Biosynthesis of GlyCAM-1, a Mucin-like Ligand for L-Selectin*

Deirdre Crommie and Steven D. Rosen§

From the Department of Anatomy and Program in Immunology, University of California, San Francisco, California 94143-0452

L-selectin, a member of the selectin family of leukocyte-endothelial adhesion proteins, mediates the initial attachment of lymphocytes to lymph node high endothelial venules during lymphocyte recirculation. One of the endothelial-associated ligands for L-selectin is GlyCAM-1, a mucin-like glycoprotein, which presents novel sulfated, sialylated and fucosylated O-glycans. In order to understand the generation of these glycans, we have examined the biosynthesis of GlyCAM-1 in lymph node organ culture. Using peptide-specific antibodies, lectins, and recombinant L-selectin, we detected the following carbohydrate structures on GlyCAM-1: modified with GalNAc only (28–33 kDa); modified with sialic acid, fucose, and sulfate but lacking L-selectin reactivity (40–50 kDa); and mature (L-selectin-reactive) ligand (50–60 kDa). Pulse-chase labeling at 15 °C suggested that GalNAc is added in a pre-Golgi compartment. Treatment with brefeldin A almost completely blocked sulfation, indicating that this modification occurs in the trans-Golgi network. Two distinct sialylation events occurred in the presence of brefeldin A, while fucosylation was partially blocked. We conclude that sialylation precedes both fucosylation and sulfation during biosynthesis. This ordering will help to identify the critical acceptor structures recognized by lymph node glycosyltransferases and sulfotransferases.

Received for publication, June 13, 1995, and in revised form, July 14, 1995

© 1995 by The American Society for Biochemistry and Molecular Biology, Inc.

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 270, No. 38, Issue of September 22, pp. 22614–22624, 1995

Printed in U.S.A.

* This research was supported by National Institutes of Health Grant GM23547 and by a grant from Genentech Inc. (to S. D. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Anatomy, University of California, San Francisco, CA 94143-0452. Tel: 415-476-1579; Fax: 415-476-4845.

‡ Present address: Div. of Tumor Virology, Dana-Farber Cancer Institute, 44 Binney St., Boston, MA 02115.

§ To whom correspondence should be addressed: Dept. of Anatomy, University of California, San Francisco, CA 94143-0452. Tel: 415-476-1579; Fax: 415-476-4845.

1 The abbreviations used are: HEV, high endothelial venules; AAA, Aleuria aurantia agglutinin; BFA, brefeldin A; C-type, calcium type; Fuc, fucose; Gal-6S, galactose-6-sulfate; GlcNAc-ol, alditol of GlcNAc; LEC-IgG, mouse L-selectin human IgG1 chimeric receptor; MAA, Maackia amurensis agglutinin; N-acetylactosamine, Galβ1–4GlcNAc; PNA, peanut agglutinin; Sia, sialic acid; sialyl Lewis x or sLe x, 3GalNAc-β1–4(Fucα1–3)Galβ1–4(Fucα1–3)GlcNAc; SNA, Sambucus nigra agglutinin; T-antigen, ultimately extravasate across the endothelium into secondary lymphoid organs. L-selectin is essential for the initial adhesive interaction of lymphocytes with HEV of lymph nodes and also appears to be involved in the recruitment of lymphocytes to Peyer’s patches (1, 3, 8, 9). Leukocyte integrins and their endothelial counter-receptors participate in later steps of recruitment (10–14).

A fundamental property of L-selectin is that it recognizes carbohydrate-based ligands via its lectin-like domain (reviewed in 7, 15). An L-selectin/g chimera has been used to identify two HEV-associated ligands in mouse as GlyCAM-1 and CD34 (formerly designated Sgp50 and Sgp90) (16–18). More recently, GlyCAM-1 has been observed in HEV-like vessels that are induced at sites of chronic inflammation (19).2 MAdCAM-1, a member of the Ig superfamily, exists in mouse mesenteric lymph node HEV in a glycoform that is recognized by L-selectin (20). GlyCAM-1, CD34, and MAdCAM-1 are found at other sites as glycoforms (19, 21, 22) that do not exhibit high affinity binding with L-selectin. A fourth distinct ligand, Sgp200 is also present in mouse lymph nodes (23) but has not been identified at the molecular level. All of these HEV-associated glycoproteins possessing ligand activity for L-selectin are recognized by the function-blocking monoclonal antibody known as MECA 79 (16, 23, 24). Finally, a heparin-like ligand for L-selectin has been identified intracellularly in cultured endothelial cells (25).

Both GlyCAM-1 and CD34 are sialylated, fucosylated, and sulfated glycoproteins, and their primary sequence indicates that they are serine/threonine-rich mucin-like glycoproteins with many potential sites for O-linked glycosylation (16–18). MAdCAM-1 also possesses a short mucin domain (26) which is proposed to bear the carbohydrate recognition determinants for L-selectin. In the case of GlyCAM-1, all of the oligosaccharides are O-linked, adding approximately 35 kDa to a predicted core protein of 14 kDa (17, 27). GlyCAM-1 is present at high levels in the conditioned medium of lymph node organ cultures and in serum (17, 28). By EM immunocytochemistry, GlyCAM-1 is undetectable on the apical plasma membrane of the endothelial cells of HEV but is found in large cytoplasmic vesicles (29). Taken together, these observations suggest that GlyCAM-1 is a secreted product. In contrast, CD34 is an integral membrane protein (30).

Sialic acid and sulfate are critical components of the oligosaccharide ligands for L-selectin (16, 31), and an essential role for fucosylation is strongly suspected (32). L-, E, and P-selectin recognize the sialyl Lewis x tetrasaccharide (sLe x, Neu5-Acα2–3Galβ1–4(Fucβ1–3)GlcNAc), and related structures (reviewed in Refs. 15, 33), although each selectin has preferred biological ligands (34–36). There has been recent interest in...
the possibility that sulfation may define a unique modification of L-selectin ligands, which greatly enhances their interaction with L-selectin. Direct structural analysis of GlyCAM-1 (37) has identified Gal-6-sulfate and GlcNAc-6-sulfate as the major sulfated monosaccharides in the context of N-acetyllactosamine, i.e. Gal β1-4GlcNAc. Further structural studies revealed that 6-sulfato sialyl Lewis x, i.e. Sia α2→3(SO4-6)Gal β1→4(Fucα1→3)GlcNAc and 6-sulfato sialyl Lewis x, i.e. Sia α2→3Gal β1→4(Fucα1→3)GlcNAc-6SO4 are major capping groups of this ligand (27, 38). Structures of two of the simplest O-glycans of GlyCAM-1 are predicted (38) as Structures 1 and 2:

These oligosaccharides contain the T-antigen, i.e. Gal β1→3GalNAc, which is incorporated into the core-2 structure (39), i.e. Gal β1→3GlcNAcβ1→6GalNAc.

Although there is increasing information about the carbohydrate structure of the biological ligands of L-selectin, as well as those for the two endothelial selectins (40, 41), there have been no reports on the biosynthesis of these structures. The present study investigates the biosynthesis of O-linked oligosaccharides of GlyCAM-1 as it relates to the elaboration of functional ligand activity. We employ lectin analysis, pulse-chase labeling, and the inhibition of membrane transport (via reduced temperature and brefeldin A) for this analysis. We report the identification of GlyCAM-1 biosynthetic intermediates that represent distinct stages of the O-linked biosynthetic pathway. These studies provide a view of how L-selectin binding activity may be regulated at the level of O-glycan biosynthesis.

**EXPERIMENTAL PROCEDURES**

Reagents
L-[3H]Serine, L-[3H]methionine, and carrier-free [35S]sodium sulfate were obtained from ICN (Costa Mesa, CA). d-[^3H]Galactose was from DuPont NEN, brefeldin A, sodium chloride, phenylmethylsulfonyl fluoride, aprotinin, lactose, N-acetyl neuraminic acid were purchased from Sigma. Arthrobacter ureafaciens sialidase was obtained from Calbiochem (La Jolla, CA) and Vibrio cholerae sialidase was from Oxford GlycoSystems (Rosedale, NY). Streptomyces α1→3/4fucosidase was obtained from Takara Shuzo (Berkeley, CA). Diplodocus pneumoniaeexo-β1→4galactosidase, pepstatin, leupeptin, and Triton X-100 were obtained from Boehringer Mannheim. Protein A-Sepharose 4B was purchased from Zymed (South San Francisco, CA). Arachis hypogaea (peanut) agglutinin-agarose (5 mg PNA/ml gel), Vicia villosa agglutinin-agarose (3 mg VVA/ml gel), Sambucus nigra (elderberry bark) agglutinin-agarose (3 mg SNA/ml gel), and Lycopersicon esculentum agglutinin agarose (2 mg LEA/gel) were purchased from Vector (Burlingame, CA). Limax flavus agglutinin was obtained from Calbiochem. Aleuria aurantia agglutinin (AA) was from Boehringer Mannheim, and Maackia amurensis agglutinin (MAA) was from Sigma. The latter three lectins were immobilized on CNBr-activated Sepharose 4B (Sigma) at a concentration of 2 mg lectin/ml gel (for Limax agglutinin and A) or 10 mg/ml gel (for MAA) following the protocol recom-
ice. The lysate was prepared as described in steady-state labeling and subjected to preclearing and immunoprecipitation in parallel with the conditioned medium, as described below.

Protein Normalization—When comparing multiple samples within the same experiment, the protein concentration of each detergent lysate was determined by a standard colorimetric protein assay (Pierce). The conditioned medium and detergent lysates were normalized for total protein based on the detergent-lysate protein concentrations.

Immunoprecipitation and Peptide Elution—Lysate and conditioned medium samples were precleared by incubation with Protein A-Sepharose beads (50 μl of packed beads/25 ml of lysate or conditioned medium) on a rocker overnight at 4 °C for GlyCAM-1 (as described previously (27)). For the experiment in which the lymph nodes were radiolabeled with [3H]Gal labeling, subjected to fluorography with EN3HANCE (DuPont NEN). The beads on 10% SDS-polyacrylamide gels under non-reducing conditions were directly analyzed by SDS-PAGE with protease inhibitors) on a rocker for 3–4 h at 4°C. Samples solubilized in Laemmli sample buffer were diluted to 0.25 ml with 0.25% lysis buffer (PBS with 0.25% Triton X-100) and incubated in parallel with lectins (VVA, lactose, PNA, MAA, and SNA; N-acetylneuraminic acid, Limax agglutinin; fucose, AAA, or SNA) or LEC-IgG immobilized on Sepharose or agarose beads (25 μl of packed beads/sample; see above). After the incubation, the beads were centrifuged and washed three times with 0.25% lysis buffer. The material bound by anti-peptide 3 or preimmune serum beads was directly solubilized in Laemmli sample buffer for analysis by SDS-PAGE or 5% SDS for direct liquid scintillation counting. The material bound by lectins was eluted with 100 mM of the appropriate mono- or disaccharide competitor (GalNAc, VVA, lactose, PNA, MAA, and SNA; N-acetylneuraminic acid, Limax agglutinin; fucose, AAA) in 0.3 ml of 0.25% lysis buffer containing protease inhibitors on a rocker for 4 h at 4 °C. The material bound by LEC-IgG was eluted in parallel with 10 μl EDTA in 0.3 ml of 0.25% lysis buffer containing protease inhibitors. Eluates were directly counted by liquid scintillation or precipitated with 4 volumes of acetone on ice for 30 min with 5 μg of bovine serum albumin carrier protein, centrifuged at 10,000 x g for 15 min at 4 °C, and solubilized in Laemmli sample buffer for analysis by SDS-PAGE or fluorography. In some cases, aliquots of the anti-peptide 3, preimmune serum, lectin, and LEC-IgG precipitates prepared for SDS-PAGE were directly counted.

RESULTS

Identification of GlyCAM-1 Biosynthetic Intermediates—In order to characterize intermediates along the GlyCAM-1 biosynthetic pathway, the protein core of GlyCAM-1 was radiolabeled with [3H]Ser/Thr. Mouse lymph node samples were radiolabeled in organ culture, and GlyCAM-1 was isolated from the lymph node detergent lysate and conditioned medium with a rabbit polyclonal antibody (Ab) raised against peptide 2 from the deduced GlyCAM-1 protein core (17). Bound material was specifically eluted with peptide 2 and precipitated with a second anti-peptide Ab directed against the C terminus of GlyCAM-1 (peptide 3; 17). As shown in Fig. 1A (anti-peptide 3 lanes), multiple [3H]Ser/Thr-labeled GlyCAM-1 proteins were isolated from the detergent lysate. The pattern of precipitated proteins was highly reproducible, consisting of a broad band at 40–60 kDa and strongly labeled low molecular mass proteins migrating between 28–33 kDa (28, 29, 31, 32, and 33 kDa proteins, collectively denoted by *). Faintly labeled proteins also were visible at 22–27 and 15 kDa, the latter of which closely approximates the predicted mass of the core protein. These proteins were all precipitable by an independent anti-peptide Ab against the N terminus of GlyCAM-1 (peptide 1, 17, 31)). We also determined that individual electroeluted bands could be reprecipitated with anti-peptide 2 Ab (data not shown). The
Biosynthesis of GlyCAM-1, a Ligand for L-Selectin

22617

Fig. 1. Precipitation of GlyCAM-1 and characterization of O-linked oligosaccharides. Lymph nodes were metabolically labeled with [3H]Ser/Thr, and GlyCAM-1 was immunoprecipitated from the detergent lysate (A) or conditioned medium (B) with anti-GlyCAM-1 anti-peptide 2 Ab. The material bound was eluted with peptide 2 Ab, dialyzed against sialidase buffer, and treated with and without A. ureafaciens and V. cholerae sialidase. Equal aliquots of sialidase-treated (+) or untreated (−) GlyCAM-1 were then reprecipitated with premune serum, anti-GlyCAM-1 peptide 3 Ab, VVA, PNA, MAA, Limax agglutinin, AAG, SNA, or LEC-IgG. The material bound was specifically eluted as described under “Experimental Procedures,” acetonetreated, and solubilized in Laemmli sample buffer. Aliquots of each sample were counted by liquid scintillation (see Table II) and subjected to analysis by SDS-PAGE on 10% gels under non-reducing conditions (band profiles did not differ with reduction), and fluorography using ENHANCE. Discrete low molecular mass proteins migrating between 28–33 kDa are denoted by *.

TABLE I

| Lectin | Primary recognition structure(s) | Refs. |
|--------|---------------------------------|-------|
| VVA (B4 lectin) | GalNAcα1-3Galβ1 (terminal α-linked and β-linked GalNAc) | 76, 77 |
| PNA | Galβ1–3GalNAc | 78 |
| MAA | Siaα2–3Galβ1–4GlcNAc | 79, 80 |
| Limax agglutinin | Sialic acid | 81 |
| AAA | α1–2, α1–3 and α1–6 linked fucose | 82 |
| SNA | (SO4-6)Galβ1–4GlcNAc, Siaα2–6Galβ1–4GlcNAc and Siaα2–6GalNAc | 83–85 |

Recognition of these discrete proteins by anti-peptide Abs raised against three independent deduced GlyCAM-1 peptides indicated that they all contained the GlyCAM-1 core protein. When conditioned medium was analyzed as above (Fig. 1B, anti-peptide 3), a major broad band was seen at 40–60 kDa while the low molecular mass proteins were detected only at trace levels.

Evaluation of O-Linked Oligosaccharides of GlyCAM-1—As observed previously (16, 31), functional GlyCAM-1, defined as the subset that binds to the L-selectin-human IgG1 chimera (LEC-IgG), migrated as a broad band at 50–60 kDa (designated by #, Fig. 1A and B). In order to characterize the oligosaccharide structures on the multiple forms of GlyCAM-1 precipitated by the anti-peptide Abs, we examined the binding of these proteins to a panel of lectins with defined carbohydrate specificities (Table I). VVA, which preferentially binds GalNAcα1–3Ser/Thr, was used to identify the first glycosylation step in the biosynthesis of O-linked oligosaccharides for mucin-type glycoproteins (reviewed in Ref. 45). PNA was used to detect the presence of the core Galβ1–3GalNAc structure (referred to as T-antigen or core-1), commonly found on mucin-like proteins, and Limax agglutinin was used to identify sialylated species. AAG, MAA, and SNA recognize discrete features of the Siaα2–3(SO4-6)Galβ1–4Fucα1–3GlcNAc capping structure of GlyCAM-1 (27). These lectins require fucosylation, sialylation, and sulfation, respectively, for their binding (Table I).

For the lectin and LEC-IgG binding studies, [3H]Ser/Thr-labeled GlyCAM-1, isolated from detergent lysates or conditioned medium with anti-peptide 2 Ab, was treated with or without sialidase. Equal aliquots were subsequently incubated with the lectin panel, anti-peptide 3 Ab, or LEC-IgG, all immobilized on agarose or Sepharose. The material bound was analyzed by SDS-PAGE (Fig. 1) and was quantified by scintillation counting (Table II). Fig. 1A presents the analysis of precipitates of GlyCAM-1 from the detergent lysates. We established that precipitation was efficient, since a second round of precipitation yielded only 10–20% of the initial values (Table II). VVA precipitated proteins up to 45 kDa, including the discrete low molecular mass species migrating between 28–33 kDa (denot-
The data are the mean of duplicates from two independent experiments (deviations were <5% of the mean). The percent of \(^{3}H\)Ser/Thr-labeled GlyCAM-1 recovered from the detergent lysates in a second round of precipitation was as follows: VVA (–), 1.2; PNA (+), 1.6; MAA (–), 0.9; Limax agglutinin (–), 3.2; AAA (–), 1.5; SNA (–), 1.8; LEC-IgG (–), 0.8. These values indicate that the recovery of GlyCAM-1 in the first round of precipitation was almost quantitative (80–90%). –, indicates without sialidase treatment; +, indicates with sialidase treatment.

| Sialidase | Lysate | Conditioned medium |
|-----------|--------|--------------------|
| VVA       | 13.1   | 17.5               |
| PNA       | 4.2    | 23.5               |
| MAA       | 4.7    | 0.9                |
| Limax     | 21.7   | 1.4                |
| AAA       | 16.8   | 23.2               |
| SNA       | 2.8    | 10.5               |
| LEC-IgG   | 7.2    | 1.8                |

The percent binding of GlyCAM-1 to lectins and LEC-IgG.

Aliquots of \(^{3}H\)Ser/Thr-labeled GlyCAM-1 with or without prior desialylation were incubated with lectins, LEC-IgG or anti-peptide 3 antibody as described in Fig. 1 and under "Experimental Procedures." The percent of GlyCAM-1 bound was calculated as described in Fig. 5. The data are the mean of duplicates from two independent experiments (deviations were <5% of the mean). The percent of \(^{3}H\)Ser/Thr-labeled GlyCAM-1 recovered from the detergent lysates in a second round of precipitation was as follows: VVA (–), 1.2; PNA (+), 1.6; MAA (–), 0.9; Limax agglutinin (–), 3.2; AAA (–), 1.5; SNA (–), 1.8; LEC-IgG (–), 0.8. These values indicate that the recovery of GlyCAM-1 in the first round of precipitation was almost quantitative (80–90%). –, indicates without sialidase treatment; +, indicates with sialidase treatment.

Table II

Percent binding of GlyCAM-1 to lectins and LEC-IgG.

| Sialidase | Lysate | Conditioned medium |
|-----------|--------|--------------------|
| VVA       | 13.1   | 17.5               |
| PNA       | 4.2    | 23.5               |
| MAA       | 4.7    | 0.9                |
| Limax     | 21.7   | 1.4                |
| AAA       | 16.8   | 23.2               |
| SNA       | 2.8    | 10.5               |
| LEC-IgG   | 7.2    | 1.8                |

The effect of reduced temperature on processing of GlyCAM-1. Lymph nodes were pulse-labeled with \(^{3}H\)Ser/Thr and chased for various times in a 200 molar excess of unlabeled serine and threonine to give final chase times of 5, 10, 20, 45, 90, 180, and 300 min. Detergent lysates (lys) and conditioned medium (CM) were generated for each time point and pooled from two independent experiments. The samples were normalized for total protein, and GlyCAM-1 was isolated by immunoprecipitation with anti-peptide 2 Ab. The material bound was eluted with peptide 2, dialyzed against PBS, and reprecipitated with preimmune serum and anti-peptide 3 Ab. Aliquots of each sample were counted in duplicate by liquid scintillation (see Fig. 3) and subjected to analysis by SDS-PAGE. The anti-peptide 3 Ab immunoprecipitates are shown; the preimmune serum immunoprecipitates were completely negative (data not shown). Each lane contains intracellular (lysates) or secreted (conditioned medium) pulse-labeled GlyCAM-1 from the lymph nodes of 1.5 mice.

Fig. 2. Time course of the synthesis and secretion of GlyCAM-1. Lymph nodes were pulse-labeled for 5 min with \(^{3}H\)Ser/Thr and chased for various times in a 200 molar excess of unlabeled serine and threonine to give final chase times of 5, 10, 20, 45, 90, 180, and 300 min. Detergent lysates (lys) and conditioned medium (CM) were generated for each time point and pooled from two independent experiments. The samples were normalized for total protein, and GlyCAM-1 was isolated by immunoprecipitation with anti-peptide 2 Ab. The material bound was eluted with peptide 2, dialyzed against PBS, and reprecipitated with preimmune serum and anti-peptide 3 Ab. Aliquots of each sample were counted in duplicate by liquid scintillation (see Fig. 3) and subjected to analysis by SDS-PAGE. The anti-peptide 3 Ab immunoprecipitates are shown; the preimmune serum immunoprecipitates were completely negative (data not shown). Each lane contains intracellular (lysates) or secreted (conditioned medium) pulse-labeled GlyCAM-1 from the lymph nodes of 1.5 mice.

Table II

Percent binding of GlyCAM-1 to lectins and LEC-IgG.

| Sialidase | Lysate | Conditioned medium |
|-----------|--------|--------------------|
| VVA       | 13.1   | 17.5               |
| PNA       | 4.2    | 23.5               |
| MAA       | 4.7    | 0.9                |
| Limax     | 21.7   | 1.4                |
| AAA       | 16.8   | 23.2               |
| SNA       | 2.8    | 10.5               |
| LEC-IgG   | 7.2    | 1.8                |
of membrane and secretory proteins. Transport of viral glycoproteins from the TGN to the plasma membrane is blocked at 20 °C (47, 48), and transport from the rough endoplasmic reticulum to the Golgi apparatus is blocked at 15 °C (48). Treatment at 15 °C causes the accumulation of proteins in transitional elements between the endoplasmic reticulum and cis-Golgi (48, 49).

We undertook temperature block studies in order to localize the GlyCAM-1 glycosylation intermediates to particular intracellular compartments. Lymph nodes were pulsed with \(^{{[3H]}}\)Ser/Thr for 5 min, as described for Fig. 2; however in this case, the chase was performed at 37, 20, or 15 °C for both a short (20 min) and long interval (90 min). As shown in Fig. 4, at 37 °C a small fraction of the 28–33 kDa duster was chased to 40–60 kDa by 20 min. By 90 min, the 40–60 kDa proteins increased in the lysate and accumulated in the conditioned medium, recapitulating what was demonstrated in Fig. 2. Reducing the temperature to 20 °C significantly slowed the processing of the 28–33 kDa proteins. At 20 °C, the 40–60 kDa proteins were completely absent at 20 min and substantially reduced at 90 min. Secretion of GlyCAM-1 was completely blocked. Furthermore, the level of the 50–60 kDa protein within the lysate was increased at 20 °C relative to 37 °C at 90 min, suggesting that mature GlyCAM-1 was accumulating, presumably in the TGN. With further reduction of the temperature to 15 °C, processing of the 28–33 kDa proteins to 40–60 kDa, as well as secretion, were completely blocked at both time points. The 28–33 kDa proteins were generated within the 5-min pulse-labeling period at 37 °C (see Fig. 2). The demonstration that these proteins were not further processed at 15 °C strongly suggested that they acquired their additional molecular mass before being transported to the Golgi cisterna. The 28–33 kDa proteins that accumulated at 15 °C comigrated precisely with the GlyCAM-1 proteins precipitated by VVA in Fig. 1A. These results suggest that the initiation of O-linked glycosylation on the GlyCAM-1 core protein occurs in a pre-Golgi compartment.

Time Course of the Biosynthesis of Oligosaccharides of GlyCAM-1—The pulse-chase studies were extended by evaluating the time course of the biosynthesis of the oligosaccharides of GlyCAM-1. Lymph nodes were pulse-labeled with \(^{{[3H]}}\)Ser/Thr and chased for various lengths of time (as in Fig. 2), and GlyCAM-1 was immunoprecipitated with anti-peptide 2 Ab. In this case, the material recovered from the lysate and conditioned medium were combined, and aliquots were reprecipitated with the anti-peptide 3 immunoprecipitates of Fig. 2 pulse-labeled with \(^{{[3H]}}\)Ser/Thr and chased in an excess of unlabeled Ser/Thr for 0–300 min were subjected to scintillation counting. The values shown are the mean of duplicate counts for each sample (standard deviations were less than 1% of the mean value). CM, conditioned medium.

**Fig. 3. Quantitation of the time course of GlyCAM-1 secretion.** Aliquots of the anti-peptide 3 immunoprecipitates of Fig. 2 pulse-labeled with \(^{{[3H]}}\)Ser/Thr and chased in an excess of unlabeled Ser/Thr for 0–300 min were subjected to scintillation counting. The values shown are the mean of duplicate counts for each sample (standard deviations were less than 1% of the mean value). CM, conditioned medium.

**Fig. 4. The effect of temperature on the oligosaccharide processing and secretion of GlyCAM-1.** Lymph nodes were pulse labeled for 5 min with \(^{{[3H]}}\)Ser/Thr and chased at 37, 20, and 15 °C to give final chase times of 20 and 90 min. Detergent lysates and conditioned medium were obtained for each condition, and the samples were normalized for total protein and subjected to immunoprecipitation with pre-immune serum and anti-peptide 2 Ab. The bound material was solubilized in Laemmli sample buffer and analyzed by SDS-PAGE and fluorography. The anti-peptide 2 immunoprecipitates and a representative preimmune immunoprecipitate are shown; all preimmune samples were completely negative.
of GlyCAM-1 \(\left[^{35}S\right]SO_4\)-lysate values) was completely blocked, thus accounting for the augmented accumulation in the cells, presumably in a pre-TGN compartment. Strikingly, the incorporation of \(\left[^{35}S\right]SO_4\) into oligosaccharides of intracellular GlyCAM-1 \(\left[^{35}S\right]SO_4\)-lysate values) was inhibited by 85% at 2.5 \(\mu\)g/ml BFA, which strongly suggests a role for the TGN in the sulfation of GlyCAM-1.

The Effect of BFA on GlyCAM-1 Fucosylation, Sialylation, and LEC-1Gg Binding—To evaluate the effect of BFA on other post-translational modifications, aliquots of the \(\left[^{3}H\right]\)Ser/Thr-labeled intracellular GlyCAM-1, with or without sialidase treatment, were reprecipitated with PNA, AAA, MAA, Limax agglutinin, SNA, LEC-1Gg, or anti-peptide 3 Ab. The material bound by these matrices was counted by liquid scintillation and computed as the fraction of total GlyCAM-1 bound by the lectins and LEC-1Gg as described under "Experimental Procedures" and counted by liquid scintillation. The data were plotted as the percent of total GlyCAM-1 bound by the lectins and LEC-1Gg over time. The percent of GlyCAM-1 bound was calculated by dividing the counts bound with the lectin or LEC-1Gg by the counts bound with anti-peptide 3 Ab. For Limax agglutinin, AAA, and LEC-1Gg, data are shown for precipitates of non-sialidase-treated GlyCAM-1, whereas for PNA and SNA, data are shown for precipitates of sialidase-treated GlyCAM-1. Data are derived from the mean of duplicate reprecipitations and have a standard deviation of less than 5% of the mean value. Inset shows the percent of GlyCAM-1 bound by AAA and SNA in an independent experiment with chase times up to 360 min. These data are the mean of reprecipitations performed in triplicate with a standard deviation of less than 1% of the mean value.

The maturation of \(\left[^{3}H\right]SO_4\)-lysate values) was completely inhibited by 85% at 2.5 \(\mu\)g/ml BFA, which strongly suggests a role for the TGN in the sulfation of GlyCAM-1 as described under "Experimental Procedures" and counted by liquid scintillation. The counts bound by the lectins and LEC-1Gg were normalized for the total available GlyCAM-1 at each BFA concentration by dividing the counts obtained with anti-peptide 3 Ab, peptide-eluted, and counted by liquid scintillation. The data, reported as "relative biosynthesis" are plotted as the percent of GlyCAM-1 recovered in the presence of BFA as compared to the absence of BFA. The data are based on the mean of duplicate values from two independent experiments. B, aliquots of the GlyCAM-1 peptide 2 eluate from the \(\left[^{3}H\right]SO_4\)-lysate values) were treated with or without Arthrobacter and cholerae sialidase and reprecipitated with anti-peptide 3 Ab, PNA ( ), MAA ( ), Limax agglutinin ( ), AAA ( ), SNA ( ), or LEC-1Gg ( ). The material bound was eluted as described under "Experimental Procedures" and counted by liquid scintillation. The counts bound by the lectins and LEC-1Gg were normalized for the total available GlyCAM-1 at each BFA concentration by dividing the counts obtained with anti-peptide 3 Ab. The data, reported as "relative biosynthesis" are plotted as the percent of GlyCAM-1 recovered in the presence of BFA normalized to the percentage recovered in the absence of BFA. The percentages recovered in the absence of BFA were as follows for each precipitating reagent: PNA, 18.2; MAA, 3.8; Limax agglutinin, 25.9; AAA, 17.0; SNA, 13.1; LEC-1Gg, 8.4. For MAA, Limax agglutinin, AAA, and LEC-1Gg, data are shown for precipitates of non-sialidase-treated GlyCAM-1, whereas for PNA and SNA, data are shown for precipitates of sialidase-treated GlyCAM-1. All values are derived from the mean of duplicate reprecipitations (deviations were less than 5% of the mean value).

minations for AAA, MAA, Limax agglutinin, and LEC-1Gg binding were made without prior sialidase treatment. As shown in Fig. 6B, the reactivity of GlyCAM-1 with SNA decreased by 99% at 2.5 \(\mu\)g/ml BFA (Fig. 6B), paralleling the inhibition of \(\left[^{35}S\right]SO_4\) incorporation demonstrated in Fig. 6A. The reactivity of GlyCAM-1 with AAA decreased by 50%, indicating that BFA had a partial inhibitory effect on overall fucosylation. In contrast, reactivity for PNA, MAA, and Limax agglutinin increased to 495, 335, and 195%, respectively. PNA binding was negligible without sialidase treatment (data not shown) demonstrating that the T-antigen was efficiently sialylated in the presence of BFA as it was in its absence (Fig. 6B).
The Generation of BFA-induced Glycosylation Intermediates—The nearly complete inhibition of sulfation by BFA implied that BFA would cause the accumulation of distinct glycosylation intermediates. To identify such species, GlyCAM-1 was radiolabeled with $[^3]H$Ser/Thr in the presence or absence of BFA. Equal aliquots of GlyCAM-1 were then treated with or without sialidase, recipreplicated with anti-peptide 3 Ab or lectins as in Fig. 6B, and then analyzed by SDS-PAGE. As shown in Fig. 7 (anti-peptide 3 lane), BFA treatment dramatically altered the size distribution of the immunoprecipitated GlyCAM-1 precursors. The relative amount of the 28–33 kDa protein cluster was considerably reduced, and a new dominant 40 kDa protein (range 35–46 kDa) and a faint 50 kDa species were generated (lane 3). These latter two species were sialylated as indicated by their binding to Limax agglutinin (lane 15); additionally, sialidase treatment reduced the new 40 and 50 kDa proteins by 5 kDa, producing a broad 35 kDa band and a much fainter 45 kDa band, respectively (lane 4). In addition, these two new species were fucosylated as shown by precipitation with AAA (data not shown). In accord with the results above, neither of the proteins were precipitated by LEC-IgG or SNA. Therefore, these proteins represented sialylated and fucosylated but non-sulfated biosynthetic intermediates of GlyCAM-1. We are not certain what accounts for the difference in size between the 40 and 50 kDa BFA intermediates. Both of these proteins were precipitated by MAA (lane 11), and by PNA after sialidase treatment (lane 8), indicating that they contained sialylated N-acetyllactosamine (Sia$_{a2–3}$Gal$_{b1–4}$GlcNAc, as well as sialylated T-antigen (Sia$_{a2–3}$Gal$_{b1–3}$Gal). It is possible that differences exist in oligosaccharide branching or the number of elongated chains on these two species.

The Effect of BFA on the Generation of Gal$_{b1–4}$GlcNAc—Gal$_{b1–4}$GlcNAc (N-acetyllactosamine) is modified by sulfate, sialic acid, and fucose on the major oligosaccharide capping groups of GlyCAM-1, i.e. 6-sulfo sLe$^a$ and 6-sulfo sLe$^b$. This disaccharide is a component of the MAA epitope (Sia$_{a2–3}$Gal$_{b1–4}$GlcNAc). The ability of MAA to bind the BFA intermediates at a level similar to undersulfated GlyCAM-1 (chlorate-generated) indicates that this structure was synthesized normally in the presence of BFA. As an independent approach for detecting this disaccharide, we analyzed the ability of Diplococcus exo-$eta$1–4galactosidase to release Gal from the BFA intermediates. This enzyme specifically hydrolyzes Gal in a $\beta1–4$ linkage to GlcNAc (51), an activity that is blocked by sulfation at the 6-position of Gal (38). Based on the knowledge that chlorate blocks the sulfation of GlyCAM-1 but does not diminish its galactosylation (38), we used chlorate-generated GlyCAM-1 as a positive control to establish the maximum possible release of Gal by the $\beta(1–4)$galactosidase. GlyCAM-1 was radiolabeled with $[^3]H$Gal in the presence of chlorate, BFA, or no inhibitor and isolated from lysates with anti-peptide 2 Ab. The $\alpha2–3$ sialylation of Gal and the $\alpha1–3$ fucosylation of GlcNAc within the capping structures of GlyCAM-1 also inhibited Gal release by Diplococcus exo-$\beta$1–4galactosidase (27). Accordingly, aliquots of the $[^3]H$Gal-labeled GlyCAM-1 were treated with or without sialidase and digested with the exo-$\beta$1–4galactosidase with or without $\alpha1–3$ galactosidase treatment. As shown in Table IV, without chlorate or BFA treatment, exo-$\beta$1–4galactosidase released 4% of $[^3]H$Gal from desialylated/defucosylated GlyCAM-1. With chlorate treatment, the amount of $[^3]H$Gal released from desialylated/defucosylated GlyCAM-1 increased to 15%, and this hydrolysis was completely dependent on prior sialidase treatment. With BFA treatment, exo-$\beta$1–4galactosidase released 12% of $[^3]H$Gal, indicating that $\beta1–4$linked Gal was only slightly reduced (20%) in the presence of BFA. Moreover, this hydrolysis was also completely dependent on prior desialylation, confirming that Gal$_{b1–4}$GlcNAc was efficiently sialylated in the presence of BFA, in agreement with the MAA binding data shown in Fig. 6B and Table III. The effect of $\alpha1–3$galactosidase on exo-$\beta$1–4galactosidase release of Gal allowed an independent assessment of the degree of fucosylation of GlyCAM-1 in the presence of BFA. As shown in Table IV, there was a substantial increase (7–12%) in the amount of Gal released by this enzyme when the GlyCAM-1 intermediates were treated with $\alpha1–3$galactosidase plus sialidase as compared to sialidase alone. This result concurs with the AAM analysis (Fig. 6B) in establishing that a substantial degree of fucosylation occurred in the presence of BFA.

**DISCUSSION**

GlyCAM-1 is an HEV-derived, secreted ligand for L-selectin. Its functional role in lymphocyte-HEV binding has not been determined as yet. Nonetheless, detailed biochemical analysis of GlyCAM-1 is warranted, since it shares a carbohydrate-based recognition determinant with the other known HEV ligands for L-selectin (23) and is associated with sites of chronic inflammatory processes.
The eluates were acetone-precipitated and subjected to analysis by SDS-PAGE and fluorography. reprecipitated with anti-peptide 3 Ab, PNA, MAA, Limax agglutinin, SNA, or LEC-IgG, and eluted as described under "Experimental Procedures." The eluates were acetone-precipitated and subjected to analysis by SDS-PAGE and fluorography.

---

**Table IV**

Percent release of \(^{3}H\)Gal from GlyCAM-1 by exo-\(1\rightarrow4\)galactosidase

| Treatment       | Sialidase | Fucosidase |
|-----------------|-----------|------------|
| No treatment    | 0         | 0          |
| Chlorate        | 1         | 2          |
| BFA             | 1         | 1          |

---

Fig. 7. The effect of BFA on the maturation of O-linked oligosaccharide structures. Lymph nodes were preincubated with (+) or without BFA (−) at 2.5 μg/ml for 1 h and then metabolically labeled with \(^{3}H\)Ser/Thr in the presence or absence of BFA. Detergent lysates were generated and equalized for total protein. GlyCAM-1 was isolated from the lysates with anti-peptide 2 Ab, eluted with peptide, and dialyzed against sialidase buffer. The GlyCAM-1 preparations were treated with (+) or without (−) Arthrobacter and V. cholerae sialidase. Equal aliquots of each sample were reprecipitated with anti-peptide 3 Ab, PNA, MAA, Limax agglutinin, SNA, or LEC-IgG, and eluted as described under "Experimental Procedures." The eluates were acetone-precipitated and subjected to analysis by SDS-PAGE and fluorography.

---

Inflammation (19).

We have defined the following discrete stages in biosynthesis of GlyCAM-1: 1) unglycosylated species of < 28 kDa; 2) discrete 28–33 kDa proteins containing GalNAc-terminating chains; 3) a broadly migrating 40–50 kDa species containing the T-antigen, sialic acid, fucose, and sulfate, but not reactive with LEC-IgG; and 4) a 50–60 kDa sialylated, fucosylated, and sulfated protein, reactive with LEC-IgG.

By pulse-chase analysis, we established that the first three groups of proteins were biosynthetic intermediates of mature GlyCAM-1. The low molecular mass proteins (28–33 kDa) were synthesized within 5 min and processed to 40–50 kDa with a halftime of approximately 30 min. The half-time for the acquisition of L-selectin binding was ~65 min. An unexpected finding was that up to 75% of the 40–50 kDa species was secreted into the medium without attaining the capacity to bind L-selectin.

Using temperature blocks, we have gained information about the initiation of O-glycosylation in GlyCAM-1. A reduction of the temperature to 15°C during the chase period completely blocked the processing of the rapidly synthesized 28–33 kDa cluster. In multiple cell types, membrane transport into the Golgi compartment. In some systems, the addition of GalNAc to nascent proteins occurs in the endoplasmic reticulum or transitional elements of the endoplasmic reticulum (55–57), whereas in others, initiation appears to take place in the Golgi apparatus (58–60). Thus, the site for the initiation of O-linked glycosylation appears to differ for different cell types and perhaps for different core proteins.

The metabolic inhibitor BFA is a valuable experimental tool that permits discrimination of processing events in the ER/Golgi compartment from those in the TGN. We employed this drug to dissect the terminal processing events for GlyCAM-1, which could not be adequately resolved by pulse-chase analysis. As expected, BFA completely blocked the secretion of GlyCAM-1 into conditioned medium. BFA caused the accumulation of biosynthetic intermediates of GlyCAM-1 with oligosaccharides that were efficiently sialylated, partially fucosylated, and almost completely lacking in sulfate.

The epitope for PNA (the T-antigen) increased approximately 5-fold with BFA treatment, and it was fully sialylated since prior desialylation was required for binding. The increased level of sialylated T-antigen with BFA may have been due to the increased contact of GlyCAM-1 with the appropriate glycosyltransferases in the BFA-induced compartment. BFA treatment also allowed the formation of N-acetyllactosamine (Galβ1→4GlcNAc) within GlyCAM-1 and its efficient sialylation. Thus with BFA, the total amount of \(^{3}H\)Gal released by Diplococcus exo-β(1→4)galactosidase decreased only marginally relative to the control. Additionally, the release of \(^{3}H\)Gal completely depended upon the prior desialylation of GlyCAM-1, consistent with a fully sialylated state of the terminal Galβ1→4 residues. Finally, MAA reacted with BFA-generated GlyCAM-1 comparably to chlorate-generated GlyCAM-1, indicating the formation of Siaα2→3Galβ1→4GlcNAc.

Taken together, these results argue that the sialyltransferases that form the Siaα2→3Galβ1→3GalNAc and Siaα2→3Galβ1→4GlcNAc structures are localized in a pre-TGN compartment. This conclusion is consistent with previous studies on several glycoproteins in which BFA treatment does not impede sialylation of O-linked oligosaccharides (61–63). The β1→4 galactosyltransferase involved in the formation of...
N-acetyllactosamine is localized to the trans-Golgi cisternae in a number of cells (64, 65). Thus, the Galβ1→4GlcNAc α2→3 sialyltransferase pertinent to GlyCAM-1 is likely to reside in the trans-Golgi cisternae, in distinction to the apparent TGN localization of sialyltransferases that act on N-linked oligosaccharides (63, 66, 67). Our data cannot distinguish the subcellular localization of the Galβ1→4GlcNAc α2→3 sialyltransferase relative to the Galβ1→4GlcNAc α2→3 sialyltransferase. However, the T-antigen-specific α2→3 sialyltransferase involved in the synthesis of another sialomucin has been mapped to a compartment proximal to the trans-Golgi cisternae (65).

Fucose is included in an α1→3 linkage to GlcNAc in the N-acetyllactosamine of GlyCAM-1. BFA inhibited fucosylation of GlyCAM-1 by ~50% as determined by direct precipitation with AAA and by the defucosylation requirement for exoα(1→4)galactosidase action. Thus, the accessibility of nascent oligosaccharides to fucosyltransferases is clearly affected by BFA. In contrast to the sialyltransferases, the fucosyltransferase appears to reside in a compartment that was partially redistributed by BFA. Given the apparent greater efficiency in the redistribution of the sialyltransferases, the fucosyltransferase is likely to reside in a more distal region of the biosynthetic pathway. This conclusion is consistent with the general finding that α2→3 sialylation precedes α1→3 fucosylation during the synthesis of sLex (68, 69).

Gal-6-sulfate and GlcNAc-6-sulfate occur equally in GlyCAM-1 (37). In the presence of BFA, the sulfation of GlyCAM-1 was almost completely suppressed, as demonstrated by the 85% reduction in 35S-Sulfate labeling of GlyCAM-1 and the 99% reduction in SNA binding. Since BFA allowed the synthesis of the Galβ1→4GlcNAc structure, the inhibition of sulfation is likely attributable to the inaccessibility of GlyCAM-1 biosynthetic intermediates to the TGN where the relevant sulfotransferases reside. In a number of other systems, BFA has been employed to reach the same conclusion about the subcellular localization of sulfotransferases that modify 0-glycans (70–73). Taken together, our biosynthetic analysis of GlyCAM-1 argues that the sialylation events precede both fucosylation and sulfation. As noted above, the ordering of sialylation versus fucosylation is consistent with previous studies. The relationship of fucosylation to sulfation is more problematic. BFA produced partial inhibition of fucosylation (~50%) and almost complete inhibition of sulfation, which would argue for fucosylation occurring before the two sulfation modifications. In support of this possibility, Jain et al. (74) have reported that several of the known α1→3/4 fucosyltransferases are unable to fucosylate Siaα2→3(SO4-6)Galβ1→4GlcNAc to form the 6′-sulfido sLex carrying enzyme, whereas these enzymes are active on the non-sulfated structures. However, Scudder et al. (75) have reported that a lymph node N-acetylgalcosaminase-6-O-sulfotransferase is unable to add sulfate to GlcNAc-containing oligosaccharides if the C-3 position of GlcNAc is substituted with fucose, arguing that sulfation cannot precede fucosylation on this sugar. Clearly, further studies are necessary to define the temporal relationship of the two sulfation events to fucosylation during the biosynthesis of GlyCAM-1.

The present study has identified glycosylation intermediates of GlyCAM-1 as it is synthesized in mouse peripheral lymph nodes. Our analysis helps to elucidate acceptor structures for the endothelial enzymes that form the ligand. An important future challenge is to determine the molecular identity of these enzymes and to understand how their activities are regulated in lymphoid organs and at sites of inflammation.

Acknowledgments—We are grateful to Mark Singer, Carolyn Bertozzi and Samuel Green for helpful advice. We thank Larry Lasky and Susan Watson of Genentech for their generous contribution of LEC-IgG.
