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Ligand-Specificity of the Selectins

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Abstract The selectins are carbohydrate-binding cell adhesion molecules acting in the vascular system. They mediate the docking of leukocytes to the blood vessel wall and the rolling of these cells along the endothelial cell surface. These adhesion phenomena initiate the entry of leukocytes into sites of inflammation as well as the migration of recirculating lymphocytes into secondary lymphoid tissues. Blocking selectin function with antibodies or oligosaccharides has proven to be beneficial in various animal models of inflammation and models of ischemia/reperfusion damage. This has raised much interest in the identification of the physiological ligands of the selectins. Several glycoprotein ligands have been identified, some of which can even be selectively isolated from cellular detergent extracts using a selectin as an affinity probe. Four of these "high affinity" ligands have been cloned. The structural requirements of their interaction with the selectins is discussed. © 1996 Wiley-Liss, Inc.

Key words: selectins, vascular system, leukocytes, sialomucins, fucosylation

Leukocytes cross the blood vessel wall in order to enter into inflamed tissue. In addition, certain subsets of lymphocytes constantly recirculate and enter lymphoid tissues in a process called lymphocyte homing. In both cases, leukocytes in the bloodstream need to attach to the vessel wall, a process which is achieved by multiple sets of different cell adhesion molecules [Springer, 1994]. The selectins are among the first adhesion molecules acting in this process. They are involved in the docking of leukocytes to the endothelial cell surface, as well as in the rolling along this surface. Upon docking and rolling, the leukocyte-integrins become activated, which is probably triggered by factors present on the surface of activated endothelium. This initiates the stabilization of the leukocyte-endothelial cell contact and enables leukocytes to migrate on the endothelial cell surface and finally through the blood vessel wall.

The selectins are a family of carbohydrate-binding cell adhesion molecules comprised of three glycoproteins designated by the prefixes L (leukocytes), E (endothelium), and P (platelets). Only L-selectin is found on leukocytes, while the other two selectins are expressed by endothelial cells. The selectins share a similar structural organisation, having an N-terminal lectin domain, a single EGF-type domain and various numbers of consensus repeats with sequences similar to those in complement regulatory proteins.

L-selectin was originally found on lymphocytes as a homing receptor, and later was also found on monocytes and neutrophil granulocytes. The two endothelial selectins are induced on the surface of endothelial cells upon stimulation with various inflammatory mediators. All three selectins are involved in the binding of neutrophil granulocytes to activated endothelium. In in vivo studies of selectin deficient mice and of various animals treated with adhesion blocking antibodies against the selectins, the importance of the selectins for the entry of neutrophil granulocytes into sites of inflammation has been documented. Such studies have been reviewed in an excellent article providing much insight into the various mechanisms of leukocyte extravasation by Albelda et al. [1994]. Various other aspects of the selectins have been summarised in several excellent reviews [Kishimoto, 1993; Bevilacqua and Nelson, 1993; Rosen, 1993]. This review will focus on the ligands of the selectins and their binding determinants.

Carbohydrate Ligands

The lectin character of L-selectin was already discovered, before sequence information revealed the existance of a C-type lectin-domain

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[Drickamer, 1988] and before studies with recombinant forms of L-selectin were possible. L-selectin-mediated binding of lymphocytes to endothelium was found to be blocked by phosphorylated monosaccharides, a mannose-6-phosphate rich polysaccharide (PPME) and the sulfated fucose polymer fucoidin [Yednock et al., 1987; Imai et al., 1990]. Cloning of the selectins and identification of the lectin domain initiated the search for the carbohydrate ligands of all three selectins.

The tetrasaccharide sialyl Lewis X (sLe^x, Neu5Acα2–3Galβ1-4[Fucα1–3]GlcNAc) was first *suspected to* be a ligand for E-selectin. Its expression on PMNs, monocytic cell lines, and other cells correlated with the binding of these cells to E-selectin [Phillips et al., 1990; Walz et al., 1990]. Antibodies which recognized sLe^x could inhibit cell adhesion in these assays and neoglycoproteins consisting of bovine serum albumin loaded with sLe^x, or its stereoisomer sLe^a, could support binding of E-selectin transfected cells [Berg et al., 1991a]. Soon it was found that sLe^x could indeed bind to all three selectins [Foxall et al., 1992] and could even block neutrophil migration into inflamed tissue in some models in vivo [Mulligan et al., 1993]. Thus, it is likely that the carbohydrate structures on the physiological selectin ligands somehow resemble the sLe^x structure. However, whether they are identical with sLe^x is not known, since several glycoproteins are known which are strongly modified with sLe^x, yet do not bind to P-selectin [Moore et al., 1992]. In addition, various mammals and nonhuman primates do not express sLe^x although selectin recognition functions across these species barriers [Ito et al., 1994]. Various derivatives of sLe^x were shown to bind with considerably higher affinity [Nelson et al., 1993]. Common to most selectin-binding oligosaccharides is fucose and a charged group such as sialic acid, a sulfate group or uronic acid.

Carbohydrates could be presented to selectins on glycolipids or on protein backbones. Glycoproteins would have the advantage of being more accessible on the cell surface than the shorter glycolipids [Springer, 1990]. Accessibility and well exposed location on the cell surface is likely to be important for adhesion molecules which mediate rolling, as was shown for L-selectin which was localized on the tips of pseudopods of neutrophil granulocytes [Picker et al., 1991]. The same subcellular distribution was found for the P-selectin ligand PSGL-1 [Moore et al., 1995].

Glycoprotein Ligands

The use of soluble forms of the selectins as affinity probes has been a successful approach to identify glycoprotein ligands. With antibody-like fusion proteins in which the Fab part of human IgG1 was replaced by the extracellular part of mouse L-selectin, the two ligands GlyCAM-1 [Lasky et al., 1992] and CD34 [Baumhueter et al., 1993] were identified on the endothelium of high endothelial venules of lymph nodes. Both glycoproteins are recognized by the monoclonal antibody MECA 79, which blocks lymphocyte-binding to high endothelial venules [Imai et al., 1991]. Therefore, these two ligands are good candidates for vascular addressins involved in lymphocyte homing to peripheral lymph nodes. A third glycoprotein of 200 kD was recently found in the same tissue by affinity isolation with L-selectin-IgG [Hemmerich et al., 1994]. This glycoprotein, as well as GlyCAM-1 and CD34 was identified by labelling with $^{35}SO_4$. Other candidates for vascular addressins include additional glycoproteins which are also recognized by the anti-carbohydrate mAb MECA 79 in lymph node tissue and which have not yet been further characterized [Berg et al., 1991b].

The expression of the protein backbones of GlyCAM-1 and CD34 is not restricted to lymph node high endothelial venules. CD34 is also expressed on hematopoetic progenitor cells in the bone marrow, as well as on most endothelial cells throughout the body. GlyCAM-1, which is a secreted protein, was also found in milk [Dowbenko et al., 1993]. However, in those sites outside lymph node tissue both ligands lack the correct carbohydrate-modifications which function as selectin-recognition motifs. Sulfation of GlyCAM-1, which was found to be essential for binding to L-selectin [Imai et al., 1993], was not found for GlyCAM-1 in milk. Analysing the carbohydrate capping structures on GlyCAM-1 revealed the presence of a sulfate in position 6 on galactose in sLe^x [Hemmerich and Rosen, 1994]. In general, for none of the identified selectin ligands, the expression of their protein backbone is restricted to those cells where they serve as ligands (see below). However, only on those cells which are able to bind to a selectin the ligands carry the correct carbohydrate-modifications which define them as selectin ligands. This demonstrates the central importance of the modifying enzymes and their cell type-specific

regulation for the selectin-mediated adhesion phenomena.

GlyCAM-1 and CD34 are both sialomucins which carry large clusters of sialic acid-rich O-linked carbohydrate side chains. These carbohydrate clusters are essential for the binding to L-selectin. Other ligands are also sialomucins, or at least contain a sialomucin domain, for example the vascular addressin MAdCAM-1. Although MAdCAM-1 usually serves as a ligand for the lymphocyte integrin $\alpha_4\beta_7$, a small percentage of these molecules which carry MECA 79 epitopes also carry O-linked carbohydrate modifications which can be recognized by L-selectin [Berg et al., 1993].

Another sialomucin-type ligand is the human P-selectin ligand PSGL-1. This ligand was identified by expression cloning: COS-7 cells cotransfected with PSGL-1 and fucosyl transferase III were able to bind to human P-selectin-IgG fusion protein coated onto a surface [Sako et al., 1993]. The same protein was also found by affinity isolation using purified P-selectin from human platelets as an affinity probe [Moore et al., 1992]. Using an adhesion blocking monoclonal antibody against human PSGL-1, it was shown recently that PSGL-1 mediates rolling of leukocytes on P-selectin [Moore et al., 1995]. A 230/130 kD pair of proteins, which was affinity-purified with mouse P-selectin-IgG from mouse neutrophils [Lenter et al., 1994], is probably the mouse homolog to human PSGL-1. A similar pair of proteins which was detected with mouse P-selectin-IgG on human HL60 cells is recognized with a serum against a human PSGL-1-IgG fusion protein [Borges and Vestweber, unpublished].

Human PSGL-1 was also shown to support binding of cells transfected with human E-selectin [Sako et al., 1993]. A quantitative comparison of the binding of PSGL-1 transfected cells to immobilized P- or E-selectin, revealed that PSGL-1 binds with higher affinity to P- than to E-selectin [Moore et al., 1994]. However, the affinity between PSGL-1 (mouse or human) and mouse E-selectin is sufficient to survive extensive washings in affinity isolation experiments [Lenter et al., 1994].

In contrast to the sialomucin type ligands, the E-selectin ligand ESL-1 requires N-linked carbohydrates for binding. ESL-1 was found as a 150 kD glycoprotein ligand by affinity isolation with E-selectin-IgG on mouse neutrophil granulocytes [Levinovitz et al., 1993]. This ligand is not susceptible to degradation with O-sialoglycoprotease (a protease which specifically degrades sialomucins) [Lenter et al., 1994]. In contrast to the sialomucin PSGL-1 which binds to P-selectin and to E-electin, ESL-1 binds exclusively to E-selectin. This suggests that both ligands, while overlapping in some of their functions, may also serve different functions.

ESL-1, transfected into CHO cells, is only bound by E-selectin when co-transfected with a fucosyl transferase [Steegmaier et al., 1995]. Thus, fucosylation of ESL-1 and PSGL-1 is essential for selectin-binding. This is in good agreement with the necessity of fucose for selectin-mediated cell adhesion processes in vivo, which is well documented by studies on patients suffering from a genetic defect in fucose metabolism. These patients lack fucose containing glycoconjugates and one of the consequences are defects in selectin-mediated adhesion of neutrophils to endothelium and in neutrophil infiltration into sites of infection. Therefore, this disease was named leukocyte adhesion deficiency II (LAD II) [Etzioni et al., 1992].

Sequencing of ESL-1 [Steegmaier et al., 1995] revealed that it is highly homologous to a novel type of cysteine-rich fibroblast growth factor receptor (CFR) [Burrus et al., 1992]. The amino acid sequence of ESL-1 is 94% identical (over 1,078 amino acids) to CFR, except for a unique 70 amino acid amino-terminal domain of mature ESL-1. Whether ESL-1 is a splicing variant of the putative exact mouse equivalent of the chicken CFR is not yet known.

The strong structural homology to the chicken FGF-receptor raises the question whether ESL-1 could have some signalling function. It is intriguing to speculate that ESL-1 may be involved in triggering signals which lead to integrin activation. Indeed, evidence has been reported that soluble E-selectin can induce activation of the integrin Mac-1 on PMNs [Lo et al., 1991]. In this context, it may be of interest that interactions between neuronal cell adhesion molecules such as N-CAM, N-cadherin, and L1 with FGF-receptors were found recently, which are responsible for growth regulatory functions of the cell adhesion molecules [Doherty and Walsh, 1994]. However, so far no evidence has been presented that ESL-1 or the chicken CFR are able to mediate signal transduction.

While a receptor-like function of ESL-1 is still speculative, its function as a cell adhesion molecule has been demonstrated [Steegmaier et al.,

1995]. An immunoglobulin fusion protein of ESL-1 containing the complete extracellular part of ESL-1 and modified by fucose supported binding of E-selectin transfected CHO cells. No binding was seen to a fucose-containing L-selectin-Ig fusion protein. Furthermore, affinity-purified polyclonal antibodies against ESL-1 blocked the binding of the mouse neutrophilic cell line 32Dcl3 to immobilized E-selectin-IgG. Similarly, the binding of these cells and of mouse PMNs to cytokine-induced mouse endothelioma cells could be blocked by the anti-ESL-1 antibodies.

GlyCAM-1, CD34, PSGL-1, and ESL-1 are the selectin ligands which have been cloned and for which binding to a selectin has been directly shown by isolation with a selectin affinity probe. In addition to these, many ligands have been described for which affinity isolation with a selectin was not shown or which were identified in this way, but have not yet been cloned. Three such ligands were identified by affinity isolation with L-selectin: A 200 kD ligand for mouse L-selectin [Hemmerich et al., 1994], a heparan sulfate containing proteoglycacn [Norgard-Sumnicht et al., 1993], and several sulfated proteins *in* rat lymph nodes [Tamatani et al., 1993]. For P-selectin, a 160 kD glycoprotein (reduced 80 kD) was affinity isolated from mouse neutrophil granulocytes with mouse P-selectin-IgG [Lenter et al., 1994], but not yet cloned. Analogously to ESL-1, which does not bind to P-selectin, the 160 kD P-selectin-ligand does not bind to E-selectin and requires N-linked carbohydrates for binding. The heat stable antigen (HSA), purified from different mouse leukocytes and immobilized on a surface, supports binding of P-selectin-IgG but not E-selectin-IgG [Sammar et al., 1994]. Many ligands were described for E-selectin, of which only one was identified by direct affinity isolation. This is a 250 kD (reduced 280 kD) glycoprotein on bovine peripheral $\gamma|\delta$ T-cells [Walcheck et al., 1993]. The other E-selectin ligands were defined either by adhesion blocking antibodies or by testing their capacity to support the binding of E-selectin-expressing cells to the purified, immobilized ligand: These proteins are L-selectin from human neutrophils [Picker et al., 1991], members of the NCA-family [Kuijpers et al., 1992], a subpopulation of sLe^x carrying β_2-integrins [Kotovuori et al., 1993], the sLe^x carrying lamp-1 lysosomal protein on the surface of carcinoma cells [Sawada et al., 1993], and not yet characterised glycoproteins on human T-lymphocytes which carry sLe^a-like carbohydrate structures defined by the mAb HECA 452 [Berg et al., 1991c].

At present it is impossible to decide which of the known selectin-ligands is most likely to be of physiological relevance. The best candidates probably are those for which specific binding to a selectin has been directly demonstrated by affinity isolation.

Selectin Ligand Recognition

The cloning of some of the selectin ligands now allows the study of various questions about the structural requirements for the selectin-ligand-interactions and about the function of the ligands. It is still not known in detail what structural motif(s) on the ligands determine the recognition by a selectin. Since most of the well defined selectin ligands are sialomucins, Norgard et al. [1993] have suggested that clusters of common oligosaccharides could present uncommon "clustered saccharide patches" generated by multiple oligosaccharides that are spaced closely enough to restrict their motion. This hypothesis has been described in more detail in an excellent recent review by Varki [1994]. Since the carbohydrate binding site in the lectin domain is too small to accomodate large patches of carbohydrate side chains [Graves et al., 1994], Varki suggests that a cluster of common carbohydrate side chains generates carbohydrate epitopes which are unique (by forcing a single side chain into an unusual conformation or by combining certain groups of different side chains into one binding epitope). This is at present the best model for the binding of sialomucins to selectins.

Binding of ESL-1 to E-selectin is based on different structural requirements. There are only five widely spaced potential N-glycosylation sites on this glycoprotein and there is no evidence for O-linked carbohydrates. It was shown that N-linked carbohydrates, sialic acid, and fucose are essential for the binding to E-selectin. It is possible that ESL-1 carries complex carbohydrate side chains which are unique for this protein and determine the high affinity binding motif on this ligand. Indeed complex tetraantennary carbohydrate structures could be affinity isolated with a human E-selectin affinity matrix from carbohydrate extracts that had been released from membranes of myeloid cells [Patel et al., 1994]. This work demonstrates that single

carbohydrate side chains can bind to E-selectin with sufficient affinity to withstand extensive washing. However, ESL-1 is very selectively recognised by E-selectin. Beside ESL-1, only a sialomucin-ligand of 230/130 kD (most likely mouse PSGL-1) and a yet unidentified protein of 250 kD is recognised by E-selectin on mouse neutrophil granulocytes. On the myeloid cell line 32Dcl3, ESL-1 was even the only identified ligand. If E-selectin-binding to ESL-1 is determined by unique forms of carbohydrate side chains, there must be a mechanism which allows the repertoire of glycosyltransferases in a cell to create such unique types of side chains on only a single protein backbone. It is an open question, which structural entities of a protein backbone would be the determinants for such exclusive modifications.

Another possibility to explain the striking selectivity of the E-selectin interaction with ESL-1 would be that a part of the protein backbone of ESL-1 is directly involved in the binding. This can now be tested using recombinant ESL-1.

ESL-1 is exclusively recognised by E-selectin while PSGL-1 binds to E- and P-selectin. This raises the question, what distinguishes ESL-1-binding to E-selectin from PSGL-1-binding to E-selectin? One possibility would be that both ligands bind to different sites. However, only one carbohydrate binding site was identified on the E-selectin lectin domain of the crystal structure [Graves et al., 1994] and since ESL-1 as well as PSGL-1 were shown to require sialic acid and fucose for selectin binding, it is more likely that the carbohydrate determinants of both ligands bind to the same site in the lectin domain. This is also supported by structure-function studies of E- and P-selectin [Erbe et al., 1993] and a recent report on three mAbs which recognise both selectins and block their function [Berg et al., 1995]. If the carbohydrate binding site on E-selectin is the same for ESL-1 and PSGL-1, an alternative explanation for the E-selectin specificity of ESL-1 would be that a second binding site on E-selectin recognizes a different part (possibly a protein epitope) of ESL-1. According to this hypopthesis, recognition of ESL-1 by E-selectin would require the carbohydrate binding site and the second binding site. Indeed, a second binding site on the EGF domain of P-selectin has been suggested from experiments using L-selectin/P-selectin chimeras [Kansas et al., 1994]. In these experiments, the EGF-domain of P-selectin combined with the lectin domain of L-selectin could transfer P-selectin-specificity to the chimeric molecule. Additional evidence for a second binding site in the EGF-domain of P-selectin is based on peptide inhibition studies [Murphy and McGregor, 1994].

Analysis of the binding sites on the selectins and of the recognition motifs on the physiological selectin ligands will provide the basis for understanding selectin function as well as for the rational design of selectin blocking compounds. The availability of recombinant forms of the selectin ligands will facilitate these studies.

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