

Identification of human complement Factor H as a ligand for L-selectin

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The selectin family of adhesion molecules (E-, P- and L-selectins) is involved in leukocyte recruitment to sites of inflammation and tissue damage. Recently it has been shown that L-selectin is involved not only in leukocyte tethering and rolling, but also plays an important role in leukocyte activation. For example, glycosylation-dependent cell-adhesion molecule 1 (GlyCAM-1), a known ligand for L-selectin, has been shown to enhance β 2-integrin function. GlyCAM-1 is a secreted protein and is present in mouse serum at a concentration of approx. 1.5 μ g/ml. There is no obvious GlyCAM-1 homologue in man and, to date, L-selectin ligand(s) from human serum have not been characterized. Therefore we have used L-selectin affinity chromatography, followed by ion-exchange chromatography, to isolate specific ligand(s) for L-selectin. Using this procedure, we have isolated three major glycoproteins of apparent molecular masses 170 kDa, 70 kDa and 50 kDa. The 170 kDa protein band was digested with

trypsin and peptides were analysed by delayed extraction matrix-assisted laser desorption ionization MS and protein database searching. The 170 kDa protein was identified as the human complement protein Factor H. Human Factor H, isolated by a different method, was shown to bind specifically to L-selectin in the presence of CaCl_2 , and binding was inhibited by anti-L-selectin antibodies, fucoidan and lipopolysaccharide. Only a part of the purified Factor H preparation bound to immobilized L-selectin. The interaction of Factor H with leukocyte L-selectin was shown to induce the secretion of tumour necrosis factor- α (TNF- α). Pretreatment of Factor H with sialidase reduced both the binding of L-selectin to Factor H and the Factor H-induced L-selectin-mediated TNF- α secretion by leukocytes. Taken together, these results demonstrate that a post-translationally modified form of human plasma Factor H is a potential physiological ligand for L-selectin.

INTRODUCTION

Selectins are cell-surface integral membrane glycoproteins involved in leukocyte trafficking, thrombosis and inflammation [1]. The three known selectins (E-, P- and L-selectin) each possess an N-terminal carbohydrate-recognition domain followed by an epidermal growth factor domain, between two and nine complement control protein domains, a single membrane-spanning region and a cytoplasmic C-terminal cytoplasmic domain. E- and P-selectins are expressed on activated endothelial cells, whereas L-selectin is expressed constitutively on essentially all blood leukocytes [1]. On the basis of either direct binding or inhibition experiments, it has been shown that the three selectins bind to fucosylated, sialylated oligosaccharides, such as sialyl-Lewis x [sLe^x: NeuAc α 2-3, Gal β 1-4(α 1-3Fuc)GlcNAc] in the presence of Ca^{2+} ions. Although the binding affinity of the selectins for sLe^x is in the low millimolar range, it has been shown that higher avidity is achieved either through multiple binding or through the interaction of the selectin (P- or L-selectin) with a sulphated residue on the carbohydrate or protein ligand [2,3]. P- and/or L-selectins also bind to sulphated polysaccharides, such as heparan sulphate and dextran sulphate, as well as to sulphatides, cardiolipin and lipopolysaccharide (LPS) [4,5]. These ligands lack sialic acid and fucose and can interact with selectins in the presence of EDTA, suggesting that they bind at a site distinct from the Ca^{2+} -dependent carbohydrate-recognition domain [4,5].

Insights into the physiological roles of selectin-mediated adhesion have come from studies with selectin-deficient (knockout) mice and from *in vivo* studies with anti-selectin antibodies (for review see [6]). These results indicate that L- and P-selectins

mediate the initial capturing of leukocytes from the flowing blood, while the synergistic action of L- and E- or P-selectin is required for optimal and stable leukocyte rolling [6]. Recently, L-selectin has also been established as a receptor capable of signal transduction [7,8]. The interaction of sulphatides, LPS or anti-L-selectin antibodies with neutrophil cell surface L-selectin triggers an increase in intracellular free calcium, the release of oxygen radicals, tyrosine phosphorylation and an enhanced expression of cytokine mRNA [such as tumour necrosis factor- α (TNF α) and interleukin-8] [7]. These results indicate that L-selectin is involved not only in the tethering and rolling of leukocytes on endothelium, but it also plays an important role in the activation of leukocytes. Hwang et al. [8] have shown that treatment of naive (CD45RA⁺) T-cells with glycosylation-dependent cell-adhesion molecule 1 (GlyCAM-1), a natural mouse glycoprotein L-selectin ligand, results in the appearance of a neoepitope on β 2-integrins which is associated with a high-avidity state. However, neither GlyCAM-1, nor an obvious homologue, has been reported in man. Consequently, we have used L-selectin affinity chromatography and MS to isolate and identify L-selectin ligands from human plasma.

EXPERIMENTAL

Reagents

A plasmid containing the human L-selectin gene was provided by Professor B. Seed (Department of Genetics, Harvard Medical School, Boston, MA, U.S.A.) and Dr Bernard Allet (Glaxo

Abbreviations used: BCECF, 2,7-bis-(2-carboxyethyl)-5-(and -6)-carboxyfluorescein acetoxymethyl ester; GlyCAM-1, glycosylation-dependent cell-adhesion molecule 1; HRP, horseradish peroxidase; LPS, lipopolysaccharide; PSGL-1, P-selectin glycoprotein ligand-1; TNF- α , tumour necrosis factor- α ; MAdCAM, mucosal addressin cell-adhesion molecule; DE-MALDI, delayed extraction matrix-assisted laser desorption ionization.

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Institute of Molecular Biology, Geneva, Switzerland) produced recombinant human selectins lacking the transmembrane and cytosolic domains as chimaeras with the ZZ-domain of Protein A (referred to as 'selectin-ZZ') attached to the C-terminus of the selectin coding sequence. The ZZ domain binds tightly to human IgG. A baculovirus/insect cell expression system was used and the proteins, purified by adherence to immunoglobulin IgG, were partially characterized in cell adhesion assays [5]. Two anti-L-selectin monoclonal antibodies were used: LAM1-3 and TQ1 (subtype IgG1; Coulter Immunology, Coulter Corporation, Hialeah, Finland). Horseradish peroxidase (HRP)-conjugated swine-(anti-rabbit) IgG (Cat. No p217; Lot No 111) was from Dako-Immunoglobulin, Denmark. IgG-agarose beads were obtained from ACL (Isle of Man, U.K.). Protein A-agarose, plasminogen, ovalbumin, fucoidan and BSA were obtained from Sigma, Poole, U.K. Purified *Arthrobacter ureafaciens* sialidase or α -mannosidase (Jack bean) were obtained Oxford GlycoSystems, Abingdon, Oxon., U.K.

Solid-phase binding assays

Microtitre plate wells (Maxisorb, Nunc, Roskilde, Denmark) were coated with potential ligands (protein or fucoidan) in 50 mM sodium bicarbonate buffer (pH 9.6) at ambient temperature for 16 h [4]. Non-specific binding sites were blocked with Tween 20 (1% v/v) in 10 mM Tris/HCl buffer (pH 7.4) for 2 h at ambient temperature. Selectin-ZZ in the presence or absence of competitor ligands was added to the coated wells in a 10 mM Tris/HCl buffer (pH 7.4) containing 2 mM CaCl₂ or 2 mM EDTA, 0.1% (v/v) Tween 20 and HRP-conjugated IgG (1/500 dilution). The plates were incubated for 2 h at ambient temperature. After extensive washing, the amount of bound selectin-ZZ-IgG complex was determined by adding the HRP substrate *O*-phenylenediamine dihydrochloride (Fast enzyme system; Sigma, cat. no. P-9187). The reaction was stopped after 5 min with 3 M HCl and the A_{490} was measured.

Purification of L-selectin ligands

Human plasma (20 ml) was obtained by centrifugation of fresh human blood in the presence of the anti-coagulant EDTA (4 mM). The human plasma was diluted with an equal volume of 10 mM Tris/HCl (pH 7.4) containing 5 mM CaCl₂, 2 mM benzamidine, 2 mM Pefabloc-SC (Boehringer Mannheim, Lewes, U.K.), 2 mM PMSF and 50 μ g/ml pepstatin A (Sigma) (buffer A) and then incubated overnight at 4 °C with Protein A-agarose beads (4 ml; binding capacity 25 mg IgG/ml). After centrifugation (1000 g, 20 min), the pellet was washed with 20 ml of buffer A. The supernatant and the first wash from Protein A-agarose beads was further precleared by incubating overnight at 4 °C with IgG-agarose beads (2 ml). The resulting supernatant was then incubated overnight at 4 °C with L-selectin-ZZ-IgG-agarose beads (2 ml; L-selectin concentration 200 μ g/ml). After removing the supernatant, the beads were washed three times with 10 mM Tris/HCl buffer (pH 7.4) containing 2 mM CaCl₂. Proteins bound to the L-selectin-ZZ-IgG-agarose beads were eluted with 10 mM Tris/HCl buffer (pH 7.4) containing 5 mM EDTA. Eluted proteins were dialysed against 10 mM Tris/HCl buffer (pH 7.4) containing 2 mM CaCl₂ and re-incubated overnight with L-selectin-ZZ-IgG-agarose beads (2 ml; L-selectin concentration 200 μ g/ml). The bound proteins were eluted as described above. Eluted proteins were dialysed against 10 mM Tris/HCl buffer (pH 7.4) and were then tested for their ability to bind to L-selectin-ZZ using the solid-phase binding assay system described above. Further purification of L-selectin-binding proteins was conducted on a Resource-Q FPLC column (1 ml,

Pharmacia, Milton Keynes, U.K.), pre-equilibrated with 10 mM Tris/HCl buffer (pH 7.4). The column was washed extensively and bound proteins were eluted with a linear gradient (25 ml) of 0–1 M NaCl in 10 mM Tris/HCl buffer (pH 7.4). The eluted fractions were tested for L-selectin-binding activity and positive fractions were pooled. The pooled L-selectin-binding proteins were analysed by SDS/PAGE and further characterized as described below.

SDS/PAGE

SDS/PAGE was carried out as described by Laemmli [9] (10% acrylamide gel). Samples were prepared under reducing conditions and protein gels were stained with either Coomassie Brilliant Blue or a silver stain.

In-gel enzymic digestion

Protein bands of interest were excised from a Coomassie stained gel, reduced, alkylated and digested with trypsin according to the procedure described by Wilm et al. [10]. Briefly, the gel pieces were excised and shrunk by dehydration in acetonitrile. The acetonitrile was removed and the gel pieces were dried in a vacuum centrifuge. The dried gel pieces were re-swollen in digestion buffer (10 μ l) composed of 50 mM ammonium bicarbonate (pH 8.5), 5 mM CaCl₂ and 12.5 μ g/ml trypsin (Boehringer Mannheim, sequencing grade). Digestion was allowed to proceed overnight at 37 °C. After digestion with trypsin, the peptides were extracted using 100 mM ammonium bicarbonate (2 \times 50 μ l) and acetonitrile (2 \times 50 μ l) followed by 5% formic acid in 50% methanol (2 \times 50 μ l). Each step involved vortexing for 10 min to promote efficient extraction. The samples were then centrifuged before removing the liquid into labelled tubes. The combined extracts from each sample were then dried using a centrifugal evaporator.

Peptide analysis by delayed extraction matrix-assisted laser desorption ionization (DE-MALDI) MS [11]

The dried extract from the protein digest was redissolved in 5% formic acid containing 5% methanol (10 μ l). An aliquot (0.4 μ l) was spotted onto a stainless steel target pre-coated with α -cyano-4-hydroxycinnamic acid and nitrocellulose. The target was allowed to air dry before being washed with 1% aqueous trifluoroacetic acid (2 μ l). Excess wash solution was blown off and the target was dried using compressed air. The MALDI mass spectrum was obtained using a ToFSpec SE instrument (Micromass, Manchester, U.K.) fitted with a 337 nm nitrogen laser. The mass spectrum was calibrated using a matrix-related ion signal (m/z 1060.10) and a trypsin autolysis peptide (m/z 2163.057). Monoisotopic masses were assigned for each peptide and these were used collectively as a 'peptide mass map' [12] to search an in-house non-redundant protein sequence database. This database currently contains more than 2×10^5 entries and was searched using PeptideSearch[®] software [13]. No restriction was placed on the species of origin of the protein or on its isoelectric point and a protein mass range from 0 to 300 kDa was allowed.

Peptide analysis by nano-electrospray MS

The remainder of the sample was desalted using a pulled-out glass capillary containing a small amount of POROS R2 resin (PerSeptive Biosystems, Framingham, MA, U.S.A.). Peptides were then eluted directly into the nanospray needle using 1% formic acid in 50% methanol (2 μ l). A PE-Sciex API III

Ionspray[®] mass spectrometer (PE-Sciex, Thornhill, Ontario, Canada) fitted with a nano-electrospray source [14] was used to acquire tandem MS/MS mass spectra for several peptides. In MS/MS mode the first quadrupole is used to select the precursor ion which is then passed into a collision cell where fragmentation is induced by collision with argon gas molecules. The energy of collision is typically between 30 and 60 eV, depending on the mass and charge of the precursor ion. Ions formed by the cleavage of backbone bonds are designated a, b, c if the charge is retained on the N-terminal fragment and x, y, z if the charge resides on the C-terminal fragment (nomenclature according to [13]). High mass-product ions (observed by scanning the third quadrupole above the m/z value for the doubly or triply charged precursor ion) are often part of a series of y' ions [15]. A partial amino acid sequence can be determined from the pattern of y' ions. This, together with the masses bracketing the sequence, forms a peptide sequence tag [16], which was used to confirm the identity of the peptide, and hence the protein.

Purification of Factor H

Factor H was purified from human plasma by the method of Sim et al. [17]. Briefly, the method consists of affinity isolation using an immobilized anti-Factor H monoclonal antibody (MRC OX23) [18]. Traces of IgG in the purified Factor H were removed by passage through a Hi-Trap Protein G column (Pharmacia).

Enzymic treatment of Factor H

The *A. ureafaciens* sialidase used was highly purified and analysis provided by GlycoSystems showed no detectable protease activity and, furthermore, incubation with corresponding phosphonitrophenyl glycosides confirmed the absence of activity for α -mannosidase, β -mannosidase, α -*N*-acetylgalactosaminidase, β -*N*-acetylhexosaminidase, β -galactosidase, α -galactosidase, α -fucosidase and β -fucosidase. Factor H (150 μ g/ml; 30 μ l) was incubated with *A. ureafaciens* sialidase (0.1 units) or with α -mannosidase (Jack bean) (1 unit) for 18 h at 37 °C in incubation buffer provided by Oxford GlycoSystems. The reactions were stopped by adding 500 μ l of 100 mM sodium bicarbonate buffer (pH 9.6). As a control, Factor H protein was incubated for 16 h at 37 °C in reaction buffer in the absence of enzyme, and enzyme was then added just before the addition of sodium bicarbonate buffer. Factor H, pre-treated with or without enzyme, was coated onto microtitre plate wells and the plate was incubated overnight at 4 °C. Non-specific binding sites were blocked with Tween 20 (1% v/v) in 10 mM Tris/HCl buffer (pH 7.4) for 2 h at ambient temperature. L-Selectin-ZZ (100 μ l, 10 μ g/ml) was added to coated wells in a 10 mM Tris/HCl buffer (pH 7.4) containing 2 mM CaCl₂, 0.1% (v/v) Tween 20 and HRP-conjugated IgG (1/500 dilution). The plates were incubated for 2 h at ambient temperature. After extensive washing, the amount of bound selectin-ZZ-IgG complex was determined as described above.

Cytokine secretion assay

Factor H, pre-treated with or without sialidase enzyme (as described above), was coated onto microtitre plate wells as described above. Non-specific binding sites were blocked with BSA (3 mg/ml) in PBS (pH 7.4) for 2 h at ambient temperature. Fresh human blood was collected in the presence of the anti-coagulant EDTA (1.5 mg/ml). Human leukocytes (buffy coat cells) were isolated by centrifugation of whole blood at 800 g for 5 min. The top plasma layer was discarded and the middle layer containing leukocytes was collected. This process was repeated

and the leukocyte-containing fraction (approx. 75% leukocytes, 25% erythrocytes by vol.) was washed three times in PBS at 600 g for 5 min. The cell pellet was suspended in RPMI 1640 culture medium (Sigma, Irvine, U.K.) containing 2 mM glutamine. The leukocyte cell suspension (200 μ l: 1×10^7 cells per ml of RPMI 1640 culture medium) was incubated in Factor H- or BSA-coated wells for 3 h in a 5% CO₂/95% air incubator at 37 °C. As a control, leukocyte cell suspension was incubated with LPS or Factor H in solution for 3 h in a 5% CO₂/95% air incubator at 37 °C. Supernatants were collected and analysed for TNF α content using a Quantikine human TNF α kit (R&D Systems, Abingdon, Oxon., U.K.; Cat. number DTA50).

In another set of experiments microtitre plate wells were coated with Factor H and blocked with BSA as described above. Isolated human leukocytes were labelled by incubation with 2,7-bis-(2-carboxyethyl)-5-(and -6)-carboxyfluorescein acetoxy-methyl ester (BCECF) obtained from Cambridge Bioscience, Cambridge, U.K. (final concentration 10 μ M) for 20 min at ambient temperature. The BCECF-labelled cells (200 μ l: 1×10^7 cells per ml of RPMI culture medium) were incubated with Factor H-coated microtitre plate wells in the presence of an anti-L-selectin antibody (LAM 1-3; 20 μ g or 5 μ g per well) or isotype control antibody (20 μ g or 5 μ g per well) or anti-CD14 antibody (20 μ g per well) for 45 min at ambient temperature. After three washes with RPMI 1640 culture medium, the bound cells were lysed with Triton-X100 (6%) and A_{530} was measured using a fluorescence plate reader (excitation 485 nm; emission 530 nm).

RESULTS

Purification of L-selectin ligands from human plasma

L-selectin-binding proteins were isolated from fresh human plasma using two cycles of affinity chromatography, followed by ion-exchange chromatography. Figure 1(A) (lane 1) shows the SDS/PAGE profile of proteins eluted from the second L-selectin affinity column. Four major proteins of molecular masses between 80 kDa and 170 kDa were observed, together with several minor components of lower molecular masses. A solid-phase adhesion assay was used to confirm that the eluate from the affinity column contains (glyco)protein ligands for L-selectin. Aliquots of the column eluate were adsorbed onto microtitre plate wells. After blocking non-specific sites with Tween 20, L-selectin-ZZ was added in the presence of serial dilutions of anti-L-selectin antibody (LAM 1-3) or isotype control antibodies. Figure 2 shows that binding of L-selectin-ZZ to the immobilized ligands occurred and was inhibited by the anti-L-selectin antibody, but not by the control antibody. Furthermore, fucoidan and LPS (Table 1) inhibited the binding of L-selectin-ZZ to its ligands. No binding of E-selectin-ZZ to the immobilized ligands was observed under similar conditions (results not shown). These results indicate that the column eluate contains specific (glyco)protein ligand(s) for L-selectin. The affinity purified pooled L-selectin-binding proteins were then further purified using Resource-Q FPLC. Individual fractions were tested as above for their ability to bind L-selectin. Figure 1(B) shows the protein elution profile and L-selectin-binding activity of the different fractions. L-Selectin-binding activity was observed in fractions 1–5 (pool 1), fractions 7–13 (pool 2) and fractions 15–20 (pool 3). On further analysis, pool 3 was found to contain the fraction with maximum Ca²⁺-dependent L-selectin binding activity. The proteins in pool 3 were subjected to SDS/PAGE (Figure 1A, lane 2), which revealed the presence of three major proteins corresponding to molecular masses of 170 kDa, 70 kDa and 50 kDa and a minor protein of molecular mass 90 kDa. The protein bands were excised from a Coomassie-stained gel,

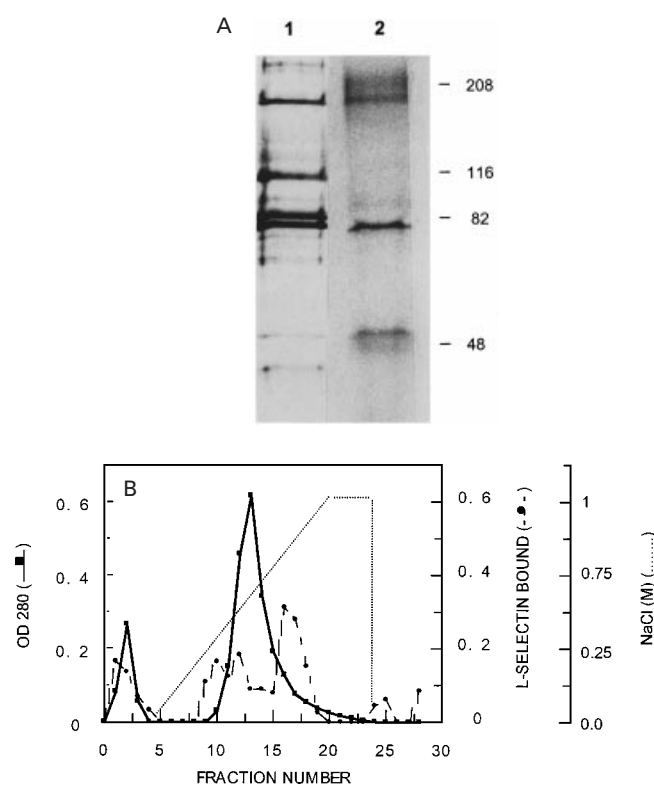


Figure 1 Purification of L-selectin-binding proteins from plasma

(A) SDS/PAGE analysis of L-selectin-binding proteins. Lane 1, proteins eluted with EDTA from the second L-selectin-ZZ-IgG-agarose affinity column; lane 2, proteins purified from the Resource-Q FPLC column. The 10% acrylamide gels were stained with Coomassie Blue. Molecular mass values are shown on the right. (B) Profile of the L-selectin affinity purified ligands eluted from a Resource Q FPLC column. Individual fractions (100 μ l; 1:4 dilution in 100 mM sodium bicarbonate buffer) were coated onto microtitre plate wells and the binding of soluble L-selectin-ZZ was determined. Pool 3 (fractions 15–20) contained ligands exhibiting maximum Ca^{2+} -ion-dependent L-selectin-specific binding. OD 280 is A_{280} .

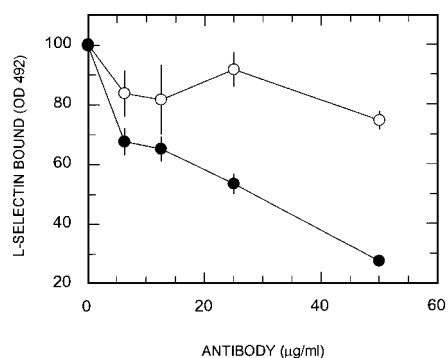


Figure 2 Binding characteristics of the proteins eluted from L-Selectin-ZZ-IgG-agarose

The binding of L-selectin-ZZ (50 μ l; 50 μ g/ml) to immobilized potential ligands from a second L-selectin-ZZ-IgG-agarose affinity column was inhibited by serial dilutions of anti-L-selectin antibody (●) (LAM 1-3; maximum concentration 100 μ g/ml; 50 μ l final assay volume) but was not inhibited by an isotype control antibody (○). OD 492 is A_{492} .

reduced, alkylated and digested with trypsin as described in the Experimental section [10]. Peptide mass and sequence information were obtained by analysis by DE-MALDI MS [11].

Table 1 Binding characteristics of the proteins eluted from L-Selectin-ZZ-IgG-agarose

The binding of L-selectin-ZZ to potential L-selectin ligands was inhibited in the presence of fucoidan (200 μ g/ml) or LPS (200 μ g/ml). L-selectin-ZZ bound to potential ligands in the presence of Ca^{2+} , but not in the presence of EDTA. The results shown are the means of two separate experiments (values for the individual experiments are given in parentheses).

Inhibitors	Binding to L-selectin (A_{490})
Ca^{2+} (2 mM)	0.541 (0.525, 0.557)
Ca^{2+} + LPS (200 μ g/ml)	0.175 (0.215, 0.135)
Ca^{2+} + fucoidan (200 μ g/ml)	0.080 (0.084, 0.076)
EDTA (2 mM)	0.171 (0.183, 0.159)

Protein sequence

Several peptides were obtained after trypsin digestion of the 170 kDa protein band (Figure 3). The peptide(s) corresponding to a molecular mass of 1340 Da had an m/z of 671.0 (Figure 4). This peptide, together with data from 12 other peptides, was assigned to human complement protein Factor H (Table 2). Figure 5 shows the distribution of peptides found in the Factor H sequence from the DE-MALDI spectrum. From this analysis, it was confirmed that the protein with an apparent molecular mass of 170 kDa is the human complement protein Factor H. The band corresponding to 70 kDa was identified as human serum albumin and the 90 kDa minor band was identified as human plasminogen (Figure 1A, lane 2). We were unable to identify the 50 kDa band.

Complement Factor H as a ligand for L-selectin

Human complement Factor H was purified to homogeneity as described by Sim et al. [17]. Microtitre plate wells were coated with Factor H and the binding of L-selectin-ZZ was investigated. Table 3 shows that L-selectin-ZZ binds to Factor H in the presence of Ca^{2+} ions. No binding of L-selectin-ZZ to BSA was observed under the same conditions. Furthermore, binding of L-selectin to Factor H was inhibited by a known, functional-blocking anti-L-selectin antibody (LAM 1-3) (Table 3). To investigate the inhibitory efficacy and specificity of soluble Factor H, an additional solid-phase assay was used in which immobilized fucoidan was employed as the ligand for L- and P-selectins. Figure 6 shows that binding of L-selectin-ZZ to fucoidan was inhibited by soluble Factor H in a concentration-dependent manner. The binding of P-selectin-ZZ to fucoidan was not significantly affected by Factor H. Similarly, an irrelevant protein, ovalbumin, had no effect in this assay.

Effect of variation in ionic strength on L-selectin-ZZ binding to Factor H

Avery and Gordon [19] reported that the binding of Factor H to human neutrophils is Ca^{2+} -ion and NaCl-concentration dependent. A sharp decrease in binding was observed above approx. 110 mM NaCl. To establish whether the binding of L-selectin to Factor H shows similar characteristics, we investigated the effect of NaCl on the binding of L-selectin-ZZ to immobilized fucoidan or Factor H. Figure 7 shows that L-selectin-ZZ binding to

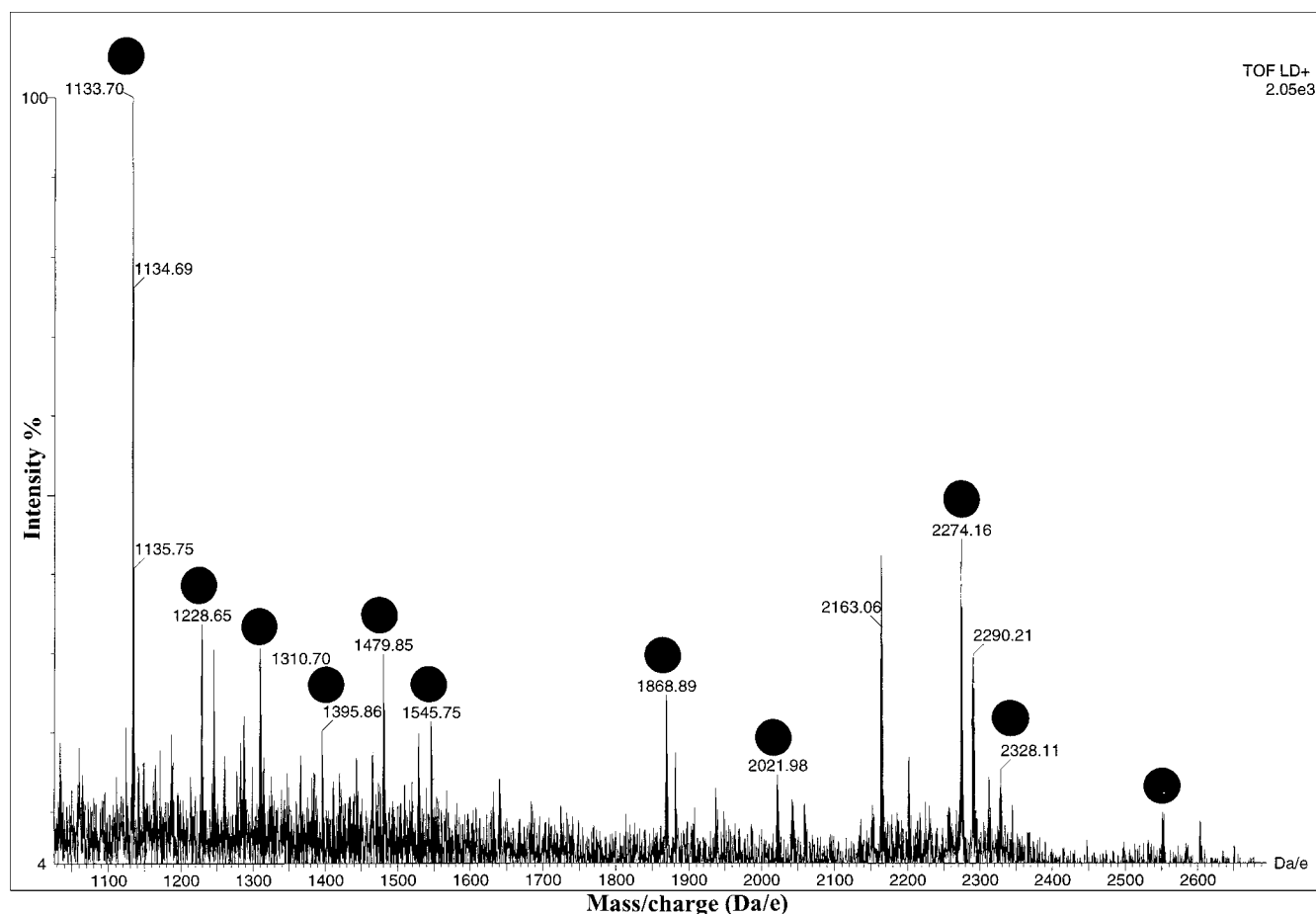


Figure 3 Identification of peptides from the 170 kDa protein

The DE-MALDI spectrum for the tryptic digest of the 170 kDa protein is shown. Ion signals whose measured masses match calculated masses of protonated tryptic peptides of Factor H [$M + H$]⁺ within 0.3 Da are indicated with closed circles. Twelve peptides (of which eleven are shown) match the SwissProt database entry P08603 (human Factor H), covering 13% of the total protein sequence. Note that some of the unmarked peaks are matrix related, the others are trypsin autolysis products.

Factor H or fucoidan is sensitive to NaCl concentration, and that a sharp decrease in binding is observed between 75 and 150 mM NaCl.

Binding of leukocytes to Factor H

The results presented above clearly establish the binding of recombinant L-selectin-ZZ to Factor H and that the binding characteristics of L-selectin-ZZ to Factor H are similar to those reported by Avery and Gordon [19] for the binding of Factor H to an unidentified leukocyte surface molecule. To further establish Factor H as a ligand for L-selectin on leukocytes, we investigated the binding of freshly isolated leukocytes to solid-phase-immobilized Factor H. Table 4 shows that the binding of BCECF-labelled leukocytes to Factor H was inhibited by anti-L-selectin antibody, but not by isotype control antibody or anti-CD-14 antibody.

Fractionation of purified Factor H on L-selectin-ZZ-IgG-agarose affinity column

Human Factor H purified by immunoaffinity chromatography

consists of two different isoforms, ϕ_1 and ϕ_2 [20]. The two forms have an identical polypeptide backbone, but are thought to differ in charge because of a post-translational modification [20,21]. Therefore to explore further what proportion of Factor H from human plasma has affinity for L-selectin, immunoaffinity purified Factor H was fractionated on an L-selectin affinity column. Immunoaffinity purified human Factor H (130 μg), preabsorbed with IgG-agarose, was incubated overnight at 4 °C on an L-selectin-ZZ-IgG-agarose column (1 ml packed volume; 490 $\mu\text{g}/\text{ml}$ L-selectin-ZZ) in 10 mM Tris/HCl buffer containing 4 mM CaCl_2 . After extensive washing with incubation buffer, bound Factor H was eluted with 10 mM Tris/HCl buffer containing 4 mM EDTA. Figure 8 shows the SDS/PAGE analysis of the starting material (left-hand lane) and proteins eluted with EDTA (right-hand lane). The L-selectin affinity column retained a relatively small fraction of the Factor H and the majority of protein was recovered in the flow-through. Interestingly, the Factor H that was retained and subsequently eluted from the affinity column had a higher capacity to bind L-selectin-ZZ than the starting material (Table 5). Table 5 also shows that L-selectin-ZZ does not bind to commercially available human plasminogen or human serum albumin.

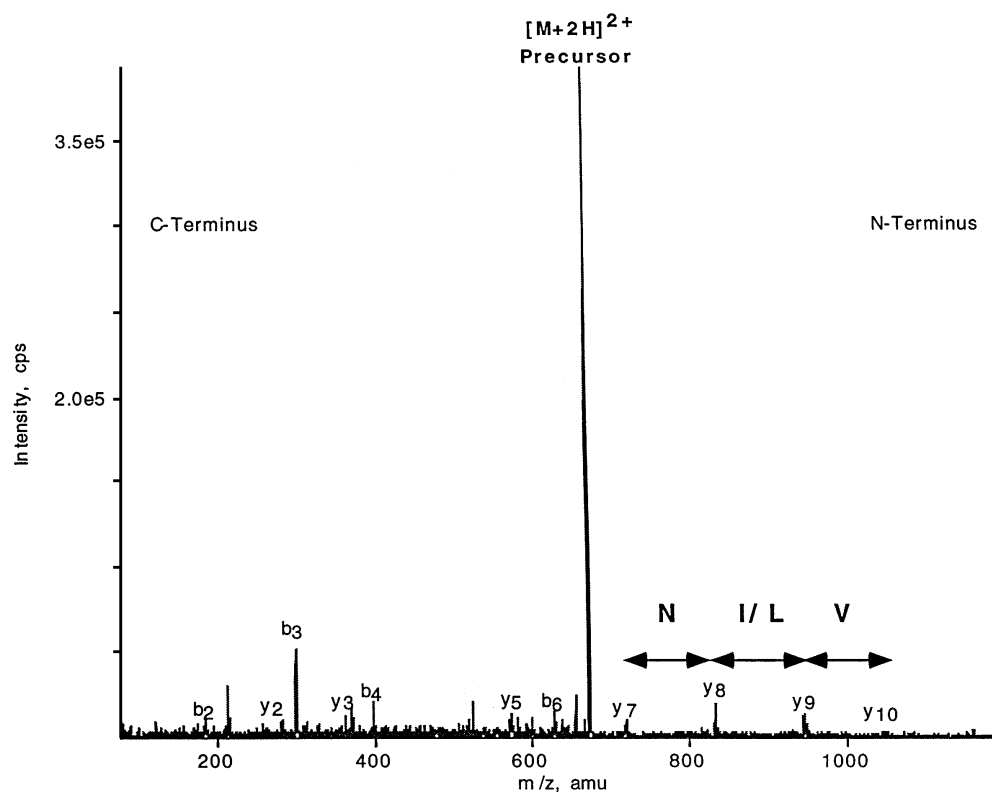


Figure 4 Analysis of the 1340.0 Da peptide

Tandem MS/MS data were obtained for the doubly charged molecular ion at m/z 671.0 for the peptide of molecular mass 1340.0 Da. The spectrum was acquired with Q1 set to transmit a mass window of 2 Da and spectra were accumulated with a 0.2 Da mass step. Resolution was set so that fragment masses could be assigned to better than 1 Da. Ions formed by the cleavage of backbone bonds are designated a, b, c, if the charge is retained on the N-terminal fragment and x, y, z, if the charge resides on the C-terminal fragment (nomenclature according to [13]). In this case it is possible to read part of the sequence from the pattern of 'y' ions. This, together with the masses bracketing the sequence, forms a peptide sequence tag [16], which can be used unambiguously to identify the protein. Here the tag is written as (718)N(IL)V(1045).

Table 2 Identification of complement Factor H as a potential ligand for L-selectin by DE-MALDI MS and database searching

Measured (Da)	Calculated* (Da)	Residues	Sequence
1133.70	1133.647	342–351	RPYFPVAVGK
1228.65	1228.548	332–341	HGGLYHENM†R
1310.70	1310.636	1193–1203	TGESVEFVCKR
1340.70	1340.69	212–224	SPDVINGSPISQK‡
1395.86	1395.697	296–308	NGFYPATRGNTAK
1479.86	1480.709	959–972	CFEGFGIDGPAIAK
1545.75	1545.682	920–932	WSSPPQCEGLPCK
1867.89	1867.828	266–281	SCDNPIYPNGDYSPLR
2020.98	2020.976	1045–1062	DTSCVNPPTVQNAVYIVSR
2274.16	2274.111	146–166	CLPVTAPENGIKIVSSAMEPDR
2328.11	2328.182	332–351	HGGLYHENMRRPYFPVAVGK
2553.17	2553.168	497–518	DGWSAQPTCIKSCDIPVFMNAR

* Monoisotopic mass of the predicted $[M+H]^+$ molecular ion.

† Oxidized methionine residue.

‡ Tandem MS/MS data obtained for the doubly charged molecular ion at m/z 671.0.

Effect of sialylation on binding of L-selectin to Factor H or fucoidan

It is well established that the binding of L-selectin to its ligands requires sialylation, fucosylation or sulphation of the ligands

[22]. Since the binding of L-selectin to Factor H is inhibited by EDTA and by antibodies recognizing the lectin domain of L-selectin, it is possible that the binding of L-selectin to Factor H involves an interaction between the lectin domain of L-selectin and a glycan structure on Factor H. To further explore whether the binding of L-selectin to Factor H involves protein-carbohydrate interaction, we investigated the effect of pre-treatment of Factor H with a non-selective sialidase on its binding to L-selectin. Table 6 shows that the pre-incubation of Factor H with sialidase reduces the binding of L-selectin to desialylated Factor H, whereas another glycosidase, mannosidase, did not affect the binding.

Recently, it has been reported that cross-linking of L-selectin on leukocyte surface with anti-L-selectin antibody or L-selectin ligands induces TNF- α release (reviewed in [1,7,8]). Similarly, incubation of human leukocytes with immobilized Factor H induces TNF- α release from the leukocytes (Table 6), whereas soluble Factor H did not induce TNF- α secretion (Table 6). Pre-treatment of Factor H with sialidase reduces the secretion of TNF- α into the medium (Table 6). As a positive control, leukocytes were incubated with LPS, which resulted in TNF- α release (Table 6). Results presented in Table 6 indicate that sialylation of Factor H is necessary for its binding to L-selectin, and sialylation and aggregation of Factor H is necessary for it to induce TNF- α release via L-selectin.

MRLAKIICMLWAICVA
 EDCNELPPRRNTEILLTGSWSDQTYEGTQALYKCRPGYRSLGNVHVCRKGEWALNPLRKC
 QRRCGHPGDTFFGTFTLTGGNVFEYGVKAVYTCNKGQQLLGEINRYRCDTDDGWTNDIPICE
 VVKCLPVTAPENKIVSSAMEPDRREYHFGQAVRVCNCSGKIEGDEEMHCSDDGFWSEKPKCV
 ELSCKSPDVI~~NGS~~PI~~SK~~IIYKENE~~RFQ~~YKCNMGYYSERGDVAVCTESGWRPLPSC
 EKSCDNPIYIPNGDYSPLRKHRTGDEITTYQCRNGEYPTARGNTAKCTSTGWI~~PAP~~RCT
 LKPCDYDIKHGGLYHENMRRFPYVAVGKYYSYCDHEFTFSGSYWDHIHCTQDGGWSPAVPCL
 RRCYFPYLENGYNQHGRKFKVQGSIDVACHPGYALPKAQTFTVTCMENGWSPTRCIRV
 KTCCKSSIDIENGFISESYTYALKEKAKYQCKLGVVTDAGETS~~SGS~~IRCGKDGWSAQPTCI
 KSCDI~~PVFN~~ARTKNDFTWFK~~ND~~LDYECHDGYESNTGSTTGSIVCGYNGWSDLPICY
 ERECELPKIDVHLVDRKKDQYKVEVLKESCKPGPTIVGPNVQCYHFLGSLPDLPICK
 EQVQSCGFPBELLNGN~~VKE~~KTKEEYGHSEV~~VY~~YCNPRFLMKGENKIQCVDEWTTLPVCI
 VEESTCGDIPELEHGWAQLSSPPYYGDSVEF~~NCS~~ESFTMIGHRSITCIHGVTQLPQC
 ATDRKKKSSNLIILEHLLRNKKEFDHNSNIYRRCRCKEGWIHTVCINGRWDPEV~~NCS~~
 MAQIQLCPPPPQIPNSH~~ND~~TTLNRYDGEKVS~~VLC~~OVNLYIQEGETTCKDKGRWQSIPLCV
 EKI~~PC~~SPQPIEHGT~~NSS~~FSQESYAHGTLKLSYTCEGGFRISE~~ND~~TCYMGKWSPPQCE
 GLPCKSPPEISHGVVA~~FMS~~DSYQYGEVYTR~~IF~~EGFGIDGPAIAKCLGKWSHPPSCI
 KIDCLSPLSFENAI~~PM~~GKEDVYKAGEQVYTCATY~~YK~~MDGAS~~SV~~TCINSRWTRGPTCR
 DTSVNVPTVQNAIYISRQMSKYPGERVRYQCRSPYEMFDEEVMCLNG~~NW~~TEPPQCK
 DSTGKCGPPPIIDNGDITSFPLSVYAPASSVEYQCNLYQLEGNKRITCRNGQWSEPPKCL
 HPCVISREIMENYNIALRWAKQKLYSR~~TGES~~VEFVCKRGYRLSSRSHTLRTTCDWGKLEYPCTCAK

Figure 5 Distribution of peptides in the Factor H sequence

The primary sequence of human complement Factor H is found under the SwissProt accession number P08603. Peptides observed in the DE-MALDI spectrum are underlined. The signal sequence is composed of residues 1–18 and is shown on the first line. The rest of the sequence is shown as 20 lines each corresponding to one complement control protein (short consensus repeat) domain. N-linked glycosylation sites are shown in bold. The N-linked glycosylation sites known to be occupied are circled [36,37]. Potential sulphation sites are indicated by an arrow and the sulphation consensus sequences are boxed. These sulphation sites fulfil the previously noted consensus sequence for tyrosine sulphation [39,40].

Table 3 Binding of L-selectin-ZZ to purified Factor H

Microtitre plate wells were coated with purified Factor H (100 μ l; 30 μ g/ml) or BSA (100 μ l; maximum concentration 30 μ g/ml) in 100 mM sodium bicarbonate buffer (pH 9.6). The binding of soluble L-selectin-ZZ (10 μ g/ml) was determined as described in the Experimental section. The results shown are the means of two separate experiments (values for the individual experiments are given in parentheses). L-selectin-ZZ bound to Factor H-coated wells in the presence of 2 mM Ca^{2+} ions. Binding of L-selectin-ZZ to Factor H was inhibited by anti-L-selectin antibody (LAM 1-3; 20 μ g/ml), but not by an isotype control antibody. No binding of selectin to BSA was observed.

Inhibitors	Binding of L-selectin ($A_{490} \pm$ range) to	
	BSA	Factor H
Ca^{2+} (2 mM)	0.108 (0.102, 0.114)	1.033 (1.023, 1.044)
Ca^{2+} + anti-L-selectin	0.071 (0.064, 0.078)	0.366 (0.336, 0.396)
Ca^{2+} + isotype control	0.093 (0.073, 0.113)	0.805 (0.735, 0.875)
EDTA (2 mM)	0.197 (0.155, 0.239)	0.198 (0.138, 0.204)

DISCUSSION

It is widely accepted that selectins have an important function in the initial tethering and rolling of leukocytes during the process of leukocyte migration into inflammatory sites and lymphoid organs. These processes are mediated by interactions between selectins and their ligands. Several ligands for L-selectin from mouse lymphoid organs have been characterized, including GlyCAM-1, CD34, Sgp200 and MAdCAM-1 [1]. GlyCAM-1 is a 50 kDa glycoprotein and is one of the best-characterized ligands for L-selectin [22]. Recently GlyCAM-1 was shown to be a secreted protein which is present in mouse serum at a concentration of $\sim 1.5 \mu$ g/ml [8,23]. Although GlyCAM-1 and other murine selectin ligands have been identified and characterized, the only well-characterized ligand for selectins in human systems is P-selectin glycoprotein ligand-1 (PSGL-1) [24]. PSGL-1 is expressed on leukocytes and was initially characterized as a P-selectin ligand, although recently PSGL-1 has been shown to

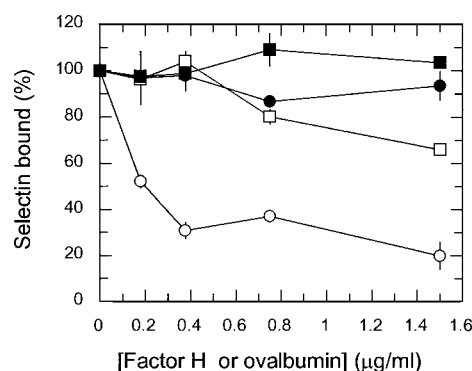


Figure 6 Binding of L- or P-selectin-ZZ to fucoidan

Microtitre plate wells were coated with fucoidan (100 μ l; 50 μ g/ml) in 100 mM sodium bicarbonate buffer (pH 9.6) and the binding of soluble L- or P-selectin-ZZ (10 μ g/ml) was determined as described in the Experimental section. \circ , L-selectin + factor H; \square , L-selectin + ovalbumin; \bullet , P-selectin + factor H; \blacksquare , P-selectin + ovalbumin. The values shown are the means and ranges of two separate experiments with the 'uninhibited' values (zero Factor H or ovalbumin) taken as 100%. Binding of L-selectin-ZZ to fucoidan was inhibited by purified soluble Factor H, whereas the binding of P-selectin-ZZ to fucoidan was unaffected.

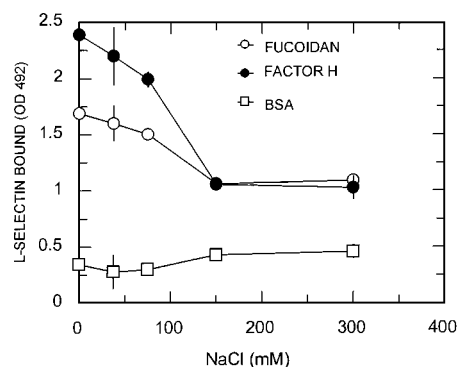


Figure 7 Effect of ionic strength on L-selectin binding to Factor H and fucoidan

Microtitre plate wells were coated with Factor H, fucoidan or BSA, as described in the legend to Figure 6. Soluble L-selectin-ZZ (10 μ g/ml, final assay volume 100 μ l) was added to the wells in 10 mM Tris/HCl (pH 7.4) buffer containing 2 mM $CaCl_2$ and serial dilutions of NaCl (maximum concentration 300 mM). The binding of L-selectin was determined as described above. The values shown are the mean and range of two separate experiments. OD 492 is A_{492} .

Table 4 Binding of leukocytes to solid-phase-immobilized Factor H

Microtitre plate wells were coated with immunoaffinity purified Factor H or BSA. Non-specific binding sites were blocked with BSA, and BCECF-labelled leukocytes in the presence of different antibodies were added to the wells in RPMI culture medium. Binding of cells to Factor H was inhibited by anti-L-selectin antibody (LAM 1-3), but not by isotype control antibody or anti-CD14 antibody. Binding of cells to BSA-coated wells is shown as background binding. The results shown are the means of two separate experiments (values for the individual experiments are given in parentheses).

Inhibitors	Binding of leukocytes to Factor H (A_{530})
Isotype control antibody (20 μ g/ml)	322 (301, 343)
Isotype control antibody (5 μ g/ml)	366 (343, 389)
Anti-L-selectin antibody (20 μ g/ml)	100 (88, 112)
Anti-L-selectin antibody (5 μ g/ml)	94.0 (71, 117)
Anti-CD14 antibody (20 μ g/ml)	281 (267, 295)
Background	20.5 (5, 36)

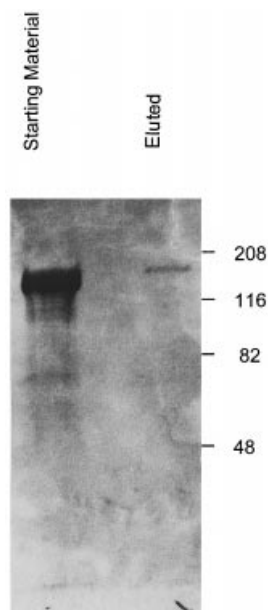


Figure 8 Fractionation of immunoaffinity purified Factor H using an L-selectin-ZZ-IgG-agarose affinity column

SDS/10%-PAGE profile of the immunoaffinity purified Factor H (lane 1) and the proteins eluted with EDTA from the L-selectin-ZZ-IgG-agarose column (lane 2). Samples loaded onto the SDS/PAGE were adjusted to the same volume. Molecular mass values are shown on the right.

Table 5 Binding of L-selectin to different fractions of Factor H and control proteins

Equal amounts of either purified Factor H (starting material) or purified Factor H specifically eluted from the L-selectin affinity column were adsorbed onto microtitre plates. The protein eluted from the L-selectin affinity column showed increased ability to bind soluble L-selectin-ZZ. In the same assay, L-selectin-ZZ showed very low binding to ovalbumin- or plasminogen-coated wells. The results shown are the means of two separate experiments (values for the individual experiments are given in parentheses).

Protein fraction	L-selectin bound (A_{490})
Factor H (starting material)	0.262 (0.237, 0.287)
Factor H (eluted from L-selectin column)	0.847 (0.798, 0.896)
Albumin	0.046 (0.01, 0.083)
Plasminogen	0.124 (0.034, 0.214)

bind L- and E-selectins as well (for review see [25]). In the current study, we have isolated and identified ligands for L-selectin from human plasma. Three major proteins of molecular masses 170 kDa, 70 kDa and 50 kDa, and one minor protein of 90 kDa, were isolated. The 70 kDa protein was sequenced and identified as serum albumin. Solid-phase binding assays demonstrated that L-selectin did not bind to purified albumin from a commercial source. We were unable to obtain sufficient sequence to identify the 50 kDa band. The minor band (90 kDa) was identified as plasminogen. It is interesting to note that tetranectin, a C-type lectin, has been reported to bind plasminogen [26]. Tetranectin is a homotrimer forming a triple helical coiled coil. Each monomer consists of a C-type lectin. Furthermore, tetranectin binds to sulphated polysaccharides in the presence of Ca^{2+} ions [27]. But in our hands, using purified human plasminogen

Table 6 Effect of sialidase treatment of Factor H

Immunoaffinity purified Factor H was incubated with mannosidase or sialidase for 18 h at 37 °C. Microtitre plate wells were then coated with Factor H which had been pretreated either with glycosidase or incubation buffer. Pre-treatment of Factor H with sialidase reduced the binding of L-selectin to Factor H. Binding of selectin to Factor H was not affected by the pretreatment of Factor H with the incubation buffer (shown as buffer alone) and α -mannosidase had only a small effect. Similarly pre-treatment of Factor H with sialidase reduced Factor H-induced TNF- α secretion by leukocytes. TNF- α released from leukocytes by immobilized BSA was used as background and cytokine released from leukocytes in the presence of soluble LPS was used as a positive control. Soluble Factor H did not induce cytokine release. The results shown are the means of two separate experiments (values for the individual experiments are given in parentheses). ND, not done.

Conditions	L-selectin bound (A_{490})	TNF- α release (pg/ml)
Factor H + buffer alone	0.592 (0.560, 0.624)	109 (105, 113)
Factor H + sialidase	0.218 (0.170, 0.266)	61 (57, 65)
Factor H + mannosidase	0.437 (0.377, 0.497)	ND
BSA	0.120 (0.107, 0.133)	34 (30, 38)
LPS (100 μ g/ml), soluble	ND	490 (484, 496)
LPS (10 μ g/ml), soluble	ND	332 (328, 336)
LPS (1 μ g/ml), soluble	ND	354 (348, 360)
LPS (0.1 μ g/ml), soluble	ND	374 (369, 379)
LPS (0.01 μ g/ml), soluble	ND	38 (34, 42)
Factor H (150 μ g/ml), soluble	ND	31 (26, 36)

obtained from Sigma, no specific binding of L-selectin to plasminogen was observed. It is possible that only a small fraction of human plasminogen expresses the post-translation modifications (glycosylation or sulphation) required for high-affinity binding to L-selectin, and that the commercial plasminogen provided by Sigma lacks the appropriate modification.

The other major glycoprotein of apparent molecular mass 170 kDa was identified as Factor H. The complement protein Factor H plays an essential role in the regulation of the alternative pathway of the complement system (for review see [28]). It has been established that Factor H binds to the alternative pathway convertase, C3bBb, and induces dissociation of the convertase. Factor H also acts as a non-enzymic cofactor for the proteolytic cleavage of C3b to iC3b by the plasma protease Factor I [29,30]. In addition, several workers have provided direct or indirect evidence for a Factor H receptor on leukocytes. For example, it has been shown that Factor H stimulates the release of interleukin-1 β from monocytes [31], promotes rosette formation of C3b-coated erythrocytes with phagocytic cells [32], functions as a chemotactic protein for monocytes [33] and displays cell-attachment activity [34]. Erdei and Sim [35] and Ripoche et al. [21] showed direct binding of Factor H to Raji cells at physiological ionic strength. Avery and Gordon [19] demonstrated that the binding of Factor H to polymorphonuclear leukocytes is ionic strength-dependent, requires divalent cations and is not inhibited by RGD peptides.

In the current study, we have shown that L-selectin-ZZ binds to Factor H and that the binding is dependent on divalent cations and ionic strength, which is consistent with the reported characteristics for Factor H binding to polymorphonuclear leukocytes and U937 cells [19]. Complement proteins are soluble proteins, which are maintained at a relatively constant concentration in blood or other body fluids (for review see [36]). Receptors for proteins of this type, unlike those for protein hormones, do not in general bind the native monomeric ligand strongly, as this might result in depletion of the ligand from the fluid phase and its sequestration on a cell surface. Instead, receptors detect an altered form of the protein, which is formed

in response to a specific stimulus (e.g. complement activation, formation of immune complexes). The alteration may take many forms, but one of the commonest in immunological systems is aggregation or polymerization of the ligand, such that multiple copies of the ligand, fixed to a surface, can interact with multiple copies of the receptor, situated on a cell surface [36]. In the last case, monomer–monomer interaction may be very weak, but the multiplicity of interaction provides strong binding. Since Factor H can become ‘aggregated’ by binding to C3b-coated surfaces or acidic phospholipids, it is possible that *in vivo*, significant binding to receptors occurs only after aggregation of the ligand. In order, therefore, to detect significant binding with monomeric ligand *in vitro*, a departure from strictly physiological conditions is necessary. This is seen with many immunological receptors, such as the C3b receptor (CR1), C1q receptor and some Fc receptors, in which binding to monomeric ligand is weak, but can be measured very readily *in vitro* at lower ionic strength [36]. Although most binding assays were done in 10 mM Tris, the binding of plasma proteins to L-selectin agarose was done at half physiological ionic strength. In Figure 7, binding is still readily detectable at 150 mM NaCl and above. Furthermore, as shown in Table 6, the binding of leukocytes to immobilized Factor H via L-selectin increased TNF- α release, indicating that the interaction between L-selectin and Factor H does have physiological significance. As soluble Factor H did not induce TNF- α secretion, it would appear that aggregation of Factor H is crucial for this biological function.

We have shown that binding of L-selectin-ZZ to Factor H is Ca²⁺-ion dependent and is inhibited by fucoidan, LPS and a functional-blocking anti-L-selectin antibody. Furthermore, pre-treatment of Factor H with sialidase reduced the binding to L-selectin. Taken together, these results indicate that the binding involves an interaction between the lectin domain of L-selectin and appropriate glycans on Factor H. The Factor H polypeptide chain contains about 9.3% carbohydrate [37,38] and analysis of the primary amino acid sequence reveals seven potential N-linked glycosylation sites [38]. However, a contribution from O-linked glycans cannot be ruled out.

It was observed that only a small proportion of plasma Factor H has appropriate characteristics for binding to L-selectin. It would appear that post-translational modification, such as sulphation (see Figure 5 for a potential sulphation site), differential glycosylation or aggregation of Factor H might be important for binding L-selectin. Preliminary results from our laboratory indicate that pre-treatment of Factor H with a bacterial arylsulphatase reduces the binding of L-selectin to Factor H (results not shown).

Further analysis and characterization of the carbohydrate and protein residues involved in the binding of L-selectin to Factor H will provide greater insight into the role of Factor H in

modulating L-selectin-mediated interactions in inflammatory processes.

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