The Complement Binding-like Domains of the Murine Homing Receptor Facilitate Lectin Activity

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Abstract. The leukocyte homing receptor (HR), the endothelial leukocyte adhesion molecule, and gmp140/platelet activation-dependent granule membrane protein are members of a family of adhesion molecules, termed the lectin cell adhesion molecules (LEC-CAMS) which are unified by a multi-domain structure containing a lectin motif, an epidermal growth factor-like (egf) motif, and variable numbers of a complement binding-like (CB) motif. Previous data have indicated a predominant role for the lectin motif in cell adhesion directed by the LEC-CAMS, although the egf-like domain of the HR may also play a potential role in cell binding. While the role(s) of the CB domains in the LEC-CAMS is currently not understood, they have been hypothesized to act as rigid spacers or stalks for lectin and perhaps, egf domain presentation. In this paper, we analyze the functional characteristics of murine HR-IgG chimeras containing the lectin, lectin plus egf, and lectin plus egf plus CB domains. The Mel 14 mAb, an adhesion blocking antibody which recognizes a conformational determinant in the N-terminus of the HR lectin domain, shows a significantly decreased affinity for a HR construct which lacks the CB motifs, consistent with the possibility that the CB domains are involved with lectin domain structure. In agreement with this conjecture, HR mutants lacking the CB domains show a profound decrease in lectin-specific interaction with the carbohydrate polyphosphomannan ester, suggesting that the changes in Mel 14 affinity for the lectin domain are reflected in lectin functionality. Various assays investigating the interactions between the HR deletion mutants and the peripheral lymph node high endothelium, including cell blocking, immunohistochemical staining, and radioactively labeled ligand binding, all showed that removal of the CB domains results in a lack of HR adhesive function. These results imply that the CB domains of the HR, and, by analogy, the other members of the LEC-CAM family, may play important structural roles involving induction of lectin domain conformation and resultant functionality.

THE lectin cell adhesion molecule (LEC-CAM)[1] (40) family currently contains three members; the leukocyte homing receptor (HR) (25, 34, 43), the endothelial leukocyte adhesion molecule (ELAM) (3), and gmp140/platelet activation dependent granule membrane protein (PADGEM) (19). The leukocyte HR is found on all leukocytic cells and appears to be involved with the trafficking of lymphocytes to peripheral lymph node (pln) high endothelial venules (HEV) (11, 50) as well as the influx of neutrophils to inflammatory sites (20, 49). ELAM is an endothelial cell surface glycoprotein that is transcriptionally induced by various mediators of inflammation and which may be involved in the adhesion of neutrophils, T cells, and monocytes to endothelium at certain inflammatory sites (2). gmp140/PADGEM is a platelet and endothelial cell storage granule glycoprotein whose cell surface expression is induced by thrombin activation and which allows for neutrophil and monocyte adhesion, perhaps to thrombotic sites (4, 13, 21, 27). All three members of this family of adhesion molecules contain a multi-domain structure consisting of an NH₂-terminal type C or calcium-dependent lectin motif (10) followed by an epidermal growth factorlike (egf) domain and either two (HR), six (ELAM), or eight to nine (gmp140/PADGEM) copies of a motif homologous to the short consensus repeats of complement binding (CB) proteins such as complement receptor 1, decay accelerating factor (DAF), or the C3d/Epstein-Barr virus receptor (3, 19, 25, 34, 43). Genomic structural analysis and chromosomal localization have revealed that these domains are all encoded by separate exons and that the LEC-CAM genes all appear to map to a relatively small region of human and murine chromosomes 1, adjacent to the regulation of complement activation locus

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1. *Abbreviations used in this paper:* CB, complement binding; DAF, decay accelerating factor; egf, epidermal growth factor; ELAM, endothelial leukocyte adhesion molecule; HEV, high endothelial venules; HR, homing receptor; L, lectin; LE, lectin-egf; LEC, lectin-egf-CB; LEC-CAM, lectin cell adhesion molecule; PADGEM, platelet activation dependent granule membrane; pln, peripheral lymph node; PPME, polyphosphomannan ester.

which encodes a family of complement binding proteins containing various numbers of homologous CB domains (9, 29, 43, 47).

The relative functional contributions of the protein mofits found in the LEC-CAMS have been analyzed most directly for the murine HR and somewhat indirectly for ELAM and PADGEM/gmp140. Initial data clearly demonstrated the role of a lymphocyte surface carbohydrate receptor(s) in lymphocyte-pln HEV cell adhesion (40, 41, 42, 51, 52). Subsequent results demonstrating the identity of this carbohydrate receptor with an antigen recognized by the Mel 14 mAb, an antibody which binds to the HR and which blocks lymphocyte-pln HEV adhesion (12), were consistent with the conclusion that the HR was a carbohydrate-binding adhesion protein (14, 17, 52). These results, thus, indicated a major role for the HR lectin domain in cell adhesion, presumably by virtue of its ability to recognize a carbohydrate structure located on the pln HEV luminal surface. Epitope mapping experiments confirmed this idea by demonstrating that the Mel 14 adhesion-blocking antibody mapped to a conformational determinant within the lectin domain of the murine HR (6). More recently, it has been shown that the binding of a recombinant form of the HR to pln HEV could be completely abolished by competition with carbohydrates such as fucoidin (48) or by treatment of pln endothelium with sialidase (45). In addition, two recently discovered 50 and 90 kD pln HEV glycoprotein ligands for the homing receptor have been shown to have an absolute sialic acid requirement for HR binding, consistent with the possibility that the carbohydrate recognized by the HR lectin domain contains sialic acid and that this sugar is required for receptor-ligand interaction (18). The complete calcium dependance of cell adhesion directed by ELAM (16) and gmp140/PADGEM (13) initially suggested that the calcium-dependent lectin domains of these adhesion molecules probably also played a major role in cell adhesion. Recently, ligands for both ELAM (15, 26, 30, 44, 46) and gmp140/PADGEM (8, 22, 28) have been characterized and have been shown to be related carbohydrates in the Lewis^x family: sialyl Lewis^x (ELAM) and lactosyl-N-fucopentose III (gmp140/PADGEM) (reviewed in 37). The fact that either soluble or glycolipid forms of these carbohydrates could completely inhibit adhesion is consistent with a lectin-carbohydrate interaction as a major mediator of cell binding directed by these LEC-CAMs. Interestingly, it appears that sialic acid is also required for both ELAM and gmp140/PADGEM adhesion, consistent with results found for the homing receptor-ligand interaction. In summary, the bulk of evidence strongly suggests that the major known interaction involved with LEC-CAM-mediated cell adhesion involves the recognition of carbohydrates by the NH_2-terminal lectin domains found in these glycoproteins.

The potential roles for the egf-like and CB motifs in LEC-CAM function are less clear-cut. In the case of the murine HR, examination of the LY-22 allotypic antibody binding site indicated that this antibody recognized an epitope contained within the egf-like domain (35). In addition, this antibody appeared to block lymphocyte-pln HEV adhesion, apparently without disrupting carbohydrate (polyphosphomannan ester; PPME) binding, suggesting that the egf-like domain may also directly participate in cell adhesion. Previous epitope mapping by radio-immunoprecipitation demonstrated that the reactivity of the Mel 14 antibody for the HR lectin domain appeared to be abolished when this domain was ex-

pressed in the absence of the egf-like domain, consistent with the possibility that the structure of the Mel 14 epitope was dependent upon the egf-like motif (6). A similar egf dependence has recently been reported for a mAb directed against the ELAM lectin domain (46). The functional role(s) of the LEC-CAM CB domains is currently not understood. The exact duplication (at the nucleotide level) of this domain in the murine genome strongly suggests an important functional requirement for duplicate copies of this domain in the HR (9). Homology comparisons between human and murine forms of the HR have shown that the CB motifs are less well conserved than either the lectin or egf-like domains, although their inter-species degree of conservation is still relatively high ($\sim$60–80%) when compared to other CB domains ($\sim$20– 30%), again implying an important role for these domains in HR function (5, 32, 43).

In this paper we describe the efficient production of murine HR–immunoglobulin G chimeras containing the lectin, lectin plus egf, and lectin plus egf plus CB domains. We have analyzed these proteins for Mel 14 binding, carbohydrate recognition, and endothelial cell adhesion. The results establish a potential role for the CB motifs in HR lectin function.

Materials and Methods

Construction and Purification of Truncated mHR-IgG Chimeras

Cell lines producing either the entire extracellular domain (lectin-egf-CB, LEC) or truncated forms of the receptor (lectin, L; Lectin-egf, LE) were produced as previously described (6, 48). High level protein production was accomplished by methotrexate amplification (23, 24). Cells were grown to large scale in roller bottles, and serum-free cell supernatants were produced. The various IgG chimeras were purified by protein A sepharose as previously described (48). Protein concentrations were determined using a previously described ELISA assay with purified recombinant CD4-IgG as a standard (7, 48).

Analysis of Mel 14 Reactivity

The ability of the various IgG chimeras to react with the Mel 14 mAb was analyzed using a modification of the previously described ELISA format (48). Purified Mel 14 or anti-human IgG 1 Fc mAb was coated onto the wells of microtitre plates. Dilutions of serum-free cell supernatants or purified IgG chimeras were incubated with the plates, the plates were washed, and the amounts of IgG chimeras captured by either the Mel 14 or anti-human IgG 1 mAbs were determined by reaction of the wells with HRP-conjugated goat polyclonal anti-human Fc antibody.

Analysis of PPME Reactivity

HR chimera carbohydrate interactions were analyzed using either the previously published ELISA assay or a modification of it (17, 48). In the modified procedure, microtitre plates were coated with the yeast cell wall carbohydrate PPME by overnight incubation at 4°C of a 10 μg/ml PPME solution 0.2 M Na_2PO_4 (pH 7.6). The wells were blocked with 1% BSA in Dulbecco's PBS for 2 h at 37°C. Protein A sepharose-purified HR-IgG chimeras were added to the wells and allowed to react for 2 h at room temperature. The plates were washed and an HRP-conjugated goat anti-human IgG Fc antibody was reacted with the wells for 30 min at room temperature. The plates were washed and substrate added as previously described (17, 48).

Analysis of Cell Blocking, Immunohistochemical Staining, and Ligand Precipitation by the IgG Chimeras

Blocking of mesenteric lymphocyte binding to lymph node endothelial cells and immunohistochemical staining of these cells by the IgG chimeras was analyzed as previously described (45, 48). Immunoprecipitation of a lymph

node specific ~50-kD glycoprotein with each chimera was done by a modification of a previously described method (18). Na^{35}SO$_4$-labeled mesenteric lymph node lysates were precleared with protein A sepharose and then incubated with 30 μg of each purified chimera or with 100× concentrates of cell culture supernatants from IgG chimera producing lines for 4 h at 4°C. The complexes were then precipitated with protein A sepharose, boiled in SDS–beta mercaptoethanol, and resolved on 5–20% acrylamide gradient gels.

Results

Production of Truncated mHR-IgG Chimeras

We previously described the production of large quantities of a HR–IgG chimeric protein containing the entire extracellular domain of the murine HR (mHR-LEC+IgG) (48). This work was extended here to two other previously described truncated HR–IgG chimeras which contained either the lectin domain alone (mHR-L+IgG) or the lectin plus egf domains (mHR-LE+IgG) (6) (Fig. 1 A). Human kidney cell lines producing high levels of each of these glycoproteins were produced by methotrexate amplification of a cotransfected dihydrofolate reductase plasmid (23). The serum-free supernatants of these permanent cell lines were analyzed using a previously described ELISA assay (40) specific for the human IgG Fc region, and high level producers were expanded. Material from cell conditioned medium was passaged over protein A sepharose columns, and the resultant bound material was eluted at low pH and immediately neu-

tralized. As can be seen in Fig. 1 B, highly purified preparations of each form of the HR could be obtained in this manner. The purified and tissue culture derived materials were both capable of forming dimers under nonreducing conditions (data not shown) (40).

Analysis of Mel 14 Reactivity

Previous work has shown that the Mel 14 mAb appears to recognize a determinant within the first 53 amino acids of the lectin domain of the murine HR (6). This also showed that this recognition appeared to be dependent upon both the CB and egf domains, since removal of the CB domains appeared to result in a diminution of Mel 14 reactivity, while removal of the egf domain resulted in a loss of antibody recognition. While these results were clearcut, the use of immunoprecipitation to assay the reactivity of each chimera for Mel 14 binding was not quantitative. We have therefore measured the reactivity of each chimera (L, LE, LEC) here using a quantitative ELISA-based assay. This assay uses either the Mel 14 mAb or an anti-human IgG 1 Fc mAb to capture each construct, after which the relative amounts captured by each antibody are analyzed by goat anti–human IgG 1 polyclonal antiserum binding to the captured antigens.

As shown in Fig. 2 A, all three chimeras appeared to be captured by the anti-human IgG 1 mAb with similar efficiency. These results suggested that the different NH$_2$-terminal sequences on these three chimeras appeared to have little effect on the efficiency of IgG-1 recognition. In contrast to
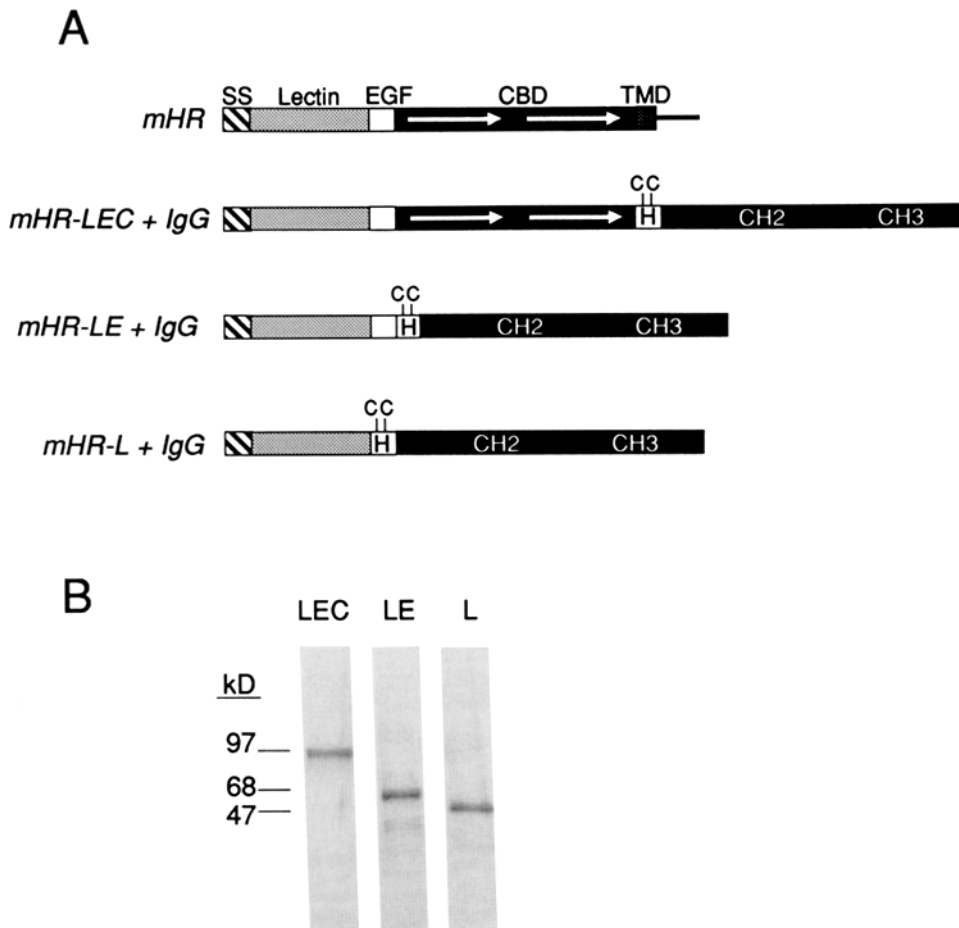

Figure 1. Construction and purification of HR-IgG chimeras. (A) The structure of the murine HR is shown with an NH$_2$-terminal signal sequence (SS), lectin domain, egf domain, duplicate complement binding-like domains (CBD), transmembrane binding domain (TMD), and cytoplasmic sequence. The various IgG chimeras including the full extracellular domain (mHR-LEC + IgG), the lectin plus egf domains (mHR − LE + IgG), and lectin domain alone (mHR − L + IgG) are shown ligated to the hinge (H) and CH2 and CH3 domains of the human IgG 1 heavy chain Fc region. The cysteine residues (C) in the hinge region involved with dimer formation are also illustrated. (B) The three protein A sepharose-purified HR-IgG chimeras are shown as visualized by Coomassie staining of a 7.5% polyacrylamide-SDS gel.
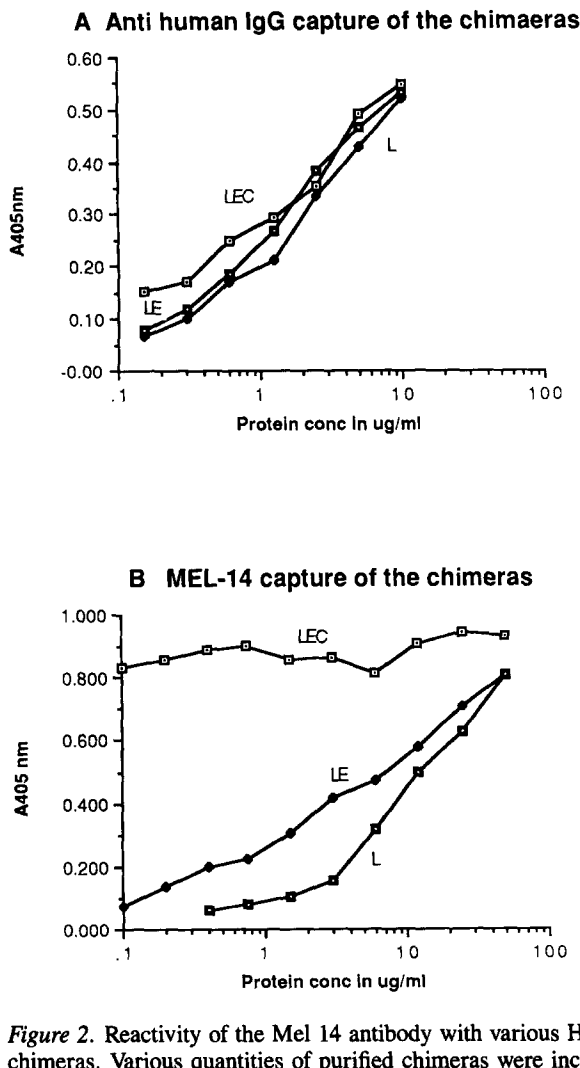
A Anti human IgG capture of the chimaeras

B MEL-14 capture of the chimeras

Figure 2. Reactivity of the Mel 14 antibody with various HR-IgG chimeras. Various quantities of purified chimeras were incubated in microtitre wells coated with either an anti-human IgG 1 mAb (*A*) or the Mel 14 antibody (*B*). After incubation, the wells were washed and incubated with a goat polyclonal anti-human IgG 1 (HRP-conjugated) antibody, after which the wells were washed and the amount of bound goat anti-human antibody measured by peroxidase reactivity. Incubation of chimeras in wells containing no capture antibody gave background signals.

these results, Fig. 2 *B* illustrates that capture with the Mel 14 antibody showed widely different efficiencies of binding. Thus, although all three constructs contained the lectin domain, the full-length LEC chimera effectively bound to Mel 14 at extremely low chimera concentrations (<100 ng/ml) while both the LE and L forms appeared to give similar levels of binding at much higher chimera concentrations (∼50 μg/ml). Comparison of the Mel 14 recognition of the LE and L forms showed that the LE form was similarly recognized at protein levels two- to threefold lower than the L form, consistent with previous immunoprecipitation experiments (6). Similar results were obtained with unpurified material from tissue-culture supernatants, suggesting that the purification procedure did not disrupt Mel 14 antibody recognition. In summary, these results were consistent with a role for the duplicated CB domains in the recognition of the Mel 14 epitope in the HR lectin domain.

Analysis of PPME Reactivity

Previous work has demonstrated that both the natural (17) or IgG chimeric recombinant forms (48) of the HR react with the yeast cell wall carbohydrate PPME in a specific and apparently lectin-directed manner. Thus, this interaction appeared to be calcium dependent and blocked by the lectin-binding mAb, Mel 14 (17, 48). To determine the relative abilities of each chimera to recognize this carbohydrate, PPME was coated onto microtitre plates and reacted with each IgG chimera. The amount of bound chimera was then assayed by incubation with a peroxidase-conjugated polyclonal anti-human Fc antibody. As can be seen from Fig. 3 *A*, the purified LEC chimera recognized PPME in a calcium-dependent manner, in agreement with previous experiments (17, 48). However, the L or LE chimeras showed very limited reactivity with coated PPME, and this reactivity was often not calcium dependent. These results suggest that the removal of the CB domains results in a loss of lectin-directed carbohydrate-binding activity. In addition, they also imply that the decrease in Mel 14 reactivity was mirrored in a concomitant loss of lectin function, consistent with the notion that the antibody recognizes a conformational determinant within the lectin domain.

To determine if the L form could react with carbohydrate under different assay conditions, a second PPME binding assay was performed (17). In this assay, the LEC and L chimeric forms were coated onto microtitre plates, reacted with PPME in solution, after which a polyclonal antibody directed against PPME was used for determining carbohydrate binding. As can be seen in Fig. 3 *B*, a very low, but significant, level of carbohydrate binding was seen for the L chimera. This binding activity was completely calcium dependent as evidenced by its sensitivity to EGTA. This was in contrast to the much higher level of binding seen for the LEC form of the molecule. These results were, again, consistent with the assays shown in Fig. 3 *A*, but suggested that the L form alone could bind PPME, albeit at relatively low levels.

Analysis of Cell Blocking, Immunohistochemical Staining, and Ligand Interaction

While the PPME binding results were consistent with the possibility that the removal of the CB domains inhibited lectin function, they did not address the interactions between the HR and any ligand(s) expressed on the endothelial cell surface. Previously we demonstrated that either the natural (14) or recombinant forms (49) of the HR could effectively inhibit endothelial cell binding by lymphocytes in vitro and neutrophils in vivo (49). Fig. 4 shows that while the LEC chimera could effectively inhibit the in vitro binding of mesenteric lymphocytes to pln HEV, neither the LE nor L forms of the receptor were effective in inhibiting cell binding. These results suggested that the profound decrease of lectin-carbohydrate (PPME) binding function as well as Mel 14 reactivity upon deletion of the CB domains correlated with the loss of endothelial cell binding and resultant cell blocking activity.

While the cell blocking experiments implied that HR lacking the CB domains were incapable of interacting with pln HEV, the relative insensitivity of this assay was a cause for concern. We, therefore, carried out immunohistochemical studies using the LEC, LE, and L containing IgG chimeras
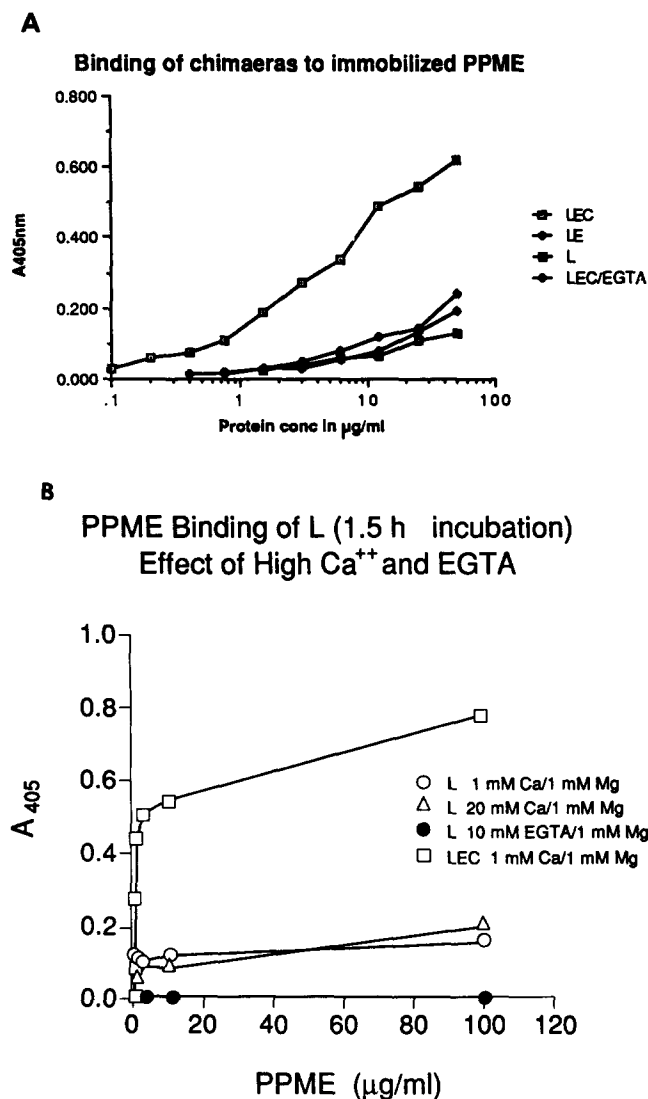
A

Binding of chimaeras to immobilized PPME

B

PPME Binding of L (1.5 h incubation)
Effect of High Ca^{++} and EGTA

Figure 3. Reactivity of HR-IgG chimeras with immobilized PPME. (*A*) The yeast cell wall carbohydrate polyphosphomannan ester (*PPME*) was coated onto the wells of microtitre plates, after which the ability of each HR-IgG chimera to interact with immobilized carbohydrate was determined by incubation of each chimera for 1 h. Bound chimera was detected by incubation with HRP-conjugated goat anti-human Fc serum as described in Fig. 2 (*B*) Purified IgG chimeras equivalent to one A405 U of either the LEC- or L-IgG chimeras as measured by anti-Fc ELISA assay were coated onto the wells of a microtitre plate. Increasing amounts of PPME were incubated for 1.5 h in the wells. In the case of the L-IgG chimera, either 1 or 20 mM calcium was included. The wells were washed, and the amount of bound PPME was measured using a rabbit polyclonal anti-PPME antiserum. The specificity of the signal obtained with the LEC or L chimeras was demonstrated by incubating the reaction in the presence of EGTA to chelate calcium ions.

which allowed for a more sensitive analysis for endothelial cell binding (48). This assay uses the IgG chimeras as immunohistochemical probes for detecting a HR ligand(s) expressed on HEV in an approach which is similar to immunohistochemical staining using mAbs (48). LEC chimera binding has been shown to be calcium dependent, blocked by Mel 14 and various carbohydrates, and sialidase sensitive,
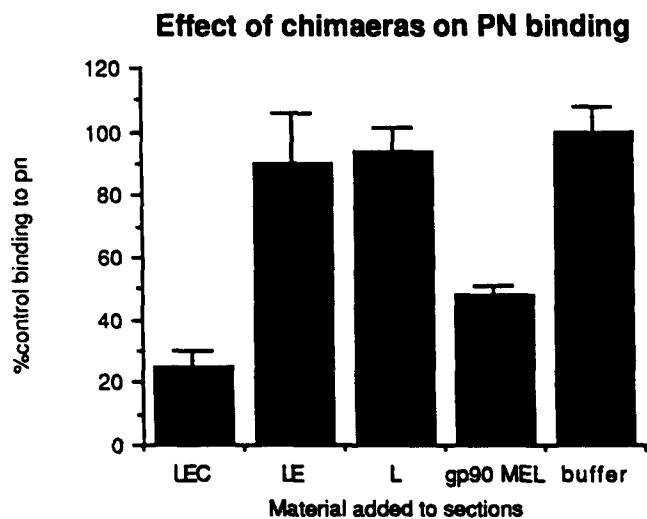
Effect of chimaeras on PN binding

Figure 4. Ability of HR-IgG chimeras to block lymphocyte–endothelial cell adhesion. Mesenteric lymphocytes were incubated with frozen sections of peripheral lymph nodes in the presence of PBS (*buffer*), each of the chimeras at 1 µg per frozen section, or purified spleen cell-derived HR (*gp90 MEL*). Sections were washed and visualized by microscopy, after which the numbers of lymphocytes bound to endothelial venules was determined.

implying that the HR lectin domain was intimately involved with the staining interaction. As can be seen in Fig. 5, the LEC chimera was clearly effective in staining pln HEV cells, while both the LE and L containing chimeras showed no visible HEV staining, even when added at 10-fold higher levels than LEC-IgG. Experiments using the unpurified cell culture supernatants showed identical results, although the general background staining was somewhat higher in these studies (data not shown). Thus, as with cell blocking studies, the removal of the CB domains results in truncated molecules that appeared to be incapable of specifically interacting with HEV.

While the immunohistochemical approach suggested that truncation of the CB domains completely abolished HEV interactions, it may be argued that a more sensitive analysis would show some residual HEV ligand(s) binding activity. Recently, we demonstrated that the LEC chimera could be used as an immunoprecipitating reagent to bring down a glycosylated, sulfate-labeled component of ∼50 kD that was specific for mesenteric and pln (17). In addition, a much more weakly labeled ∼90-kD component was also occasionally seen. The precipitation of these putative HR ligands is calcium dependent, inhibited by specific polysaccharides such as PPME and fucoidin and dependent upon sialylation. From these results, it was clear that the lectin domain played a major role in the binding of the HR to these putative ligands. We, therefore, analyzed the ability of each of the HR-IgG chimeras to immunoprecipitate material prepared from mixed mesenteric and pln metabolically labeled with Na^{35}SO$_4$ in organ culture. As can be seen from Fig. 6, the purified LEC chimera specifically immunoprecipitated the ∼50-kD glycoprotein, while neither the LE nor L forms could precipitate this glycoprotein (the ∼90-kD component was not seen in these experiments). Identical results were ob-
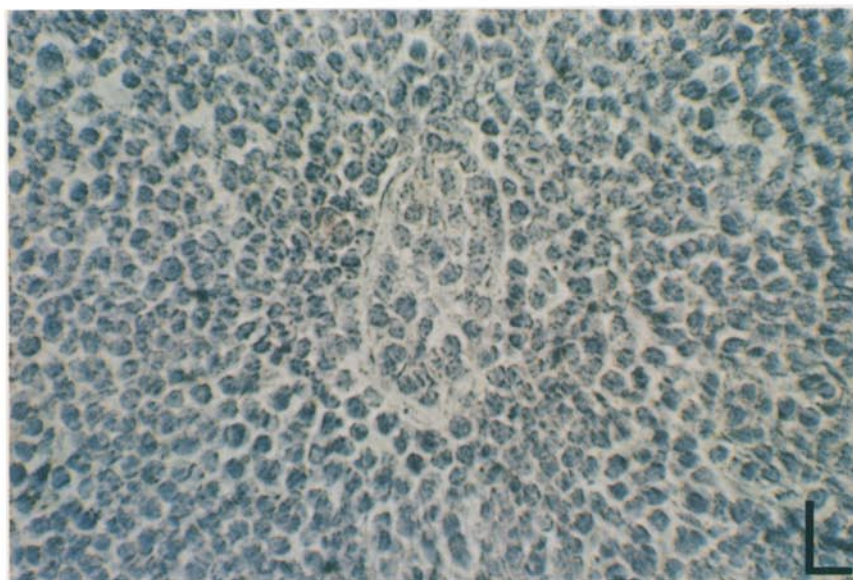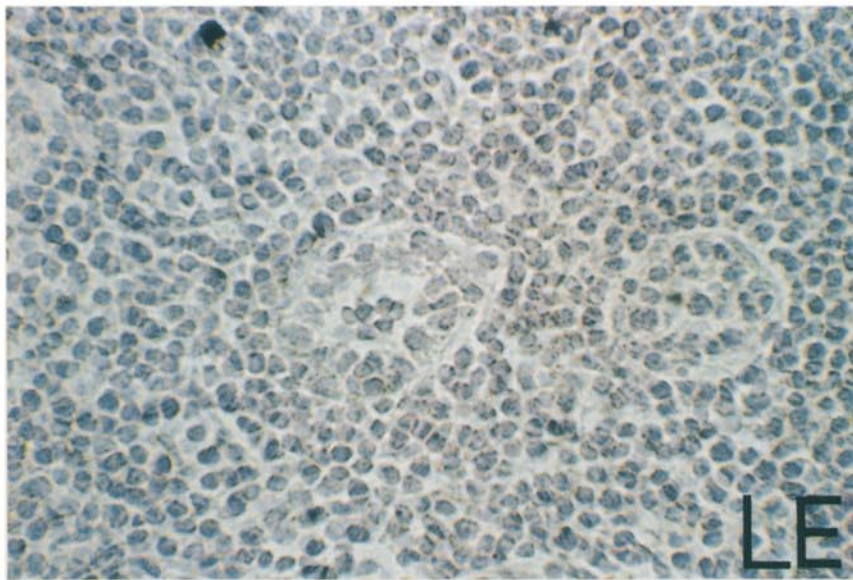
Figure 5. Staining of peripheral lymph node (*pln*) high endothelial venules (*HEV*) with HR-IgG chimeras. pln were sectioned and stained with 30 μg per section of each HR-IgG chimera as previously described (36, 38). The bound chimeras were detected with a mAb directed against the human Fc region conjugated to HRP. Shown are micrographs (560×) of HEV stained with the LEC, LE, and L IgG chimeras.
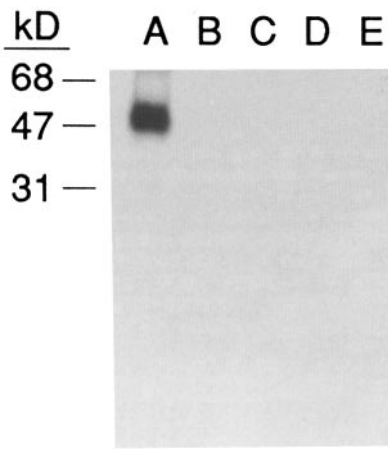
Figure 6. Precipitation analysis of a ~50,000 dalton glycoprotein with the HR-IgG chimeras. A mixture of murine peripheral and mesenteric lymph nodes was sliced and incubated in organ culture with $Na^{35}SO_4$ as previously described (16). The tissues were detergent extracted and cell-free extracts were incubated with the LEC, LE, or L IgG chimeras. Bound material was pelleted after reaction with protein A sepharose beads, and the material was eluted by boiling in SDS and betamercaptoethanol and analyzed by 7.5% polyacrylamide-SDS gels. Radiolabeled glycoproteins precipitated with: (*A*) LEC-IgG chimera; (*B*) LEC-IgG chimera in the presence of EGTA; (*C*) LE-IgG chimera; (*D*) L-IgG chimera; (*E*) CD4-IgG chimera. Identical results were obtained when 100× concentrated tissue culture supernatants rather than purified IgG chimeras were used as the precipitating agents.

tained when tissue culture supernatants were used for the precipitation experiments. Thus, in agreement with the other assays performed, the removal of the CB domains appears to have a profoundly negative effect on the function of the HR.

Discussion

The data reported in this paper introduce two novel but complementary concepts about the structure–function relationships of the protein motifs found in the HR. First, the apparent correlation between the loss of Mel 14 antibody reactivity and lectin domain function is consistent with the notion that the lectin-localized epitope recognized by this antibody is an important structural determinant for carbohydrate recognition and cell adhesion. The almost complete correlation between carbohydrate binding and cell adhesive activities mediated by the HR, together with a large body of literature on the role of similar lectin domains in carbohydrate binding (10), are entirely consistent with this hypothesis, although it is still formally possible that other domains in the LEC-CAMs also bind carbohydrates. In addition, these data also support the hypothesis that the CB domains play an important role in facilitating appropriate lectin domain structure, as determined by Mel 14 antibody recognition and resultant adhesive functions. While previous discussions have proposed that the LEC-CAM CB domains may function as rigid spacer sequences for presentation of the lectin and egf domains to other cells (36), the data reported here argue for a more complex role for these motifs in HR function. The results are, therefore, consistent with a mosaic protein whose

various protein motifs interact with each other to produce a functionally active carbohydrate recognition domain.

The broad distribution of the CB domain in a number of proteins suggests that this motif has the potential for a diversity of functions (1, 31). The use of these domains as complement binding structures has been clearly demonstrated for members of the regulation of complement activity family which includes such proteins as complement receptor 1A, C4 binding protein, and decay accelerating factor. Their function(s) in other members of the complement activation family, including the serine proteases and terminal components, may also be for complement binding, although this is less clear-cut. Attempts to demonstrate complement binding activity with recombinant forms of the HR have been unsuccessful (B. Bowen and L. Lasky, unpublished data). It is interesting that the CB domain is often found juxtaposed to an egf-like domain, especially in the terminal component family, a domain structure which is reminiscent of that found for the LEC-CAMS, although the functional relevance of this association is not understood (31). The role of these domains in other proteins, such as the interleukin 2 receptor, thyroid peroxidase, etc., is currently unknown. While it may be argued that these domains may play structural roles in these proteins analogous to that found here for the HR, it should be noted that not only is the overall sequence conservation between these diverse CB domain motifs weak, there are also six cysteine residues in the domains found in the LEC-CAMS while there are only four in the CB domains in other proteins. This finding argues for a structural role for the LEC-CAM CB domains which may be specific to these adhesion molecules.

The wide variety in CB domain repeat numbers is especially interesting with respect to the LEC-CAM family. If it is assumed that the presence of the CB domains in ELAM and gmp140/PADGEM is also critical for lectin structure and function, then it is puzzling why these proteins have either six (ELAM) or eight to nine (gmp140/PADGEM) CB repeats, respectively, as compared to only two in the HR. One possibility is that these repeats fulfill multiple roles in these molecules, including structural, spacer, oligomer formation, and complement binding functions. Indeed, it is conceivable that one of the CB domains in the HR interacts with the lectin domain while the other may have a separate function, such as, for example, the formation of oligomers. The relative conservation of CB domain sequences between the human and murine HRs (~60–80%) is far greater than the conservation between the various repeats in the human LEC-CAM family members (~40%) (5), implying that the HR CB domains serve functions specific for the HR. It will therefore be interesting to examine these questions in the future by deleting one of the two HR CB domains, and by replacing one or more of these domains with CB domains from other LEC-CAMS. In this way, the relative importance of CB repeat number as well as primary sequence may be analyzed.

The quantitative decrease in Mel 14 antibody reactivity upon deletion of CB domains is consistent with the previous observations (6), which argued that the epitope recognized by the antibody was localized within the lectin domain but was structurally complex. Indeed, the residual Mel 14 reactivity found here for the L chimeric construct confirms the previous mapping studies in a more quantitative manner.

Other studies have claimed that the Mel 14 epitope might be an unusual form of ubiquitin, which can be expressed in the bacterium E. coli. Given the conformational nature of the Mel-14 epitope in the HR, it appears unlikely that this structure can be generated in a bacterial system where it has often proven difficult to produce appropriately folded mammalian proteins (11, 33, 38). Thus reactivity to ubiquitin may represent a fortuitous crossreactivity of the antibody. In the absence of compelling evidence for the presence of ubiquitin in the primary sequence of the HR (25), this modification of the HR is in question. We prefer the interpretation that the Mel 14 epitope is a structurally complex region of the lectin domain, whose structure depends on interactions with the other domains of the molecule.

The complete correlation between the disruption of the lectin domain as determined by Mel 14 binding and HR function confirms previous work demonstrating that the lectin domain plays a major role in cell binding mediated by both the HR as well as by the other two LEC-CAM family members, ELAM and PADGEM/gmp140 (6, 40, 42, 51, 52). The residual carbohydrate-binding activity found for the L chimera also could be correlated with the residual Mel 14 antibody binding of this protein, suggesting that the L domain alone maintained some low level of carbohydrate-binding conformation. The lack of cell blocking, HEV staining, and ligand precipitation by this construct, however, suggests that, this low level of activity is insufficient for appropriate HR recognition of the endothelial cell ligand. The role of the egf domain in HR function remains to be elucidated. As was previously found (6), the consistently greater Mel 14 reactivity found here for LE vs L containing chimeras suggests that at least one role for the egf domain is to somehow influence the conformation of the lectin domain, although this enhancement is clearly insufficient to allow for efficient carbohydrate recognition. Similar findings with the ELAM glycoprotein (46) suggest that this type of interdomain dependence may be a general aspect of LEC-CAM structure. The findings of Siegelman et al. (35) implying a direct role for the egf domain in cell adhesion are not incompatible with these results. However, the complete lack of endothelial recognition by the CB deletion mutant that lacks lectin function suggests that, if the egf domain does directly interact with a ligand on the endothelial cell surface, this interaction may not be sufficient for HR binding. Alternatively, it is possible that the removal of the CB domains also affects the structure of the egf motif such that it is no longer functional.

In summary, the work reported here suggests that the CB domains of the HR and, by analogy, those of the other LEC-CAMS, appear to serve an important role(s) in facilitating the structure and resultant function of the lectin domain. Conceivably, these interactions could be intramolecular or could be because of oligomer formation induced by the CB domains. The exact CB domain sequence requirements for this interaction(s) remain to be elucidated, as does the importance of duplicated CB domains. In addition, the possibility that the CB domains are also directly involved in carbohydrate binding has not been eliminated by this study, and this important point must also be examined. It seems clear that in spite of the mosaic structure of this protein, the lectin domain does not function as a wholly separate entity but appears to be dependent upon the presence of the other domains for appropriate structure and function.

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