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Subsets of sialylated, sulfated mucins of diverse origins are recognized by L-selectin. Lack of evidence for unique oligosaccharide sequences mediating binding

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Previous studies have shown that the mucin-type polypeptides GlyCAM-1, CD34, and MAdCAM-1 can function as ligands for L-selectin only when they are synthesized by the specialized high-endothelial venules (HEV) of lymph nodes. Since sialylation, sulfation, and possibly fucosylation are required for generating recognition, we reasoned that other mucins known to have such components might also bind L-selectin. We show here that soluble mucins secreted by human colon carcinoma cells, as well as those derived from human bronchial mucus can bind to human L-selectin in a calcium-dependent manner. As with GlyCAM-1 synthesized by lymph node HEV, α2–3 linked sialic acids and sulfation seem to play a critical role in generating this L-selectin binding. In each case, only a subset of the mucin molecules is recognized by L-selectin. Binding is not destroyed by boiling, suggesting that recognition may be based primarily upon carbohydrate structures. Despite this, O-linked oligosaccharide chains released from these ligands by beta-elimination do not show any detectable binding to L-selectin. Following protease treatment of the ligands, binding persists in a subset of the resulting fragments, indicating that specific recognition is determined by certain regions of the original mucins. However, O-linked oligosaccharides released from the subset of non-binding mucin fragments do not show very different size and charge profiles compared to those that do bind. Furthermore, studies with polylactosamine-degrading endoglycosidases suggest that the core structures involved in generating binding can vary among the different ligands. Taken together, these data indicate that a single unique oligosaccharide structure may not be responsible for high-affinity binding. Rather, diverse mucins with sialylated, sulfated, fucosylated lactosamine-type O-linked oligosaccharides can generate high-affinity L-selectin ligands, but only when they present these chains in unique spacing and/or clustered combinations, presumably dictated by the polypeptide backbone.

Key words: L-selectin/mucins/sialic acid/sulfate/adhesion

Introduction

The selectins are a family of mammalian lectins involved in the recruitment of leukocytes in normal and pathologic conditions (see Bevilacqua and Nelson, 1993; Rosen and Bertozzi, 1994; Varki, 1994b; Lasky, 1995; McEver *et al.*, 1995; Tedder *et al.*, 1995, for reviews). While E- and P-selectin are expressed by activated endothelia and P-selectin is presented by activated platelets, L-selectin is constitutively expressed on leukocytes. Physiological glycoprotein ligands of selectins have been described, most of which are sialomucins (reviewed in Rosen and Bertozzi, 1994; Varki, 1994b; Lasky, 1995; McEver *et al.*, 1995; Tedder *et al.*, 1995). In mice, a 50 kDa ligand for L-selectin is synthesized by the specialized high endothelial venules (HEV) of peripheral and mesenteric lymph nodes (Imai *et al.*, 1991; Lasky *et al.*, 1992). The O-linked glycans in this small mucin-like molecule (GlyCAM-1) are sialylated, fucosylated, and heavily sulfated (Imai and Rosen, 1993; Hemmerich and Rosen, 1994; Hemmerich *et al.*, 1994, 1995), and these modifications are required for binding. An additional, 90 kDa membrane-bound mouse HEV ligand was identified as a glycoform of CD34 (Imai *et al.*, 1991; Baumhueter *et al.*, 1993). Mucosal Addressin-Cell Adhesion Molecule-1 (MAdCAM-1), an adhesion molecule which interacts with integrins can also function as a ligand for L-selectin (Berg *et al.*, 1993). In all cases, correct glycosylation by HEV endothelium is required to create ligands for L-selectin. Likewise, the sialomucin PSGL-1 can generate a high affinity P-selectin ligand only when it is expressed in myeloid cells (Moore *et al.*, 1992, 1994, 1995; Norgard *et al.*, 1993b; Sako *et al.*, 1993), certain subsets of T lymphocytes (Alon *et al.*, 1994), or in COS cells co-transfected with a cDNA encoding an α(1–3/4) fucosyltransferase (Sako *et al.*, 1993). PSGL-1 can also present high affinity ligands for E-selectin (Asa *et al.*, 1995). A recent report (Steegmaier *et al.*, 1995) of a non-mucin-type ligand for E-selectin (ESL-1) is confounded by the fact that it is identical to a previously described medial Golgi marker, MG-160 (Gonatas *et al.*, 1995).

The selectins all recognize the tetrasaccharide sialyl-Lewis^x (sLe^x; Siaα2–3Galβ1–4(Fucα1–3)GlcNAcβ1-R) and its isomer sialyl-Lewis^a (sLe^a; Siaα2–3Galβ1–3(Fucα1–4)GlcNAcβ1-R), and differentially interact with other related oligosaccharides (Green *et al.*, 1995). The combined results of many studies indicate that sialic acid and the fucose residues are usually essential, and that a sulfate ester can replace the sialic acid in some cases. However, the affinity of the selectins for such oligosaccharides is poor–most reports give values in the high micromolar to low millimolar range based on IC_{50} values and binding-inhibition assays (Brandley *et al.*, 1993; Nelson *et al.*, 1993a,b; Narasinga Rao *et al.*, 1994; Yoshida *et al.*, 1994a; Jacob *et al.*, 1995). In contrast, the physiological ligands show strong binding that survives stringent washing under the conditions used for precipitation, blotting, or flow cytometry analyses. When direct measurements have been

made, these improved affinities were confirmed, e.g., a K_d of 70 nM for monomeric, soluble P-selectin interacting with its native ligand on HL-60 cells (Ushiyama *et al.*, 1993), and a similar affinity predicted for monovalent E-selectin (Hensley *et al.*, 1994). With L-selectin, it has been suggested that the novel oligosaccharide 6'-sulfo-sialyl Lewis^x on GlyCAM-1 might explain high affinity binding (Hemmerich and Rosen, 1994; Hemmerich *et al.*, 1994, 1995). However, chemically synthesized quantities of this molecule (Jain *et al.*, 1994) do not show greatly superior inhibition of L-selectin interactions (Koenig,A., Jain,R., Vig.,R., Norgard-Sumnicht,K.E., Matta,K.L. and Varki,A, manuscript in preparation).

To further complicate the picture, P- and L-selectin can also bind with high affinity to some heparin/heparan sulfate chains, despite the fact that these do not carry sialic acids and fucose (Skinner *et al.*, 1989; Nelson *et al.*, 1993a; Norgard-Sumnicht *et al.*, 1993; Green *et al.*, 1995; Norgard-Sumnicht and Varki, 1995). It has been difficult to study the precise structure of the natural high-affinity ligand(s) for L-selectin, because the relevant polypeptides must be expressed in the specialized HEV of lymph nodes, whose phenotype is not well preserved in *in vitro* culture systems (Tamatani *et al.*, 1993). We reasoned that unless HEV are capable of some unique glycosylation not found in any other tissues, sulfated sialylated mucins from other sources might also bind L-selectin. Here we show that this is the case, and present data suggesting that recognition by L-selectin involves not unique oligosaccharide chains, but unique spacing and/or clustered combinations, presumably dictated by the polypeptide backbone of the mucins.

Results

Mucins from human colon carcinoma and from human bronchial mucus can be recognized by L-selectin

We studied mucins from a colon carcinoma and from human bronchial mucus, both of which have sialylation, sulfation, and fucosylation (Klein *et al.*, 1988, 1993; Kitagawa *et al.*, 1991; Lamblin *et al.*, 1991; Lo-Guidice *et al.*, 1994; van Halbeek *et al.*, 1994). The colon carcinoma cell line LS 180 was recently shown to bind to immobilized L-selectin (Nelson *et al.*, 1993b; Cecconi *et al.*, 1994; Mannori *et al.*, 1995) in a manner sensitive to O-sialoglycoprotease, suggesting the presence of mucin-type ligands on these cells (Mannori *et al.*, 1995). As cell surface and secreted molecules are exposed to similar glycosyltransferases during biosynthesis, we reasoned that L-selectin ligands might also be secreted into the medium. Mucins from the medium of LS 180 cells metabolically labeled with [^{3}H]glucosamine or [^{35}S]sulfate were enriched for molecules with O-linked oligosaccharides by jacalin affinity chromatography (Hortin and Trimpe, 1990) (Figure 1A). To isolate high-molecular weight mucins, this material was treated with Heparin lyase II and Chondroitinase ABC (to eliminate contaminating glycosaminoglycans), and then fractionated on a Sephacryl S-200 gel filtration column. The excluded material (Figure 1B) contains all of the high-molecular weight mucins that can bind to L-selectin (fractionation by Sepharose CL-4B column gave losses of some of the smaller mucin-type ligands, data not shown). As shown in Figure 1C, a portion of the labeled mucin bound to an L-selectin affinity column in the presence of calcium, and was eluted

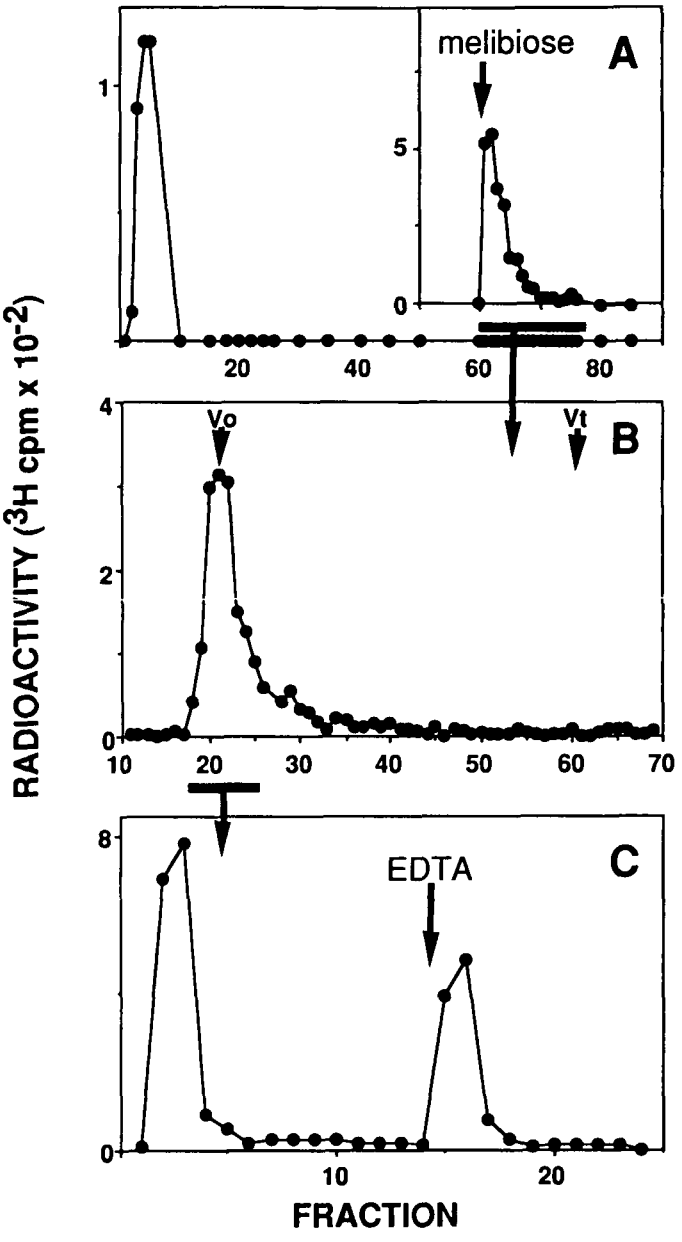

Fig. 1. Metabolically labeled mucins secreted by LS 180 colon carcinoma cells bind to L-selectin. Mucins from the culture medium of LS 180 cells metabolically labeled with [6-^{3}H] glucosamine were affinity purified by jacalin-agarose affinity chromatography as described under Materials and methods (inset in **A** shows a scale-up view of the elution fractions), and after digestion with glycosaminoglycan-degrading enzymes, were size-fractionated on Sephacryl S-200, and pooled as indicated by the bar (**B**). The purified labeled mucin preparation was applied to an L-selectin affinity column in the presence of calcium, and the bound material eluted with EDTA (**C**). $^{35}SO_4$-labeled GlyCAM-1 derived from metabolically labeled mouse lymph nodes served as a positive control for binding to the L-selectin column (data not shown).

with EDTA. This fraction can rebind after calcium reconstitution (data not shown, see tables below), demonstrating the C-type lectin-dependent specificity of the interaction. It was usually necessary to re-pass the radioactivity over the L-selectin affinity column several times to collect all of the binding material. Typically, ~60 % and ~90 % of the ^{3}H and ^{35}S radioactivity from the S-200 column was able to bind to L-selectin, respectively.

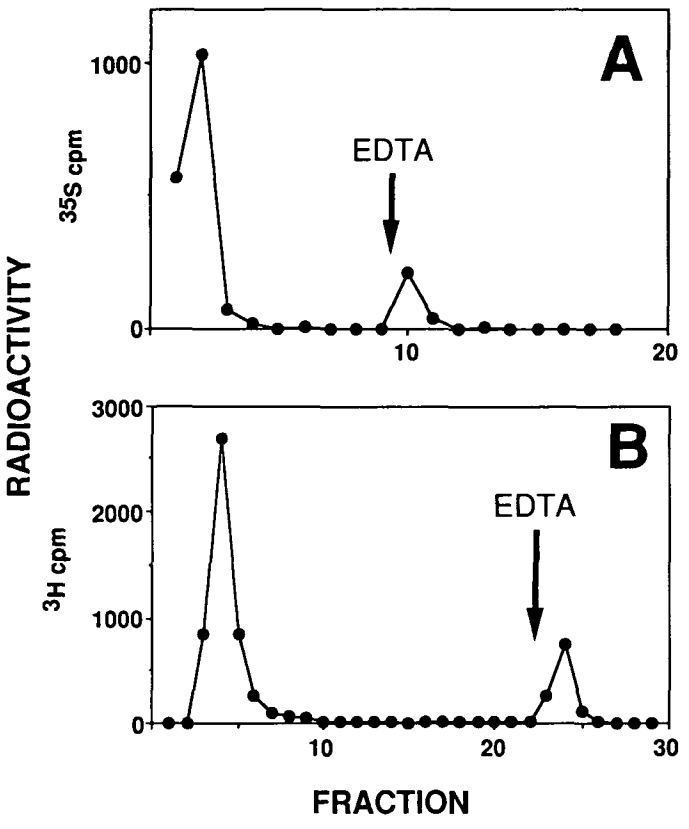

Fig. 2. Large mucin fragments from bronchial mucins bind to L-selectin. The mucin fragments were labeled as described in Materials and methods via the polypeptide chain (using ^{35}S sulfur labeling reagent, **A**) in the sialic acid side chain (using periodate/NaB^3H_4, **B**), or in terminal Gal residues (via galactose oxidase/NaB^3H_4, not shown). The purified labeled glycopeptides were applied to an L-selectin affinity column in the presence of calcium, and the bound material eluted with EDTA. $^{35}SO_4$-labeled GlyCAM-1 derived from metabolically labeled mouse lymph nodes served as a positive control for binding to the L-selectin column (data not shown).

Previous studies have reported a variety of O-linked sugar chains in the bronchial mucus of a patient with bronchiectasis, including several that are sialylated and/or fucosylated (Breg *et al.*, 1988; Klein *et al.*, 1988, 1991; van Halbeek *et al.*, 1988; Van Kuik *et al.*, 1991; Klein *et al.*, 1993), and sulfated structures are predicted to be present (Klein *et al.*, 1988). Because the starting material (bronchial mucus) is a very complex mixture, we studied the large mucin-glycopeptides from this source (gift of Dr. A. Klein, INSERM, Lille, France), i.e., fraction 'P2' which is included in a CL-2B column (Klein *et al.*, 1988). These large mucin-type glycopeptides were labeled via the polypeptide chain (using an *in vitro* [^{35}S] sulfur protein-labeling reagent), in terminal Gal residues (via galactose oxidase/NaB^3H_4, without prior desialylation), or in the sialic acid side chain (using mild periodate/NaB^3H_4). Although the latter gives truncation of the sialic acid side chain, we had previously shown that this does not affect recognition of the GlyCAM-1 ligand by L-selectin (Norgard *et al.*, 1993a). As shown in Figure 2, a portion of these labeled mucin fragments binds to L-selectin in a calcium-dependent manner. Typically, ~13% of the [^{35}S]-, ~11% of the [^3H]Gal-, and 12–18% of the [^3H]Sia-labeled material were bound. As with the carcinoma-derived mucin, the bound fractions rebind in the presence of calcium (data not shown; see also tables).

$^{35}SO_4$-labeled GlyCAM-1 from metabolically labeled mouse lymph nodes (Imai *et al.*, 1991; Lasky *et al.*, 1992) served as a positive control; about 18% of the material enriched on a WGA lectin column bound to L-selectin (data not shown). For many of the subsequent studies, the three ligands (colon carcinoma, bronchial mucin, and GlyCAM-1) were compared with one another.

Susceptibility to O-sialoglycoprotease confirms the mucin-type structure of these molecules

SDS–PAGE and autoradiography showed that the affinity purified L-selectin ligands from both colon carcinoma and bronchial mucin give diffuse bands of high apparent molecular weight (>200 kDa), with a substantial portion of the carcinoma mucin not entering the gel (data not shown). This is expected for large mucins or glycosaminoglycans (the latter had been enzymatically eliminated from our preparations). The enzyme O-sialoglycoprotease (O-SGPase) specifically recognizes glycoproteins with large numbers of closely-spaced sialylated O-linked chains (Abdullah *et al.*, 1992; Norgard *et al.*, 1993b). We have previously shown that O-SGPase can cleave GlyCAM-1 and PSGL-1 (Norgard-Sumnicht *et al.*, 1993; Norgard *et al.*, 1993b), as well as the surface ligands on LS180 carcinoma cells (Mannori *et al.*, 1995). As shown by Figure 3, the soluble L-selectin ligands from colon carcinoma and bronchial mucin are also degraded by O-SGPase. For the colon carcinoma derived mucins and for GlyCAM-1 (Figure 3A,C), the shift towards smaller fragments produced by O-SGPase was less pronounced than that seen with proteinase K, a broad-spectrum protease, indicating that O-SGPase cleaves only in discrete sites within the polypeptide. In contrast, the bronchial mucin fragments were more affected by O-SGPase than by proteinase K, with only the very large material being degraded by the latter enzyme (Figure 3B). This is not unexpected, since the bronchial mucin glycopeptides are derived from a prior pronase digest of mucin (Klein *et al.*, 1988, 1991, 1993). Interestingly, sequential digestion with the two proteases gave even further degradation (data not shown), indicating that O-SGPase can access sites initially protected from proteinase K.

Binding of these mucins to L-selectin is partly sialic acid-dependent

We confirmed the requirement for α2–3 linked sialic acids of GlyCAM-1 in binding (Imai *et al.*, 1992) using sialidase L, which specifically cleaves sialic acids in α2–3 linkage (Sinnott *et al.*, 1993; Chou *et al.*, 1994). Similar treatment of colon carcinoma and bronchial mucin ligands also gave diminished binding to an L-selectin column (Table I). The effects of sialidase L and of the broad-spectrum sialidase from *A. ureafasciens* on L-selectin rebinding were comparable, emphasizing the predominance of the α2–3 linkage in binding. However, even prolonged incubation with sialidases did not abolish binding. In particular, the rebinding of the bronchial mucin ligand was affected to a limited extent. This may be explained either by the lack of accessibility of the sialidase or by a sub-component of sialic acid-independent binding, as previously suggested for GlyCAM-1 (Imai *et al.*, 1992,1993, see also Norgard-Sumnicht

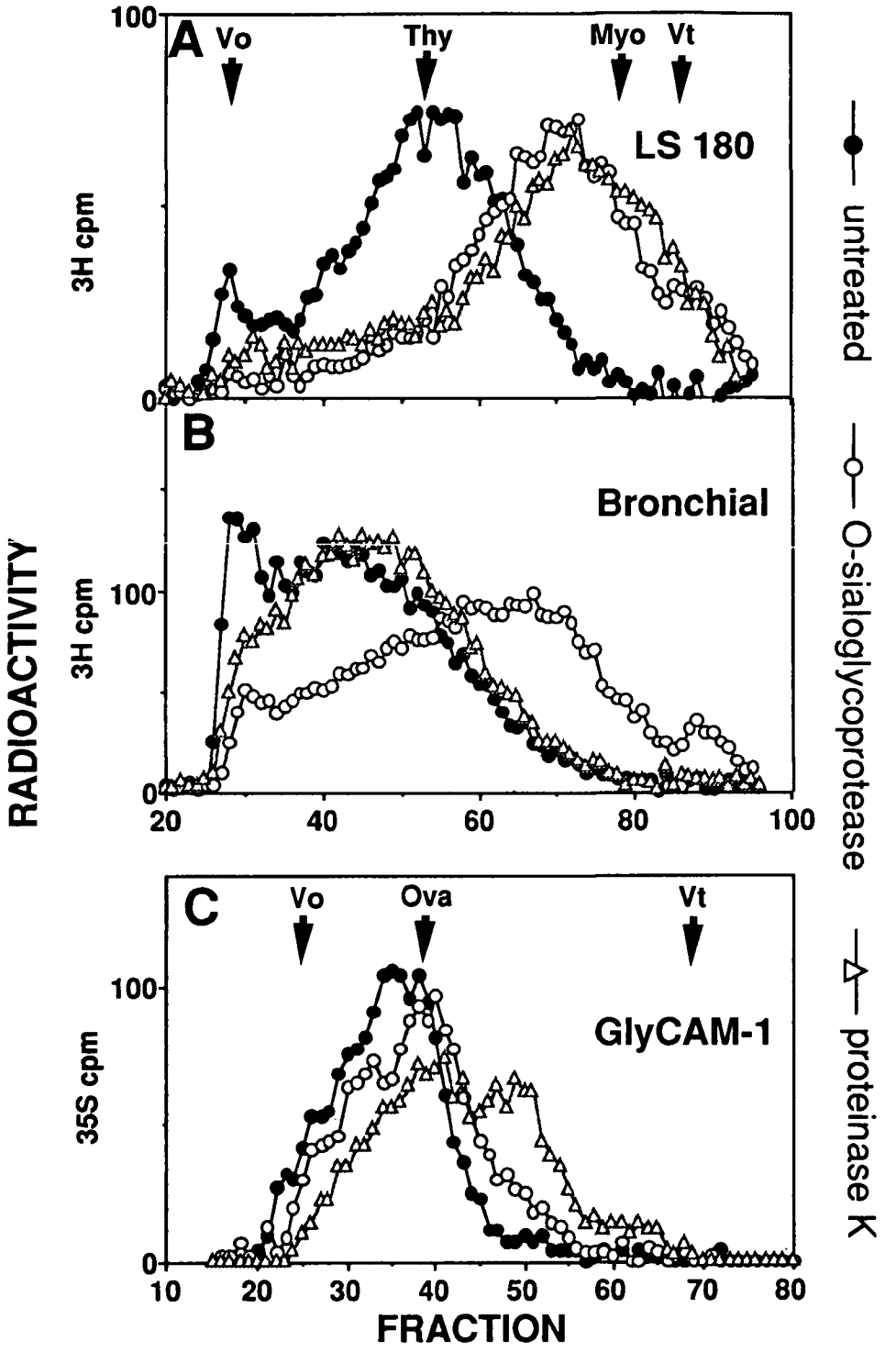

Fig. 3. The L-selectin ligands are susceptible to degradation by O-sialoglycoprotease. Radiolabeled molecules were digested with O-sialoglycoprotease (open circles) or with proteinase K (triangles), or not treated (solid circles). The effect of the enzyme treatment was evaluated on a Sepharose CL-4B column for the carcinoma and bronchial mucins (**A** and **B**, respectively), and on Sephacryl S-200 for GlyCAM-1 (**C**). The void (Vo) and total (Vt) volumes of the columns are indicated, as well as the elution volumes of the following markers: Thy, thyroglobulin (669 kDa); Myo, myosin (200 kDa); Ova, ovalbumin (46 kDa).

et al., 1993). Digestion of the [^{3}H]glucosamine-labeled colon carcinoma ligand was judged to be extensive since 26% of the radioactivity was reproducibly released by the enzyme from *Arthrobacter*, and 21% by sialidase L, as compared to 33% after mild acid treatment (2 h at 80°C in 2 M acetic acid, releasing the total sialic acids). However, the presence of some inaccessible and/or α2–6 linked sialic acids in the LS 180 ligand cannot be completely ruled out. Not surprisingly, the truncated [^{3}H] sialic acids on the bronchial mucin ligands were resistant to the enzymatic treatments (respectively, 17, 21, and 85% of release by the two enzymes versus acid treatment). This mucin prepara-

Table I. Effects of sialidase treatment upon the binding of mucin-type ligands to L-selectin

Treatment	% of re-binding to LS-Rg[a]		
	LS 180 mucin[b]	Bronchial mucin	GlyCAM-1
Untreated control	99	97	74
pH 4.5 buffer control	99	87	84
A. ureafasciens sialidase	35	67	31
Sialidase L	41	74	37

[6-^{3}H]glucosamine labeled LS 180 mucin, [^{3}H]Gal-labeled bronchial mucin, and [$^{35}SO_4$]GlyCAM-1 were treated as indicated (see Materials and methods for details), the samples neutralized with a 10-fold excess of LS-Rg column buffer, boiled for 5 min, and then reapplied to a column of LS-Rg-PAS.

[a] The data are presented as a % of radioactivity bound by L-selectin and released with 5 mM EDTA relative to the total radioactivity recovered in the run-through, wash, and eluate. Recoveries were quantitative in all runs.

[b] The released sialic acids were removed by gel filtration before the rebinding experiment.

Table II. Effects of chlorate treatment upon mucin binding to L-selectin

Treatment	$^{35}S/^3H$ ratio in mucin	L-Selectin affinity chromatography	
		Unbound	Bound
None	0.16	17%	83%
Chlorate	0.09	79%	21%

Parallel plates of LS 180 Cells were metabolically labeled with $^{35}SO_4$ or [^{3}H]glucosamine in the presence or absence of 10 mM chlorate, the labeled mucins isolated, and studied for the ratio of incorporation of $^{35}SO_4$ and ^{3}H as described under Materials and methods. The binding of the ^{3}H-labeled mucins to L-selectin was studied in each case.

tion is known to contain both α2–3 and α2–6 linked sialic acids (van Halbeek *et al.*, 1988; Klein *et al.*, 1993). Taken together with the data above, the ligands from colon carcinoma and bronchial mucin share many similarities with conventional L-selectin ligands, being mucin-type O-linked glycoproteins, and requiring some α2–3 linked sialic acids for calcium-dependent recognition.

Diminished sulfation causes decrease in binding of colon carcinoma mucins to L-selectin

Chlorate treatment was previously employed to inhibit sulfation during the biosynthesis of GlyCAM-1 in lymph node HEV (Imai *et al.*, 1993), and was accompanied by significant loss of binding to L-selectin. We treated the LS 180 cells with chlorate under similar conditions, giving a partial loss of sulfation in the secreted mucins, without a marked effect upon their synthesis (by parallel $^{35}SO_4$ and [^{3}H]glucosamine labeling). The extent of sulfation loss varied in different experiments. As shown in Table II, in an experiment where there was ~50% decrease in sulfation, this was accompanied by a ~75% decrease in L-selectin binding. These data show that the colon carcinoma mucins also require sulfation for optimal binding to L-selectin. Similar studies could not of course be done for the bronchial mucin, since it was derived from a patient.

Only a subset of these mucins are ligands for L-selectin

Review of prior published data (Dowbenko *et al.*, 1993a,b; Imai *et al.*, 1993) indicates that even when derived from lymph node HEV, only a subset of GlyCAM-1 molecules can be bound by L-selectin. We confirmed this observation by sequential purification of $^{35}SO_4$-labeled GlyCAM-1 using WGA and L-selectin columns, monitored by SDS–PAGE (data not shown). Figure 4C shows that the WGA-enriched, L-selectin unbound material (after three rounds of depletion) is also sensitive to O-SGPase. Notably, these glycoforms do show a smaller apparent molecular weight (compare to Figure 3C), as also seen on previously reported SDS–PAGE analyses (Dowbenko *et al.*, 1993b; Imai *et al.*, 1993). Some of the high-molecular weight molecules from colon carcinoma and bronchial mucin that do not bind to L-selectin are also degraded by O-SGPase (Figure 4A,B). Thus, as with GlyCAM-1, only a subset of these mucins bind to L-selectin (again, these have smaller median apparent molecular weight, compare to Figure 3, A and B, respectively). This could be explained either by differences in the structure of the oligosaccharides on the different subsets or by differential spacing and/or presentation of similar oligosaccharides. With the mucins from colon carcinoma and bronchial mucus, the two subsets could also represent distinct apomucin carriers. Notably, enzymatic and chemical release of sialic acids from the non-binding pools of the carcinoma and bronchial mucins yielded similar values to those obtained for their respective ligand counterparts (data not shown).

Fragments derived from the mucin-type ligands by proteolysis can still bind to L-selectin, even after denaturation

In some experiments above, it was necessary to inactivate enzymes before re-passing the samples over L-selectin columns. Inactivation by boiling could be used, because the native ligands were insensitive to this denaturing treatment. In fact, GlyCAM-1 ligand activity is known to survive purification by SDS–PAGE as well as organic extraction (Imai *et al.*, 1991; Lasky *et al.*, 1992). To explore the minimum requirements for high-affinity recognition, we subjected these ligands to extensive proteolysis with O-SGPase and subsequent denaturation by prolonged boiling, and then re-examined their binding to L-selectin. As shown in Figure 5, many of the mucin fragments continued to bind L-selectin in a calcium-dependent manner, indicating that their native structure is either resistant to boiling, or not required for recognition. Similar results were obtained with proteinase K, i.e., about 50% rebinding was observed in all cases (data not shown). Given the broad-spectrum action of proteinase K, the resistant fragments are likely to be densely packed clusters of O-linked glycans,

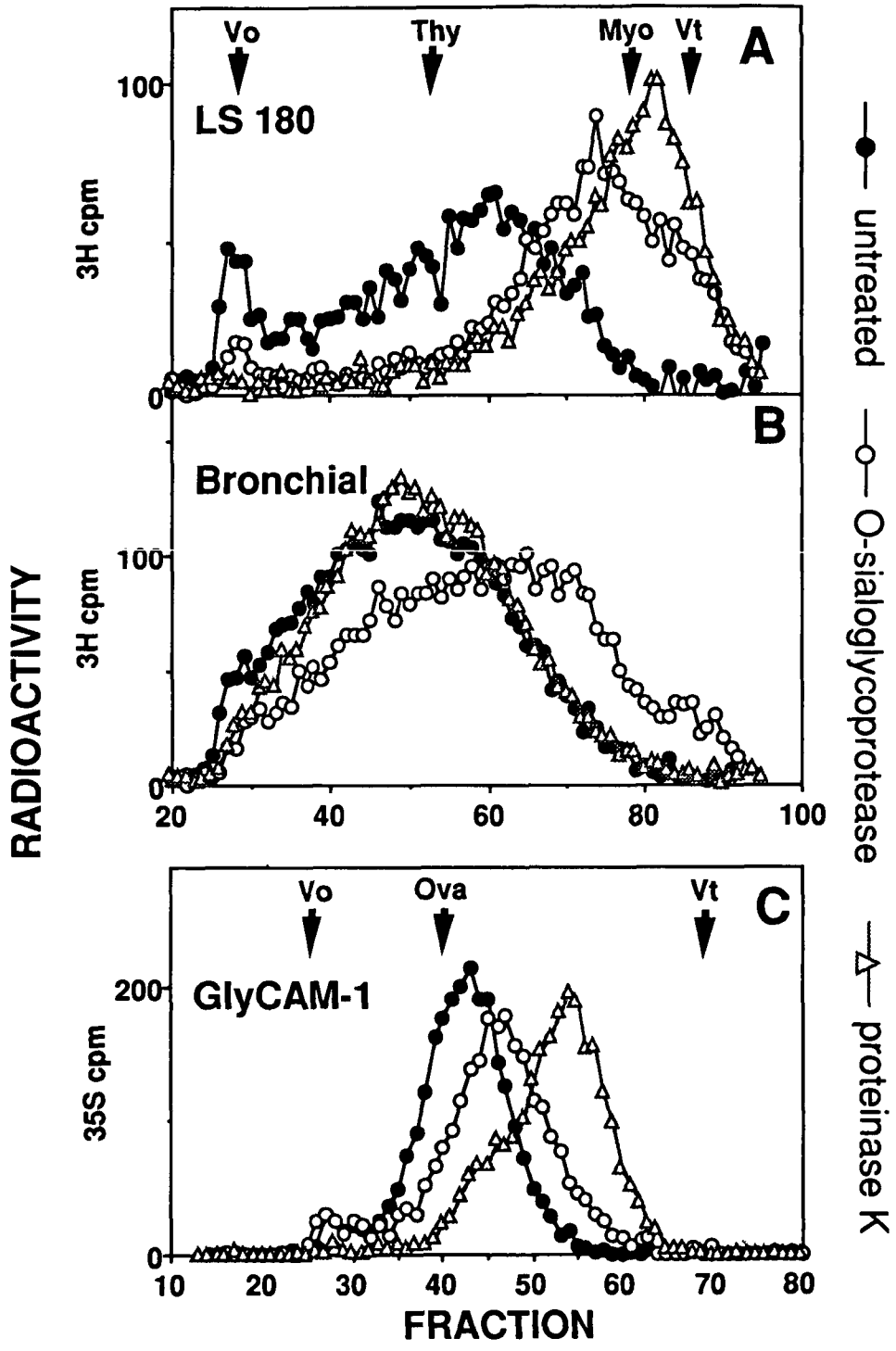

Fig. 4. A subset of the mucins are not ligands for L-selectin. Radiolabeled molecules that did not bind to L-selectin were digested with O-sialoglycoprotease (open circles) or with proteinase K (triangles), or not treated (solid circles) . The effect of the enzyme treatment was evaluated on a Sepharose CL-4B column for the carcinoma and bronchial mucins (**A** and **B**, respectively), and on Sephacryl S-200 for GlyCAM-1 (**C**) exactly as described in Figure 3.

which protect the polypeptide from further cleavage. In this regard, others have previously reported that trypsin digests of GlyCAM-1 also retain some binding (Imai *et al.*, 1992). These effects of proteases on ligand activity could be explained either by the presence of non-binding portions of the ligands, or else by the separation of two separate binding sugar chains that together would build a ligand.

Oligosaccharides released from the mucin-type ligands do not show detectable binding to L-selectin

An alternative explanation for preservation of binding after extensive proteolysis and denaturation could be the presence of one or more unique oligosaccharide ligand structures selectively present on the fragments which continue to bind. Indeed, this has been suggested to be the

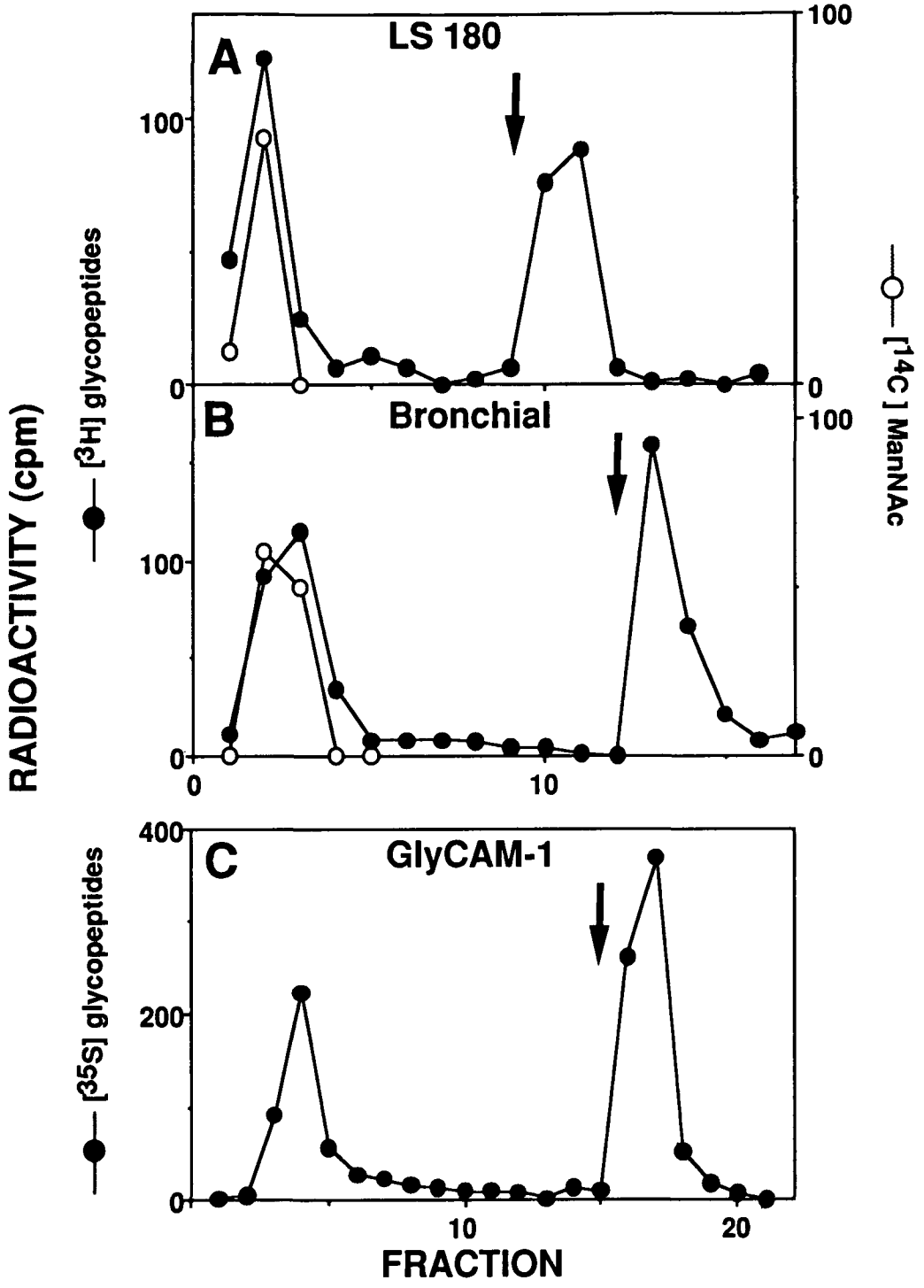

Fig. 5. A subset of glycopeptides derived from mucins by proteolysis can still bind to L-selectin, even after denaturation. [6-^{3}H]glucosamine-labeled LS 180 mucin (**A**), [^{3}H]Sia-labeled bronchial mucin (**B**), and $^{35}SO_4$ GlyCAM-1 (**C**) that bound to L-selectin were treated with O-SGPase followed by boiling. Aliquots were compared to sham-treated samples for re-binding to L-selectin (as described in Figure 1) and for the change in size by gel-filtration on Sepharose CL-4B (as described in Figure 3). In A and B, [^{14}C] N-acetylmannosamine was used as an internal marker which does not interact with the column (open circles). Arrows indicate the point of addition of EDTA. Control samples showed >90% of binding to the column (not shown).

case for GlyCAM-1 (Hemmerich *et al.*, 1995). To explore this, each ligand was subjected to beta-elimination in alkaline borohydride, to release the O-linked chains from the polypeptide backbone. These released chains were studied for rebinding to L-selectin. However, as shown in Figure 6, none of these glycans showed any detectable binding to a high density L-selectin column (10 mg/ml), i.e., they are not even retarded in elution compared to a non-binding internal marker. The lack of binding of free chains from GlyCAM-1 confirms previous observations (Imai and Rosen, 1993; Norgard-Sumnicht *et al.*, 1993). Under similar conditions of affinity chromatography, interactions involv-

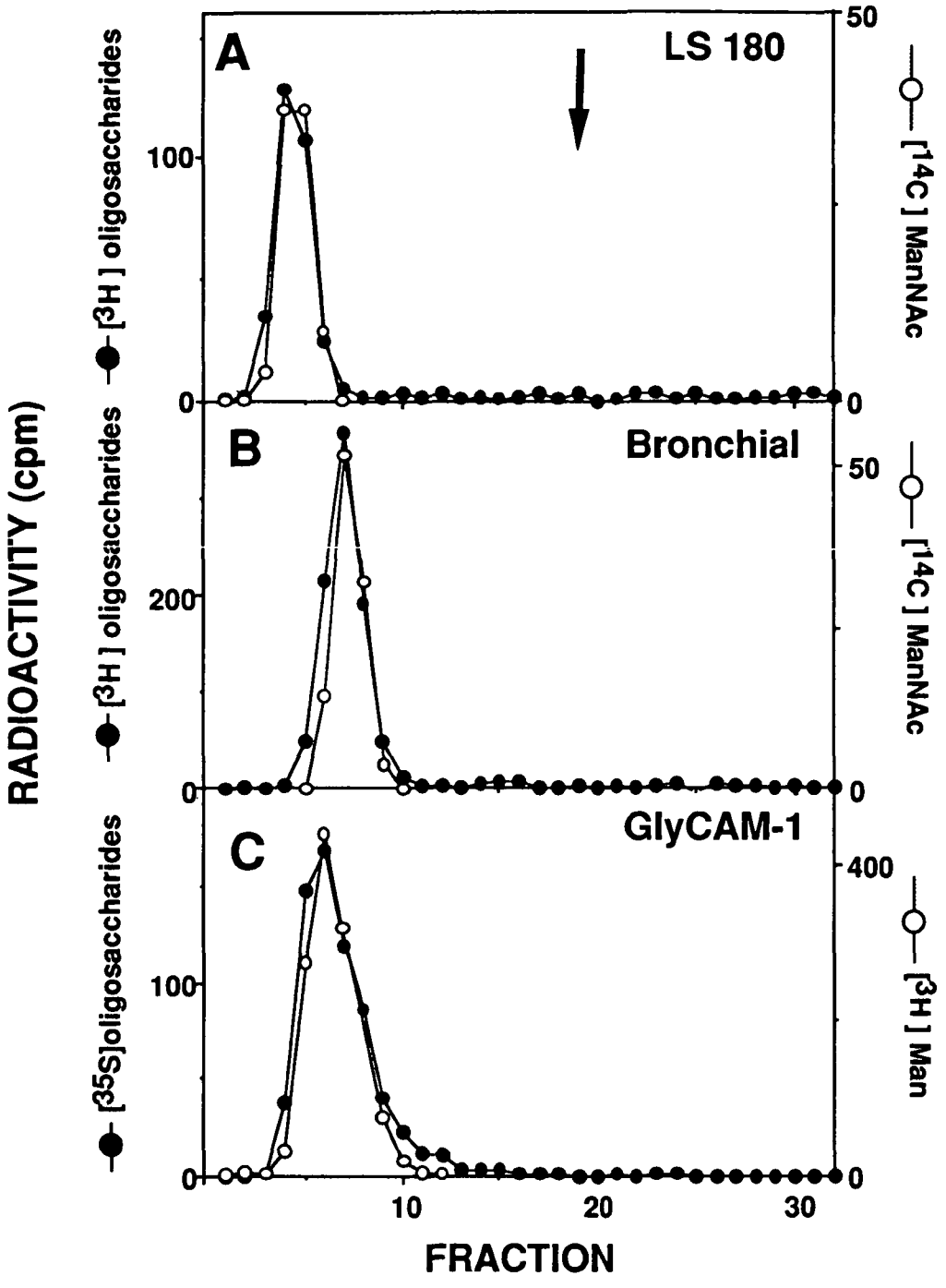

Fig. 6. Oligosaccharides released from the mucin-type ligands do not show detectable binding to L-selectin. O-linked oligosaccharides from L-selectin binding mucins were released by beta-elimination, isolated by gel filtration as described in Materials and Methods, and then studied for binding to an LS-Rg-Protein A Sepharose column carrying a high density (10 mg/ml) of the selectin. (**A**) [6-^{3}H]glucosamine-labeled LS 180 mucin; (**B**) [^{3}H]Sia-labeled bronchial mucin; (**C**) $^{35}SO_4$ GlyCAM-1. The samples were pre-mixed with the internal marker [^{14}C]ManNAc or [^{3}H]Man (open circles). Both markers are completely included by the column, but do not interact with it. Elution volumes reflect the slight differences in the bed volumes of the LS-Rg columns. Control samples (intact mucins) showed >90% of rebinding to the column (not shown). The arrow indicates the point of addition of EDTA.

ing oligosaccharide ligands with very weak binding constants (in the low micromolar range) were easily detectable with two other mammalian lectins, the mannose 6-phosphate receptor (Varki and Kornfeld, 1983) and CD22β (Powell *et al.*, 1993; Powell and Varki, 1994; Sjoberg *et al.*, 1994). Thus, the strong binding of the subset of the original mucins or of the subset of proteolytic fragments does not appear to be explained by the presence of one or more unique high affinity oligosaccharide ligands. Rather, the spacing and/or arrangement of the oligosaccharides on these fragments may be critical. It is also unlikely the base treatment damaged some component of the sugar chains

required for binding. The critical components (sialic acid, sulfate esters, and probably fucose) would not be released by these treatments. Of course, we cannot rule out a very minor oligosaccharide with high affinity (< 1% of the total) which could have escaped detection. Likewise, we cannot rule out that the opening of the glycosidic ring of the linkage region GalNAc residue caused loss of a required structural conformation in a branched O-linked chain.

Effects of polylactosamine-degrading endoglycosidases on the binding of mucins to L-selectin

GlyCAM-1 contains the disaccharides Galβ1–4(6-SO_4)GlcNAc and (6-SO_4)Galβ1–4 GlcNAc (Hemmerich *et al.*, 1994), which are reminiscent of partially sulfated poly-N-acetyllactosamine (keratan sulfate). Moreover, desialylated and defucosylated GlyCAM-1 binds to the *Lycopersicon esculentum* lectin, which recognizes lactosamines (Hemmerich and Rosen, 1994). As shown recently for the P-selectin ligand PSGL-1 (Moore *et al.*, 1994), polylactosamine chains are likely to carry determinants critical for binding. We therefore studied the effect of three different polylactosamine-degrading enzymes on the L-selectin-binding mucins. In each case, release of labeled chains was detected by gel filtration on Bio-Gel P-6. Following this, the undegraded portion of the mucins were collected from the void volume fraction and their ligand activity re-evaluated by affinity chromatography on an L-selectin column. We observed a highly variable range of effect of these enzymes. Some examples are shown in Figure 7, and the overall results are summarized in Table III. Endo-β-galactosidase from *E. freundii* did not give detectable release of label from any of the substrates. The corresponding enzyme from *Bacteroides fragilis* (Scudder *et al.*, 1983) also yielded negative results (data not shown). Neither of the keratanases released significant amounts of radioactivity from GlyCAM-1 labeled either with $^{35}SO_4$ (see Table III) or with [^{3}H] glucosamine (e.g., see Figure7F). Both keratanase I and keratanase II did give release of label from the LS 180 and bronchial mucin ligands (Figure 7B–E). Each of these enzymes liberated a pair of small fragments of similar size from the [^{3}H]Sia-labeled bronchial mucin (Figure 7D,E), which may arise from two distinct capping structures. The more effective cleavage by keratanase II of the LS 180 and bronchial mucins (Figure 7C,E) could reflect the presence of fucose and/or galactose-6-sulfate residues along some poly-N-acetyllactosamine units, which would block the action of keratanase I and the endo-β-galactosidases. The cleavage by keratanase I despite the lack of effect of endo-β-galactosidase cannot be explained by any known specificities of the enzymes (see Discussion). We verified that all enzymes used were active, using keratan sulfate from bovine cornea as a substrate (data not shown). Also, when the respective L-selectin-nonbound fractions from each mucin preparation were treated with the same enzymes, the patterns of release by all these enzymes were essentially the same (data not shown). Thus, no particular polylactosamine structure(s) is specifically enriched in the L-selectin binding subset of the mucins.

The effects on L-selectin binding were not very striking in most instances and could not be predicted from the extent of release of label (Table III). This is particularly true for GlyCAM-1 where re-binding was diminished after treatment by keratanase I or II despite the fact that no released label could be detected. These results suggest that in this case the sensitive chains responsible for about half of the binding must represent a very minor fraction. This also fits our prior report that treatment with keratanase I (or with *E.freundii* endo-β-galactosidase) does not substantially alter the migration of GlyCAM-1 on SDS-PAGE (Norgard-Sumnicht *et al.*, 1993). Because 6′-sulfated sLe^x (Siaα2–3(6SO_3)Galβ1–4(α1–3Fuc)GlcNAcβ1-) represents a major capping group in GlyCAM-1 (Hemmerich and Rosen, 1994), the bulk of this structure must reside on chains that are not susceptible to the enzymes we have tested. For the LS 180 mucin the extent of fragmentation by the three enzymes (e.g., compare Figure 7A–C) was not paralleled by the diminution of the re-binding, suggesting that the most crucial chains are not substrates for the enzymes tested. Finally, despite the considerable effect of keratanase II on the [^{3}H]Sia-labeled bronchial mucin, the ligand activity of the surviving intact mucin is only modestly affected by this treatment (Table III).

Commercial keratanases are known to be contaminated by sialidases. However, we used a high concentration of a potent sialidase inhibitor in these reactions. Indeed, under these conditions no labeled sialic acids were released from LS 180 samples metabolically labeled with [^{3}H]glucosamine, which contain ~33% of the label in sialic acids (e.g., see Figure 7B).

Oligosaccharides on the subset of mucin fragments that bind to L-selectin are not markedly different from those that do not bind

As described above, we found no significant enrichment for sialic acids or particular polylactosamine structures in the ligand fraction as compared to the non-binding mucins. It is however possible that some high affinity structures are presented preferentially in sections of the ligand recognized by L-selectin. To test this, aliquots of the L-selectin binding mucin from LS 180 was treated with O-SGPase, and then fractionated again into fragments that continued to bind to L-selectin and those that no longer did (as in Figure 5, these properties were confirmed by reapplication to the L-selectin column, data not shown). The O-linked oligosaccharides were then released from these two groups of mucin fragments by beta-elimination, and compared for their relative size and charge distribution. There was no obvious difference in size (by gel-filtration on Sephadex G-50, data not shown). The profile of negative charge was studied (after desalting) by MonoQ Sepharose FPLC chromatography with and without prior mild acid treatment to remove sialic acids. As shown in Figure 8, there were no major differences in the profiles of the oligosaccharides derived from the two fractions. Similar results were obtained with oligosaccharides released by hydrazinolysis (data not shown). Most of the mild acid resistant negatively charged molecules ran in the position expected for chains carrying one sulfate ester or that of free sialic acid. Since our earlier results had indicated that about one-third of the ^{3}H-label is in sialic acid residues, acid-released sialic acid likely accounts for most, if not all, of this peak. Regardless, the O-linked oligosaccharides derived from the bind-

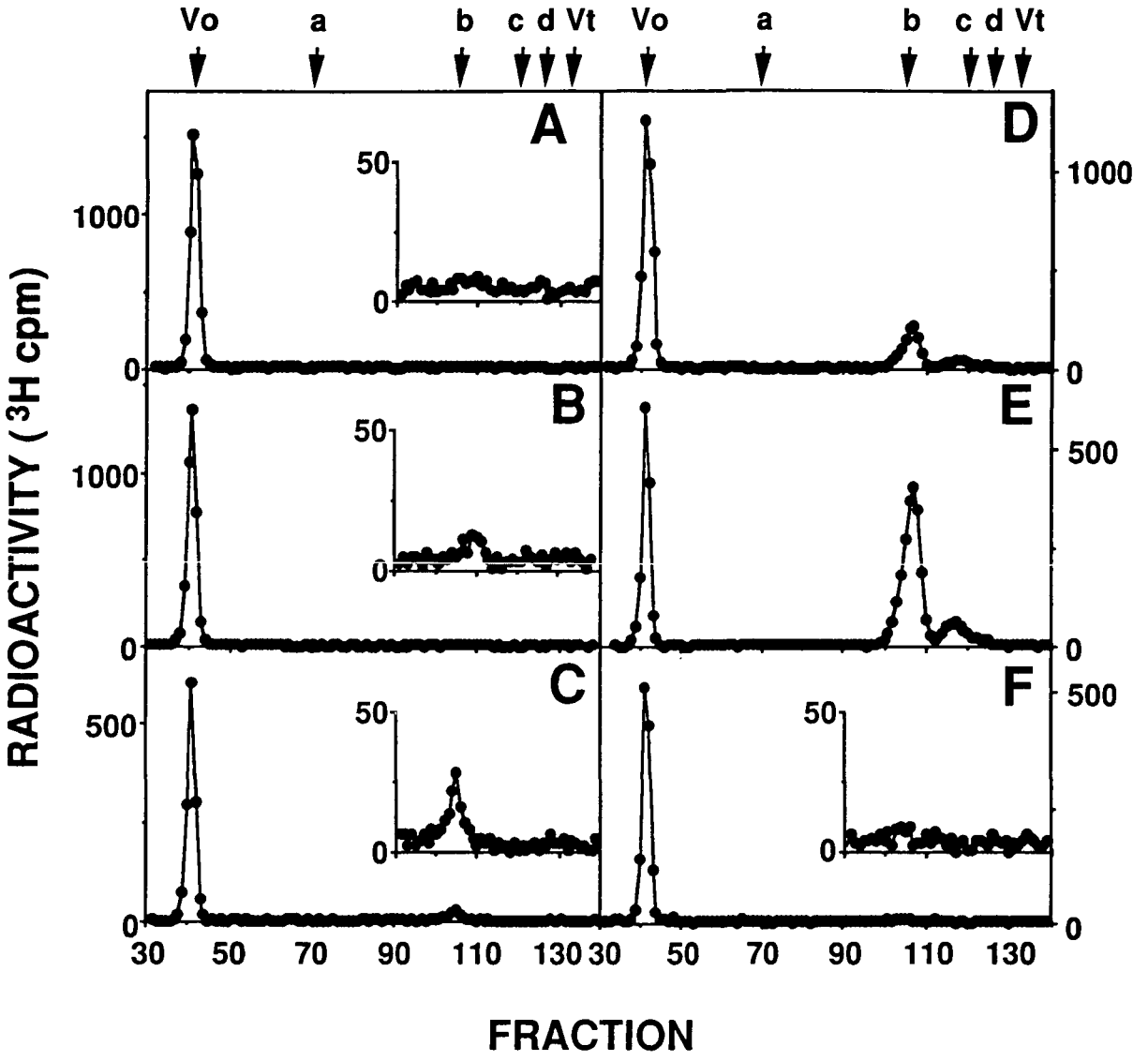

Fig. 7. Effects of polylactosamine-cleaving endoglycosidases on the binding of mucins to L-selectin. [6–³H]glucosamine-labeled LS 180 mucin, [³H]Sia-labeled bronchial mucin, and [6–³H]glucosamine-labeled GlyCAM-1 that bound to L-selectin were degraded by the polylactosamine-cleaving enzymes, boiled, and then subjected to gel filtration on Bio-Gel P-6. Examples are shown for the effects of *E.freundii* endo-β-galactosidase on LS 180 mucin (**A**); of keratanase I on LS 180 mucin (**B**), bronchial mucin (**D**), or GlyCAM-1 (**F**); and of keratanase II on LS 180 mucin (**C**) and bronchial mucin (**E**). Complete results are presented in Table III. The void (Vo) and totally included (Vt) volumes are indicated, as well as the elution volumes of standards: a, α2–6 bisialylated octa- and nonasaccharide alditols (oligosaccharides 6 and 7 in Powell and Varki, 1994); b, Neu5Ac; c, ManNac; d, Man. The insets in (A–C) and (F) show a blow-up of the relevant fractions for comparison.

ing and non-binding mucin fragments do not show markedly different charge or size profiles.

Discussion

The affinity of various fucosylated, sialylated (and/or sulfated)oligosaccharides for the selectins is generally poor (IC_{50} values in the high micromolar to millimolar range), while binding of the natural ligands is much stronger (surviving stringent washing conditions in all cases, and showing direct binding constants in the nanomolar range in the instances studied). Since protease digestions of the mucin ligands for P- and L-selectin diminish ligand activity, the avidity of these interactions is thought to rely upon tight clustering of O-linked oligosaccharides on the polypeptide backbones. Such clustering could explain improved binding in a variety of different ways, which are discussed in detail in Varki, 1994b. Simple avidity, caused by oligosaccharide multivalency is the most parsimonious explanation (Lasky *et al.*, 1992; Baumhueter *et al.*, 1993; Berg *et al.*, 1993; Norgard *et al.*, 1993b). There is ample precedence for this 'cluster' effect with other mammalian lectins (Kornfeld, 1992; Lee, 1992; Lee and Lee, 1994; Powell *et al.*, 1995). However, chemically synthesized divalent sLe^x does not show a greatly improved affinity for E-selectin (DeFrees *et al.*, 1995), P- and L-selectin do not recognize some cell types with considerable amounts of sLe^x, and myeloid cell recognition by P-selectin is destroyed by O-sialoglyco-

Table III. Effects of various endoglycosidases upon mucin binding to L-selectin

Ligand	Treatment			
	Endo-β-gal	Keratanase I	Keratanase II	Buffer control
[^{3}H]LS 180 mucin				
% release of label	<1	2	8	<1
% rebinding[a]	80	95	88	>90
[^{3}H]Bronchial mucin				
% release of label	<1	27	64	<1
% rebinding[a]	98	86	72	>90
[^{35}S]GlyCAM-1				
% release of label	<1	<1	<1	<1
% rebinding[a]	96	61	41	>97

[6–^{3}H]glucosamine-labeled LS 180 mucin, [^{3}H]Sia-labeled bronchial mucin and [$^{35}SO_4$] GlyCAM-1 were treated with the indicated endoglycosidases as described under Materials and methods. Samples were neutralized if necessary, boiled for 5 min to inactivate the enzymes, and then studied by gel filtration on Bio-Gel P-6. The percentage of release of label is shown in each case. The high molecular weight material was recovered and studied for rebinding to L-selectin.

[a] Radioactivity that was bound by L-selectin and released with 5 mM EDTA relative to the total radioactivity recovered in the run-through, wash, and eluate. Recoveries were quantitative. Every repeat experiment tested was reproducible (individual values within 5% of the mean value).

protease (Steininger *et al.*, 1992; Norgard *et al.*, 1993b; Ushiyama *et al.*, 1993), despite retention of the vast majority of surface sLe^x (Norgard *et al.*, 1993b). In contrast, high affinity interactions can be generated when neoglycoproteins are created carrying clustered sLe^x oligosaccharides (Welply *et al.*, 1994). Multivalency generated by selectin aggregation also been suggested as an explanation (Rosen and Bertozzi, 1994), but there is as yet no published evidence for naturally occurring multimeric states of any of the selectins. Furthermore, the high affinity binding of soluble monomeric P-selectin (Ushiyama *et al.*, 1993) or E-selectin (Hensley *et al.*, 1994) to cell surfaces indicates that selectin aggregation is not essential. The possibility of multiple oligosaccharide binding sites interacting with a single selectin lectin domain is made unlikely by epitope mapping, mutagenesis, homology criteria and X-ray crystallography studies (Erbe *et al.*, 1992, 1993; Hollenbaugh *et al.*, 1993; Bajorath *et al.*, 1994; Graves *et al.*, 1994), which indicate a very small carbohydrate binding site. With most selectin ligands, including the mucins studied here, the combination of a single oligosaccharide sequence and an adjacent peptide recognition sequence also seems unlikely, since high affinity recognition persists after extensive denaturation or proteolytic digestion (Imai *et al.*, 1992; Lasky *et al.*, 1992; Moore *et al.*, 1992; Norgard *et al.*, 1993b; Norgard-Sumnicht *et al.*, 1993). By exclusion, we therefore suggested that at least some high affinity selectin binding might involve recognition of a 'clustered saccharide patch' (Norgard *et al.*, 1993b; Varki, 1994b) created by a mucin polypeptide carrying closely spaced O-linked chains with limited conformational freedom (Butenhof and Gerken, 1993) that can build an unique high affinity site for the cognate selectin. This could occur either because a single oligosaccharide with low affinity (e.g., sLe^x) is forced by other surrounding chains into an unusual conformation favored for recognition, or by combinations of side chains and groups from multiple oligosaccharides, i.e., a 'discontiguous saccharide epitope'. Recent reports indicate that tyrosine sulfate residues can co-operate with immediately adjacent sialylated oligosaccharides to generate P-selectin recognition of PSGL-1 (Wilkins *et al.*, 1995; Sako *et al.*, 1995; Pouyani and Seed, 1995). In this instance the clustered patch is not be purely made up of saccharides, but would be a composite 'clustered anionic patch' with the oligosaccharide and the adjacent tyrosine sulfate(s) as participants. In all these instances the polypeptide backbone need not be part of the selectin contact site, but would be crucial for presenting the sugar chains and sulfates in the correct arrangement. This hypothesis could explain why the free oligosaccharides released from the three mucin-type ligands studied here do not bind with recognizable affinity to an L-selectin column even at high density of receptor, where interactions with binding constants in the low micromolar range should be picked up (Powell and Varki, 1994; Powell *et al.*, 1995). Also consistent with this model is the lack of obvious differences between the O-linked oligosaccharides released from glycopeptides that bind or do not bind to L-selectin.

The few striking exceptions to this hypothesis are the direct binding of certain N-linked oligosaccharides from human leukocytes to a high density E-selectin column (Patel *et al.*, 1994), the interaction of certain heparan sulfate chains to L-selectin (Norgard-Sumnicht *et al.*, 1993), and the interaction of some fucoidans and glucan sulfates with L-selectin (Yoshida *et al.*, 1994a,b). However, in the first case, the ligands are complex tetra-antennary N-linked chains that might generate a cluster within the context of the highly branched structure emanating from a small core region. With heparan sulfate chains, we have suggested (Norgard-Sumnicht *et al.*, 1993; Norgard-Sumnicht and Varki, 1995) that these generate a recognition patch without the aid of sialic acid or fucose, by utilizing their dense concentration of sulfate esters and uronic acid residues. This fits well with the last case, in which certain subpopulations of highly sulfated fucoidans or sulfated glucans (which are not natural ligands) achieve high affinity binding.

Since sialic acids, sulfate esters and fucose residues are frequently found on polylactosamines with a type 2 repeating unit, we treated the three mucin-type ligands with enzymes known to cleave such chains. Shown below is a composite structure of potential substrates for endo-β-galactosidase from *Escherichia freundii* (Fukuda and Matsumura, 1976), keratanase I from *Pseudomonas sp.*(Naka-

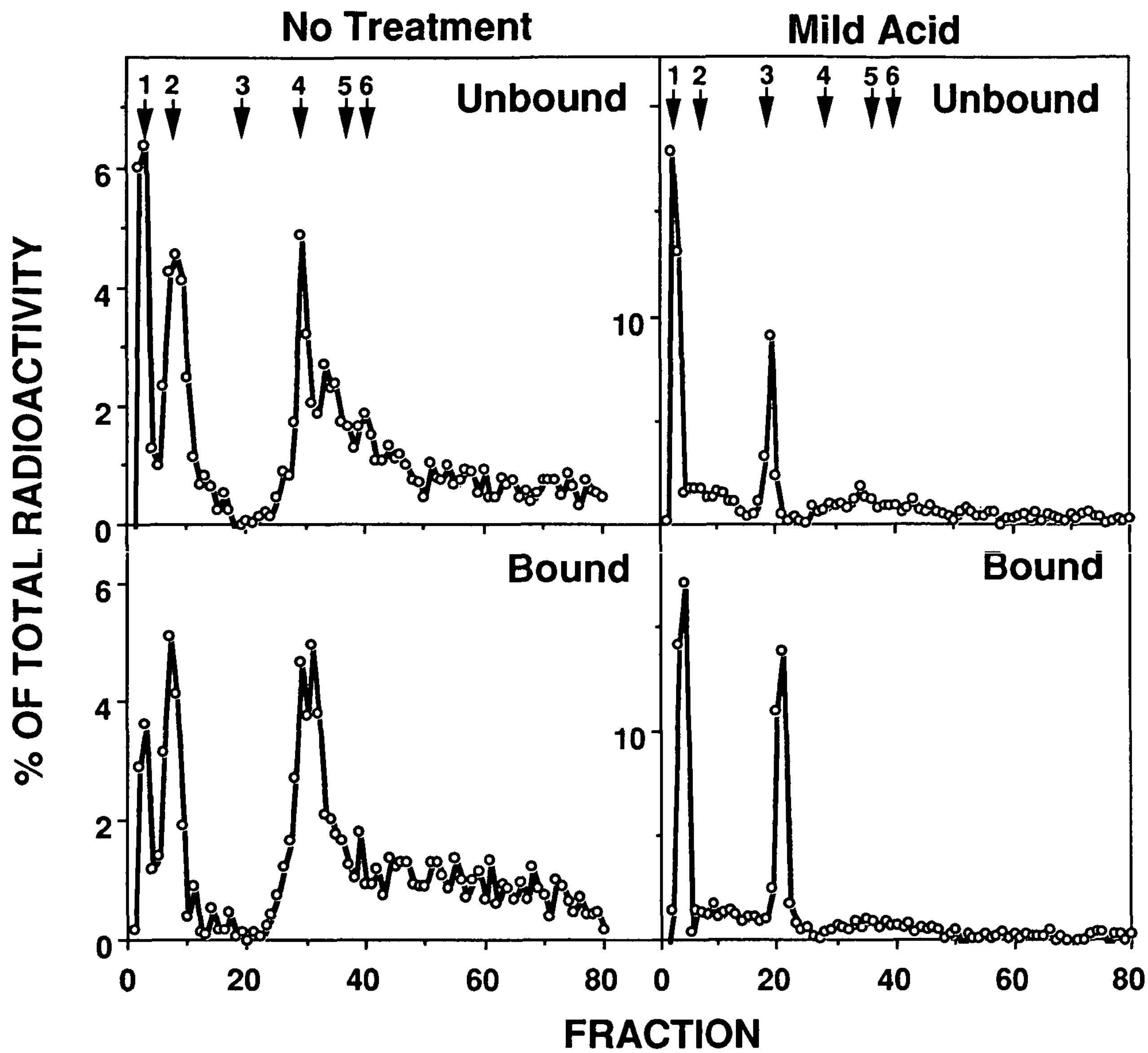

Fig. 8. Comparison of negative charge on oligosaccharides released from subsets of mucin glycopeptides that do or do not bind to L-selectin. Aliquots of the L-selectin binding mucin from LS 180 were treated with O-SGPase, and fractionated into fragments that continued to bind to L-selectin and those that no longer did. The O-linked oligosaccharides were then released from these two groups of mucin fragments by beta-elimination, and studied for negative charge by MonoQ Sepharose FPLC chromatography with and without prior mild acid treatment as described under Materials and Methods. The elution position of the N-linked oligosaccharides standards are as indicated: 1, neutral; 2, monosialylated ; 3, monosulfated; 4, disialylated; 5, monosulfated/monosialylated; and 6, disulfated.

zawa and Suzuki, 1975; Tai *et al.*, 1993), and keratanase II from *Bacillus sp.* (Nakazawa *et al.*, 1989; Brown *et al.*, 1994b).

$$\begin{array}{ccc} SO_3 & SO_3 & SO_3 \\ \pm 6 & \pm 6 & \pm 6 \end{array}$$
$$\pm\ Sia\alpha 2\text{-}3Gal\beta 1\text{-}4GlcNAc\beta 1\text{-}3Gal\beta 1\text{-}$$
$$\begin{array}{c} \pm\ \alpha 1\text{-}3 \\ Fuc \end{array}$$

$$\begin{array}{ccc} SO_3 & SO_3 & SO_3 \\ \pm 6 & \pm 6 & \pm 6 \end{array}$$
$$4GlcNAc\beta 1\text{-}3Gal\beta 1\text{-}4GlcNAc\beta 1$$
$$\begin{array}{cc} \pm\ \alpha 1\text{-}3 & \pm\ \alpha 1\text{-}3 \\ Fuc & Fuc \end{array}$$

Depending on surrounding groups, the internal β-galactosyl bonds (for the first two enzymes) or β-N-acetylglucosaminyl bonds (for keratanase II) are more or less susceptible to the digestion. The *E.freundii* endo-β-galactosidase cleaves only internal β-galactosyl bonds, and is unaffected by sulfation of GlcNAc residues. However, it is sensitive to sulfation at the Gal residue (Fukuda and Matsumura, 1976), and is blocked by fucosylation of the next GlcNAc residue (Scudder *et al.*, 1984), while working slower if a fucose is present on the non-reducing side of the susceptible bond (Spooncer *et al.*, 1984). Branching of the Gal residue by GlcNAc in β1–6 linkage (not indicated in the scheme) also seems to adversely affect action, more so in the context of a glycolipid substrate (Scudder *et al.*, 1983, 1984). The specificity of keratanase I and II have only been

studied using keratan sulfate. Keratanase I is an endo-β-galactosidase whose action requires the presence of at least one sulfate group on adjacent GlcNAc residues (Nakazawa *et al.*, 1984). Cleavage of an internal β-Gal bond is inhibited by sulfation of the galactose or by fucosylation of either of the flanking GlcNAc residues (Tai *et al.*, 1993, 1994). In contrast, Keratanase II is an endo-N-acetylglucosaminidase that can only cleave at N-acetylglucosamine-6-sulfate residues, and is not influenced by fucosylation, nor by the presence of galactose-6-sulfate. However, a tetrasaccharide may be too small a substrate, and terminal sialylation may inhibit the enzyme (Brown *et al.*, 1994a).

We show here that GlyCAM-1 and the L-selectin binding mucins from colon carcinoma and bronchial mucins possess oligosaccharides susceptible to keratanase I and II (Table III). With GlyCAM-1, susceptibility is indirectly inferred based upon loss of binding activity, while labeled fragments are seen released from the other two mucins. These results indirectly demonstrate that all three ligands display some GlcNAc-6-SO_4 residues in the context of poly-N-acetyllactosamine, a feature already known to be present in GlyCAM-1 (Hemmerich *et al.*, 1994). A puzzling result is the release of label from the bronchial mucin by keratanase I in the absence of release by endo-β-galactosidase. Since all structures susceptible to keratanase I should be theoretically susceptible also to *E.freundii* endo-β-galactosidase, these results indicate new or unexpected sequence motifs in these L-selectin ligands. Interestingly, a differential action of these two enzymes on mucins from cat airway secretions has been previously reported (Davies *et al.*, 1991). Assuming the digestions went to completion (the material treated was present only in radioactive tracer amounts), several alternative explanations can account for the inability of any treatment to completely abolish binding. First, some chains important for recognition by L-selectin could lack poly-N-acetyllactosamine. Second, modifications and/or branching of some critical lactosamine units (other than sulfation of galactose or fucosylation) could confer resistance even to keratanase II. Endoglycosidases alone might be insufficient tools to solve these two issues. Third, some important chains could be inaccessible to the enzymes due to their close packing in a cluster of O-linked chains. Thus, for example the suggested 'major capping group' of GlyCAM-1 viz. Siaα2–3(6SO_3)Galβ1–4(α1–3Fuc)GlcNAcβ1- (Hemmerich and Rosen, 1994) must evidently not be released by any of the enzymes from ^{35}S-labeled GlyCAM-1. On the other hand, the release by keratanase II of about 65% of the [^{3}H]Sia label from bronchial mucin (Table III) seems very extensive, considering the variety of non-substrate, sialylated oligosaccharides known to be present in this mucin preparation (van Halbeek *et al.*, 1988; Klein *et al.*, 1993).

Regardless, certain conclusions emerge from these studies. First, there is no consistent pattern for the presence or absence of specific susceptible chains on the three different ligands; second, the extent of release does not correlate with the extent of loss of binding to L-selectin; finally, mucin fractions that bind or do not bind to L-selectin display no obvious difference with respect to the presence of susceptible chains. These results could be taken as evidence that sulfated polylactosamine chains do not play a major role in L-selectin recognition. However, they are also compatible with the possibility that recognition involves a clustered patch that can be generated by more than one combination of closely spaced oligosaccharides. Thus, a single enzymatic treatment alone may not be sufficient to completely destroy binding. Of course, we cannot rule out the possibility that the differential susceptibility to endoglycosidases merely shows that a single critical determinant recognized by L-selectin can be carried on different oligosaccharide scaffolds.

In contrast to the L-selectin ligands studied here, endo-β-galactosidase digestion of PSGL-1 affects most of the oligosaccharide chains carrying sLe^x or Lewis x, but only modestly diminishes binding to P-selectin (Moore *et al.*, 1994). Again, some chains not susceptible to a polylactosamine-degrading enzyme and/or the tyrosine sulfate residues must contribute to the binding. If sulfation of PSGL-1 occurs not only on tyrosine residues but also on the oligosaccharides, it would be interesting to study the effect of the keratanases on this ligand. Also unlike PSGL-1, the binding of mucin-type ligands to L-selectin seems to possess a 'sialidase-insensitive' component (Table I). Sulfated and polylactosamine structures attached to O-linked chains are known to be present in several other sources, sometimes in association with fucose residues. For example, in keratan sulfate II, heavily sulfated and fucosylated type II lactosaminyl arms are presented by branched core 2 O-linked chains (Nakazawa *et al.*, 1989; Tai *et al.*, 1993, 1994; Brown *et al.*, 1994a,b). Recent reports describe the presence in porcine zona pellucida glycoproteins of sialylated, partially sulfated type II repeats present on core 1 O-linked glycans (Hokke *et al.*, 1993, 1994), in which GlcNAc-6-SO_4 represent the sole sulfated residues. Finally, the presence in some other mucins of sulfated polylactosamine repeats can also be inferred from their susceptibility to keratanase I digestion (Davies *et al.*, 1991; Wu *et al.*, 1991). It would be interesting to know if subsets of such molecules can also bind to L-selectin.

All three L-selectin binding mucins studied here are secreted. With GlyCAM-1, the initial proposal that it is the ligand for binding of lymphocytes to HEV has been altered to suggest that it might actually be an anti-adhesion molecule (Lasky, 1995). The other mucins studied here would not normally gain access to the blood stream. However, bronchial mucins are present in the airways of the lung, a site to which neutrophils and monocytes (which bear L-selectin) frequently migrate in various infectious and inflammatory states. Also, the tenacity of the sputum in conditions like cystic fibrosis and bronchiectasis arises partly from DNA released from neutrophils that were initially bound up in the bronchial mucus. The potential importance of L-selectin in these situations needs to be explored. Regarding the secretion of soluble mucin ligands by colon cancer cells, these are known to gain access to the blood stream in the setting of invasive and/or metastatic epithelial tumors (Irimura *et al.*, 1991; Matsushita *et al.*, 1991; Saitoh *et al.*, 1992; Sawada *et al.*, 1994). These circulating molecules may well act as false ligands, binding to L-selectin on circulating neutrophils, monocytes, and lymphocytes. This in turn could alter the trafficking of these important cells that mediate the inflammatory and immune response. Furthermore, it has recently been shown that L-selectin is not just an adhesion receptor, but a signalling molecule (Waddell *et al.*, 1995). Thus, colon cancer mucins could ligate L-selectin on leucocytes, and explain part of

the depressed immunity seen in patients with disseminated cancer. In addition, the soluble mucins could act as decoys, protecting the cancer cells themselves (which also have cell surface mucins) from recognition by L-selectin on the neutrophils and monocytes. While these are obviously speculative possibilities, the potential significance of these L-selectin interactions is worth exploring further. Since a variety of MUC genes are expressed in epithelia (Porchet *et al.*, 1991; Ho *et al.*, 1993), it will be interesting to know if any specific polypeptide is selectively capable of generating L-selectin binding, and if the ectopic expression of mucin polypeptides like CD43 in colon carcinoma (Baecksström *et al.*, 1995) is relevant. It would also be worthwhile to see if the MECA-79 epitopes which decorate the L-selectin ligands GlyCAM-1, CD34, MAdCAM-1, and PNAd (Berg *et al.*, 1991, 1993; Imai *et al.*, 1991) are present on these epithelial ligands. Since MECA-79 reactivity is sialylation-independent (Berg *et al.*, 1991) but sulfation-dependent (Rosen and Bertozzi, 1994), it could be informative to study the effect of the various enzymatic treatments reported here upon MECA-79 reactivity of L-selectin ligands.

Regardless of their physiological or pathological significance, the production of L-selectin binding mucins by the LS 180 cells is of practical interest. To date, the correct glycosylation of mucins required for binding to L-selectin was thought to occur only in organ cultures of lymph nodes (Imai *et al.*, 1991, 1993; Lasky *et al.*, 1992), in short-term cultures of certain primary endothelial cell cultures (Tamatani *et al.*, 1993), and in a single HEV-derived cell line (Toyama-Sorimachi *et al.*, 1993; Sueyoshi *et al.*, 1994; Yoshida *et al.*, 1994b). The LS 180 cell line is well studied, freely available, and capable of sustained growth in long-term culture, and can now be used as a further model system for study of L-selectin ligand biosynthesis. It has the added advantages of including type I and type II lactosamine chains (Mannori,G. *et al.*, unpublished observations) as well as being from a blood group O individual (Kitagawa *et al.*, 1991) i.e., devoid of terminal blood group A and B structures. We are also currently exploring the interactions of these mucins with P- and E-selectin, and of other colon carcinoma mucins with selectins in general.

Materials and methods

Materials

Most of the materials used were obtained from the Sigma Chemical Company. The following materials were obtained from the sources indicated: [^{35}S] protein-labeling reagent (800 Ci/mmol), Amersham Corp. (Arlington Heights, IL); [6-^{3}H] glucosamine hydrochloride (20 Ci/mmol) and [^{3}H] $NaBH_4$ (60 Ci/mmol), ARC Inc. (St. Louis, MO); Bio-Gel P-2 and Bio-Gel P-6, Bio-Rad (Hercules, CA); 2-deoxy-2,3-dehydro-N-acetylneuraminic acid and endo-β-galactosidase from *Bacteroides fragilis* (EC 3.2.1.103), Boehringer Mannheim (Indianapolis, IN); neuraminidase from *Arthrobacter ureafasciens* (EC 3.2.1.18), Calbiochem (La Jolla, CA); Dulbecco's Modified Eagle Media and proteinase K (20 milliAnson units/mg), Gibco BRL (Gaithersburg, MD); fetal bovine serum (HyClone, Logan, UT); carrier-free [^{35}S] Na_2SO_4 (43 Ci/mg S), ICN Biomedicals (Costa Mesa, CA); Ecoscint scintillation cocktail, National Diagnostics (Mayville, NJ); Protein A-Sepharose 4 Fast Flow, Sephadex G-50-80 Fine, and Sephacryl S-200 Superfine, Pharmacia LKB (Piscataway, NJ); Triton X-100, USB (Cleveland, OH); keratanase II from *Bacillus sp.* Ks 36, Seikagaku America (Rockville, MD); chondroitinase ABC from *Proteus vulgaris* (EC.4.2.2.4), Sigma, St. Louis; and agarose-bound jacalin, Vector Laboratories (Burlingame, CA). The following were generous gifts: bronchial mucin glycopeptides from a patient with bronchiectasis (blood group O, secretor with Core 1, 2, 3, and 4 O-linked chains) (Breg *et al.*, 1988; Klein *et al.*, 1988, 1991, 1993; van Halbeek *et al.*, 1988; Van Kuik *et al.*, 1991), Dr. A.Klein, INSERM unit 377, Lilles, France; O-sialoglycoprotease from *Pasteurella haemolytica* (EC 3.4.24.57), Dr. A.Mellors, University of Guelph, Ontario, Canada; sialidase L from *Macrobella decora*, Dr. Y.T.Li, Tulane University, New Orleans, LO; endo-β-galactosidase from *Escherichia freundii* (EC 3.2.1.103), Dr. M.N.Fukuda, La Jolla Cancer Research Foundation, La Jolla, CA; heparin-lyase II, Dr. R.J.Linhardt, University of Iowa, Iowa City, IA; two [^{3}H] α2-6 bisialylated alditols from bronchial mucin (oligosaccharides 6 and 7 in Powell and Varki, 1994), Dr. L.D.Powell, UCSD, La Jolla, CA. The production and purification of LS-Rg (for L-selectin recombinant globulin) has been described elsewhere (Norgard *et al.*, 1993a; Norgard-Sumnicht *et al.*, 1993). This fusion protein consists of the complete extracellular domain of human L-selectin coupled to the Fc region of human IgG2. All other chemicals were of reagent grade or better, and were from commercial sources.

Cell culture

LS 180 cells (ATCC CL 187) were grown in culture in Dulbecco's Modified Eagle Media containing 20% FBS, and split at 1:5 or 1:10 ratio.

Metabolic labeling of LS 180 cells

Cells were split at 1:10 ratio into 100 mm petri dishes, and washed with PBS the next day, and then incubated for 4 d in 10 ml of Dulbecco's Modified Eagle Media containing 10–20% FBS and 66 mCi/ml of [6-^{3}H] glucosamine or 133 mCi/ml of [^{35}S] Na_2SO_4. The media and a first wash with PBS were combined and clarified by centrifugation.

Isolation of radiolabeled L-selectin ligand(s) from the culture media of LS 180 cells

O-linked glycoproteins of the media were enriched by passage over a 5 ml jacalin-agarose column run at 4°C in 150 mM Tris–HCl, pH 7.5, and bound material was eluted with 150 mM melibiose in the same buffer (Hortin and Trimpe, 1990), dialyzed extensively against water, and freeze-dried. Since glycosaminoglycans were occasionally found as contaminants, all preparations were digested in buffer A (see below) with 0.025 units of heparin lyase II (Lohse and Linhardt, 1992) and 0.020 units of chondroitinase ABC for 3.5 h at 37°C. After boiling for 10 min and clarification, the large mucins were isolated from the void volume of a Sephacryl S-200 column as described below, dialyzed against water, and lyophilized. L-selectin ligand was enriched by several passages over a 5 × 0.3 cm column of LS-Rg-PAS (Norgard *et al.*, 1993a) run at 4°C in 20 mM 3-[N-morpholino]propane-sulfonic acid, 100 mM NaCl, 0.02 % sodium azide, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4 (buffer A), washed in the same buffer, and eluted with 5 mM EDTA (replacing the divalent cations). The bound and run-through materials were dialyzed and freeze-dried.

Labeling of bronchial mucin glycopeptides

Thirty micrograms of bronchial mucin glycopeptides (Klein *et al.*, 1988) were labeled with 50 μCi of [^{35}S] sulfur labeling reagent according to the manufacturer's instructions. Another 2 mg were labeled by oxidizing the C-6 position of terminal galactose residues with 10 units of galactose oxidase (EC 1.1.3.9) from *Dactylium dendroides* (390 units/mg protein, Sigma lot 5OH6813), followed by reduction with 14 mCi of [^{3}H] $NaBH_4$ as described (Fukuda, 1989). Mild periodate oxidation of the sialic acids (2 mg of mucin) and reduction with 14 mCi of [^{3}H] $NaBH_4$ were performed as previously described (Norgard *et al.*, 1993b). These preparations were cleaned-up over a 17 × 1 cm Sephacryl S-200 column run in 50 mM sodium acetate, pH 5.5, dialyzed against water, and lyophilized. L-selectin ligand was purified as described above.

Metabolic labeling of mouse lymph nodes and isolation of radiolabeled GlyCAM-1

Labeling of axillary lymph nodes of 6-week-old male Balb/c mice (Harlan, Sprague–Dawley) with [^{35}S] Na_2SO_4 or [6-^{3}H]glucosamine was performed as described (Norgard-Sumnicht *et al.*, 1993), using minimally deficient media (Varki, 1994a) that was supplemented with all amino acids but L-cysteine, and with 100 μM glucose and 15 μM Na_2SO_4. The clarified organ culture media was enriched for sialoglycoconjugates by passage over a WGA-agarose column (Lasky *et al.*, 1992) and concentrated using a Centricon-30 cartridge (Amicon) with addition of Triton X-100 sufficient to obtain a final concentration of 0.01% (w/v). GlyCAM-1 ligand

was then purified by L-selectin affinity chromatography as described above, but included 0.01% (w/v) of Triton X-100 in the buffers. The samples were again concentrated on a Centricon unit, and the EDTA removed by washing with buffer A.

Enzyme digestions

Unless stated otherwise, 0.1 ml reactions were performed at 37°C under a toluene atmosphere for 16–24 h, neutralized, and stopped by boiling for 5 min. Mucins or oligosaccharides were treated with 3 mU of *Arthrobacter* sialidase in 0.02 ml of 0.1 M sodium acetate buffer, pH 4.5, for 3.5 h. Digestions with 2 mU of sialidase L (from leech) were carried out at pH 5.5 as described previously (Sinnott *et al.*, 1993; Chou *et al.*, 1994). Digestions with 2 mU of endo-β-galactosidase from *E.freundii* (Fukuda and Matsumura, 1976) were accomplished in 0.1 M sodium acetate, pH 5.8, containing 50 μg/ml of BSA. Digestions with 1 mU of keratanase from *Pseudomonas* sp. (Sigma) were performed in buffer A in the presence of 20 mM 2-deoxy-2,3-dehydro-Neu5Ac as sialidase inhibitor. Keratanase II (1 mU) was incubated in 10 mM sodium phosphate, pH 6.5, containing 20 mM of sialidase inhibitor. Reactions with 1 μl of O-sialoglycoprotease from *P. haemolytica* (able to digest 5 μg of glycophorin A per hour (Abdullah *et al.*, 1992) were incubated in buffer A, and reactions were stopped by boiling for 30 min. Reactions with proteinase K at 0.5 mg/ml in buffer A were stopped with 1 mM diisopropylfluorophosphate for 30 min at room temperature and then boiled.

Release and purification of O-linked oligosaccharides from mucins

Release was carried out by beta-elimination or by hydrazinolysis. Beta-eliminations (Iyer and Carlson, 1971) performed in 0.1 M NaOH containing 1–2 M $NaBH_4$ for 24 h at 45°C were stopped with glacial acetic acid, and treated three times with methanol containing 1% glacial acetic acid followed by evaporation. Hydrazinolysis was performed in the N plus O-mode on a Glycoprep 1000 machine (Oxford Glycosystems). The desalted samples were lyophylized into the reactor vials, and subjected to automated hydrazinolysis, peptide removal, glycan recovery and desalting. The machine mimics previously described conditions (Patel *et al.*, 1993), in which O-linked chains survive the release reaction without undergoing peeling. The samples were recovered in 10% acetic acid, which was removed by lyophylization. In each case, the released oligosaccharides were separated over a Sephadex G-50–80 column run as described below. The included, unresolved oligosaccharide peak was pooled, evaporated, and when needed, desalted by using a 15 × 0.7 cm Bio-Gel P-2 column run in the same buffer. In some cases, the Sephadex G-50 step was omitted.

Rebinding of mucins onto L-selectin after various treatments

Digestions carried out as described above in a small volume (20–50 μl) were neutralized with 0.5 ml of buffer A and boiled for 5 min. The core mucins were then separated from the released fragment over a 8 × 0.7 cm Bio-Gel P-6 column, run in buffer A in the presence of 0.01% (w/v) Triton X-100, and their rebinding was assessed at 4°C by L-selectin affinity chromatography (5 × 0.3 cm, with 10 mg/ml of LS-Rg). Desalted oligosaccharides were pre-mixed with the internal non-binding markers [^{14}C] ManNAc or [^{3}H] Man, applied to the column in 0.2 ml of buffer A, washed with the same buffer, eluted with 5 mM EDTA in the same buffer, and 0.1 ml fractions were collected and counted. For each run, the crossover of ^{14}C or ^{35}S into the ^{3}H channel of the scintillation counter (Beckman LS1801) was determined, and the appropriate correction made when plotting the elution profiles.

Gel filtration

Samples were supplemented with blue dextran (to mark the void volume) and with a suitable radiolabeled monosaccharide (to mark the total volume). Sepharose CL-4B (25 × 1.5 cm) was equilibrated and eluted in 0.1 M Tris–HCl, pH 8.0 with 0.05 % (w/v) Triton X-100 at a flow rate of 0.86 ml/min, and 0.5 ml fractions were collected and mixed with 5 ml of scintillation cocktail for counting. Sephacryl S-200 Superfine (18 × 1.5 cm) was run in buffer A with 0.01 % (w/v) Triton X-100 at a flow rate of 1 ml/min, and 0.5 ml fractions were collected. Sephadex G-50–80 (39.5 × 0.7 cm) was run in 100 mM pyridine/acetate, pH 5.6, at a flow rate of 0.46 ml/min, and collected in 0.23 ml fractions. Bio-Gel P-6 (47 × 1.5 cm) was run in the same buffer, using a 15.8 ml/h flow rate and 0.55 ml fractions.

MonoQ FPLC fractionation of O-linked oligosaccharides

A preparation of secreted LS 180 mucins that bound to L-selectin was treated with O-sialoglycoprotease overnight at 37°C as described above. The resulting mucin glycopeptides were then separated into bound and unbound species on the same L-selectin column. The glycopeptides were desalted by voiding on a Sephadex G-50 column, and aliquots were reloaded onto the same L-selectin column to verify this separation. O-linked oligosaccharides from the bound and unbound fractions were then released with β-elimination or hydrazinolysis as described above. Reaction completion was checked by profiling an aliquot on Sephadex G-50 (the majority of the radioactivity now ran in the included volume). After desalting, the final samples were dissolved in water. An aliquot of these oligosaccharides (2000 c.p.m.) was desialylated with 2 M acetic acid at 80°C for 2 h, followed by evaporation of the acid. The untreated or desialylated oligosaccharides were studied for negative charge on a MonoQ FPLC (Pharmacia) column, by injection in 200 μl of water. Column elution at 1.0 ml/min was initiated with a 5 min wash with 2 mM Tris–HCl pH 7.5, and followed with a linear gradient from 0–100 mM NaCl in 2 mM Tris-HCl over 55 min (Smith *et al.*, 1993). Mono-, di-, and tri- sialylated standards were PNGase-F released N-linked oligosaccharides from α_1-acid glycoprotein labeled with [^{3}H]Neu5Ac (Powell and Varki, 1994). Sulfated/sialylated standards were PNGase-F released N-linked oligosaccharides from bovine lutropin radiolabeled at the reducing terminus (Smith *et al.*, 1993). Standards run before and after the sample injections gave consistent retention times. Recovery of radioactivity was consistently around 70–80% of injected material.

Chlorate treatment of colon carcinoma cells to reduce sulfation of mucins

LS 180 cells were split in 1:10 dilution into four 35 mm plates and grown overnight with 20% serum. Cells were incubated with fresh 10 mM chlorate (Aldrich) in culture medium for 2 h, at which time either 100 μCi of $Na_2{}^{35}SO_4$ (ICN) or 100 μCi of [6–^{3}H]-glucosamine (Amersham) was added. Control cells were also radiolabeled identically without the preincubation of chlorate. The media was collected the following day, the secreted mucins prepared as described above and studied by L-selectin affinity chromatography as described above. An aliquot of the same media was also run over Sephacryl S-200 directly, and the void volume regions monitored for incorporation of ^{35}S and ^{3}H radioactivity.

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Abbreviations

BSA, bovine serum albumin; FBS, fetal bovine serum; GlyCAM-1, glycosylation-dependent cell adhesion molecule 1; HEV, high endothelial venules; LS-Rg, L-selectin-recombinant globulin; MAdCAM-1, mucosal addressin cell adhesion molecule-1; Neu5Ac, N-acetyl-neuraminic acid; O-SGPase, O-sialoglycoprotease; PAS, protein A-Sepharose; PBS, phosphate-buffered saline; PNAd, peripheral lymph node addressin; PSGL-1, P-selectin glycoprotein ligand-1; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; sLe^x, sialyl-Lewis^x (Siaα2–3Galβ1–4(Fucα1–3)GlcNAcβ1-R); Sia, sialic acid type unspecified; WGA, wheat germ agglutinin; FPLC, fast protein liquid chromatography.

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