CD45 (7–23) were pooled, diluted in 0.5% Triton X-100, 140 mM NaCl, 10 mM Tris-HCl, pH 8, containing protease inhibitors to 25 and 50 ml, respectively, and applied to an 800-μl CR-Fc-protein A-Sepharose column (2 mg CR-Fc/ml). After extensive washing in lysis buffer containing 0.5% (v/v) Triton X-100, bound proteins were eluted using 0.5% (v/v) diethylamine in 0.5% (v/v) Triton X-100. Collected fractions (binding forms, Sn-b and CD45-b) and flow-through (non-binding forms, Sn-nb and CD45-nb) were analyzed by Coomassie Blue staining (not shown) and tested for CR-Fc and antibody binding (Fig. 5). Approximately 50% of Sn present in spleen bound to CR-Fc in agreement with the high expression of Sn detected in CR-Fc+ MZM/Ms. Two forms of Sn can be detected in the unbound fraction, a CR-Fc non-binding glycoform (upper band) and probably an unglycosylated precursor (lower band). As expected from the fact that most splenic cells are CD45+ and a minor proportion is CD45+ CR-Fc+, only a small fraction of the total CD45 present in spleen-bound CR-Fc (data not shown). Fractions containing the CR-Fc binding forms of CD45 were enriched in the lower isoforms (~180 kDa). These results confirm the specificity of the selection procedure and the results obtained by immunoprecipitation.

Comparison of N-Glycan Pools Associated with Binding and Non-binding Forms of Sn

The preparations of Sn used in this study include the initial preparation obtained by CR-Fc affinity chromatography shown...
flow-through (non-binding forms) were collected, analyzed by Western affinity chromatography, as described under "Experimental Procedures," and fractionated into CR-Fc binding and non-binding forms using CR-Fc-protein A-Sepharose. Eluted fractions (binding forms) and flow-through (non-binding forms) were collected, analyzed by Western blot using digoxigenin-labeled 3D6 (α-Sn) or YBM42.2.2 (α-CD45), and tested for CR-Fc binding by ligand blot. Binding was detected using peroxidase-conjugated anti-digoxigenin F(ab')2 (α-Sn), anti-rat IgG (α-CD45), or anti-human Fc F(ab')2, respectively.

in Fig. 1 (a), and the CR-Fc-binding (b) and non-binding forms (c) of Sn described in the previous section. Similar results were obtained with samples a and b (data not shown).

Analysis of the N-Linked Glycan Pools Released from Sn-b by NP-HPLC—The 2-AB-labeled N-glycan pool released from Sn-b was resolved by NP-HPLC (Fig. 6). 14 major peaks were assigned on the basis of their elution positions, measured in glucose units, obtained by comparison with the elution profile of a series of oligomers from a standard dextran hydrolysate and from previously determined incremental values for the addition of monosaccharides residues to glyccan cores (29). The preliminary assignments were confirmed by exoglycosidase digestion of aliquots of the glyccan pool (30). Each assigned peak eluted at its predicted position following digestion of the entire pool with the specific exoglycosidases shown in each panel. The effect of the enzyme arrays on 4 specific sugars (A2G2FS2, A4G4, A3G3FS, A3G3FS2) is illustrated by the arrows. Further digestions with arrays containing almond meal α-mannosidase, or Streptococcus pneumoniae β-N-acetylhexosaminidase indicated that there are no significant amounts of outer arm fucose residues nor are there polylactosamine or oligomannose structures (data not shown). Thus Sn-b contained a range of bi-, tri-, and tetra-antennary complex type sugars most of which were core fucosylated and sialylated. The major structure was the disialylated bi-antennary core fucosylated glycan A2G0F. The enzyme arrays in panel iv of Fig. 6 digests all non-substituted unsimplified complex sugars to the core glycans A2G0, A3G0, and A4G0. Glycans that are not digested to these core structures, including unassigned peaks in panels i–iii, could contain sulfated sugars. WAX analysis of Sn-b glycan pool + Abs (Fig. 7, WAX, Sn-b, pool B') suggests that these structures might be monosulfated since their elution profile corresponds to those with charge equivalent to 1 sialic acid residue. 

Analysis of Sn-b and Sn-nb Glycans on the Basis of Charge Using WAX HPLC—2-AB-labeled N-glycan pools of Sn-b (A–C) and Sn-nb (D–F) untreated (A–F) or treated with sialidase (A’–F’) were separated according to charge by WAX chromatography (Fig. 7, WAX). The column was calibrated with fetuin standard sugars (neutral, mono- (S1), di- (S2), and tri- (S3)sialylated). Three pools were collected from each sample: A, A’, D, and D’, 0–5 min, contained neutral polysaccharide; B, B’, E and E’, 5–20 min, contained charged glycans eluting at positions equivalent to mono- to tri-sialylated structures; and C, C’, F and F’, 20–30 min, containing putative highly charged structures. Subsequent analysis of each pool by NP-HPLC chromatography revealed that almost all of the material in pools A and A’ consisted of a contaminant polymer (data not shown).

NP-HPLC analysis of pool B confirmed that most N-linked glycans in Sn-b were charged. Most of the material in pools C and F was non-carbohydrate. Only Sn-b-derived glycans in pool C contained any glycans when the pools were analyzed by NP-HPLC, and these were a minor component (data not shown).

Whereas most of the peaks observed in pool E (5–20 min, from Sn-nb) disappeared after sialidase digestion (Fig. 7, NP-HPLC, Sn-nb, compare panels E and E’), two major components in the corresponding fraction from Sn-b were resistant to this enzyme (Fig. 7, NP-HPLC, Sn-b, compare B and B’). This was consistent with the possibility that terminally sulfated glycans were major components of the Sn-b glycan pool (compare Fig. 6, ii, abs treatment, and Fig. 7, NP-HPLC, Sn-b, panel B’). In contrast, NP-HPLC analysis of pool E’ confirmed that the majority of the charged sugars present in pool E from Sn-nb contained sialic acid. Accordingly, an increase in the amount of neutral sugars was observed after sialidase digestion of this sample (data not shown).
Sulfation of Sn Correlates with CR-Fc Binding

Analysis of Sn-b and Sn-nb N-linked glycan pools suggested the presence of sulfated structures in Sn-b. To investigate this possibility, Sn was immunoprecipitated from $^{35}$SO$_4$-labeled spleen protein lysates, prepared as described under “Experimental Procedures,” and fractionated into Sn-b and Sn-nb using CR-Fc-protein A-Septahose. In agreement with the sugar analysis described in the previous section, $^{35}$SO$_4$ was only incorporated into Sn-b (Fig. 8A, left panel). This labeling could be inhibited in the presence of chlorate, an inhibitor of ATP-sulfurylase, the first enzyme in the synthesis of 3’-phosphoadenosine 5’-phosphosulfate, the high energy donor of sulfate (31) (Fig. 8B). These results suggest that sulfated structures in Sn-b mediate binding to the CR domain of the MR.

DISCUSSION

In this study we present the identification of two of the counter-receptors recognized by the CR domain of the MR in secondary lymphoid organs, Sn and CD45. Neither of these proteins is exclusively expressed by CR-Fc+ cells. Sn is an Ms-specie specific surface molecule highly expressed by MZM and subcapsular sinus Ms (CR-Fc+ cells) but also present in splenic red pulp Ms, medullary Ms in LN, and bone marrow stromal Ms (CR-Fc+) (17, 33). CD45 is present in all nucleated cells of hemopoietic origin (14, 15). Characterization of CR-Fc binding to the isolated proteins by ligand blotting demonstrated that this chimeric protein is able to discriminate between proteins obtained from different sources (in the case of Sn) and between the different isoforms of CD45 present in spleen and LNs. These results suggested that CR-Fc recognized a post-translational modification and underlined the correlation between the ligand blot and in situ labeling studies. Based on this it can be predicted that only the CR-Fc+ cells detected in situ are able to modify Sn and CD45 to generate ligands for CR-Fc. The fact that only low molecular weight isoforms of CD45 bind to CR-Fc is consistent with the MR binding in spleen and LNs since these are the forms found in cultured Ms in vitro.2 CR-Fc binding is lost after peptide N-glycosidase F digestion and, at least in the case of Sn, is structure-independent. These data confirm that the CR domain of the MR itself has lectin-like binding activity (34) and indicate that cell-specific glycosyltransferase(s), responsible for the transformation of Sn and CD45 (among others) into CR-Fc ligands, are present in CR-Fc+ cells. The influence of site of synthesis on the binding properties of a molecule has been previously described. Only the form of GlyCAM 1 expressed by endothelial cells of peripheral and mesenteric lymph nodes contains the sulfate-modified carbohydrate required for L-selectin binding. Different carbohydrate modifications have been found in GlyCAM 1 expressed by lactating mammary gland epithelial cells, a non-binding form (35).

Inhibition assays were performed in an attempt to define the sugar moiety recognized by the CR domain of the MR. CR-Fc

\[^2\] L. Martínez-Pomares, unpublished observations.
binding (2 μg/ml) to tissues could not be competed for by t-mannose (10 mM), mannann (1 mg/ml), fucose (10 mM), D-galactose (10 mM), N-acetylglucosamine (10 mM), 6’N-acetylneuramin-lactose (1 mM), 3’N-acetylneuramin-lactose (1 mM), or dextran sulfate (10 mg/ml) (data not shown). Only the presence of fucoidan (10 mg/ml) inhibited this interaction, although the large amounts of sugar required and the undefined components present in this reagent make interpretation difficult. Involvement of sialic acid in this system was ruled out by the ability of Sn treated with sialidase from Vibrio choleræ to bind CR-Fc (data not shown).

Fiete et al. (34) recently reported that the CR domain of the MR domain recognizes the sugar structure SO4-4-GalNAcβ1,4GlcNAcβ1,2Manα- present in lutropin and other glycoprotein hormones (36). The two enzymatic activities required for this modification have been found in several bovine tissues including spleen (37). Analysis of the glycan pool associated with the CR-Fc binding and non-binding forms of Sn is compatible with these data since it revealed the presence of putative sulfated structures in the Sn-b isoform that could be responsible for MR CR domain recognition (Figs. 7 and 8). The putative sulfated structures in the Sn-b isoform that could be compatible with these data since it revealed the presence of putative sulfated structures in the Sn-b isoform that could be responsible for MR CR domain recognition (Figs. 7 and 8). The motif required for recognition by the PXIKR-specific GalNAc-transferase (38) is not present in Sn or CD45. Sn and CD45 do not account for all the CR-Fc ligands detected by ligand blots on total tissue lysates (10) (Figs. 1 and 3A). Affinity purification on Sn-, CD45-depleted lysates should yield additional counter-receptors.

In agreement with the reported presence of CR domain ligands in glycoprotein hormones produced in anterior pituitary (34), CR-Fc labeled isolated cells in this organ (data not shown), and our CR-Fc preparation bound to bovine lutropin-Sepharose in a salt-dependent, Ca2+-independent fashion (data not shown). These results emphasize the close correlation between tissue labeling and biochemical analysis.

Sn in MZMMøs and subcapsular sinus Ms is a major membrane molecule thought to be involved in cell-cell adhesion (12, 17, 39). The presence of Sn in CD45 preparations is suggestive of an interaction between these molecules (Fig. 4A, left panel). No evidence for signal transduction motifs is found in the cytoplasmic tail of Sn (12), but the intracellular region of CD45 contains a tyrosine phosphatase domain (14, 15). These results open the possibility for signal transduction in CR-Fc+ cells following binding of the CR domain of the MR.

Glycoprotein hormones secreted by CR-Fc+ cells in anterior pituitary could be endocytosed by MR+ cells in liver (sinosoidal endothelium or Kupffer cells) through the recognition of SO4-4-GalNAcβ1,4GlcNAcβ1,2Manα- by the CR domain of the MR (40). In the case of the spleen and LN, the two ligands found for this domain (Sn and CD45) are integral membrane proteins for which no cleaved, soluble forms have been described. We proposed a soluble form of the MR (sMR), found in Ms-conditioned media and mouse serum (11), as the counter-ligand for the specialized forms of Sn and CD45 (among others) present in the CR-Fc+ cells of secondary lymphoid organs. This interaction could mediate delivery of native antigen to follicular areas (10). Assessment of the biological relevance of these findings will require the characterization of the CR-Fc+ cells in situ and in vitro, study of the effects that sMR or CR-Fc have on their endocytic and phagocytic activity, and analysis of their interaction with other cell populations (MR− or MR+).

In this study we provide further evidence for the important role that post-translational modifications play in modulating protein function. MR has been shown to undergo proteolytic cleavage to release a soluble form (11), and the binding properties of its CR domain seem to be influenced by the site of synthesis (liver versus lung) (40) probably due to differential post-translational modification(s). Accordingly, a heterogeneous population of MR molecules with different binding activities was obtained in CHO cells transfected with MR-specific cDNA (41). These results highlight the versatility of the MR and the importance of in vivo studies to unveil its multiple roles in immunological and physiological processes.

Ligands for the CR domain of the MR are themselves produced by post-translational modification(s) that take place in specific cell populations located in anterior pituitary (secreted glycoprotein hormones), secondary lymphoid organs (membrane-bound glycoforms of Sn and CD45 among others), and thymus (5). In particular, the characterization of the glycan pools associated with the CR-Fc binding and non-binding forms of Sn demonstrate that the same glycoprotein expressed in different cells of the same organ can display distinct sugar epitopes that determine its binding properties.

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Ligands for the Cys-rich Domain of the Mannose Receptor

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Cell-specific Glycoforms of Sialoadhesin and CD45 Are Counter-receptors for the Cysteine-rich Domain of the Mannose Receptor
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