Anionic phospholipids bind to L-selectin (but not E-selectin) at a site distinct from the carbohydrate-binding site

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It is known that L-selectin binds to glycoconjugates containing the tetrasaccharide sialyl Lewis X in a Ca^{2+} -dependent manner. In addition, a number of other acidic oligosaccharides (for example heparin or chondroitin sulphate) or glycolipids (for example sulphatides) bind to L-selectin independent of cations. In this paper we have established that L-selectin binds to charged phospholipids, such as cardiolipin and phosphatidylserine, but not to neutral phospholipids such as phosphatidylcholine. No interaction between E-selectin and any phospholipid was observed. The interaction between L-selectin and cardiolipin was inhibited by dextran sulphate, fucoidan, mannose 6-phosphate and monoclonal antibodies previously reported to block the interaction between L-selectin and its natural ligands. Analysis

of the amino acid sequence of the selectins indicated that L-selectin, but not E-selectin, contains a sequence homologous to the putative cardiolipin-binding epitope found in plasma glycoprotein $\beta_2\text{I}$. Glycoprotein $\beta_2\text{I}$ and a peptide corresponding to the putative cardiolipin-binding epitope in $\beta_2\text{I}$ inhibited the binding of L-selectin to cardiolipin or fucoidin. Based on the binding characteristics, sequence analysis and structural modelling of L-selectin, we suggest that the amino acid sequence KKNKED (residues 84–89) is a novel site for the binding of acidic species to L-selectin. This motif is localized close to the putative carbohydrate-binding domain of L-selectin and may be a second site within the lectin domain for the interaction of leucocyte L-selectin with its natural endothelial ligands.

INTRODUCTION

Selectins are cell-surface integral membrane glycoproteins reported to be involved in leucocyte trafficking, thrombosis and inflammation. The three known selectins (E-, P- and L-selectin) are characterized by an N-terminal carbohydrate recognition domain (CRD) 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. On the basis of either direct binding or inhibition experiments, it has been shown that several simple and complex carbohydrates interact with selectins in a calcium-dependent manner (for a review see [1]). The common feature of these ligands is a lactosamine backbone of either type-1 (Gal β 1-3GlcNAc) or type-2 (Gal β 1-4GlcNAc) glycans [2,3]. Fucosylated, sialylated oligosaccharides, such as sialyl-Lewis-X [sLex; NeuAc α 2-3, Gal β 1-4(α 1-3Fuc)GlcNAc], are considered to be minimal ligands for the selectins, although some groups have reported additional ligands for L- or P-selectin that lack sialic acid or fucose. The latter ligands, such as sulphatides, heparins and polyphosphomannan ester, interact with selectins in the presence of EDTA [4–6], suggesting that they bind at a site distinct from the classical Ca^{2+} -dependent CRD.

Glycoprotein $\beta_2\text{I}$ has been shown to bind to DNA, mitochondria, platelets and negatively charged phospholipids (for reviews, see [7–9]). It has been proposed that $\beta_2\text{I}$ forms a complex with charged phospholipids and that this complex is recognized by autoantibodies often associated with autoimmune diseases such as systemic lupus erythematosus. $\beta_2\text{I}$ glycoprotein is composed of five repeating domains of 60–80 amino acid residues and each domain contains the consensus sequence of the comp-

lement control protein. Several pieces of evidence indicate that the sequence KNKEKK, present in the fifth domain of $\beta_2\text{I}$, may be responsible for the binding of $\beta_2\text{I}$ to charged phospholipids [9]. For example a soluble form of this domain inhibited the binding of $\beta_2\text{I}$ to immobilized cardiolipin, and molecular modelling of the fifth domain of $\beta_2\text{I}$ indicated that the sequence KNKEKK is exposed on the surface of the $\beta_2\text{I}$ [10]. Subsequently, a peptide containing the sequence CKNKEKKC was shown both to bind to a cardiolipin affinity column and to inhibit the binding of $\beta_2\text{I}$ to cardiolipin [11]. Chemical modification of lysine residues in $\beta_2\text{I}$ have further established the role of the sequence KNKEKK in cardiolipin binding [8]. In addition, the complement system protein factor H was shown also to bind to cardiolipin as well as to inhibit cardiolipin-dependent binding [8]. A sequence similar to KNKEKK (KNKKEFD) is present in the 13th domain of factor H.

Examination of the primary sequence of L-selectin reveals a stretch of sequence KKNKED within the lectin domain (residue numbers 84–89) that shares sequence similarity with the sequence KNKEKK found in $\beta_2\text{I}$. In this article we provide evidence to indicate that KKNKE is a novel, putative site in L-selectin for the binding of negatively charged phospholipids. This site may be important for the binding of L-selectin to both its natural endothelial ligands and to acidic non-sialylated oligosaccharides.

MATERIALS AND METHODS

Recombinant selectins and proteins

A plasmid containing the human L-selectin gene was kindly provided by Professor B. Seed. Recombinant human selectins,

Abbreviations used: CRD, carbohydrate recognition domain; HRP, horseradish peroxidase; sLex, sialyl Lewis X.

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Table 1 Inhibition of binding of L-selectin-ZZ to cardiolipin or fucoidan

Microtitre-plate wells were coated with cardiolipin or fucoidan and then incubated for 2 h (cardiolipin) or 16 h (fucoidan) at room temperature with 50 μ l of L-selectin-ZZ (0.72 μ g in antibody experiments, otherwise 0.4 μ g) in the presence or absence of various inhibitors. The amount of bound L-selectin-ZZ was determined as described in the Materials and methods section. The values are means $\pm$ S.D. of two separate experiments.

Additions	Binding of L-selectin-ZZ [$A_{492} \pm$ S.D. (% inhibition)]	
	Cardiolipin	Fucoidan
CaCl ₂ (5 mM)	0.71 $\pm$ 0.07 (0)	0.29 $\pm$ 0.01 (0)
EDTA (5 mM)	0.73 $\pm$ 0.07 (0)	0.14 $\pm$ 0.01 (53)
Fucoidan (200 μ g/ml)	0.28 $\pm$ 0.003 (60)	0.06 $\pm$ 0.01 (80)
LAM 1-3 (12.5 μ g/ml)	0.11 $\pm$ 0.05 (85)	0.02 $\pm$ 0.007 (92)
MCA 1076 (12.5 μ g/ml)	0.35 $\pm$ 0.14 (51)	0.22 $\pm$ 0.03 (24)
Anti-(E-selectin) (BBA) (12.5 μ g/ml)	0.69 $\pm$ 0.06 (4)	0.22 $\pm$ 0.03 (23)

lacking the transmembrane and cytosolic domains, were produced by Bernard Allet (Glaxo Institute of Molecular Biology, Geneva, Switzerland) as C-terminal chimeras with the ZZ domain

of Protein A. The ZZ domain binds tightly to human IgG. A baculovirus/insect cell expression system was used and the IgG-purified proteins were partially characterized in cell adhesion assays [12]. β_2 I was purified by the method described by Kertesz et al. [8] and was kindly provided by Dr. R. B. Sim (MRC Immunochemistry Unit, Department of Biochemistry, Oxford, U.K.).

Two anti-(L-selectin) monoclonal antibodies were used: LAM 1-3 (subtype IgG1; Coulter Immunology, Coulter Corporation, Hialeah, FL 33010, U.S.A.) and MCA 1076 (subtype IgG 2, clone no. FMA46; Serotec, U.K.). The anti-(E-selectin) monoclonal antibodies used were: clones BBA and ENA2 (subtypes IgG1; cat. no. MON 6011, Bradsure Biological Ltd, Loughborough, Leics., U.K.). Horseradish peroxidase (HRP)-conjugated pig anti-(rabbit IgG) (cat. no. p217; Lot no. 111) was from Dako-Immunoglobulin, Denmark. sLex was from Dextra Laboratories Ltd, Reading, U.K.

Binding of L-selectin to fucoidan

Microtitre-plate wells (Maxisorb, NUNC, Denmark) were coated with 100 μ l of fucoidan (200 μ g/ml) (Sigma) in 50 mM sodium bicarbonate buffer (pH 9.6) at ambient temperature for 16 h.

Figure 1 Characteristics of binding of L-selectin-ZZ to cardiolipin

Microtitre-plate wells were coated with lipids and then incubated with L-selectin-ZZ or E-selectin-ZZ in the presence or absence of potential inhibitors. Following a 2 h incubation at room temperature, the amount of bound selectin was determined as described in the Materials and methods section. Each experiment was conducted two or three times, and a representative result is shown. Values are means $\pm$ S.D. for triplicate experimental points. (A) Serial dilutions of L-selectin-ZZ (■, □, ▲) or E-selectin-ZZ (●, ○, ▲) were incubated with lipid-coated wells: cardiolipin (■, ●), phosphatidylserine (□, ○) or phosphatidylcholine (▲). (B) Various concentrations of L-selectin-ZZ in the presence of 5 mM CaCl₂ (○) or 5 mM EDTA (●) were incubated with cardiolipin-coated wells. (C) A constant amount of L-selectin-ZZ (0.4 μ g) was incubated in the presence of various concentrations of dextran sulphate (●), dextran (○) or fucoidan (□) with cardiolipin-coated wells. (D) L-selectin-ZZ in the presence of serial dilutions of mannose 6-phosphate (○) or mannose (●) was incubated with cardiolipin-coated wells.

Table 2 Inhibition of binding of L-selectin-ZZ to cardiolipin

Microtitre-plate wells were coated with cardiolipin and then incubated for 2 h at room temperature with 50 μ l of L-selectin-ZZ (0.4 μ g) in the presence or absence of various inhibitors. The amount of bound L-selectin-ZZ was determined as described in the Materials and methods. The values are means $\pm$ S.D. of three separate experiments.

Additions	Binding of L-selectin-ZZ to cardiolipin [$A_{492} \pm$ S.D. (% inhibition)]
No inhibitor	0.659 $\pm$ 0.04 (0)
Fucoidan (500 μ g/ml)	0.119 $\pm$ 0.02 (82)
Sialyl Lewis X (1 mM)	0.604 $\pm$ 0.01 (8)
Mannose 6-phosphate (1 mM)	0.428 $\pm$ 0.01 (35)

Non-specific binding sites were blocked with BSA (3 mg/ml) in 10 mM Tris/HCl buffer (pH 7.4) for 2 h at ambient temperature. L-selectin-ZZ (14 μ g/ml; 50 μ l) in the presence of 5 mM EDTA or 5 mM CaCl_2 was added to the fucoidan-coated wells in a buffer containing 10 mM Tris/HCl (pH 7.4), BSA (1 mg/ml) and HRP-conjugated IgG (1/1000 dilution). The plates were incubated for 16 h at 4 $^\circ\text{C}$. After extensive washing with 10 mM Tris/HCl buffer (pH 9.6), the amount of bound L-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_{492} was measured.

Binding of selectins to phospholipids

Microtitre-plate wells were coated with phospholipids according to a standard published method [11]. Briefly, phospholipids (100 μ l of a 50 μ g/ml solution in ethanol) were added to the wells and the ethanol was evaporated at ambient temperature. Non-specific binding sites were blocked with ovalbumin (3 mg/ml) in 10 mM Tris/HCl buffer (pH 7.4) containing 150 mM NaCl. Serial dilutions of E- or L-selectin-ZZ protein (100 μ l; maximum concentration 7 μ g/ml) were added to the phospholipid-coated wells in a buffer containing 10 mM Tris/HCl (pH 7.4), 150 mM NaCl, ovalbumin (1 mg/ml), HRP-conjugated IgG (1/1000 dilution) (Buffer A) and 5 mM EDTA or 5 mM CaCl_2 . The amount of bound selectin was determined as described above.

Competition assays

The concentrations of selectins, compounds under test and antibodies are indicated in the legends to the appropriate Figures and Tables. Three peptides NCKNKEKKCVEIY (from β_2 I), NNKKNKEDCVEIY (from L-selectin) and KCNVDNIIKYEK (scrambled peptide from β_2 I) were synthesized, purified and characterized (results not shown). Where indicated, the peptide was treated with dithiothreitol (final concentration 10 mM) at 37 $^\circ\text{C}$ for 1 h. The dithiothreitol-treated or untreated peptide was incubated with cardiolipin-coated wells at room temperature for 2 h. After extensive washing, L-selectin-ZZ (100 μ l; 4 μ g/ml) was added to the microtitre wells in buffer A for 2 h. The bound L-selectin-ZZ was determined as described above.

Homology modelling of the lectin domain of L-selectin

A three-dimensional model of the lectin domain of L-selectin was constructed based on sequence similarity with E-selectin [13] using the program Insight II (Biosym Technologies Inc., San

Figure 2 Effect of β_2 I or β_2 I-derived peptide on binding of L-selectin-ZZ to cardiolipin

Microtitre-plate wells were coated with cardiolipin. The binding of L-selectin-ZZ was determined as described in the legend to Figure 1. (A) A constant amount of L-selectin-ZZ (0.4 μ g) in the presence of serial dilutions of β_2 I (●) or ovalbumin (○) was incubated with cardiolipin-coated wells. (B) Serial dilutions of untreated peptide (NCKNKEKKCVEIY; ○), peptide pretreated with 10 mM dithiothreitol (NCKNKEKKCVEIY; ●) or 10 mM dithiothreitol (□) were incubated with cardiolipin-coated wells for 2 h at room temperature. After extensive washing of the plate, a constant amount of L-selectin-ZZ (0.4 μ g) was added. After a further 2 h incubation at room temperature, the amount of bound selectin was determined as described in the Materials and methods section. Each experiment was conducted twice, and a representative result is shown. Values are means $\pm$ S.D. for triplicate experimental points. (C) A constant amount of L-selectin-ZZ (0.4 μ g) in the presence of serial dilutions of peptides NCKNKEKKCVEIY (□), NNKKNKEDCVEIY (○) or KCNVDNIIKYEK (△) was incubated with cardiolipin-coated wells.

Diego, CA, U.S.A.). The high degree of identity between the two sequences (62%) enabled us to use the complete fold of E-selectin as a starting approximation for L-selectin. Chi torsion angles for non-conserved amino acids were assigned values such that the side-chain conformation was comparable with the

Figure 3 Structure of the lectin domain of L-selectin

(a) Stereo-view of the model structure for the lectin domain of L-selectin. The backbone atoms comprising the KKNKED motif (residues 84 to 89) and the calcium ion which forms part of the carbohydrate-binding region are highlighted. The four amino acid residues which co-ordinate the calcium ion in L-selectin (Glu-80, Asn-82, Asn-105 and Asp-106), whereas residues 84 to 87 are not. (b) The molecular surfaces for E- and L-selectin are colour-coded by electrostatic potential: red indicates a potential of -5 kT; white, 0 kT; and blue, 5 kT. The KKNKED region in L-selectin can be seen to be positively charged whereas the homologous region in E-selectin is not.

original residue. All of the non-secondary structural elements of L-selectin were checked against the contents of Iritis (a protein structure database that is useful for checking consistency in model protein structures) [14]. The model structure was also compared with the crystal structure of the lectin domain of mannose-binding protein [15], particularly around the predicted carbohydrate-binding site and the KKNKED loop.

RESULTS

Binding characteristics of recombinant selectins

The properties of recombinant E-selectin-ZZ in cell adhesion assays have been described previously [12]. The binding characteristics of L-selectin-ZZ were examined using fucoidan, which is a fucose sulphate-rich polysaccharide from *Fucus vesiculosus* that has been shown previously to interact with L-selectin [16,17]. Table 1 shows that L-selectin-ZZ binds to immobilized fucoidan in the presence of Ca^{2+} and that the binding is partially inhibited by EDTA (53 %) and soluble fucoidan (80 %). The binding of L-selectin-ZZ to immobilized fucoidan was inhibited also by LAM 1-3 (92 %), whereas only partial inhibition was observed with MCA 1076 (Table 1). Antibody LAM 1-3 has been shown previously to block the binding of L-selectin-expressing cells to fucoidan [18], whereas MCA 1076 only partially blocks the binding of L-selectin to its ligands [19]. An anti-(E-selectin) antibody (same subtype as LAM 1-3) had no effect on the binding of L-selectin-ZZ to fucoidan. The above results indicate that the recombinant L-selectin-ZZ is functionally active and has characteristics indistinguishable from those of natural L-selectin.

Characteristics of binding of L-selectin-ZZ to charged phospholipids

The observation that L-selectin contains a sequence (KNKE) homologous to the putative cardiolipin-binding site in $\beta_2\text{I}$ (KNKEKK) suggests that L-selectin may contain a binding site for cardiolipin. This hypothesis was tested by incubating L- or E-selectin-ZZ with immobilized phospholipids in the presence of CaCl_2 . L-selectin-ZZ bound to the charged phospholipids, cardiolipin and phosphatidylserine, but not to the neutral phosphatidylcholine (Figure 1A). E-Selectin-ZZ, which does not contain the KNKE sequence, did not bind to any of the phospholipids.

The binding of L-selectin-ZZ to cardiolipin was unaffected by the presence of 5 mM EDTA, indicating that the binding is Ca^{2+} -independent (Figure 1B). However, the binding was inhibited by acidic saccharides such as fucoidan and mannose 6-phosphate, but not by the neutral compounds dextran and mannose (Figures 1C and 1D). Dextran sulphate only partially inhibited the binding (Figure 1C). This inhibitory profile is similar to that reported for the binding of L-selectin to its natural ligands [16,17]. In a separate set of experiments binding of L-selectin-ZZ to cardiolipin in the presence of sLex was studied. sLex did not inhibit the binding of L-selectin-ZZ to cardiolipin (Table 2), whereas in the same assay fucoidan and mannose 6-phosphate inhibited the binding (Table 2).

The nature of the cardiolipin-binding site was then investigated using antibodies whose epitopes are reasonably well defined. Antibody LAM 1-3, which blocks the interaction of L-selectin with its natural ligands [18], completely inhibited the binding of L-selectin-ZZ to cardiolipin, whereas antibody MCA 1076 only partially blocked the interaction [19] (Table 1). As expected, two antibodies to E-selectin (BBA and ENA2) had little or no effect

on the binding of L-selectin-ZZ to cardiolipin. Similar results with antibodies were obtained when the binding of L-selectin-ZZ to fucoidan was examined (Table 1).

Effect of $\beta_2\text{I}$ on the binding of L-selectin-ZZ to cardiolipin

Since glycoprotein $\beta_2\text{I}$ binds to cardiolipin, it was postulated that $\beta_2\text{I}$ may inhibit the binding of L-selectin-ZZ to cardiolipin. Indeed, a concentration-dependent inhibition of binding by $\beta_2\text{I}$ was observed (Figure 2A). As discussed above, the sequence KNKE within the lectin domain of L-selectin is homologous to the $\beta_2\text{I}$ sequence CKNKEKKC, which has been identified as a binding site for cardiolipin. A synthetic peptide including this sequence inhibited the binding of L-selectin-ZZ to cardiolipin (Figures 2B and 2C). Prior treatment of the peptide with dithiothreitol abolished the inhibitory effect of the peptide (Figure 2B), suggesting that disulphide bridging of the Cys-Cys-containing peptide is required for inhibition. Similar results have been obtained with a related $\beta_2\text{I}$ -derived peptide in binding experiments with cardiolipin and $\beta_2\text{I}$ [11]. An homologous peptide from L-selectin, which is unable to form intramolecular disulphide bonds, failed to inhibit the binding of L-selectin-ZZ to cardiolipin (Figure 2C).

Structure of the lectin domain of L-selectin

Our results demonstrated that the complete secondary structure of the lectin domain of E-selectin was a suitable model to use for L-selectin. Structure checking employing Iritis indicated that non-conserved motifs in loop regions were not expected to form alternative conformations. Figure 3(a) illustrates the three-dimensional model of L-selectin highlighting the calcium ion of the predicted carbohydrate-binding site and the KKNKED motif (residues 84–89). The KKNKED sequence in L-selectin was predicted to be part of a loop which includes a β -turn (Lys-85 O—HN Glu-88). A related β -turn conformation was predicted in the KNKEKK motif in the fifth domain of $\beta_2\text{I}$ [10]. Analysis of the electrostatic potential of L-selectin using Grasp (developed by A. Nicholls, Department of Biochemistry and Molecular Biophysics, Columbia University, NY, U.S.A.) demonstrated the protein surface around the KKNKED motif is basic unlike the homologous region in E-selectin. The electrostatic surface of L-selectin also revealed a second region of positive charge further away from the carbohydrate-binding domain. There are five lysine residues in the region and it may be an alternative site for the binding of anionic ligands. An interesting difference between E- and L-selectin is the total charge of the lectin domains; L-selectin is more positive by five units.

DISCUSSION

Examination of the primary sequence of L-selectin led us to speculate that the lectin domain contains a binding site for cardiolipin (the consensus sequence KKNKE, see the Introduction). This hypothesis was confirmed in direct binding studies. Thus acidic sugars such as mannose 6-phosphate, dextran sulphate and soluble fucoidan inhibited L-selectin-ZZ binding to both cardiolipin and fucoidan, as did a 'blocking' anti-(L-selectin) antibody. These agents are known to inhibit the binding of L-selectin to its natural ligands [16–18], presumably by interacting with the lectin domain of L-selectin, although the binding site for these inhibitors has not been investigated in detail. In addition to the above observations, a peptide derived from the cardiolipin-binding domain of glycoprotein $\beta_2\text{I}$ inhibited the binding of L-selectin-ZZ to both cardiolipin and fucoidan

(latter result not shown). On the other hand, some differences in the binding of L-selectin-ZZ to the two immobilized ligands were noted. For example, the binding of cardiolipin to L-selectin-ZZ was independent of added Ca^{2+} , whereas binding to fucoidan was partially dependent on Ca^{2+} . The interaction of L-selectin with established ligands such as sLex-containing glycoconjugates is Ca^{2+} -dependent. Taken together, these findings indicate that cardiolipin interacts with L-selectin-ZZ at a binding site distinct from, but closely associated with, the carbohydrate-binding domain.

A consensus sequence for cardiolipin binding is found in the plasma glycoprotein $\beta_2\text{I}$ (see the Introduction). Hunt and Krillis [11] showed that a peptide corresponding to the $\beta_2\text{I}$ sequence CKNKEKCC binds to cardiolipin and inhibits the binding of cardiolipin to $\beta_2\text{I}$. It was proposed that the presence of two cysteines in the peptide was important for maintaining the secondary structure required for binding to cardiolipin.

Molecular modelling of the fifth domain of $\beta_2\text{I}$ has also indicated that the sequence CKNKEKCC in the fifth domain of $\beta_2\text{I}$ forms an exposed loop-like cyclic structure in which the two cysteines do not form a disulphide bond [9,10]. Instead, the loop-like structure is stabilized by disulphide bridging either between cysteines at positions 281 and 306 or between cysteines 288 and 326 [10]. In the synthetic peptide, it is possible that the two cysteines form an intrapeptide bond, resulting in a cyclic molecule which has a loop-like structure similar to the tertiary structure of the binding domain within $\beta_2\text{I}$. In support of this assertion, it was found that dithiothreitol abolished the inhibitory properties of the peptide, indicating that intrapeptide disulphide bonding is necessary for the binding of the peptide to cardiolipin. Mass spectroscopic analysis of the peptide showed the peptide to be monomeric, thus ruling out the possibility of interpeptide bonding (results not shown).

Homology modelling of the lectin domain of L-selectin, based on the crystal structure of E-selectin, showed that the sequence KKNKED is part of a loop similar to that observed in the fifth domain of $\beta_2\text{I}$ [10]. This loop is exposed on the protein and is adjacent to the calcium-binding site predicted to co-ordinate with sLex-containing glycoconjugates (Figure 3a). The differential binding of cardiolipin to E- and L-selectin is consistent with the difference in the total charge of the two lectin domains. L-selectin is more positively charged than E-selectin. Furthermore, comparison of the primary sequences and three-dimensional structures of E- and L-selectin shows that E-selectin lacks a basic motif corresponding to the putative acidic phospholipid (cardiolipin and phosphatidylserine)-binding site in L-selectin (L-selectin has KKNKED whereas E-selectin has RQKDED). It is interesting also that a related basic sequence corresponding to RKGR is the putative heparin-binding site in acetylcholinesterase [20]. This sequence has the same charge distribution as KKNK, providing additional support that this region within L-selectin mediates the binding of anionic species.

The physiological significance, if any, of a phospholipid-binding site on L-selectin is unclear. However, the observation that cardiolipin binding is inhibited by other acidic saccharides (e.g. fucoidan and mannose 6-phosphate) suggests that this site (KKNKE) may be the binding site for ligands such as sulphatides

and glycosaminoglycans. The latter ligands are also able to interact with L-selectin in a Ca^{2+} -independent manner. Molecular modelling indicates that this second site is in close proximity to the classical Ca^{2+} -dependent carbohydrate-binding domain, and it is possible that the interaction of leucocyte L-selectin with its complex natural endothelial ligands requires occupancy of both binding sites. Indeed, a putative natural L-selectin ligand on the glycoprotein GlyCAM-1 is a branched O-linked glycan containing sialic acid, fucose and galactose sulphate [21]. Further evidence in support of the above hypothesis will come from experiments where the key basic residues are replaced using mutagenesis techniques.

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REFERENCES

- Varki, A. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 7390–7397
- Yago, K., Zenita, K., Ginya, H., Sawada, M., Ohmori, K., Okuma, M., Kannagi, R. and Lowe, J. B. (1993) *Cancer Res.* **53**, 5559–5565
- Goetz, S., Kumar, R., Potvin, B., Sundaram, S., Brikelmaier, M. and Stanley, P. (1994) *J. Biol. Chem.* **269**, 1033–1040
- Larkin, M., Ahern, T. J., Stoll, M. S., Shaffer, M., Sako, D., O'Brein, J., Yeun, C.-T., Lawson, A. M., Childs, R. A., Barone, K. M., Langer-Shafer, P. R., Hasegawa, A., Kiso, M., Larsen, G. R. and Feizi, T. (1992) *J. Biol. Chem.* **267**, 13661–13668
- Suzuki, Y., Toda, Y., Tamatani, T., Watanabe, T., Suzuki, T., Nakao, T., Murase, K., Kiso, M., Hasegawa, A., Tadano-Aritomi, A. and Miyasaka, M. (1993) *Biochem. Biophys. Res. Commun.* **190**, 426–434
- Green, P. J., Yuen, C.-T., Childs, R. A., Chali, W., Miyasaka, M., Lemonie, R., Lubineau, A., Smith, B., Ueno, H., Nicolaou, K. C. and Feizi, T. (1995) *Glycobiology* **5**, 29–38
- Galli, M. and Bevers, E. M. (1994) *Lupus* **3**, 223–228
- Kertesz, Z., Yu, B.-B., Steinkasserer, A., Haupt, H., Benham, A. and Sim, R. B. (1995) *Biochem. J.* **310**, 315–322
- Steinkasserer, A., Estaller, C., Weiss, E. H., Sim, R. B. and Day, A. J. (1991) *Biochem. J.* **277**, 387–391
- Steinkasserer, A., Berlow, P. N., Willis, A. C., Kertesz, Z., Campbell, I. D., Sim, R. B. and Norman, D. G. (1992) *FEBS Lett.* **313**, 193–197
- Hunt, J. and Krillis, S. (1993) *J. Immunol.* **152**, 653–659
- Priest, R., Nawaz, S., Green, P. M. and Bird, M. I. (1995) *Biochem. Soc. Trans.* **23**, 162S
- Graves, B. J., Crowther, R. L., Chandran, C., Rumberger, J. M., Li, S., Huang, K.-S., Presky, D. H., Familletti, P. C., Wolitzky, B. A. and Burns, D. K. (1994) *Nature (London)* **367**, 532–538
- Thornton, J. M. and Gardener, S. P. (1989) *Trends Biochem. Sci.* **14**, 300–304
- Weis, W. I., Drickamer, K. and Hendrickson, W. A. (1992) *Nature (London)* **360**, 127–134
- Imai, Y., True, D. D., Singer, M. S. and Rosen, S. D. (1990) *J. Cell Biol.* **111**, 1225–1232
- Yoshida, T., Fennie, C., Lasky, L. A. and Lee, Y. C. L. (1994) *Eur. J. Biochem.* **222**, 703–709
- Kansas, G. S., Spertini, O., Stoolman, L. M. and Tedder, T. F. (1991) *J. Cell Biol.* **114**, 351–358
- Pilarski, L. M., Turley, E. A., Shaw, A. R. E., Gallatin, W. M., Laderoute, M. P., Gillitzer, R., Beckman, I. G. R. and Zola, H. (1991) *J. Immunol.* **147**, 136–143
- Deprez, P. N. and Inestrosa, N. C. (1995) *J. Biol. Chem.* **270**, 11043–11046
- Hemmerich, S., Leffler, H. and Rosen, S. D. (1995) *J. Biol. Chem.* **270**, 12035–12047