Endoglycan, a Member of the CD34 Family, Functions as an L-selectin Ligand through Modification with Tyrosine Sulfation and Sialyl Lewis x*

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During lymphocyte homing to secondary lymphoid organs and instances of inflammatory trafficking, the rolling of leukocytes on vascular endothelium is mediated by transient interactions between L-selectin on leukocytes and several carbohydrate-modified ligands on the endothelium. Most L-selectin ligands such as CD34 and podocalyxin present sulfated carbohydrate structures (6-sulfated sialyl Lewis x or 6-sulfo-sLex) as a recognition determinant within their heavily glycosylated mucin domains. We recently identified endoglycan as a new member of the CD34 family. We report here that endoglycan, like the two other members of this family (CD34 and podocalyxin) can function as a L-selectin ligand. However, endoglycan employs a different binding mechanism, interacting with L-selectin through sulfation on two tyrosine residues and O-linked sLex structures that are presented within its highly acidic amino-terminal region. Our analysis establishes striking parallels with PSGL-1, a leukocyte ligand that interacts with all three selectins, mediating leukocyte-endothelial, leukocyte-leukocyte, and platelet-leukocyte interactions. Since the distribution of endoglycan includes hematopoietic precursors and leukocyte subpopulations, in addition to endothelial cells, our findings suggest several potential settings for endoglycan-mediated adhesion events.

The selectins are a family of three cell adhesion molecules that participate in the initial interaction of leukocytes with the vascular endothelium (reviewed in Refs. 1 and 2). They function as lectin-like receptors by virtue of C-type lectin domains. We recently identified endoglycan as a new member of the CD34 family. We report here that endoglycan, like the two other members of this family (CD34 and podocalyxin) can function as a L-selectin ligand. However, endoglycan employs a different binding mechanism, interacting with L-selectin through sulfation on two tyrosine residues and O-linked sLex structures that are presented within its highly acidic amino-terminal region. Our analysis establishes striking parallels with PSGL-1, a leukocyte ligand that interacts with all three selectins, mediating leukocyte-endothelial, leukocyte-leukocyte, and platelet-leukocyte interactions. Since the distribution of endoglycan includes hematopoietic precursors and leukocyte subpopulations, in addition to endothelial cells, our findings suggest several potential settings for endoglycan-mediated adhesion events.

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2 The abbreviations used are: HEV, high endothelial venules; C-ABC, chondroitinase ABC; Core2GlCNacT-1, core 2 GlcNAc-6-sulfotransferase; CS, chondroitin sulfate; FTVII, fucosyltransferase VII; GAG, glycosaminoglycan; GlcNAc, N-acetylgalactosamine; HEC-GlcNAc6ST, high endothelial cell GlcNAc 6-sulfotransferase; KSGal6ST, keratan sulfate galactose-6-sulfotransferase; PNA, peripheral lymph node addressin; PSGL-1, P-selectin glycoprotein ligand 1; sLex, sialyl Lewis x; WT, wild type; ELISA, enzyme-linked immunosorbent assay; CHO, Chinese hamster ovary; AD, acidic domain of endoglycan; EG, extracellular domain of endoglycan.
HEV-expressed GlcNAc-6-O-sulfotransferase, known as HEC-GlcNAc6ST (or LSST), that contributes to the formation of the MECA-79 epitope and L-selectin ligand activity within mouse lymph nodes (15–17). The fucosylation requirements of L-selectin ligands are met primarily by fucosyltransferase VII (FT VII) with some participation by FTIV (18). While MECA-79 defines a major class of HEV-expressed ligands for L-selectin, it is clear that MECA-79 unreactive ligands exist on vascular endothelium both within lymphoid organs and in inflammatory extralymphoid sites (17, 19–23). The molecular identity of these ligands is unknown. Furthermore, several studies indicate that L-selectin can mediate leukocyte-leukocyte adhesion (24–26). Such interactions could amplify primary leukocyte-endothelial interactions at inflammatory sites by facilitating the capture of additional leukocytes from the blood. These secondary L-selectin-dependent interactions are mainly supported by PSGL-1, a mucin-like glycoprotein that is broadly expressed on leukocytes where it serves as the primary ligand for P-selectin (reviewed in Ref. 27). In distinction from the PNAd interactions, which involve sulfated sLex structures within the mucin domains, the interaction of L-selectin with PSGL-1 involves recognition of a sLex determinant and tyrosine sulfates within the amino terminus of the molecule (28–30). While PSGL-1 is the predominant leukocyte ligand for L-selectin, alternative ligands apparently also exist (31, 32).

In the present study, we identify endoglycan as a novel ligand for L-selectin and show that it employs a binding mechanism similar to that of PSGL-1. Endoglycan was originally cloned based on sequence homologies in its cytoplasmic domain to CD34 and podocalyxin (33). It shares a similar overall structure with these molecules, including a mucin-like domain and a membrane proximal globular domain. Based on these structural homologies and similar genomic organizations (34–36), endoglycan, podocalyxin, and CD34 define a gene family known as the CD34 family. In contrast to CD34 and podocalyxin, endoglycan, podocalyxin, and CD34 define a gene family known as the CD34 family. In contrast to CD34 and podocalyxin, endoglycan, like CD34 and podocalyxin, is a mucin-like glycoprotein that is modified with chondroitin sulfate in the amino-terminal region of the molecule. Our analysis uncovers striking parallels between endoglycan and PSGL-1.

**EXPERIMENTAL PROCEDURES**

**Endoglycan cDNA Constructs**—The generation of the EG/IgG and AD/IgG expression constructs in vector pEF-BOS has been described (33). The AD/IgG mutants YYA and FYT were generated by site-directed mutagenesis following standard protocols. Enzymes and the appropriate buffers were purchased from NEB Inc., the dNTPs from Roche Applied Science. All other AD/IgG mutants were generated by directed mutagenesis following standard protocols. Enzymes and the appropriate buffers were purchased from NEB Inc., the dNTPs from Roche Applied Science. All other AD/IgG mutants were generated by directed mutagenesis following standard protocols.

**Generation of Recombinant Endoglycan Chimeras**—Endoglycan fusion proteins (EG/IgG, AD/IgG, and variants) were generated using transient transfection of CHO cells with the pCLP-2/pEAK10 plasmid, containing the endoglycan full-length cDNA and a selection marker for puromycin as described above. Cells were transfected in 10 cm dishes with 10 μl of anti-rat IgG-Sepharose beads (Zymed Laboratories Inc.) and used as described above to precipitate 35SO4-labeled AD/IgG from conditioned medium of COS-7 or CHO cells. The beads were reacted with 10 μl of anti-rat IgG-Sepharose beads (Zymed Laboratories Inc.) and used as described above to precipitate endoglycan constructs EG/IgG, AD/IgG, and variants.

**Immunoprecipitation with Endoglycan-specific Antibody**—Stable transfectants of endoglycan-expressing cells were generated by transfecting CHO cells with the PCLP-2/pEAK10 plasmid, containing the endoglycan full-length cDNA and a selection marker for puromycin as described above. Cells were transfected in 10 cm dishes with 10 μl of anti-rat IgG-Sepharose beads (Zymed Laboratories Inc.) and used as described above to precipitate endoglycan constructs EG/IgG, AD/IgG, and variants.

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buffer with or without 5% β-mercaptoethanol and subjected to SDS-PAGE (5%).

RESULTS

Post-translation Requirements for Endoglycan as an L-selectin Ligand—To determine if endoglycan can serve as an L-selectin ligand, we examined the rolling of L-selectin bearing Jurkat-T cells on recombinant EG/IgG (extracellular domain of endoglycan fused to the Fc region of human IgG) in a parallel plate flow chamber. The optimal binding of L-selectin to PNAd components, such as GlyCAM-1 and CD34, require specific sialylation, fucosylation, and carbohydrate sulfation (5). To determine the contributions of α1,3 fucosylation and GlcNAc-6-sulfation, endoglycan was expressed in the presence or absence of cDNAs for FTVII and HEC-GlcNAc6ST, which provide these modifications. Sialylation was provided by endogenous α2,3 sialyltransferases present in COS-7 cells. The resulting recombinant proteins were purified from conditioned medium and coated onto the bottom plate of a flow chamber at equal site densities as determined by ELISA.

As shown in Fig. 1A, appropriately modified endoglycan supported tethering and rolling of Jurkat cells over a shear stress range of 1–8 dyn/cm². Since endoglycan was not recognized by MECA-79, we wanted to determine the effect of GlcNAc-6-sulfation on its ligand activity. In contrast to GlyCAM-1 and CD34, Jurkat cell rolling on recombinant EG/IgG was not augmented by GlcNAc-6-sulfation imparted by HEC-GlcNAc6ST, as measured either by the number of rolling cells (Fig. 1A) or rolling velocity (Fig. 1B). The interactions were nevertheless L-selectin-dependent, since rolling was completely blocked by L-selectin antibody (DREG-56) or by chelation of calcium with EDTA. There was no rolling in the absence of FTVII, establishing an absolute requirement for fucosylation. Sialylation was also required, since treatment of the EG/IgG substrate with sialidase completely abrogated rolling. The rolling on FTVII-modified endoglycan was comparable to that of HEC-GlcNAc6ST- and FTVII-modified GlyCAM-1/IgG as assayed under identical conditions (38).

Ligand Activity of the Amino-terminal Domain of Endoglycan—These results suggested a different binding mechanism for endoglycan and led us to investigate the functional importance of the distinct amino-terminal region of endoglycan. We therefore employed an Fc fusion protein containing just the amino-terminal domain of the molecule (AD/IgG) (33). Recombinant EG/IgG and AD/IgG chimeras were produced in the presence or exogenously provided FTVII and used as substrates in flow chamber experiments as above. When coated at equal densities, both substrates supported the rolling of Jurkat cells to comparable extents, as measured both by the number of rolling cells (Fig. 2A) and the velocity of rolling (Fig. 2B).

Earlier experiments with AD/IgG had demonstrated that the amino-terminal domain of endoglycan is modified with CS (33). To investigate a potential contribution of CS chains toward the ligand activity, AD/IgG was treated with chondroitinase ABC (C-ABC) prior to the flow chamber testing. This treatment did not alter the L-selectin mediated rolling of Jurkat cells on AD/IgG, indicating that the CS modification, at least as provided by the COS-7 cell system, did not influence the binding activity of the amino-terminal domain of endoglycan. The interaction of AD/IgG with L-selectin was confirmed to be dependent on the modification with sialic acid and fucose as above (Fig. 3A).

To verify ligand activity of endoglycan by another means, we employed precipitation of AD/IgG with an L-selectin/IgG fusion protein, as has been used in the previous characterizations of ligands in the PNAd category (13, 35, 39, 40). 35SO4-labeled AD/IgG produced in the presence or absence of FTVII cDNA was precipitated by L-selectin/IgG coated beads. As described previously (33), AD/IgG separates into two distinct bands when subjected to SDS-PAGE: a broad relatively high molecular weight component, which represents the CS-modified form and a sharper band of 80 kDa, which lacks the CS modification.

Fig. 1. Required post-translational modifications for ligand activity of endoglycan. Endoglycan IgG-chimeras (EG/IgG) were expressed in the presence or absence of GlcNAc-6-O-sulfotransferase (ST) and/or fucosyltransferase FTVII (FT), coated at equal site densities and tested in the flow chamber. The number of rolling cells and the rolling velocity was determined for each shear stress. Values represent the mean ± S.E. for one representative experiment. A, the number of rolling cells per field on a series of EG/IgG substrates. For inhibition studies, Jurkat cells were pretreated with 5 μg/ml DREG 56 or 10 mM EDTA or the coated substrates were incubated with 10 milliunits of sialidase (V. cholerae) prior to the experiment. B, the rolling velocity of 10 cells per field was determined for each shear stress. The differences between EG/IgG produced with or without ST were statistically not significant at any shear stress tested (p > 0.05).
With FTVII included in the transfection, L-selectin bound AD/IgG in a calcium-dependent manner as shown by elution of AD/IgG by EDTA (Fig. 3B). In the absence of FTVII, the reactivity of AD/IgG with the L-selectin chimera was dramatically reduced. Sialidase treatment of the FTVII-modified AD/IgG eliminated its interaction with the L-selectin chimera.

Biochemical Analysis of Endoglycan—Since recombinant AD/IgG expressed with exogenous FTVII manifested L-selectin ligand activity by two independent assays, we sought to determine the nature of the critical post-translational modifications of this molecule. As stated above, treatment of 35SO4-labeled AD/IgG with C-ABC eliminated the broad high molecular weight component, leaving a sharper band with a molecular weight of ~80 kDa. Subsequent treatment of this component with aryl-sulfatase (Aerobacter aerogens) nearly eliminated the remaining 35SO4 signal, indicating that the amino-terminal domain of endoglycan carries tyrosine sulfation in addition to CS modification (Fig. 4A). As described below, this conclusion was substantiated by the analysis of endoglycan mutants lacking the key tyrosines.

To determine whether sialylation and fucosylation occurred together within sLex-related structures, we immunoprecipitated 35SO4-labeled AD/IgG with C-ABC and HECA-452, a sLex reactive mAb (41). HECA-452 precipitated AD/IgG when the protein was co-expressed with FTVII, but no reactivity was detected in the absence of the fucosyltransferase (Fig. 4B). sLex structures are found on both O- and N-linked carbohydrate chains (42). The elaboration of sLex structures on O-glycans, in contrast to that on N-glycans, depends on the presence of a core-2 branching enzyme such as Core2GlcNAcT-I and FTVII in various combinations. As shown in Fig. 4B, HECA-452 reactivity depended on the presence of both Core2GlcNAcT-I and FTVII, indicating that the modification of AD/IgG with sLex is restricted to O-linked chains. Additionally, we observed that AD/IgG expressed in CHO cells contained a higher proportion of the high molecular species as compared with AD/IgG produced in COS-7 cells. Since the high molecular component remained sensitive to treatment with C-ABC (not shown), this result suggests that CHO cells can produce AD/IgG with a higher degree of CS modification than COS-7 cells.

Similarities between Endoglycan and PSGL-1—Endoglycan contains three cysteines in its membrane proximal domain. Sequence alignments with family members CD34 and podocalyxin suggest that two of the cysteines are involved in intrachain stabilization of the membrane proximal globular domain. The third cysteine residue is located in close proximity to the transmembrane domain, similar to an unpaired cysteine found in PSGL-1 (44). PSGL-1 forms a disulfide-dependent dimer that is resistant to SDS denaturation (45). To determine whether endoglycan behaved similarly, we transfected CHO cells with a full-length cDNA for endoglycan. The cells were labeled with 35SO4 and the lysate was subjected to immunoprecipitation with a polyclonal antibody to endoglycan. SDS-PAGE performed in the presence of β-mercaptoethanol revealed a band of 150 kDa molecular mass, while in the absence of β-mercaptoethanol, a band of ~300 was seen, consistent with a disulfide-linked dimer (Fig. 5).

Further similarities between endoglycan and PSGL-1 were revealed by comparison of amino acid sequences of their amino-terminal domains (Fig 6A). As noted above, this region of PSGL-1 carries post-translational modifications that are criti-
cal for its interactions both with P-selectin and L-selectin (29, 30). Present in the human sequence of PSGL-1 are 3 tyrosine residues that are modified by sulfation, and a single threonine, which carries a sLex modification (highlighted by asterisks in Fig. 6A). In contrast to PSGL-1 where the 3 tyrosines span 6 amino acids, endoglycan possesses 2 tyrosine residues separated by 16 amino acids. Significant sequence similarity was found around the third tyrosine of PSGL-1. The sequence alignment shows that 5 of 9 amino acids are identical with the endoglycan sequence including the last tyrosine and the downstream threonine.

In order to determine whether the amino acids of endoglycan that align with the critical residues of PSGL-1 were subject to similar post-translational modifications, we generated several point mutants of AD/IgG by site-directed mutagenesis. The resulting proteins carried amino acid exchanges as indicated in Fig. 6B. Either one or both tyrosine residues were replaced by phenylalanine, while Thr-124 was replaced by alanine. The constructs were named according to the amino acid changes in their sequence. Thus, the WT sequence is denoted YYT with the first Y representing Tyr-97, the second Y representing Tyr-118 and the T in the third position representing Thr-124.
were generated: FYT, YFT, FFT, YYA, and FFA.

To examine tyrosine sulfation, we prepared \( ^{35} \text{SO}_4 \)-labeled forms of the WT AD/IgG and the tyrosine mutants FYT, YFT, and FFT in COS-7 cells. Equal amounts of the Fc chimeras were subjected to chondroitinase ABC treatment and analyzed by SDS-PAGE and densitometry. The \( ^{35} \text{SO}_4 \) incorporation of mutants FYT and YFT was 50% and 64%, respectively of the WT protein while the FFT was only 18% of the WT signal (Fig. 6C). The low level of residual sulfation might be attributable to sulfation of N-linked or O-linked chains within AD/IgG or to sulfation of GAG chain "stubs" after digestion with C-ABC.

To determine whether Thr-124 carried the sLex modification, we subjected equal amounts of WT, YYA, and FFA forms of AD/IgG, expressed in the presence or absence of FTVII, to immunoprecipitation with HECA-452. The reactivity of HECA-452 with sLex structures is fucose-dependent (14). Accordingly, no precipitation was observed for AD/IgG produced without FTVII, while a reduced but still detectable reactivity with mutants YYA and FFA was observed (Fig. 6D). These findings showed that the majority of sLex was associated with Thr-124 but indicated the presence of additional sLex structures within the amino-terminal region of endoglycan.

**Analysis of Ligand Activity of AD/IgG Mutants**—To investigate the functional importance of single modifications, the endoglycan mutants were analyzed for ligand activity in the flow chamber. When both tyrosine residues were mutated to phenylalanine (FFT), the rolling of Jurkat T-cells on recombinant endoglycan was reduced by more than 50% relative to the WT recombinant protein. A decrease in the number of rolling cells of up to 80% was observed when Thr-124 was mutated to alanine (YYA). Mutation of both tyrosines and the Thr-124 (FFA) did not cause any further decrease in the rolling activity (Fig. 7A). The rolling was nevertheless completely dependent on the coexpression with FTVII indicating that the residual sLex structures within the AD/IgG (shown in Fig. 6D) were contributing to the L-selectin interaction. These effects on rolling were observed over the entire range of shear stresses tested (1–8 dynes/cm²). The mutations also affected rolling velocity. At a shear stress of 1 dyn/cm², the mean rolling velocity was increased by more than 20% for the FFT mutant relative to the WT protein and 36% and 40% for YYA and FFA mutants, respectively (Fig. 7B).
In the present study, we present evidence that endoglycan, the third member of the CD34 family is a novel ligand for L-selectin. Even though endoglycan is closely related to the other members of the family, our experiments reveal that it employs a distinct binding mechanism for interacting with L-selectin. In contrast to CD34 and podocalyxin, endoglycan did not depend on the activity of carbohydrate 6-O-sulfotransferases in order to function as an L-selectin ligand. Thus, the cotransfection of FTVII alone was sufficient to impart ligand activity to endoglycan. Furthermore, the inclusion of a cDNA for HEC-GlcNAc6ST did not detectably enhance the rolling activity for endoglycan, nor did the inclusion of a cDNA for a Gal-6-O-sulfotransferase (KSGal6ST) (data not shown). Additionally, AD/IgG, lacking the mucin domain, exhibited comparable ligand activity to the chimera containing the entire extracellular domain (EG/IgG). However, both fusion proteins needed to be modified with fucose and sialic acid in order to function as L-selectin ligands. The suspicion that sLex-related structures were present within endoglycan was confirmed by showing that HECA-452 was able to precipitate AD/IgG and EG/IgG produced in the presence of FTVII but not in its absence. By using CHO cells in which the presence of the core-2 branching enzyme could be controlled by transfection, we were able to show that the sLex-related structures were restricted to O-linked glycans.

Our earlier characterization of native endoglycan in HUVEC and recombinant endoglycan (both EG/IgG and AD/IgG) in COS-7 cells has revealed a high level of $^{35}$SO$_4$ incorporation, most of which was attributable to CS chains (33). The fact that AD/IgG contains only one potential attachment side for glycosaminoglycan (GAG) chains at position 79 of the unprocessed protein sequence establishes the utilization of this site. Whether and to what extent the other potential GAG sides within the endoglycan sequence are modified by CS remains to be determined. The residual sulfation of AD/IgG remaining after C-ABC treatment was established to be due to tyrosine sulfation. First, it was greatly reduced after treatment with aryl-sulfatase. Secondly, we observed a dramatic loss of sulfate incorporation when either one of the two tyrosine residues within the AD region was replaced by phenylalanine, indicating that both tyrosines are subject to this modification.

Studies by Miyasaka and co-workers (46) have shown a subfraction of CS chains, in particular oversulfated chains such as CS-E, exhibit ligand activity for L-selectin. We found that treatment of FTVII-modified AD/IgG with C-ABC did not impair its ability to support lymphocyte rolling. Moreover, the precipitation studies indicated that both forms of AD/IgG could interact with L-selectin. Thus, the 80-kDa component lacking CS chains, bound to L-selectin just as efficiently as the high molecular weight component that contained CS chains. It should be pointed out that only a small fraction of endoglycan expressed in COS-7 cells was modified with CS, as judged by the relative sulfate incorporation of the CS modified and non-modified forms. The CS-modified portion of endoglycan was higher in AD/IgG produced by CHO cells and endogenous en-

**Fig. 7.** Rolling of Jurkat cells on endoglycan mutants. AD/IgG and mutant AD/IgG proteins were coated at equal site densities and tested in a parallel flow chamber as described in Fig. 1. Values represent the mean ± S.E. for three independent experiments performed with independent preparation of recombinant proteins. A, the number of rolling cells per field at shear stresses 1–8 dyn/cm$^2$. The rolling of each of the mutants was significantly reduced compared with the WT protein at shear stresses 1–6 dyn/cm$^2$ with probability values of $p < 0.01$ for 1 and 6 dyn/cm$^2$ and $p < 0.001$ for 2 and 4 dyn/cm$^2$ as determined by unpaired Student-Newman-Keuls Multiple Comparison. The number of rolling cells at 8 dyn/cm$^2$ was too low for a statistical analysis. The YYA and FFA mutants were consistently weaker ligands than the FFT mutant; however, this trend achieved statistical significance only for shear stresses 1 and 2 dyn/cm$^2$. B, the velocity of a minimum of three rolling cells was determined for each of the AD/IgG mutants at a shear stress of 1 dyn/cm$^2$. The differences are statistically significant between the WT and each of the mutants with $p < 0.0002$ for FFT and $p < 0.0001$ for YYA and FFA, as well for the comparison of mutant FFT with each of the threonine mutants, YYA ($p < 0.05$) or FFA ($p < 0.02$).
Endoglycan Is a Novel PSGL-1-like Ligand

Endoglycan from HUVEC did not contain detectable amounts of the non-modified form (33). It remains to be determined whether endoglycan in particular vascular beds or on leukocytes carries oversulfated CS chains, which might facilitate interactions with L-selectin or P-selectin. Intriguingly, the same modifications of CS chains that promote selectin interaction also facilitate the binding of certain chemokinies (46), raising the possibility that certain forms of endoglycan might be involved in chemokine sequestration. A dual function for a proteoglycan as a selectin ligand and chemokine presentation molecule has recently been proposed for collagen XVIII, which carries heparan sulfate chains (47).

Excluding the CS chain as critical for the ligand activity of endoglycan as produced in COS-7 cells left the tyrosine sulfation for further investigation in this respect. The presence of tyrosine sulfation and O-linked sLex within the amino-terminal region of endoglycan led us to compare endoglycan with PSGL-1. As reviewed above, PSGL-1 is a homodimeric selectin ligand that is expressed on the surface of almost all circulating leukocytes (27). Like endoglycan, it shows an absolute dependence on sialylation and fucosylation for ligand activity (1, 27). As another parallel, we established that endoglycan, like PSGL-1, formed a disulfide-dependent homodimer in SDS-PAGE. The amino-terminal anionic region of human PSGL-1 contains 3 tyrosine residues, each of which can be sulfated, and a single sLex modified O-glycan at a proximal threonine residue (Thr-57). Alignment of the two amino-terminal sequences of endoglycan and PSGL-1 revealed a striking correspondence in a critical 9 amino acid peptide region. This peptide carries in identical spacing the last tyrosine (Tyr-51 in PSGL-1 and Tyr-118 in endoglycan) in both sequences as well as the threonine residue (Thr-57 in PSGL-1 and Thr-124 in endoglycan). PSGL-1 can interact with all three selectins (27). While the presence of the sLex structure is necessary for the binding in all cases, the sulfation requirements differ among the selectins. E-selectin binding to PSGL-1 is indifferent to tyrosine sulfation (28, 48). In the case of P-selectin, all three tyrosine sulfates can contribute but the major contribution is from Tyr-48 (50).2 For L-selectin, sulfation of Tyr-51 plays the predominant role (30). Our findings established that a sLex modification of an O-glycan at Thr-124 accounts for up to 80% of the ligand activity. This is compatible with the result that the majority of HECA-452 reactivity was found to be associated with this residue. Rolling was strongly supported by sulfation on Tyr-97 and -118. However, about 50% of the activity remained when both tyrosines were mutated, indicating that the modification with sLex is capable of sustaining a degree of ligand activity in the absence of tyrosine sulfation. Parallel findings were reported for PSGL-1 (51). The small amount of ligand activity in the secondary tyrosine mutant remained fucose-dependent and is probably attributable to O-linked sLex modifications on other threonines or serines in the amino-terminal domain. The existence of these additional sLex modifications for endoglycan is supported by the HECA-452 reactivity of the threonine mutants YYA and FFA. Since both of these mutants exhibited equivalent, albeit weak ligand activity, we conclude that the secondary sLex-bearing sites are not facilitated by the tyrosine sulfates. It is worth pointing out that the binding of endoglycan (highlighted by the frame in Fig. 6A), containing both tyrosines and Thr-124, is highly conserved within the mouse sequence (GenBank accession number XM_194288). It differs only in 2 amino acids out of 33, preserving the potential attachment sites for N-and O-glycans, and tyrosine sulfation.

As reviewed in the introduction, numerous reports indicate the existence of MECA-79-independent L-selectin ligands on vascular endothelium, although their molecular identities have not been revealed so far. Based on the characterization presented herein, endoglycan represents a strong candidate for a number of these sites. Since endoglycan is also present on hematopoietic precursors and leukocyte subpopulations (33) our findings suggest the involvement of endoglycan in leukocyte-leukocyte in addition to leukocyte-endothelial interactions. If it is found that P-selectin and E-selectin can utilize endoglycan as a ligand, as is strongly suspected from its parallels to PSGL-1, the range of potential in vivo functions for this molecule would be further broadened since these selectins are found on platelets and/or activated vascular endothelium. Gene targeting approaches (52, 53) and antibody blockade studies (49) have been essential in establishing the importance of PSGL-1 as a selectin ligand. Similar approaches will be necessary to gain a further understanding of endoglycan.

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