JCI The Journal of Clinical Investigation

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J Clin Invest. 1997;99(2):158-162. https://doi.org/10.1172/JCI119142.

Perspective

Perspectives Series: Cell Adhesion in Vascular Biology

Selectin Ligands: Will the Real Ones Please Stand Up?

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Less than a decade ago, the cloning of three seemingly disparate vascular cell surface molecules uncovered the selectin family of receptors that are now known to mediate critical cell-cell interactions in processes such as leukocyte trafficking, thrombosis, acute and chronic inflammation, and ischemia-reperfusion injury (1–15). L-, E-, and P-selectin are type-1 membrane glycoproteins sharing a similar structure, comprising an NH_2-terminal calcium-dependent "C type" lectin domain, an EGF-like domain, variable numbers of short consensus repeats domains, a single-pass transmembrane-domain, and a cytosolic carboxyl terminal tail. Abundant evidence indicates that selectins recognize specific carbohydrate ligands. Extensive studies involving mutagenesis, domain swapping, antibody inhibition, etc., have helped to define the functional domains of the selectins. It appears that selectin recognition of carbohydrate ligands involves primarily the amino-terminal C-type lectin domain, influenced to a substantial extent by the EGF-like domain, and to a much lesser degree by the short consensus repeats (1–20). As with most other adhesion receptors, it is also evident that selectins are signaling molecules. This perspective focuses on one of the most puzzling and controversial aspects of selectin biology—the structure of the natural ligands recognized by these receptors.

Mammalian lectin domains typically recognize specific oligosaccharide sequences with considerable selectivity, but relatively low affinity. Thus, for example, the cation-independent M6P receptor (CI-M6PR) recognizes mannose 6-phosphate α1-2 linked to another mannose (21), CD22 recognizes an α2-6–linked sialic acid residue attached to Galβ1-4GlcNAc (22), and the selectins share the ability to recognize the tetrasaccharide sialyl-Lewis^x (SLe^x)[1] and its isomer sialyl-Lewis^a. In the first two instances, K_d values for the basic recognition sequence are in the low micromolar range, and higher affinity binding (nanomolar range) is achieved either by multiple binding sites within a single lectin molecule (CI-M6PR) or by molecular aggregation of the monovalent lectin on the cell surface (CD22). This phenomenon of multivalency-based avidity seems to apply to most other naturally occurring lectins (23). With the selectins, the binding affinities for sialyl-Lewis^x are very poor, being in the mid micromolar to millimolar range. However, molecular aggregation (e.g., demonstrable by chemical cross-linking) has not been reported for any of the selectins (regional clustering has been reported for L-selectin on the pseudopods of neutrophils). Instead, there are some reports of high affinity binding of recombinant soluble monovalent selectins to natural ligands (16, 17). Thus, the issue of how selectins generate functional high affinity binding remains unresolved (see discussion below). Regardless, the recombinant selectins used to identify biological ligands are multimeric in structure and/or are usually presented in multivalent clustered arrays on solid supports or in soluble complexes. Thus, it should not be surprising that a large number and variety of molecules have been reported to bind to these multimeric recombinant selectins in a glycosylation-dependent manner. To date, very few of these "ligands" have actually been shown to mediate biological interactions. Thus, the current challenge is to tell the difference between what can bind to a recombinant selectin in vitro, and what does bind in vivo to a cell surface selectin in a biologically relevant manner.

What should be the criteria for a real selectin ligand? First and foremost, it should be present in the right place at the right time; e.g., a true P-selectin ligand should be present on the surface of a mature circulating blood cell at a time when P-selectin is being actively expressed on an endothelial or platelet surface somewhere in the vasculature. Second, selective removal or blockade of the putative ligand on the intact cell should abrogate biologically relevant interactions. Next, absence of the ligand should abrogate the function of the relevant selectin in the specific situation in question. Finally, the ligand should be recognized with some selectivity by the specific selectin in question, with a relatively high affinity, and preferably with a well-defined stoichiometry. Of course, some of these criteria might not be fulfilled if other relevant ligands share partial functional redundancy and/or overlap in recognition by other selectins.

Let us consider as an example the potential ligands on the surface of a circulating neutrophil for P-selectin (which is itself expressed on activated endothelium or platelets). A large body of evidence indicates that sugar chains called sialylated fucosylated lactosamines (of which SLe^x is the simplest example) are critical components of the ligands (1–14). The relevant fucosyl-

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Accepted for publication 3 December 1996.

1. *Abbreviations used in this paper:* ESL-1, E-selectin ligand–1; HEV, high endothelial venules; PSGL-1, P-selectin glycoprotein ligand-1 SLe^x, sialyl-Lewis^x.

J. Clin. Invest.

0021-9738/97/01/158/05 $2.00
Volume 99, Number 2, January 1997, 158–162

transferase enzymes have recently been identified and disrupted in whole animals (24–27). Since the enzymes involved in SLe^x synthesis reside in the late part of the Golgi apparatus, many cell surface glycoproteins isolated from myeloid cells could carry some copies of this structural motif. Thus, it should not be surprising that a large number of neutrophil glycoproteins (leukosialin, L-selectin, LAMPs, CEA-like proteins, LFA-1, etc.) have been reported to act as P-selectin (and/or E-selectin) ligands when purified and presented at high density in various in vitro assays. In contrast, only one quantitatively minor neutrophil glycoconjugate (whose polypeptide backbone is called P-selectin glycoprotein ligand-1, PSGL-1) (28, 29) actually generates high affinity binding and biologically relevant interactions with P-selectin, by presenting $< 1\%$ of the surface SLe^x (30–32) in conjunction with certain tyrosine sulfate residues (33–35). This molecule is present in the right place at the right time. It does bind with relative selectivity to P-selectin (although it is also recognized by L- and E-selectin), in a calcium-dependent manner, with a relatively high (nanomolar) affinity and likely with a defined stochiometry. Selective removal by *Pasturella hemolytica* O-sialoglycoprotease (which cleaves PSGL-1 and only a few other mucin-type glycoproteins from neutrophil surfaces) abrogates binding (30), and selective blockade by specific monoclonal antibodies (with appropriate nonblocking controls) abrogates the physiologically relevant "rolling" of neutrophils on P-selectin–expressing vascular endothelium in vivo (36). Likewise, PSGL-1 blockade abrogates P-selectin–mediated biochemical responses among activated platelets and monocytes (15, 37). Thus, the many other SLe^x-bearing glycoproteins and glycolipids present in greater abundance on the neutrophil surface seem to be insufficient to provide functional redundancy for PSGL-1. The role of PSGL-1 remains to be confirmed by studying the consequences of a genetically determined absence of the polypeptide backbone; i.e., a "knock-out" mouse. Regardless, we can reasonably conclude that when correctly glycosylated and tyrosine sulfated, PSGL-1 can present a real P-selectin ligand. In this regard, an interesting recent development is that neutrophil PSGL-1 can also act as a true ligand for L-selectin, mediating the phenomenon of neutrophils rolling on other neutrophils that are already adherent to activated endothelium (38–42).

In no other instance has the criteria for a real selectin ligand been completely fulfilled. Those that come the closest are considered below. GlyCAM-1 is a small mucin-type glycoprotein that, when correctly glycosylated and sulfated (on the sugar chain), can be recognized with high affinity and relative specificity by L-selectin (4, 10, 11, 43). It is also expressed in the right place (high endothelial venules, HEV) at the right time (constitutively in lymph nodes and inducibly in inflamed tissues). However, GlyCAM-1 lacks a transmembrane domain and appears to be primarily a secreted molecule. Indeed, modulation of its secretion during immune responses suggests that it is primarily an antiadhesion molecule (44). Of course, since L-selectin is also a signaling molecule, GlyCAM-1 could be ligating this receptor on the circulating neutrophil cell surface to carry out some other functions. Indeed, soluble GlyCAM-1 has recently been shown to activate β_2-integrins (45). A more attractive candidate for an endothelial L-selectin ligand appeared to be the mucin-type polypeptide CD34. As with GlyCAM-1, CD34 can be recognized with high affinity and relative specificity by L-selectin when it is correctly glycosylated and sulfated (4, 10, 11). Furthermore, it is a transmembrane glycoprotein expressed throughout the endothelial cells of the vasculature (46). However, CD34 only appears to be correctly glycosylated for L-selectin recognition in the HEV of lymph nodes. Moreover, genetic disruption of CD34 expression in mice does not give an obvious loss of lymphocyte trafficking to lymph nodes (47). Other candidates for true L-selectin ligands include the as yet uncloned gp200 of lymph node HEV (4, 11), and the correctly glycosylated and sulfated form of MAdCAM-1 (12). The latter is a particularly interesting molecule found in Peyer's patch and mesenteric lymph node HEV, where it can function both as a protein ligand for the integrin $\alpha_4\beta_7$, and/or as a ligand for L-selectin (12, 48).

All of the ligands mentioned above are sialomucins; i.e., they have large numbers of O-linked sugar chains clustered together on the polypeptide backbone. In contrast, a polypeptide called E-selectin ligand–1 (ESL-1) was discovered by expression cloning and found to carry exclusively N-linked sugar chains, which could constitute a high affinity E-selectin ligand when decorated with sialylated fucosylated lactosamine units such as SLe^x (9, 49). However, sequence comparisons showed a strong homology of ESL-1 to a putative chicken fibroblast growth factor receptor (9, 49), and identity to a previously well-known protein marker of the medial Golgi called MG-160 (50). If this internal location holds true in leucocytes, ESL-1 would not be present "at the right place, at the right time." However, it is possible that this is a matter of cell-type specific regulation that has not yet been elucidated. Another example of a potential selectin ligand with primarily N-linked chains is the heat-stable antigen CD24, which has been reported to bind to P-selectin in vitro (51). It is also noteworthy that E-selectin has been reported to bind with relatively high affinity to certain sialylated polyfucosylated N-linked sugar chains derived from neutrophils, completely independent of any attachment to a polypeptide backbone (52). Likewise, sialylated fucosylated glycolipids from leukocytes can be effective ligands for E-selectin when presented at high density in vitro (53–55). In all of these instances, definitive proof of in vivo function is currently lacking.

Another puzzling fact is that a completely different class of sugar chains called the heparan sulfate glycosaminoglycans (lacking sialic acids or fucose) can also be recognized by L- and P-selectin (56). Calcium dependency and competition studies suggest that the binding site for these sulfated, uronic acid–containing sugar chains is identical to or closely adjacent to those for the sialylated mucin-type ligands (56–58). Although these molecules have not fulfilled all the criteria for a true selectin ligand, they are certainly expressed in the right place (i.e., the surface of endothelia and blood cells) and have the potential diversity to generate ligand specificity. Indeed, a recent study shows that aortic endothelial heparan sulfates can act as functional ligands for L-selectin (59). In this regard, it is also of interest that the heparan sulfates are closely related in structure to the commonly used intravenous therapeutic agent heparin, which has been assumed to function primarily via binding to antithrombin III (8). It is possible that some of the therapeutic effects of heparin are related to P- and/or L-selectin inhibition.

In relation to heparan sulfate recognition, it is important to note that P- and L-selectin can also bind to certain sulfated glycolipids such as sulfatides (60), and possibly to HNK-1 antibody-reactive sulfoglucuronosyl glycosphingolipids (61); again, neither sialic acid nor fucose are components of these

molecules. However, as with the sialylated fucosylated glycolipids, it is unclear if the binding seen with purified products occurs because of their clustered presentation in various in vitro assays, or if they do indeed serve as high affinity ligands in vivo. Also, not all of these binding phenomena are consistently calcium dependent. In this regard, it is of interest that some anionic phospholipids can bind to L- and P-selectin (but not to E-selectin) at a site distinct from the carbohydrate-binding site (18). Indeed, many of the confusing data concerning P- and L-selectin might be explained by a two-site model, with a calcium-dependent site that recognizes sialylated fucosylated components of ligands and a calcium-independent anion-binding site that serves to stabilize the first interaction. Overall, if it is really true that selectins can bind to mucins, heparan sulfate glycosaminoglycans, sulfatides, glycolipids, tyrosine sulfates, or complex N-linked sugar chains in vivo, the matter of defining the biologically relevant ligands assumes a daunting complexity. Future studies should focus more upon demonstrating ligand functions in vivo.

Yet another class of potential selectin ligands that should be mentioned are the mucin-type glycoproteins expressed on the cell surface and in the secretions of epithelial carcinomas. Prior evidence for the association of carcinoma mucin sialylation and fucosylation with tumor progression and metastases (62) has led to recent studies asking if selectin recognition is involved. While the last word is not in, it is clear that epithelial carcinomas can express mucin-type selectin ligands, and that these may mediate tumor cell interactions with blood cells and endothelia (63–66). In view of the well-known role of platelet and leukocyte interactions with tumor cells in the process of metastases, these "pathological" ligands bear exploration as potentially important determinants.

Another confusing issue is that, despite the selectins showing excellent cross-recognition of ligands between species, the ligands themselves may be presented by distinct polypeptide backbones. Thus, human homologues of ESL-1 and GlyCAM-1 have yet to be described, and the same is true of a 250-kD glycoprotein reported to mediate E-selectin binding to bovine γ-δ T cells (67). One possible explanation is that the glycosyltransferases that synthesize the carbohydrate components of the ligands are under independent and more severe evolutionary selection pressures (e.g., because of the binding of microbial pathogens to cell surface sugars). Thus, they may be more prone to divergence than the carrier polypeptides themselves. If so, one should be careful in extrapolating conclusions regarding selectin ligands among different species.

It is very convenient to use the names of the polypeptide backbone when referring to selectin ligands; e.g., "PSGL-1 is the ligand for P-selectin." However, it is important to remember that unless they are correctly glycosylated and/or otherwise modified (e.g., sulfated), these polypeptides are not ligands. Thus, for example, CD34 is widely expressed on vascular endothelia (46) and PSGL-1 is expressed on leukocytes other than neutrophils and monocytes (29, 68, 69); however, these polypeptides do not generate selectin ligands in most of these situations, presumably because they are not correctly modified posttranslationally. Indeed, recent studies indicate that even on neutrophils, only $\sim$ 20% of the PSGL-1 expressed is capable of binding to P-selectin (R. Cummings, personal communication). Also, as discussed above, binding of some selectin ligands may be completely independent of a polypeptide chain. Thus, these polypeptides are better thought of as carriers of the true ligands, which are made up of combinations of posttranslational modifications such as glycosylation and sulfation.

Since very few mucin-type polypeptides seem to carry high affinity selectin ligands, it was initially thought that these proteins might be targets for selective biosynthesis of unique oligosaccharide sequences that would explain the high affinity. Indeed, there are well-established examples of polypeptide-specific glycosylation reactions in other systems (70). An unusual 6′-sulfated SLe^x structure found on GlyCAM-1 (4, 11, 43) was initially thought to be a candidate; however, direct analysis of the chemically synthesized molecule indicates that it is not a particularly potent ligand in comparison with SLe^x (71, 72). A more promising candidate may be a relatively unusual Core 2 branched sialylated polyfucosylated O-linked chain found in a small copy number on PSGL-1 (32); however, this has not yet been directly shown to be essential for P-selectin recognition. No other truly unique oligosaccharides have been found attached to mucin-type selectin ligands (66). Thus, the generation of a high affinity ligand seems to require an unexplained combination of the polypeptide backbone and its modifications (glycosylation and/or sulfation). One possible explanation for this paradox, and for the recognition of so many diverse ligands by the same selectin lectin domains, is that they share a common "clustered saccharide patch" or "clustered anionic patch" that can be generated in more ways than one (3). Thus, the common features of the selectin ligands (carboxylate residues and/or sulfate esters, along with hydroxyl, methyl, or N-acetyl groups) can all be found on a variety of oligosaccharide structures. Appropriate combinations of such units might generate high affinity ligands (3). While this theory can account for many of the existing observations about selectin ligands, it is difficult to prove without having crystal structures of the selectins in complex with several of their ligands for inspection and comparison.

From the title and the content of this perspective, it will be evident to the reader that many more studies are needed to elucidate which are the "real" selectin ligands. While the final answers may be complex, their exploration is very worthwhile. Knowing the precise nature of the high affinity ligands could also aid in pursuing one of the Holy Grails of the selectin field: developing high affinity monospecific selectin inhibitors for therapy of acute and chronic inflammatory reactions and ischemia-reperfusion injury states. Of course, an alternate way to achieve the same end is the screening of randomized libraries composed of defined structural subcomponents. In this regard, it is interesting that the highest affinity ligands described to date for selectins are artificial ones that have been isolated by affinity selection from pools of randomized molecules belonging to yet another well-known category of acidic polysaccharides: single-stranded oligonucleotides (73, 74).

Acknowledgments

The author thanks Shiv Pillai and Nissi Varki for helpful comments, and others for providing unpublished data. Since the current literature on selectins is voluminous, the bibliography presented is representative rather than fully inclusive.

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