Molecular Mapping of Functional Domains of The Leukocyte Receptor for Endothelium, LAM-1

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Abstract. The human lymphocyte homing receptor LAM-1, like its murine counterpart MEL-14, functions as a mammalian lectin, and mediates the binding of leukocytes to specialized high endothelial cells in lymphoid organs (HEV). LAM-1 is a member of a new family of cell adhesion molecules, termed selectins or LEC-CAMs, which also includes ELAM-1 and PADGEM (GMP-140/CD62). To localize the regions of LAM-1 that are involved in cell adhesion, we developed chimeric selectins, in which various domains of PADGEM were substituted into LAM-1, and used these chimeric proteins to define the domain requirements for carbohydrate binding, and to localize the regions recognized by several mAb which inhibit the adhesion of lymphocytes to lymph node HEV. The binding of PPME or fucoidin, soluble complex carbohydrates that specifically define the lectin activity of LAM-1 and MEL-14, required only the lectin domain of LAM-1. The LAMI-1, LAMI-3, and LAMI-6 mAb each strongly inhibit the binding of lymphocytes to HEV in the in vitro frozen section assay, and defined three independent epitopes on LAM-1. Blocking of PPME or fucoidin binding by LAMI-3 indicated that this site is identical, or in close proximity, to the carbohydrate binding site, and analysis of the binding of LAMI-3 to chimeric selectins showed that the epitope detected by LAMI-3 is located within the lectin domain. Although the LAMI-6 epitope is also located in the lectin domain, LAMI-6 did not affect the binding of PPME or fucoidin. The LAMI-1 epitope was located in, or required, the EGF domain, and, importantly, binding of LAMI-1 significantly enhanced the binding of both PPME and fucoidin. These results suggest that adhesion mediated by LAM-1 may involve cooperativity between functionally and spatially distinct sites, and support previous data suggesting a role for the EGF domain of LAM-1 in lymphocyte adhesion to HEV.

The interaction of circulating leukocytes with vascular endothelium underlies a number of phenomena important to host defense. The recirculation of lymphocytes throughout the secondary lymphoid organs, a process which is necessary to ensure that the full complement of antigen receptors is available throughout the organism, and the recruitment of leukocytes to sites of inflammation, are each regulated principally at the level of binding of leukocytes to specially modified regions of the vessel wall. A number of leukocyte and endothelial cell surface molecules that participate in this process have been identified and characterized in both mouse and man. Among the best characterized of the leukocyte receptors is the murine lymph node homing receptor MEL-14 (10, 24, 31), and its human counterpart, the leukocyte adhesion molecule-1 (LAM-1) (21, 42, 43), which mediate the binding of lymphocytes to the specialized high walled endothelium in the postcapillary venules present in most secondary lymphoid organs (high endothelial venules or HEV).1 (LAM-1 is recognized by the Leu-8, TQI, and DREG series mAb, in addition to the ones described in this report, and has also been referred to as LEC-CAM-1 and LECAM-1.) These molecules are also expressed on other classes of leukocytes, including neutrophils and monocytes (13, 19, 43), and recent evidence suggests a role for LAM-1/ MEL-14 in the recruitment of leukocytes to inflammatory lesions (18, 25, 47). In man, LAM-1 is also expressed on the majority of circulating hematopoietic stem cells (13), raising the possibility that this molecule is also involved in stem cell traffic.

A diverse array of functional evidence has clearly demonstrated that these homing receptors function as mammalian lectins. Thus, binding of lymphocytes to LN HEV, but not Peyer's patch HEV, is specifically inhibited by certain simple sugars, including mannose-6-phosphate (M6P) and the structurally related fructose-1-phosphate, by PPME, a M6P-rich mannan core from the yeast Hansenula holstii, and by fucoidin, a fucose-rich polysaccharide (38-40, 48, 49). PPME and fucoidin bind specifically to LAM-1 and MEL-14, and this binding is inhibited by some antibodies that selectively bind to these HEV receptors and thereby inhibit the binding of lymphocytes to LN HEV (21, 34, 48). In addition, the bind-
ing of PPME or fucoidin to LAM-1 or MEL-14 and of lymphocytes to LN HEV exhibits an identical absolute requirement for Ca²⁺ (37, 40). The lectin activity of MEL-14 has recently been directly confirmed using purified MEL-14 protein (15). These data have firmly established an essential role for specific carbohydrate recognition of carbohydrate in adhesion mediated by this class of cell adhesion molecules.

Molecular cloning of MEL-14 (24, 31) and LAM-1 (3, 6, 30, 42) has recently revealed that these molecules are members of a larger family of cell adhesion molecules. Termed LEC-CAMs (36) or selectins (11, 35), this family also includes ELAM-1, which is expressed on cytokine treated endothelium and mediates the binding of leukocytes, principally neutrophils, to these cells (1, 2); and PADGEM (GMP-140, CD62), which is expressed on activated platelets and endothelium, and mediates the binding of these cells to monocytes and neutrophils (11, 16, 22). Selectins are characterized by a novel arrangement of distinct protein motifs, the most prominent of which is an amino-terminal C-type lectin domain, consistent with the functional evidence described above. This is followed by a domain homologous to EGF, a variable number (two in LAM-1 and MEL-14, six in ELAM-1, and nine in PADGEM [36]) of tandem repeats homologous to complement regulatory proteins, a transmembrane region, and a cytoplasmic tail. Although the presence of a C-type lectin domain in these molecules is an elegant confirmation of the functional data described above, the function of these various domains in the adhesive or other biological properties of the selectins remains incompletely understood.

A requisite role for activation of adhesion molecules in efficient cell adhesion has been suggested by studies of several members of the integrin family (9, 29, 35). Consistent with this hypothesis, we have recently shown that activation of leukocytes induces a rapid and transient increase in adhesion mediated by LAM-1, and that this enhancement involves an increase in affinity of LAM-1 for carbohydrate (34). In this study, we have used chimeric selectins, in which various regions of PADGEM have been substituted for those of LAM-1, to define the domains responsible for carbohydrate binding by LAM-1, and to identify other regions of the LAM-1 molecule involved in cell adhesion and receptor regulation. The results suggest that LAM-1-mediated adhesion may involve the coordinated action of multiple sites within the molecule, not all of which are directly involved in carbohydrate recognition.

Materials and Methods

Production of mAb to LAM-1

The production and detailed characterization of the anti-LAM-1 mAb used in this report will be described elsewhere (34a). Briefly, the 300.19 murine pre-B cell line was transfected with LAM-1 cDNA (42) and used to immunize mice, as described (43). Antibodies that stained the LAM-transfected but not the parent cell line were subcloned and analyzed further. This report uses three such mAbs, termed LAM-1, LAMI-3, and LAMI-6.

Effect of Anti-LAM-1 mAb on Binding of PPME or Fucoidin

Peripheral blood lymphocytes were isolated by Ficoll-hypaque gradients. The cells were incubated on ice for 15 min in PBS containing 1% FCS and excess amounts of unlabeled antibody in the form of diluted ascites, and either PPME-FITC or fucoidin-FITC, prepared as described (12), was then added at previously determined optimal concentrations. After a further incubation of 15 min, the cells were washed, and analyzed on an EPICS Profile.

Construction of Chimeric Selectins

Four new restriction endonuclease recognition sites were introduced separately into the pLAM-1 cDNA (42) by polymerase chain reaction (PCR)-based site-directed mutagenesis as follows. Oligonucleotides surrounding and including each new restriction sequence were synthesized in both the sense and antisense directions. PCR was carried out using the new restriction site sense oligonucleotide + an antisense oligonucleotide anchor located in the plasmid near the 3' end of the cDNA. In a separate reaction, the new restriction site antisense oligonucleotide plus a sense oligonucleotide anchor from the plasmid 5' end were used to amplify the 5' end of the LAM-1 cDNA. The individual PCR products from these two reactions were gel purified, subcloned into the pSP65 vector (Promega Biotec, Madison, WI), digested with the appropriate restriction enzymes, and ligated together. The nucleotide changes were as follows: (a) GA³⁶⁰ATCC to GGTATC in the lectin domain, creating a BamHI site, corresponding to amino acid number 53 in the mature protein (42); (b) A³⁵⁰TGGACG to CTGACG in the EGF domain, creating a Psd site, corresponding to amino acid number 127; (c) GAGG³⁵⁹C³⁸⁶ to GAGG³⁵⁹C³⁸⁶ in the first SCR domain, creating a SacI site, corresponding to amino acid number 164; and (d) GTCGAA¹⁰⁹³ to GTCAAC in the second SCR domain, creating a HindII site, corresponding to amino acid number 279 (Fig. 1). The fidelity of the site-directed mutagenesis was confirmed by restriction mapping and sequence analysis of the cDNAs. Each of these sites is naturally present in the PADGEM cDNA (23). Chimeric selectins were created by exchanging the indicated regions of LAM-1 and PADGEM (Fig. 1). Creation of the SacI site involved a substitution of leu for ala; creation of the HindII site involved a substitution of thr for asn. These amino acid changes did not affect the level of expression of the mutants as detected by transient transfection in COS cells (data not shown).

Domain Mapping of Binding Sites for PPME, Fucoidin, and mAb to LAM-1

The chimeric selectin cDNAs were subcloned into the ApaI expression vector and transient transfection of COS cells was performed by the DEAE dextran method. 1 d after transfection, COS cells were replated onto plastic microscope covergrips. On day 2 after transfection, COS cells were incubated at room temperature for 30 min in humidified chambers with mAb to LAM-1 in the form of ascites fluid diluted in PBS/1% FCS, gently washed with PBS/1% FCS, and incubated similarly with fluoresceinated goat antibody to mouse Ig (Tago Inc., Burlingame, CA). For carbohydrate binding studies, a similar procedure was followed, except that PPME-FITC or fucoidin-FITC were used. The cells were then mounted in Fluoromount-G (Fisher-Biotech, Pittsburgh, PA), and examined by fluorescence microscopy. Staining was considered positive only if clear membrane staining outlining the cell was seen. Scoring was as follows: 0, no staining; +, 2-10%; ++, 10-25%; ++++, 25-50%; ++++, >50%. Negative controls included transfection of COS cells with vector alone, vector with irrelevant cDNA (e.g., CD19) (41), and staining with ascites of irrelevant specificity, and these samples always exhibited <1% staining. Expression of LIP, which is not detected by any of fourteen anti-LAM-1 mAb examined, was confirmed by staining with the ACl1.2 (22) and/or KC4 (14) anti-PADGEM mAbs.

Results

Construction of LAM-1/PADGEM Chimeric cDNAs

To identify the regions of LAM-1 involved in cell adhesion, and to determine the site and domain requirements of carbohydrate binding, we constructed chimeric selectin cDNAs by substituting homologous regions of PADGEM into LAM-1. Novel restriction sites were introduced into the pLAM-1 cDNA (42) by PCR-based site-directed mutagenesis. Chimeric selectin cDNAs containing the 5' half of the lectin domain of LAM-1 (designated LIP), the entire lectin domain
(L2P), the lectin plus EGF domains (L3P), or nearly the entire extracellular portion (L4P) of LAM-1, with the remainder of the cDNA as PADGEM, were constructed (Fig. 1). In addition, a LAM-1 cDNA with only the EGF domain of PADGEM substituted for that of LAM-1 was constructed (L2P3L). Chimeric selectin cDNAs with PADGEM at the 5' end and LAM-1 at the 3' end were also constructed, but were not expressed at detectable levels in COS cells. Because the PADGEM and LAM-1 proteins share an identical domain organization (36), these chimeric molecules are likely to retain the overall topology of the parent molecules. This approach to analyzing functional domains has many advantages over deletion analysis, which may change the conformation of the resulting molecules in unpredictable ways.

**Binding of PPME or Fucoidin Requires only the Lectin Domain from LAM-1**

The chimeric selectins were subcloned into the Aprm8 COS cell expression vector and analyzed for carbohydrate binding in transient transfection assays. Both PPME and fucoidin bound to L2P, which contains the entire lectin domain of LAM-1, but not to PADGEM, or to L1P, which contains only the 5' half of the lectin domain (Table I and Fig. 2). At least part of the binding site for these carbohydrates is therefore located in, or requires, the 3' half of the lectin domain. In addition, binding of both PPME and fucoidin to L2P was equivalent to or better than binding to LAM-1, or to L3P or L4P, which contain the lectin + EGF of LAM-1 and the lectin + EGF + short concensus repeat (SCR) domains of LAM-1, respectively (see Fig. 1). These data indicate that the EGF and SCR domains of PADGEM can replace those of LAM-1 without affecting the binding of PPME or fucoidin, and therefore, that these domains of LAM-1 are not required for carbohydrate recognition and binding.

**Domain Mapping of the Binding Sites of LAMI-1, LAMI-3, and LAMI-6 mAb**

As described in detail elsewhere (34a), the LAMI-1, LAMI-3, and LAMI-6 mAb each strongly inhibit the binding of lymphocytes to lymph node HEV in the frozen section assay, and define three spatially distinct epitopes on the LAM-1 molecule. To identify the domain locations of the epitopes defined

| Table I. Domain Mapping of LAM-1 Carbohydrate and mAb Binding Sites |
| Staining of COS cells transfected with cDNAs encoding |
| LAM-1 | PADGEM | L1P | L2P | L3P | L4P | L2P3L |
| PPME | ++ | - | - | ++ | ++ | ++ | ++/++ |
| Fucoidin | ++ | - | - | ++ | ++ | ++ | + |
| LAM1-1 | ++ | - | - | - | ++ | ++ | - |
| LAM1-3 | ++ | - | - | ++ | ++ | ++ | ++ |
| LAM1-6 | ++ | - | - | ++ | ++ | ++ | + |

COS cells transfected with LAM-1, PADGEM, or the chimeric selectin cDNAs were analyzed in transient transfection assays for staining with the indicated carbohydrates or mAb, as described in Materials and Methods. PPME, fucoidin, LAM1-3, and LAM1-6 recognized the lectin domain of LAM-1, whereas LAM1-1 expression was absent in chimeras lacking the LAM-1 EGF domain.
by these mAb, we analyzed the chimeric selectins as described above. Similar to PPME and fucoidin, LAMI-3 and LAMI-6 stained COS cells transfected with L2P, L3P, L4P, and LAM-1, but not COS cells transfected with L1P or PADGEM (Table I and Fig. 2), indicating that at least part of the epitopes defined by these mAb are also located in, or require, the 3' half of the lectin domain. Because LAMI-3 and LAMI-6 do not cross-block each others binding to LAM-1 (34a), this demonstrates that LAMI-3 and LAMI-6 identify two distinct functional epitopes within the lectin domain. In contrast, binding of LAMI-1 was not detected in chimeras lacking the LAM-1 EGF domain (Table I), indicating that the LAMI-1 epitope is within (or requires) the EGF domain. In combination with the observation that both PPME and fucoidin bind to chimeric selectins lacking the LAM-1 EGF domain, these data suggest that the LAMI-1
mAb, which inhibits lymphocyte/HEV binding, recognizes a domain of LAM-1 which is not required, i.e., can be replaced with that of PADGEM, for binding of PPME or fucoidin.

**LAMI-1, LAMI-3, and LAMI-6 Have Distinct Effects on Carbohydrate Binding**

To directly relate the functional epitopes defined by these mAb to the carbohydrate binding site, the effect of mAb binding on the binding of PPME and fucoidin was assessed. Peripheral blood lymphocytes were incubated with three- to fivefold excess concentrations of each of these three mAbs, followed by PPME-FITC or fucoidin-FITC, and analyzed by flow cytometry. Prior incubation of cells with LAMI-3 completely blocked the binding of both PPME-FITC and fucoidin-FITC (Fig. 3), suggesting that the LAMI-3 epitope is at or in close proximity to the carbohydrate binding site. In contrast, LAMI-6, which also strongly inhibits lymphocyte binding to HEV (34a) and also maps to the lectin domain (Table I), had little or no effect on carbohydrate binding. Surprisingly, the binding of LAMI-1 significantly enhanced the binding of both PPME and fucoidin (Fig. 3), an effect which varied 4-10-fold over several experiments. This enhanced binding was also inhibited by the LAMI-3 mAb or EDTA (data not shown), indicating that this increased level of carbohydrate binding was also LAM-1 dependent. Thus, a mAb with antagonist activity in one functional assay of LAM-1 had agonist activity in a separate LAM-1 functional assay.

**Discussion**

The genetic mapping of functional domains of a number of proteins that are members of gene families has been facilitated by the construction and analysis of chimeric molecules, in which different regions of one family member are exchanged for the corresponding regions of a different member of the same family. Unlike deletion analysis, in which portions of a molecule are simply removed, this approach preserves the overall topology of the protein, thus preserving some of the biological properties of the parent molecules. In this report, we have used chimeric selectins to localize the carbohydrate binding site within LAM-1, and to locate the epitopes identified by three mAb which inhibit lymphocyte binding to HEV.

Our results directly demonstrate that binding of PPME or fucoidin by LAM-1 requires only the lectin domain of LAM-1, and therefore suggest that the carbohydrate binding site is contained within the lectin domain. The selective inhibition of carbohydrate binding by LAMI-3 mAb (Fig. 3) suggests that this antibody binds at or near the carbohydrate binding site, as defined by PPME and fucoidin. These results also demonstrate that neither the EGF nor SCR domains of LAM-1 are necessary for carbohydrate binding, i.e., that the EGF and SCR domains of PADGEM, which does not bind PPME or fucoidin in these assays (Table I), can subserve whatever role, if any, is played by these domains with respect to carbohydrate binding. These domains therefore probably do not play an important role in determining the specificity of carbohydrate recognition. These results do not, however, exclude an essential structural role for the EGF domain in ensuring a proper conformation for the lectin domain, as is suggested by the observation that the EGF domains of MEL-14 and ELAM-1 were necessary to preserve epitopes identified by the MEL-14 and H18/7 mAb, respectively (4, 45). That the EGF domain of PADGEM can substitute for that of LAM-1 indicates that the structural features of the EGF domain, which are apparently essential for lectin domain function, have been well conserved among the selectins.

The LAMI-3 antibody resembles the MEL-14 antibody in its inhibition of both PPME and lymphocyte/HEV binding, and in the localization of its binding site to the lectin domain (4, 10, 48). The observation that PPME, fucoidin and LAMI-3 each bind to L2P, which contains the entire lectin domain of LAM-1, but not to LIP, which contains only the amino-terminal 53 amino acid residues of LAM-1, suggests that at least part of the carbohydrate/LAMI-3 binding site is in the carboxy-terminal ~67 amino acids of the lectin domain. Although these results would appear to differ from those of Bowen et al., who used MEL-14/LAM-1 chimeras to deduce that at least part of the epitope for the MEL-14 antibody is located in the amino-terminal 53 amino acids of the lectin domain (4), the strong homology between the selectins, and the absolute conservation of the cysteine residues in the lectin and EGF domains, suggests that both the MEL-14 and LAMI-3 epitopes are actually conformationally determined epitopes formed by residues present in both "halves" of the
LAMI-6 mAb does not inhibit the binding of PPME or fucoidin (Fig. 3), it remains possible that inhibition of HEV binding by LAMI-6 may be due to steric hindrance of lectin domain activity (i.e., recognition of carbohydrate) that is not detectable by analysis of PPME or fucoidin binding. In this regard, it should be explicitly emphasized that the soluble carbohydrates used in these and other studies are unlikely to perfectly model the physiologic carbohydrate ligand. It is therefore possible that the effects of mAb on the binding of PPME or fucoidin do not accurately predict the effect of these mAb on the recognition of the natural carbohydrate ligand by the lectin domain in the course of cell adhesion in vivo.

The LAMI-1 mAb also inhibits lymphocyte/HEV interactions, and the epitope defined by LAMI-1 is within, or requires, the EGF domain (Table I). These properties are identical to what has been described for the Ly-22 mAb, which also recognizes the MEL-14 antigen (32). The observation that this antibody enhances the binding of both PPME and fucoidin (Fig. 3) makes it less likely that inhibition of HEV binding by LAMI-1 is due to inhibition of carbohydrate binding. However, as indicated above, these data do not exclude the possibility that LAMI-1 inhibits recognition of the natural carbohydrate ligand. It also seems unlikely that inhibition of HEV binding by LAMI-1 is due to general allosteric effects, because the binding of the Leu-8 and LAMI-3 mAb, which identify distinct epitopes in the lectin domain (reference 45 and Table I), is unaffected by LAMI-1 (34a and data not shown). Moreover, with respect to carbohydrate binding, the EGF domain of LAM-1 can be replaced with that of PADGEM (Table I). In addition, the degree of amino acid identity between the EGF domains of the selectins is equal to that found in the lectin domains (63–67%) (Fig. 3 b), suggesting an equally important role in the function of these molecules. Numerous examples of EGF-like domains mediating specific interactions between proteins exist (reviewed in reference 8). These observations are consistent with the existence of a second binding site, which is essential for lymphocyte binding to HEV, within the EGF domain.

A binding mechanism which involves interaction at two sites might be advantageous for several reasons. Such a mechanism might plausibly be expected to confer a high degree of specificity on the adhesive event. This consideration appears particularly relevant, given recent evidence indicating that the carbohydrate ligands of the selectins are closely structurally related: each contains sialic acid, and the ELAM-1 and PADGEM carbohydrate ligands include the sLe^a and Le^a structures, respectively (7, 23, 26, 27, 28, 44–46). Binding via two sites, especially if cooperative, would also be expected to generate an interaction of higher avidity, which would be important given the apparently low affinity of LAM-1 for PPME or fucoidin, of PADGEM for CD15/LNFIII/Le^a, and of ELAM-1 for sLe^a (23, 26, 45). In addition, a requirement for dual recognition of separate ligand components would allow the use of a limited number of carbohydrates and EGF-like domains to generate a larger number of specificities, thus obviating the need to evolve a large number of lectin domains. This last consideration may partially account for the unique mosaic of protein motifs characteristic of the selectins.

The unanticipated enhancement of carbohydrate binding by prior binding of LAMI-1 mAb raises interesting questions regarding the nature of receptor/ligand interactions. We have previously shown that activation of T cells by cross-linking CD3/TCR or CD2, or neutrophils by GM-CSF or TNF-α,
leads to a rapid and transient upregulation of PPME binding activity by a mechanism which involves the upregulation of affinity of LAM-1 for ligand (34). This enhanced binding of PPME is indistinguishable from that produced by binding of LAM-1 mAb. Taken together, these observations suggest that the EGF domain may be involved in the regulation of receptor function, including the increase in affinity of LAM-1 for carbohydrate associated with cellular activation.

The role of the SCR domains in the function of LAM-1 and the other selectins remains undefined. Our findings suggest that this region is not directly involved in cell adhesion, and therefore suggest that the SCR domains subserve primarily a structural requirement, perhaps by extending the adhesive domains sufficiently far from the glycoalyx to ensure efficient cell adhesion. This is consistent with the variable number of SCR domains found in the different selectins, and with the lower degree of conservation exhibited by SCR domains compared with the other regions of these adhesion proteins.

In the case of LAM-1 and MEL-14, this region may also be involved in the shedding of the molecule associated with cellular activation (17, 20, 33, 43).

In summary, we have demonstrated that antibodies to LAM-1 that inhibit lymphocyte adhesion to HEV have contrasting effects on the ability of LAM-1 to recognize PPME and fucoidin, soluble carbohydrates which bind to LAM-1 and inhibit adhesion to LN HEV. In addition, we have shown that these antibodies identify distinct regions in different domains of the molecule. Our findings therefore reinforce data indirectly suggesting a role for the EGF domain in cell adhesion (32). Whether the EGF domain plays a role in supporting the appropriate conformation of the lectin domain, as suggested (44), participates directly in cell adhesion, or plays some other, unexpected role, awaits further investigation. Analysis of the ability of the chimeric selectins described in this paper to confer HEV binding, currently in progress, may provide a more definitive answer to this question.

We would like to thank Steve Rosen and Larry Lasky for helpful discussions, and Bruce and Barbara Furie for generous gifts of anti-PADGEM mAb and PADGEM cDNA.

This work was supported by grants CA-34183 and AI-26872 from the National Institutes of Health.

Received for publication 6 February 1991 and in revised form 15 April 1991.

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