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Characterization of a 180 kDa molecule apparently reactive with recombinant L-selectin

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In the present study we identified a 180 kDa molecule (p180) in rat lymph nodes (LN) apparently reactive with silkworm derived recombinant L-selectin (LEC-IgG) in a Ca^{2+}-dependent manner. Analysis of amino acid sequence revealed that p180 has a strong homology to the macrophage mannose receptor (MMR), which was corroborated by the observation that p180 reacted with polyclonal anti-alveolar MMR antibody and mannosyl-BSA-agarose. In agreement with this notion, the binding of p180 to the silkworm LEC-IgG was inhibited by α-methyl-D-mannoside. However, in sharp contrast to its reactivity against the silkworm LEC-IgG, p180 failed to bind LEC-IgG produced by COS-7 cells, suggesting that p180 reacted with the silkworm LEC-IgG through the recognition of oligomannose-type oligosaccharides expressed on the silkworm products and that the lectin activity of L-selectin was not involved in the interaction. These results, together with the immunohistochemical studies showing that p180 was absent from the majority of high endothelial venules (HEV) but present in medullary macrophages, led us to conclude that p180 obtained from LN lysates by the use of the silkworm LEC-IgG is not a physiological ligand for L-selectin, warning against the use of recombinant proteins expressed in the baculovirus/silkworm expression system for the detection of carbohydrate ligands.

***Keywords*: baculovirus, L-selectin, lymph node, mannose receptor**

***Abbreviations*: BSA, bovine serum albumin; EDTA, ethylenediamine-*N,N,N′,N′*-tetraacetic acid; ELISA, enzyme-linked imunosorbent assay; HEV, high endothelial venule; IgG, immunoglobulin G; LN, lymph node; NP-40, Nonidet P-40; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; PVR, poliovirus receptor; SDS, sodium dodecylsulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; sLe^x, sialyl Lewis X; WGA, wheat germ agglutinin**

Introduction

Lymphocyte migration from bloodstream into the secondary lymphoid organs is initiated by adhesive interactions between lymphocyte homing receptors and their respective ligands on HEV. L-selectin was initially identified as a lymphocyte homing receptor and shown to be important in lymphocyte migration into the peripheral lymph nodes [1]. Subsequently, L-selectin was also found to be expressed on other types of leukocytes and involved in rolling interactions of leukocytes in the post-capillary venules of inflamed tissues [2–4]. The study in L-selectin-deficient mice also confirmed the important role of L-selectin in lymphocyte homing to peripheral lymph nodes and leukocyte migration to the sites of inflammation [5, 6].

Like other members of the selectin family, the extracellular domain of L-selectin consists of an N-terminal calcium-dependent lectin domain followed by an epidermal growth factor-like domain and then a short consensus repeat (SCR) similar to those found in complement regulatory proteins [7, 8]. So far, studies on the carbohydrate-based ligands for L-selectin have mostly focused on the molecules expressed on HEV. The production of antibodies to murine HEV [9, 10] and the construction of a chimeric L-selectin-human immunoglobulin G fusion protein (LEC-IgG) [11] allowed the molecular identification of four L-selectin ligands [12–14]. Two of the four ligands were first identified by Imai *et al.* [15] who precipitated two sulfated glycoproteins of 50 and 90 kDa, designated sulfated glycoprotein (Sgp)50 and Sgp90, using LEC-IgG as a specific probe. Sgp50 was subsequently cloned and designated GlyCAM-1 [12], while Sgp90 was characterized as the sialomucin-like glycoprotein, CD34 [13]. Both glycoproteins were recognized by MECA-79 antibody which recognizes carbohydrate-based epitopes on HEV and inhibits lymphocyte binding to HEV *in vitro* and lymphocyte migration to the peripheral lymph nodes *in vivo* [10]. In other studies, both LEC-IgG and MECA-79 antibody were found to recognize an independent molecule of 200 kDa, designated Sgp200, in a sulfation-dependent manner [16]. It was suggested that the secreted

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form of GlyCAM-1 and Sgp200 act as modulators of cell adhesion, and that cell associated CD34 and Sgp200 mediate the attachment of lymphocytes to HEV [17]. However, it is still not clear whether these ligands are sufficient to explain the specificity of lymphocyte trafficking to the peripheral lymph nodes *in vivo*. Another ligand MAdCAM-1 was originally identified as an antigen recognized by MECA-367 antibody [9], which intensely stains all HEVs in Peyer's patches but fails to react with most HEVs in peripheral (axillary brachial, popliteal and inguinal) lymph nodes. cDNA cloning of the MAdCAM-1 molecule revealed that it has multiple functional domains [14], which apparently enable MAdCAM-1 to react with $\alpha 4\beta 7$ integrin, a Peyer's patch-specific homing receptor, as well as L-selectin [18].

We have reported the cDNA cloning of rat L-selectin [19], construction of recombinant fusion protein consisting of the entire extracellular domain of the rat L-selectin molecule attached to the Fc domain of human immunoglobulin G_1 (LEC-IgG), expression of LEC-IgG in the baculovirus/silkworm expression system, and identification of several glycoprotein species of 55, 65, 120, 190 and > 250 kDa in rat lymph nodes as possible L-selectin ligands [20]. These findings prompted us to continue our search for yet unidentified ligands for L-selectin in rat lymph nodes and investigate the biological significance of interaction between L-selectin and its ligands. In the present study, we purified a 180 kDa molecule apparently reactive with recombinant soluble L-selectin (LEC-IgG) expressed in the baculovirus/silkworm expression system and identified it as a rat macrophage mannose receptor homologue. We will herein discuss the significance of our observation and warn against the use of recombinant proteins expressed in the baculovirus/silkworm expression system in the study of lectin-carbohydrate interactions.

Materials and methods

Reagents

A recombinant soluble form of rat L-selectin (LEC-IgG) has been described previously [20]. Unless otherwise indicated, LEC-IgG was expressed in the baculovirus/silkworm expression system and purified from the haemolymph supernatants of the infected silkworms by protein A-Sepharose 4 FF (Pharmacia LKB, Uppsala, Sweden). Human poliovirus receptor (PVR)-IgG expressed in the baculovirus/silkworm expression system was a kind gift from J. Aoki, Faculty of Pharmaceutical Sciences, Tokyo University (Tokyo, Japan). CD2-IgG and CD44-IgG expression plasmids were provided by Dr B. Seed, Massachusetts General Hospital (Boston, MA) and expressed in COS-7 cells according to the method of Aruffo *et al.* [21]. Rat P-selectin-IgG was provided by Dr A.M. Manning, Upjohn Co. (Kalamazoo, MI). The rabbit polyclonal antibody reactive against rat alveolar macrophage mannose receptor [22] was a kind gift from Drs R.L. Hill and A. Eckhardt, Duke University Medical Center (Durham, NC). Bovine serum albumin (BSA) and mannosyl-bovine serum albumin (Man-BSA) were purchased from Sigma (St Louis, MO). Both proteins were immobilized on CNBr-activated Sepharose 4B (Pharmacia) at a level of 2 mg protein per ml gel. All other reagents were of analytical grade.

Isolation of p180

The experimental protocol was approved by the Animal Care Committee of our institution. Eight- to 12-week-old male specific pathogen free Wistar rats were used in these experiments. They were injected subcutaneously with fetal bovine serum emulsified with incomplete Freund's adjuvant (3:1) into the footpads to induce peripheral LN swelling. Eight to 10 days after the priming, the brachial, axillary, inguinal, popliteal, cervical, para-aortic and mesenteric LN were collected from 40 rats. These LN were minced with a scalpel blade and gently pressed between glass microscopic slides to squeeze out lymphocytes. The resulting minced tissue was suspended in 400 ml of ice-cold phosphate-buffered saline (PBS) and kept on ice for 15 min. The supernatant containing a large number of lymphocytes was discarded, and the remaining stromal elements were washed again with 400 ml of ice-cold PBS. The obtained stromal elements were solubilized with 200 ml of the lysis buffer (PBS containing 1% NP-40, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM PMSF(phenylmethylsulfonyl fluoride)). After 4 h at 4 °C, the lysates were centrifuged at $31\,000 \times \mathbf{g}$ for 40 min at 4 °C followed by filtration using Whatman 3 MM chromatography paper (Whatman International Ltd, Maidstone, England) and the supernatants were passed through WGA (wheat germ agglutinin)-coupled (5 mg protein per ml gel, 5 ml) Sepharose column (HONEN Co., Tokyo, Japan). To assess the recovery of each step, biotinylated p180, as described below, was included during the purification. After washing with buffer A (PBS containing 0.05% Tween 20, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM PMSF), the bound material was eluted with 0.5 M *N*-acetylglucosamine in buffer A. Fractions were collected at every 3 ml. This chromatographic run was repeated four times to recover completely the WGA-bound material from the lysates. After the four separate runs, WGA-eluates were combined and dialysed against 3 l of buffer B (0.05% Tween 20, 0.15 M NaCl, 10 mM $CaCl_2$, 10 mM $MgCl_2$, 1 mM PMSF, 20 mM Tris-HCl, pH 7.6) and applied to 3 ml of covalently cross-linked LEC-IgG-Protein A-Sepharose (1.5 mg protein per ml gel) column prepared with dimethyl pimelimidate (Pierce Chemical Co.), as previously reported [12, 23]. The column was washed with 40 ml of buffer B followed by further wash with 40 ml of buffer C (0.1% n-octyl-β-D-glucopyranoside (octylglucoside, OG), 0.15 M NaCl, 10 mM $CaCl_2$, 10 mM $MgCl_2$, 1 mM PMSF, 20 mM Tris-HCl, pH 7.6). Then, proteins

bounded to the column were eluted with 15 ml of buffer D (0.1% OG, 50 mM EDTA, 0.15 M NaCl, 1 mM PMSF, 20 mM Tris-HCl, pH 7.6), at a flow rate of 1 ml min^{-1}. Fractions were collected every minute. This chromatographic run was repeated seven times because of the low capacity of the affinity column. A 30 µl aliquot of each fraction was analysed by SDS-PAGE under non-reducing conditions on a 7.5% SDS-polyacrylamide gel according to the method of Laemmli [24] and silver-stained. Fractions containing p180 were combined and concentrated using Centricon-30 microconcentrator (Amicon Inc., Beverly, MA) and re-analysed by SDS-PAGE. As estimated by silver staining and comparison to standard proteins, about 2–3 µg of p180 was recovered.

N-terminal sequencing of p180

Purified p180 (2–3 µg) was subjected to SDS-PAGE on a single lane of 5% SDS-gel under reducing conditions, eletroblotted onto an IPVH filter (NIHON Millipore Ltd, Yonezawa, Japan) and stained with Coomassie Brilliant Blue R-250 (Bio-Rad, Hercules, CA). A 180 kDa band of the blot was cut out and subjected to gas-phase microsequencing using model 492 protein sequencer (Perkin-Elmer Co., Foster City, CA).

Preparation of biotinylated p180

Lymph node stromal fractions from 10 primed rats were solubilized and passed through a WGA-agarose column as described above. The bound material (about 250 µg ml^{-1}; 10 ml) was dialysed against two changes of 3 l of buffer E (0.05% Tween 20, 0.1 M NaCl, 0.1 mM PMSF, 0.1 M $NaHCO_3$, pH 8.0) and coupled with 250 µg ml^{-1} of NHS-LC-biotin (Pierce Chemical Co., Rockford, IL) for 4 h. The reaction was quenched by the addition of a quarter volume of 1 M Tris-HCl, pH 7.6. The biotinylated material was dialysed against 3 l of buffer B and applied to the covalently cross-linked LEC-IgG-protein A-Sepharose column. The column was extensively washed with buffer B, and the bound material containing biotinylated p180 was eluted with 0.05% Tween 20, 10 mM EDTA, 0.15 M NaCl, 1 mM PMSF, 20 mM Tris-HCl, pH 7.6, and stored at − 80 °C until use.

Preparation of biotinylated rat alveolar macrophage mannose receptor

A flexible silicon tubing was inserted into the bronchus of each rat, and 5 ml of warm saline was instilled in aliquots into the lung and recovered by gentle aspiration through the silicon tubing connected to a disposable 10 ml syringe. The procedure was repeated ten times and the aliquots pooled in a 50 ml centrifuge tube. The obtained bronchoalveolar lavage (BAL) fluid from two rats was centrifuged at $200 \times \mathbf{g}$ (1000 rpm) for 10 min at 4 °C. The cell pellet was washed twice with PBS and suspended in 2 ml of PBS containing 50 µg ml^{-1} NHS-LC-biotin at a final cell density of 3×10^6 cells ml^{-1}. After incubation at 4 °C for 1 h, the reaction was quenched by washing the cells four times with 0.14 M NaCl, 20 mM Tris-HCl (pH 7.6). The cell pellet was solubilized with 1 ml of the lysis buffer (1% NP-40, 0.14 M NaCl, 10 mM $CaCl_2$, 10 mM $MgCl_2$, 1 mM PMSF, 20 mM Tris-HCl, pH 7.6) followed by centrifugation at $20\,000 \times \mathbf{g}$ for 40 min at 4 °C to remove NP-40 insoluble material. When the supernatant was passed directly through Man-BSA agarose column, biotinylated mannose receptor was not retained in the column, suggesting that unlabelled intracellular glycoproteins with high mannose-type oligosaccharides were bound to the biotinylated mannose receptor during the cell lysis. To reconcile this problem, the supernatant was pretreated with EDTA at a final concentration of 50 mM at 4 °C for 10 min followed by the addition of 150-fold volume of buffer B. The resultant material was passed immediately through Man-BSA-agarose column followed by extensive washing with buffer B. The biotinylated rat alveolar macrophage mannose receptor was eluted with 0.05% Tween 20, 50 mM EDTA, 0.15 M NaCl, 1 mM PMSF, 20 mM Tris-HCl, pH 7.6, and stored at − 80 °C until use.

Immunoprecipitation

Biotinylated material obtained from the above procedure was incubated with protein A-Sepharose (10 µl gel) coupled with 10 µg of LEC-IgG or other chimeric proteins, rabbit anti-alveolar macrophage mannose receptor antibody or normal rabbit IgG, or with CNBr-activated Sepharose 4B (10 µl gel) coupled with Man-BSA or BSA (2 mg ml^{-1} gel), in 1 ml of buffer B at 4 °C overnight. The matrix was washed four times with 1 ml of Buffer B and boiled in 25 µl of reducing Laemmli sample buffer. The samples were then subjected to SDS-PAGE using 4–20% gradient gels (Daiichi Pure Chemicals Co. Ltd, Tokyo, Japan) and transferred onto an IPVH filter, as described above. Non-specific binding of proteins was blocked by incubating blots in PBS containing 3% BSA and 0.1% NaN_3 at room temperature for 2 h. The blots were washed in PBS containing 0.05% Tween 20, followed by detection using an ABC-kit (Vector, Burlingame, CA) and ECL Western blotting detection reagents (Amersham, Buckinghamshire, England) according to the instructions provided by the manufacturer.

Enzyme-linked immunosorbent assay (ELISA)

Affinity purified goat anti-human IgG antibody (10 µg ml^{-1} in PBS, 50 µl per well) was added to 96-well flat-bottom microtitre plates (Sumilon MS-8596F; Sumitomo Bakelite Co. Ltd, Tokyo, Japan) overnight at 4 °C. The wells were washed three times with PBS and blocked with 200 µl of 3% BSA-PBS for 2 h at room temperature. Wells were flicked empty and incubated with various concentrations of LEC-IgG or control IgG (50 µl per well) diluted with buffer B for 2 h at room temperature. In the next step, the wells were washed three times with buffer B and incubated with 50 µl

of 5 μg ml^{-1} biotinylated sialyl Lewis^X polymeric-probe (sLe^X BP-probe; Seikagaku Corp., Tokyo, Japan) diluted with buffer B. After washing four times with buffer B, peroxidase-conjugated streptavidin (HRP-streptavidin; Zymed, South San Francisco, CA) diluted 1:500 (50 μl) was added and incubated for 60 min at room temperature followed by five washes with buffer B. Fifty μl per well of o-phenylenediamine (0.4 mg ml^{-1}) in 50 mM sodium citrate-100 mM Na_2HPO_4, pH 5.6 containing 0.012% H_2O_2 was added, and the color was developed for 10 min at room temperature. After the reaction, 50 μl per well of 8 N H_2SO_4 was added to each well to terminate the reaction and the plate was read at 490 nm in a microtiter plate reader (InterMed Co. Ltd, Tokyo, Japan).

Preparation of anti-peptide antibodies

All peptides used were synthesized by Dr M. Tomita, Faculty of Pharmaceutical Sciences, Showa University (Tokyo, Japan). Peptide 1 (CGVEDIISGASGTVR) was derived from a region near the C-terminus of rat GlyCAM-1 (25). Peptide 2 (RQFLIYNEDHKRC) and peptide 3 (QDSRQFLIYNEDHKRC) were derived from the N-terminal sequence of p180. Peptide 2 was identical to human and mouse macrophage mannose receptors [26, 27] while peptide 3 had a unique sequence for p180. Each peptide was coupled to keyhole limpet hemocyanin (KLH; Sigma) through the added cystein residue. Aliquots containing 100 μg of peptides coupled to KLH were emulsified with equal volumes of complete Freund's adjuvant (Sigma) and injected into New Zealand White male rabbits (Oriental Yeast Co. Ltd, Osaka, Japan). Fourteen days after injection, aliquots containing the antigen (100 μg peptides coupled to KLH) were emulsified with equal volumes of TiterMax research adjuvant (CytRx Co., Norcross, GA) and injected. Booster injection with incomplete Freund's adjuvant was administered on days 28 and 42 before collection of antisera on day 45. Each antibody was purified from the antisera using FMP-Cellulofine (Seikagaku, Tokyo, Japan) coupled with the peptides. Antibody titres were determined by ELISA using each peptide as immobilized antigen, and the purified antibody was stored at $-80\,^{\circ}C$ in PBS.

Immunohistochemical staining

Mesenteric LN of unprimed male Wistar rats (12 week old) were collected and fixed in PBS containing 4% paraformaldehyde at 4 °C for 4 h. Fixed tissues were then dehydrated and immersed in polyester wax [28]. The sections were cut at 6 μm thick, dewaxed and blocked by PBS containing 3% BSA. Polyclonal antibodies against the peptides as described above, diluted in PBS containing 0.1% BSA, 1 mM $CaCl_2$, 1 mM $MgCl_2$, were added to the tissue sections and incubated at room temperature for 30 min. The sections were washed in several changes of PBS containing 0.02% Tween 20, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and endogenous peroxidase was quenched with 1% H_2O_2 in methanol for 30 min. The sections were then rinsed in PBS containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, incubated with peroxidase-conjugated goat anti-rabbit IgG (American Qualex Co., San Clemente, CA) diluted 1:500 at room temperature for 30 min and washed three times in PBS containing 0.02% Tween 20, 1 mM $CaCl_2$, 1 mM $MgCl_2$. The colour was developed using a DAB substrate kit (Pierce, Rockford, IL). Sections were rinsed in distilled water, counterstained with Haematoxylin and mounted in EUKITT mounting reagent (O. Kindler, Germany).

Results

Purification of an L-selectin binding material p180

In an attempt to obtain L-selectin ligands in rat LN, we carried out sequential column chromatography using WGA-Sepharose and LEC-IgG-protein A-Sepharose columns based on the report by Lasky *et al.* [12]. LN including brachial, axillary, inguinal, popliteal, cervical, para-aortic and mesenteric LN were collected from 40 rats that had been challenged with FCS in incomplete Freund's adjuvant. The stromal fractions of the LN were solubilized in a lysis buffer containing 1% NP-40. The crude extracts were passed through WGA-Sepharose column to obtain sialic acid-containing glycoproteins, and the bound material was collected (Figure 1A). This chromatographic step was repeated four times, resulting in a 38-fold purification with more than 90% recovery rate, as assessed later by the recovery of biotinylated p180 (a major L-selectin binding material to be discussed herein) which was included in the purification step.

The WGA-bound material was then applied to a LEC-IgG-protein A-Sepharose column and the bound material eluted by EDTA (Figure 1B). This chromatographic run was repeated seven times, which resulted in a 100-fold purification with a recovery rate of approximately 10%.

SDS-PAGE analysis (Figure 2) of the eluted material showed migration of a major protein, designated p180, as a sharp band at 160 kDa under non-reducing conditions (lane 3) and at 180 kDa under reducing conditions (lane 6). The p180 was bound to protein A-beads coated with LEC-IgG (lane 4) but not to protein A-beads coated with control IgG (lane 5), indicating that p180 specifically interacted with LEC-IgG. Although p180 was the only major band detected in most cases, a broad smear between 45 and 60 kDa and a second band at 105 kDa were observed occasionally.

Identification of p180 as the rat mannose receptor homologue

We then attempted to characterize the N-terminal amino acid sequence of p180, and found that p180 was highly homologous to the murine macrophage mannose receptor [27], with an exact match of 13 out of the 15 NH_2-terminal

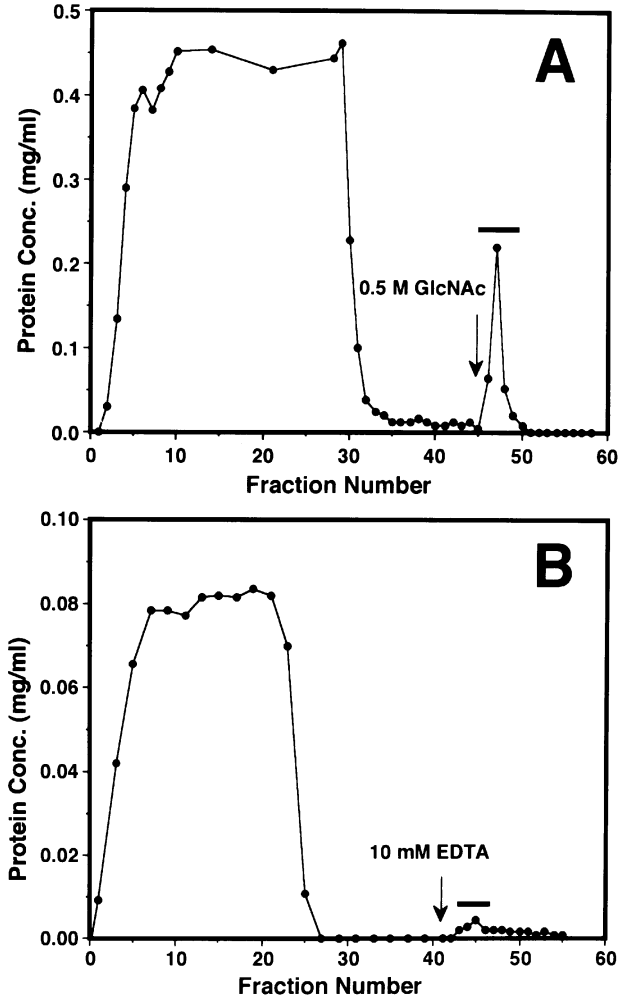

Figure 1. Elution patterns for chromatographic steps in the purification of p180. (A) Elution profile of a WGA-Sepharose column. NP-40 extracts from rat LN were loaded onto a WGA-Sepharose column equilibrated with PBS containing 0.05% Tween 20, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM PMSF (buffer A). Arrow indicates a change to buffer A containing 0.5 M GlcNAc. Thick bar: pooled fractions. (B) Elution profile of a LEC-IgG-Protein A-Sepharose column. WGA-bound pool was sequentially loaded onto a LEC-IgG-protein A-Sepharose column equilibrated with 0.05% Tween 20, 0.15 M NaCl, 10 mM $CaCl_2$, 10 mM $MgCl_2$, 1 mM PMSF, 20 mM Tris-HCl, pH 7.6. Arrow indicates a change to a buffer containing 0.1% octylglucoside, 50 mM EDTA, 0.15 M NaCl, 1 mM PMSF, 20 mM Tris-HCl, pH 7.6. Fractions indicated by the thick bar were pooled and concentrated as described in Materials and methods.

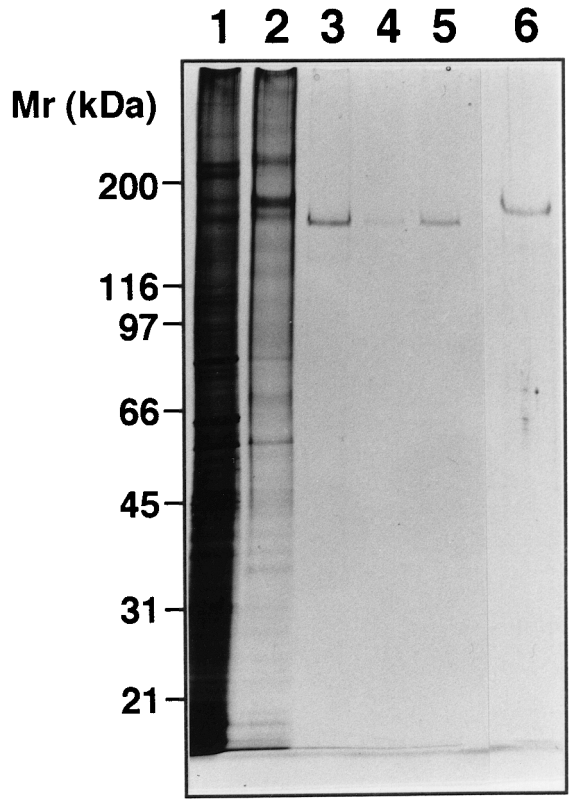

Figure 2. Analysis of aliquots from each step of purification of p180 by SDS-PAGE. Samples were separated on a 4–20% gradient gel in the absence (lanes 1–5) or presence of 2-mercaptoethanol (lane 6) and silver-stained. The molecular mass of standards is shown on the left. Lane 1, crude extracts from rat lymph nodes (1.48 μg); lane 2, WGA-Sepharose column bound pool (0.78 μg); lanes 3–6, LEC-IgG-protein A-Sepharose bound pool (0.02 μg) before (lanes 3 and 6) and after absorption with 7.5 μl of protein A-beads coated with 7.5 μg of LEC-IgG (lane 4) or with 7.5 μg of human IgG (lane 5).

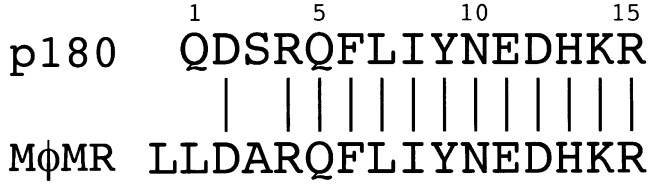

Figure 3. Comparison of NH_2-terminal amino acid sequences. Comparison of the NH_2-terminal sequence of p180 with deduced NH_2-terminus of murine macrophage mannose receptor (MϕMR) revealed an exact match of 13 out of 15 residues (indicated by the vertical lines).

residues (Figure 3). Comparison with the NH_2-terminus of the human macrophage mannose receptor [26] also revealed an exact match of 13 out of the 15 residues, suggesting that p180 was a rat homologue of the macrophage mannose receptor. Macrophage mannose receptors in human [26] and mouse [27] have a molecular size of 175–180 kDa similar to p180. Furthermore the alveolar macrophage mannose receptor purified from rats also has a comparable molecular size of 180 kDa [22]. The p180 purified in the present study reacted specifically with polyclonal anti-rat alveolar macrophage mannose receptor antibody (Figure 4C), adding further evidence that p180 is a rat homologue of the macrophage mannose receptor.

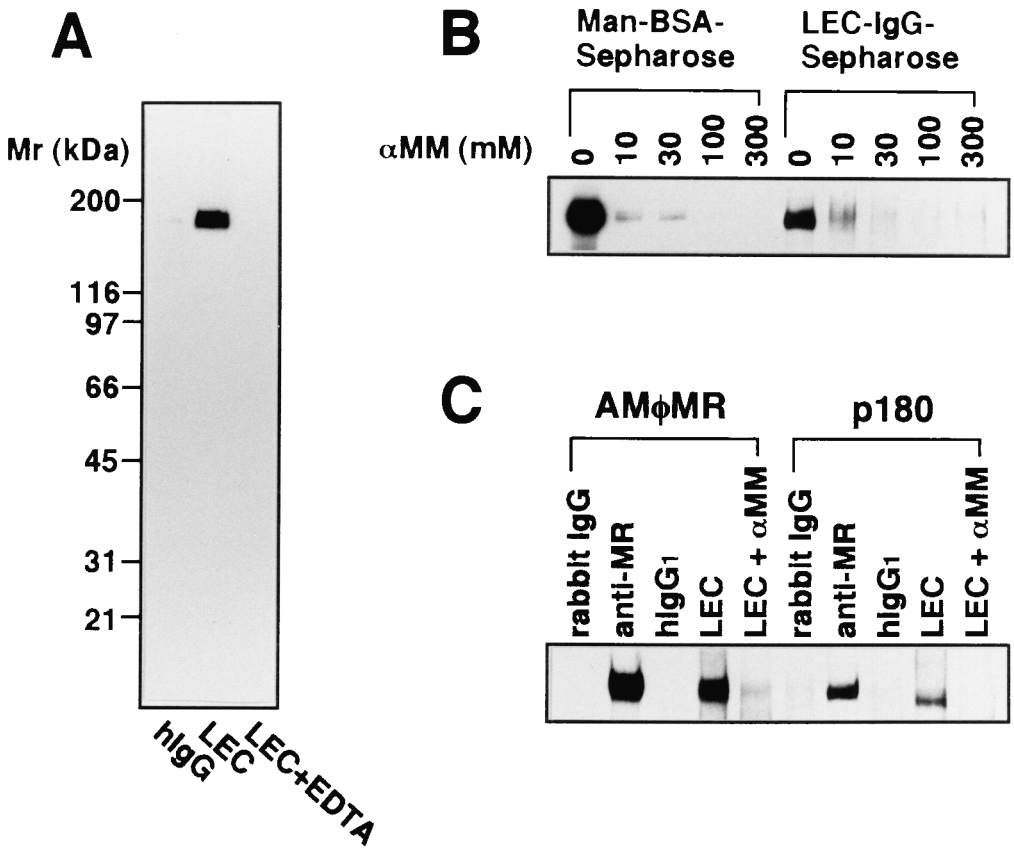

Figure 4. Immunoprecipitation analysis of biotinylated p180. (A) Biotinylated p180 was reacted with protein A-Sepharose coated with human IgG (hIgG) or LEC-IgG (LEC) in the absence or presence of 10 mM EDTA (LEC + EDTA). (B) Biotinylated p180 was reacted with CNBr-activated Sepharose coupled with mannose-BSA (Man-BSA) or protein A-Sepharose coupled with LEC-IgG in the absence or presence of indicated concentrations of *α*-methyl-D-mannoside. (C) Biotinylated rat alveolar macrophage mannose receptor (AMϕMR) and p180 were reacted with protein A-Sepharose coated with normal rabbit IgG (rabbit IgG), anti-alveolar macrophage mannose receptor antibody (anti-MR), human IgG_1 ($hIgG_1$) or LEC-IgG (LEC) in the absence or presence of 100 mM *α*-methyl-D-mannoside (*α*-MM). The precipitates were solubilized with Laemmli sample buffer under reducing conditions, subjected to SDS-PAGE on a 4–20% gradient gel and electroblotted, followed by detection with an ABC-kit and ECL western blotting detection reagents.

Involvement of lectin activity of p180 in binding to LEC-IgG

In the next step, we determined the ability of p180 to recognize mannose-containing glycoproteins. For this purpose, p180 was biotinylated and examined for its binding ability in precipitation analysis. As shown in Figure 4A, biotinylated p180 was able to bind to LEC-IgG-Sepharose, a process inhibited by 10 mM EDTA. Biotinylated p180 was also able to bind to Man-BSA-Sepharose, which was inhibited by EDTA (data not shown) as well as α-methyl-D-mannoside in a dose-dependent manner (Figure 4B, left). These findings indicate that p180 itself is a mannose-binding lectin. In support of this conclusion, the binding between p180 and immobilized LEC-IgG was also specifically inhibited by the addition of α-methyl-D-mannoside (Figure 4B, right), suggesting that p180 binds to LEC-IgG through its own lectin activity.

Inability of p180 to recognize LEC-IgG produced in COS cells

To further investigate the biochemical basis of the interaction between p180 and LEC-IgG produced in the baculovirus/silkworm expression system, we examined the binding of p180 to various recombinant proteins including LEC-IgG produced in COS cells. To our surprise, p180 was found to be reactive only with the baculovirus/silkworm-derived LEC-IgG but not with LEC-IgG produced in COS cells (Figure 5A lanes 3 and 4). Both LEC-IgG preparations were able to bind a polymeric sialyl Le^x-derivative equally well (Figure 6), indicating that the failure of p180 binding to COS LEC-IgG was not due to loss of the lectin activity of COS cell-derived LEC-IgG. P180 also failed to react with soluble CD2, CD44 and P-selectin produced in COS cells (Figure 5A, lanes 5 to 7). These results suggest that the oligomannose-type oligosaccharides specifically expressed

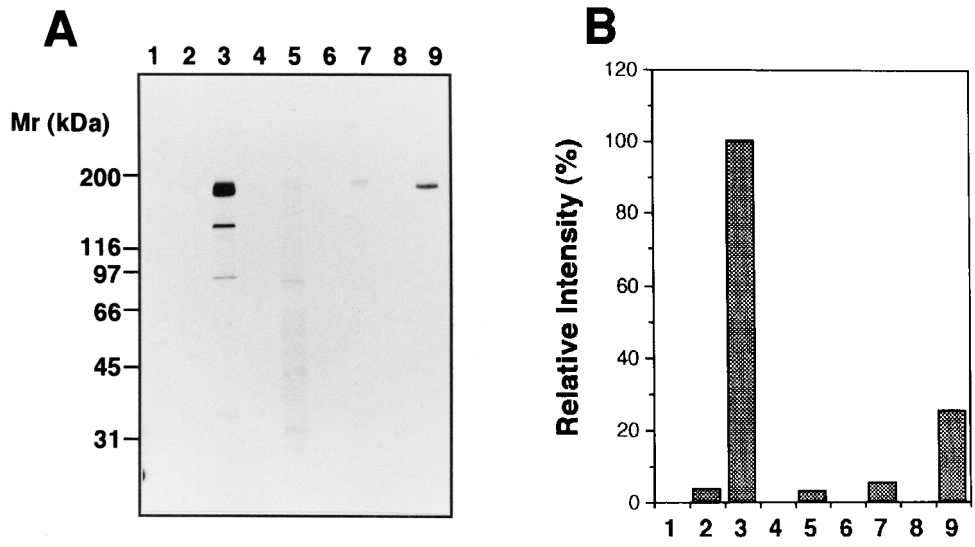

Figure 5. Immunoprecipitation analysis of p180 with various recombinant globulins. (A) Biotinylated p180 molecules were incubated with protein A-Sepharose loaded with either 10 μg of human IgG_1 (lane 1), PVR-IgG (lane 2), LEC-IgG (lane 3), LEC-IgG (lane 4), CD2-IgG (lane 5), CD44-IgG (lane 6), P-selectin-IgG (lane 7), normal rabbit IgG (lane 8) or rabbit anti-alveolar macrophage mannose receptor antibody (lane 9). Fusion proteins were expressed in the baculovirus/silkworm expression system (lanes 2 and 3) or COS-7 cells (lanes 4–7). The precipitates were analysed as described in the legend to Figure 4. (B) The relative intensity of the 180 kDa band in each lane was quantified using a Scanning Imager (Molecular Dynamics, Sunnyvale, CA) and shown as percentage of that obtained with the silkworm LEC-IgG. Each column number corresponds to the lane number in (A).

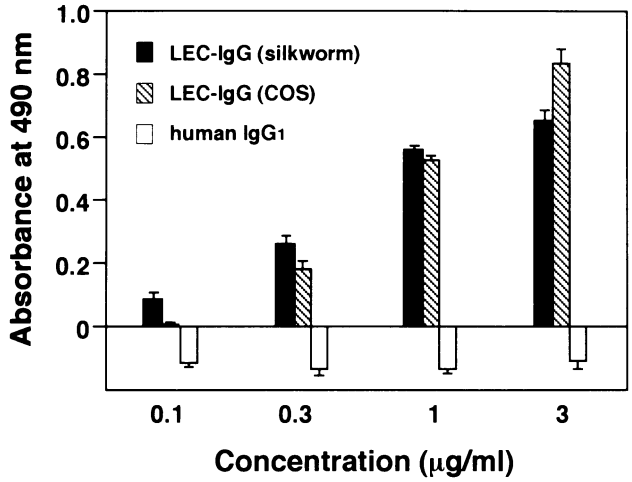

Figure 6. Binding of sLe^x BP-probe to LEC-IgG expressed in the baculovirus expression system or COS-7 cells. LEC-IgG expressed in the baculovirus/silkworm expression system (solid bar), LEC-IgG expressed in COS-7 cells (hatched bar) or human IgG_1 (open bar) at various concentrations were allowed to bind to the wells of microtitre plate coated with goat anti-human IgG, and then tested for sLe^x BP-probe binding (final concentration of 5 μg ml^{-1}) as described in Materials and methods. The abscissa indicates the optical density at 490 nm after subtraction of background value (sLe^x BP-probe not added). Bars indicate the standard deviations of triplicate determinations.

in the silkworm LEC-IgG are recognized by p180, which is a lectin itself, and that the lectin activity of LEC-IgG is not involved in the interaction. In support of this notion, neither blocking antibodies against L-selectin nor sialidase treatment affected the binding between LEC-IgG and p180 (data not shown).

P180 is absent in a majority of HEV in lymph nodes

To examine the location of p180 in LN, we prepared anti-peptide antibodies against p180 and performed immunohistochemical studies (Figure 7). When polyclonal antibodies against the N-terminal sequence of p180 were used (anti-peptide 2 and 3 antibodies), it was found that p180 was mainly localized in medullary macrophages and is absent in most of HEV. In some sections, although flat-walled venules as well as a small population of HEV appeared to be stained positively (data not shown), a large population of HEV were negative. In contrast, GlyCAM-1 was localized exclusively in HEV as revealed by the use of polyclonal antibody against the C-terminus of rat GlyCAM-1 (anti-peptide 1 antibody) as reported previously by Lasky *et al.* [12]. These results clearly demonstrate a lack of p180 in a majority of HEV and that it is mainly expressed in medullary macrophages in LN.

Discussion

Using sequential column chromatography, we have isolated p180 apparently reactive with L-selectin from rat LN lysates. The purification method used in the present study was similar to that used by Lasky and co-workers [12] except for the following differences. First, we used detergent extracts of LN stroma as the starting material to obtain the

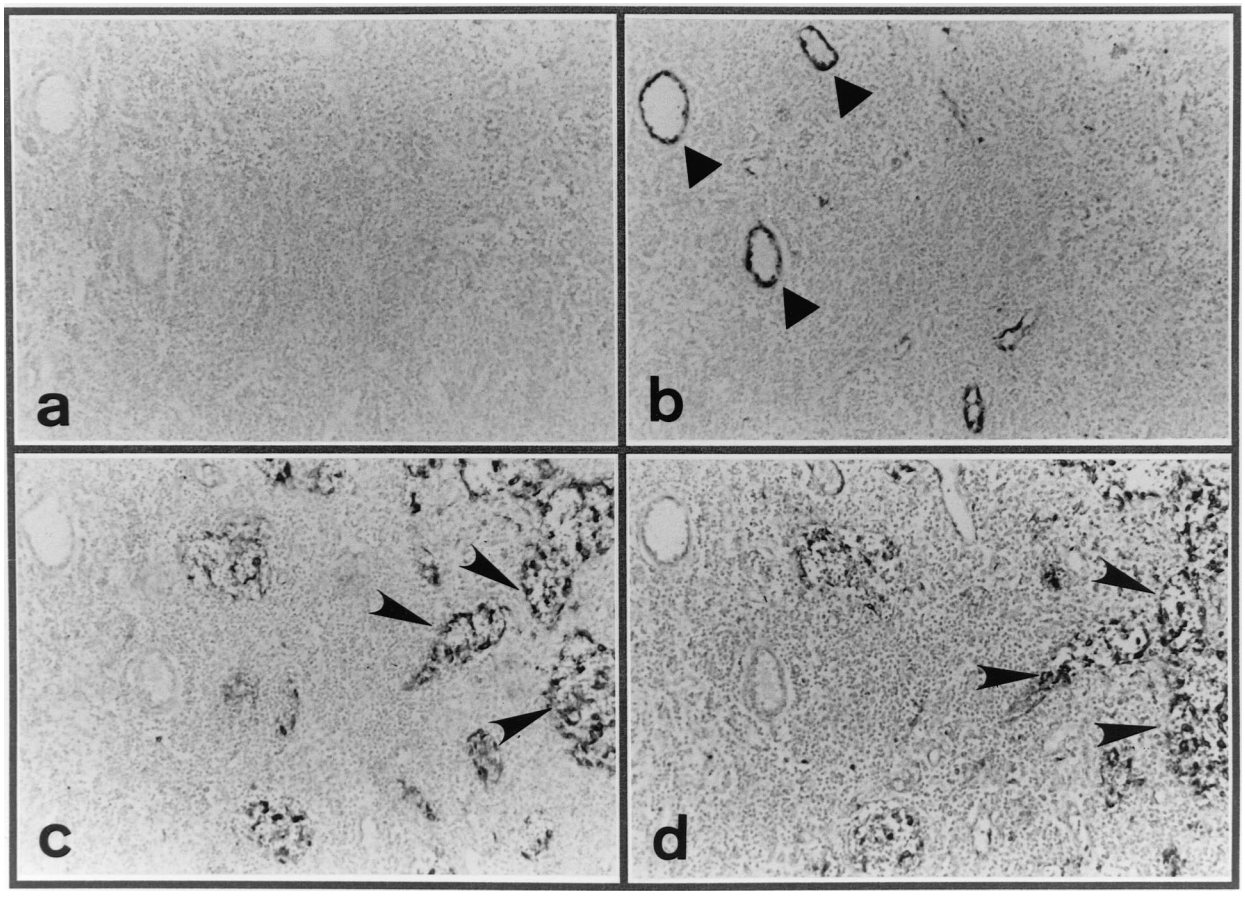

Figure 7. Immunohistochemical microphotographs of rat mesenteric lymph nodes using anti-peptide antibodies. Polyester wax sections of a rat mesenteric LN were incubated with 13 μg ml^{-1} of normal rabbit IgG (a), 2.5 μg ml^{-1} of anti-peptide 1 antibody (b), 13 μg ml^{-1} of anti-peptide 2 antibody (c) or 6 μg ml^{-1} of anti-peptide 3 antibody (d). They were subsequently treated with peroxidase-conjugated goat anti-rabbit IgG (1:500), as described in Materials and methods. The original magnification is ×100. Note that HEV (indicated by solid triangles) is positive for GlyCAM-1 but nagative for p180, whereas medullary macrophages (arrow heads) are positive for p180 but negative for GlyCAM-1.

membrane-bound ligands for L-selectin, while Lasky *et al.* used LN organ culture supernatants to purify the membrane-shed GlyCAM-1. Second, we used LEC-IgG generated in the baculovirus/silkworm expression system because of its high productivity of recombinant proteins, while Lasky *et al.* used LEC-IgG generated in COS cells.

Although a broad smear of 45–60 kDa and a second band at 105 kDa, possibly representing GlyCAM-1 and CD34, respectively, were occasionally detected in our preparations, p180 migrating at 180 kDa appeared consistently as a major band. As the 180 kDa glycoprotein was not identified in our earlier study using metabolically labelled material from rat LN with [^{35}S]-sodium sulfate [20], we initially thought p180 might be a novel type of L-selectin ligand without sulfation. As for selectin ligands with a comparable molecular size, a minor component of 170 kDa is known to be reactive with LEC-IgG and MECA-79 antibody in mouse LN [16] and a 150 kDa component with MECA-79 in human tonsils [29]. The MECA-79 recognizes carbohydrate-based epitopes on L-selectin ligands [16]. However, these molecules have not yet been characterized in detail. Another 170 kDa molecule called VAP-1 (vascular adhesion protein-1) has been reported by Salmi *et al.* [30] to be an HEV-associated sialoglycoprotein that mediates selective lymphocyte binding to HEV. However, a recent report by the same group indicates that it is not a ligand for L-selectin [31].

Determination of the partial amino acid sequence of p180 revealed that p180 is highly homologous to the murine macrophage mannose receptor (Figure 3). The observation that p180 was recognized by anti-alveolar macrophage receptor antibody (Figure 4C) and that it bound to mannosyl-BSA-agarose (Figure 4B) provided further evidence that p180 is identical or very similar to the macrophage mannose receptor. In line with this notion, the binding of p180 to the silkworm LEC-IgG was inhibited dose-dependently by α-methyl-D-mannoside. Furthermore, p180 failed to bind to the COS cell-derived LEC-IgG (Figure 5), although the

latter retained an appropriate ligand binding ability (Figure 6). It is interesting to note that the alteration of the sugar chains did not affect the binding activity of LEC-IgG to sLe^X-probe. Further study is needed to clarify whether-or-not the sugar moiety of L-selectin affects its binding ability to the other ligands. Accordingly, these findings indicate that p180 specifically reacted with the silkworm LEC-IgG by recognizing oligomannose-type oligosaccharides that are abundantly present in the silkworm LEC-IgG but absent in the COS LEC-IgG, and that the lectin activity of LEC-IgG is not involved in the recognition.

The baculovirus expression system has been used to express a variety of recombinant proteins in large quantities in insect cells under the control of polyhedrin promoter [32]. Insect cells can perform post-translational modifications such as N- and O-glycosylation [33]. In addition, recombinant proteins produced in this system are antigenically and functionally equivalent to their authentic counterparts. However, studies examining the structure of N-linked oligosaccharides of recombinant proteins contain mainly oligomannose-type structures ranging from $Man_5GlcNAc_2$ to $Man_9GlcNAc_2$ and also truncated structures with two to three mannose residues. They do not, however, contain complex-type oligosaccharides, in contrast to the mammalian cells [34–36]. The lack of complex structures in insect cell glycoproteins is not attributed to the absence of GlcNAc-transferase I and II. The recent finding of a unique membrane-bound *β-N*-acetylglucosaminidase in lepidopteran cells, which exclusively removes *β-N*-acetylglucosamine residues from the α1,3-antenna, appears to explain the absence of complex structures in insect cells [37]. Although we did not perform structural analysis, we speculate that the silkworm-derived LEC-IgG was heavily modified with oligomannose-type carbohydrate structures, and hence, recognized by p180 that has a mannose receptor activity. On the other hand, LEC-IgG produced in COS cells is devoid of these oligosaccharides, and is thus unreactive with p180. The other silkworm-derived recombinant protein used in this study, soluble poliovirus receptor-IgG (PVR-IgG), failed to react with p180 (Figure 5A, lane 2), although this protein is likely to be modified with oligomannose-type oligosaccharides. Since the numbers of potential N-glycosylation sites of rat L-selectin (seven sites) and human poliovirus receptor (eight sites) are almost the same, the differential reactivity of these proteins to p180 cannot merely be explained by the number of N-glycosylation sites. We are not sure at present whether this is due to qualitative or quantitative differences in glycosylation of this protein, and further studies are necessary to verify this point.

Finally, the immunohistochemical studies revealed that p180 is absent in the majority of HEV but is mainly localized in macrophages within the LN medulla. Therefore, it is unlikely that p180 is involved in the L-selectin-mediated initial attachment of lymphocytes to HEV based on its distribution. From these results, together with results from biochemical studies, we conclude that p180 is not a physiological ligand for L-selectin. Thus, the results of the present study are a warning against the use of baculovirus/silkworm-derived recombinant proteins for the detection of carbohydrate ligands.

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