Expression of the α(1,3)Fucosyltransferase Fuc-TVII in Lymphoid Aggregate High Endothelial Venules Correlates with Expression of L-Selectin Ligands

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Lymphocyte homing to lymph nodes and Peyer’s patches is mediated, in part, by adhesive interactions between L-selectin expressed by lymphocytes and L-selectin ligands displayed at the surface of the cuboidal endothelial cells lining the post-capillary venules within lymphoid aggregates. Candidate terminal oligosaccharide structures thought to be essential for effective L-selectin ligand activity include a sulfated derivative of the sialyl Lewis x tetrasaccharide. Cell type-specific selectin ligand activity include a sulfated derivative of N-acetyllactosamine-type precursors. The identity of the α(1,3)fucosyltransferase(s) expressed in cells that bear L-selectin ligands has not been defined. We report here the molecular cloning and characterization of a murine α(1,3)fucosyltransferase locus whose expression pattern correlates with expression of high affinity ligands for L-selectin. In situ hybridization and immunohistochemical analyses demonstrate that this cDNA and its cognate α(1,3)fucosyltransferase are expressed in endothelial cells lining the high endothelial venules of peripheral lymph nodes, mesenteric lymph nodes, and Peyer’s patches. These expression patterns correlate precisely with the expression pattern of L-selectin ligands identified with a chimeric L-selectin/IgM immunohistochemical probe and by the high endothelial venule-reactive monoclonal antibody MECA-79. Transcripts corresponding to this cDNA are also detected in isolated bone marrow cells, a source rich in the surface-localized ligands for E- and P-selectins. Sequence and functional analyses indicate that this murine enzyme corresponds to the human Fuc-TVII locus. These observations suggest that Fuc-TVII participates in the generation of α(1,3)fucosylated ligands for L-selectin and provide further evidence for a role for this enzyme in E- and P-selectin ligand expression in lymphocytes.

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Cell adhesion events between leukocytes and endothelial cells operate to facilitate the exit of blood leukocytes from the vascular tree. The selectin family of cell adhesion molecules and their counter-receptors function early in this process, mediating transient adhesive contacts between leukocytes and the endothelial cell monolayer. These selectin-dependent adhesive contacts, together with shear forces impinging upon the leukocyte, cause the leukocyte to “roll” along the endothelial monolayer. Leukocyte rolling, in turn, facilitates subsequent events that include leukocyte activation, firm leukocyte-endothelial cell attachment, and transendothelial migration (1, 2).

E- and P-selectins, expressed by activated vascular endothelial cells, recognize glycoprotein counter-receptors displayed by leukocytes. Each of these selectins can operate to mediate leukocyte rolling in the context of inflammation. L-selectin has also been implicated in mediating leukocyte adhesion to activated vascular endothelium through interactions with an as yet poorly understood endothelial cell ligand (3, 4). By contrast, lymphocyte L-selectin recognizes glycoprotein counter-receptors displayed by specialized cuboidal endothelial cells that line high endothelial venules (HEV) within lymph nodes and Peyer’s patches. L-selectin-dependent adhesive interactions in this context operate to facilitate trafficking of lymphocytes (lymphocyte “homing”) to such lymphoid aggregates.

The NH2-terminal C-type mammalian lectin domain common to each of the three selectin family members mediates cell adhesion through calcium-dependent interactions with specific oligosaccharide ligands, displayed by leukocytes (E- and P-selectin ligands) (2, 5) or by HEV (L-selectin) (6). Physiological ligand activity for E- and P-selectins is critically dependent on the expression of a nonreducing terminal tetrasaccharide termed sialyl Lewis x (SLeα) [NeuNAcα2,3Galβ1,4(Fucα1,3)-GlcnAc-R] (5) and/or its difucosylated variant (7). However, E- and P-selectin ligands recognize this oligosaccharide in different contexts. P-selectin-dependent cell adhesion is optimal when SLeα is displayed by serine and threonine-linked oligosaccharides residing on a specific protein termed P-selectin glycoprotein ligand 1 (PSGL-1) (8, 9). SLeα-modified P-selectin glycoprotein ligand 1 also appears to represent a high affinity counter-receptor for E-selectin (10, 11). A distinct leukocyte glycoprotein termed E-selectin ligand 1 (ESL-1) (12) and its α(1,3)fucosylated, asparagine-linked oligosaccharides may also function as an E-selectin counter-receptor.

Physiological L-selectin counter-receptors on HEV are rep-
resistant by the glycoproteins GlyCAM-1 (13), CD34 (14), and MAC-1 (15). Biochemical studies indicate that L-selectin ligand activity of these molecules is also critically dependent upon post-translational modification by glycosylation. Early studies documented a requirement for sialylation and sulfation (16), implied a requirement for α(1,3)fucosylation, and indicated that these modifications are components of serine-/or threonine-linked glycans. More recent oligosaccharide structural analyses extend this work and imply that high affinity L-selectin ligand activity may depend upon a sulfated variant of the SL-E determinant, NeuNACα2,3SO4(6) Galβ1,4(Fucα1,3)GlcNAc-R (17–19).

Expression of SL-E is determined by cell lineage-specific expression of one or more α(1,3)fucosyltransferases (20). These enzymes utilize the donor substrate GDP-fucose and catalyze a transglycosylation reaction involving the addition of α(1,3)-linked fucose to a common 3′-sialyl N-acetyl-lactosamine precursor. It can be presumed that expression of the sulfated variant of SL-E also depends upon lineage-specific expression of α(1,3)fucosyltransferase activities operating on sulfate-modified 3′-sialyl N-acetyl-lactosamine precursors or that create SL-E moieties modified subsequently by sulfation.

The identity of the α(1,3)fucosyltransferase(s) responsible for selectin ligand expression in leukocytes is not well defined, and HEV-specific α(1,3)fucosyltransferases have not been described. To date, five different human α(1,3)fucosyltransferases have been cloned (21–28). Northern blot and molecular cloning analyses imply that two of these, termed Fuc-TIV (24–26) and Fuc-TVII (27, 28), are expressed in leukocytic cells and represent candidates for critical participation in selectin ligand expression. The role of Fuc-TIV (also known as ELAM-1 ligand fucosyl transferase) in this process is not clear, however. Although Fuc-TIV/ELAM-1 ligand fucosyl transferase is able to efficiently utilize nonsialylated N-acetyl-lactosamine precursors to direct expression of the Le-E moiety (24, 26), this enzyme cannot determine sLex expression in all cellular contexts (29), and its ability to do so in leukocytes or in leukocyte progenitors has not been demonstrated. By contrast, Fuc-TVII is apparently able to determine sLex expression in all mammalian cellular contexts examined, where sLex synthesis is biochemically possible (27, 28). Neither enzyme has been tested for its ability to participate in the synthesis of L-selectin ligands represented by sulfated SL-E determinants.

We report here the isolation and characterization of murine cDNAs and genomic sequences encoding an α(1,3)fucosyltransferase with primary sequence similarity and catalytic properties analogous to those assigned to the human Fuc-TVII α(1,3)fucosyltransferase (27, 28). This murine locus generates alternatively spliced transcripts that differ in their respective abilities to encode α(1,3)fucosyltransferase activity. Expression of this locus is restricted largely to E- and P-selectin ligand rich bone marrow cells (30), where it may participate in the synthesis of these ligands. Transcripts derived from the Fuc-TVII locus and the corresponding α(1,3)fucosyltransferase accumulate to substantial levels in the endothelial cells lining the HEV of peripheral and mesenteric lymph nodes and of Peyer's patches. The localized and abundant expression of this α(1,3)fucosyltransferase in HEV, when considered together with the α(1,3)fucosylated oligosaccharides proposed as HEV ligands for L-selectin, imply a key role for this enzyme in the biosynthesis of L-selectin ligands.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The sources and growth conditions for COS-7 cells (31), CHO-Tag cells (32), and cultured murine blood cell lines (B cells: S107 (33); T cell line EL-4 (34); B cell hybridoma line TH2.54.63 (35); B cell hybridoma line 180.1 (36); Friend murine erythroleukaemia cell line MEL (37, 38); macrophage cell line RAW264.7 (39, 40); macrophage cell line P388D, (41); and the cytotoxic T cell line 14-7fd (42, 43).

**Antibodies**—The sources of the monoclonal antibodies used have been described previously (anti-Lewis x anti-SSEA-1 (44); anti-H and anti-Lewis a (31); anti-sialyl Lewis x/anti-CD34 (45); anti-sialyl Lewis a (46); anti-VIM-2 antibody (47); and fluorescein-conjugated goat anti-mouse IgM and IgG antibodies; Sigma). MECA-79 (48) was the generous gift of Drs. Eugene Butcher (Stanford University).

**TCR-α/β transgenic mice**—TCR-α/β transgenic mice have been described previously (anti-Lewis x/anti-SSEA-1 (44); anti-H, and anti-sialyl Lewis a, antibodies were used at 10 μg/ml). Anti-Lewis x antibodies were used at 10 μg/ml (32). Cells were then stained with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM or anti-mouse IgG and subjected to analysis on a FACScan (Becton-Dickinson) as described previously (22). Cells were also costained with the plasmid pCDM8-CAT (32), and extracts prepared from these cells were subjected to chomaropathological acetyltetranfranse assay activities (32). To allow normalization of flow cytometry and Western blot data to transfection efficiency.

**Fucosyltransferase Assays**—COS-7 cells transiently transfected with Fuc-TVII expression vectors were harvested 72 h after transfection, and extracts were prepared from these cells exactly as described previously (22, 23). These extracts were subjected to α(1,3)fucosyltransferase assay (32) asssayed in a total volume of 20 μl. Reaction mixtures contained 3 μM GDP-[3H]fucose, 20 μm acceptor (N-acetyl-lactosamine, lacto-N-biose 1, 2-fucosylactose (Sigma), or 3′ sialyl N-acetyl-lactosamine (Oxford Glycosystems)), 50 μM cacycylate buffer, pH 6.2, 5 μM ATP, 10 μM l-fucose, 15 μM MnCl2, and an amount of cell extract protein sufficient to yield approximately linear reaction conditions; typically the extraction of less than 1% of the GDP-[3H]fucose at the end of the course of the reaction (1 h). Control reactions were prepared by omitting the acceptor in the reaction mixture, and values obtained with these reactions were subtracted from the corresponding acceptor-replete reaction. This background radioactivity reproducibly represented less than 1% of the total radioactivity in the assays and corresponds to fucose present in the GDP-[3H]fucose as obtained from the manufacturer. Identical enzyme preparations were used in assays for the determination of enzyme activity with different acceptor substrates.

**Reactions containing neutral acceptors**—(N-acetyl-lactosamine, lacto-N-biose 1, 2-fucosylactose, all from Sigma) were terminated by the addition of 20 μl of ethanol and 560 μl of water. Samples were centrifuged at 15,000 × g for 5 min and a 50-μl aliquot was subjected to scintillation counting to determine the total amount of radioactivity in the reaction. An aliquot of 200 μl was applied to a column containing 400 μl of Dowex 1 × 2 × 400, formate form (21, 23). The column was
washed with 2 ml of water, and the radioactive reaction product not retained by the column was quantitated by scintillation counting. Reactions with the acceptor NeuAcα2–3Galβ1–4GlcNAc (Oxford Glyco-systems, Inc.) were terminated by adding 980 μl of 50 mM sodium phosphate buffer, pH 6.8. Samples were then centrifuged at 15,000 × g for 5 min, and a 500-μl aliquot was applied onto a Dowex 1 × 8–200 column (Bio-Rad) by electroblotting (1 mA/cm2). The membrane was then subjected to SDS-polyacrylamide gel electrophoresis. The fraction containing the insoluble material was collected and quantitated as described previously (22).

Generation of Rabbit Anti-Fuc-TVII Antibody—PCR was used to amplify a segment of the murine Fuc-TVII gene corresponding to the enzyme’s “stem” and catalytic domains (53), using PCR primers corresponding to base pairs 2194–2224 and 3053–3085 (Fig. 2, 5′ primer, gcgcggatccGACGCTGGAACCAGCTTTCAAGGTCTTC; 3′ primer, gcgcggatccGACGCTGGAACCAGCTTTCAAGGTCTTC; BamHI sites are underlined). The PCR product was purified using 20 rounds of amplification consisting of a 1.5-min 94°C denaturation step and a 2.0-min 72°C annealing/extension step. The PCR product was subsequently cloned into the BamHI site of the T7 Escherichia coli expression vector pET-3b (54). The insert in one clone (termed pET-3b-Fuc-TVIIstem) containing a single insert in the correct orientation was sequenced to confirm that no errors were introduced during DNA amplification. The recombinant Fuc-TVII fusion protein was produced by inducing mid-log phase E. coli (BL21 Lys S) carrying pET-3b-Fuc-TVIIstem with 0.4 mM IPTG for 3 h (50, 54). The bacteria were subsequently harvested and lysed by freezing and thawing the bacteria briefly with liquid nitrogen. The bacterial supernatant was sequenced to confirm that no errors were introduced during DNA amplification, followed by separation of soluble and insoluble material by centrifugation. The Fuc-TVII protein was found in the insoluble fraction, as determined by SDS-polyacrylamide gel electrophoresis (55).

Recombinant E. coli-derived Fuc-TVII was fractionated by SDS-polyacrylamide gel electrophoresis, and segments of the gel containing Fuc-TVII were excised and used subsequently as antigen for rabbit immunizations. Rabbit immunization services were purchased (Pel-Freeze Biologicals, Rogers, AR). Each of three rabbits was initially immunized subcutaneously with a total of approximately 200 μg of Fuc-TVII in pulzed polyacrylamide gel slices mixed with complete Freund’s adjuvant. Subsequent immunizations were completed in an essentially identical manner at 14-day intervals, except that antigen was administered in incomplete Freund’s adjuvant. Antiserum was harvested 10 days following the last of a total of approximately six secondary immunizations.

Antigen Affinity Purification of Anti-Fuc-TVII Antibody—The insert in pET-3b-Fuc-TVIIstem was released by digestion with BamHI and was cloned between the BamHI sites in the E. coli expression vector pET-30a (56) to yield a fusion protein derived from E. coli anti-ampicillin synthase sequence, fused in frame to Fuc-TVII sequence. This recombinant fusion protein was expressed in E. coli strain DH5α (induction for 6 h with 0.0125% indoleacrylic acid in M9 medium). The bacteria were harvested, washed, and disrupted by treating with lysozyme (3 mg/ml) in 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, with 0.65% Nonidet P-40 and 100 μg/ml of Nacetylase for 20 s at the maximal micropipet setting (Vibracell, Sonics and Materials, Inc., Danbury, CT) (56). Inclusion bodies were washed twice with 50 mM Tris-HCl, pH 7.5, and 5 mM EDTA, were solubilized by heating to 100°C in 1% SDS, 12 mM Tris-HCl, 5% glycerol, and 1% 2-mercaptoethanol, and were subjected to SDS-polyacrylamide gel electrophoresis. The fractionated proteins were transferred to polyvinylidene difluoride membrane (Bio-Rad) by electroblotting (1 mA/cm2). The membrane was then blocked for 4–6 h at 4°C with PBS containing 10% bovine serum albumin and 0.2% Tween-20. A strip of membrane containing the recombinant Fuc-TVII fusion protein was incubated overnight at 4°C with 0.5 ml of rabbit anti-mouse Fuc-TVII antiserum diluted with 2.5 ml of PBS containing 7% bovine serum albumin and 0.2% Tween-20. The membrane was then washed at room temperature in PBS with 0.05% Tween-20 and sliced into small pieces, and the bound antibody was eluted by incubating the membrane fragments on ice for 10 min in 450 μl of Tris glycine, pH 2.5. The supernatant was collected and immediately neutralized with 100 μl of 1 M Tris-HCl, pH 8.0. The elution procedure was completed a second time, and the two eluates were pooled and used subsequently for immunohistochemical procedures.

Western Blot Analysis—Cell extracts were prepared from transfected COS-7 cells 72 h after transfection. Extracts contained 50 mg Tris-HCl, pH 6.8, 1% SDS, and 10% glycerol. Extracts were boiled for 3 min immediately after preparation and were stored frozen until use. Protein content was determined using the BCA reagent procedure. Extracts were prepared for SDS-polyacrylamide gel electrophoresis by adding dithiothreitol to a final concentration of 0.1% and bromophenol blue to a final concentration of 0.05%. Samples were then boiled and fractionated by electrophoresis through a 10% SDS-polyacrylamide gel. After electrophoresis, the proteins were electrotransferred to a polyvinylidene difluoride membrane (Bio-Rad). The membrane was rinsed and then blocked for 12–14 h at 4°C in phosphate-buffered saline, pH 7.4, containing 10% bovine serum albumin and 0.2% Tween 20. The blot was washed 5 times for 10 min each with 0.1% Tween 20, followed by incubation at room temperature for 3 h with 0.2% Tween 20 and was probed with a 1:200 dilution of antigen purified rabbit anti-Fuc-TVII antibody. The blot was then washed and probed with a 1:2500 dilution of a horseradish peroxidase-conjugated anti-rabbit immunoglobulin (Sigma). The blot was then rinsed, exposed to ECL reagent (Amersham Corp.), and subjected to autoradiography.

Northern Blot Analysis—Mouse Fuc-TVII cDNA was prepared from mouse (FVB/N) tissues and cultured cell lines using published procedures (49). Oligo(dT)-purified poly(A+) RNA samples were electrophoresed through 1.0% agarose formaldehyde-dehydrate 3-phosphate dehydrogenase probe (57) to confirm that RNA samples were intact and loaded in equivalent amounts (data not shown).

Construction of a Mouse L-selectin/IgM Chimera—Histological Probe—A mouse L-selectin cDNA (58) was kindly provided by Dr. Ernie Kawasaki, Procept Inc.) was ligated into the adaptormodified end of the L-selectin sequence in a manner that fuses the open reading frame encoding L-selectin to the open reading frame encoding the CH2, CH3, and CH4 domains of an IgM constant domain. This fragment was inserted into the EcoRI site of pCMV (60) immediately downstream of the SRa promoter in the sense orientation with respect to the SRα promoter. This vector was introduced into COS-7 cells using the DEAE-dextran transfection method (21, 49).

Medium was harvested from the transfected cells 3 days after the transfection and was replaced with fresh medium (Dulbecco’s modified Eagle’s medium, 10% fetal calf serum, P/S, Q) that was collected 4 days later. The L-selectin/IgM chimera was purified and concentrated and then used at the concentration of 7.0 μg/ml in blocking solution containing 0.9–3.7 labeled 974-base pair EagI–EcoRI fragment isolated from the insert in pMUC-Fuc-TVII. The EagI site is located at nucleotides 2228–2233, whereas the EcoRI site spans base pairs 3202–3207. Blots were stripped in boiling 0.1% SDS and rehybridized with a chicken glyceraldehyde 3-phosphate dehydrogenase probe (57) to confirm that RNA samples were intact and loaded in equivalent amounts (data not shown).

Immunohistochemistry Procedures—Peripheral (axillary) and mesenteric lymph nodes and Peyer’s patches were isolated from mice immediately after sacrifice. These lymphoid tissues were embedded in OCT medium (Tissue-Tek, MILES, Elkhart, IN), sectioned with a Leica cryostat, and collected on glass microscope slides.

Sections were then washed in 2% paraformaldehyde in phosphate-buffered saline for 20 min on ice. The sections were then rinsed with phosphate-buffered saline at room temperature, and then were briefly washed with water. The tissues were then permeabilized with 100% methanol for 20 min on ice, rehydrated in phosphate-buffered saline, and then incubated for 30 min at room temperature with blocking solution A (phosphate-buffered saline containing 2% goat serum, 0.05% Triton X-100, 0.05% Tween 20). The blocking solution was aspirated, and the sections were incubated overnight at 4°C with the anti-L-selectin/IgM chimera 1G reagent (Sigma) diluted 1:200 in blocking solution A. The slides were then washed in phosphate-buffered saline, and mounted with citifluor (Citifluor Products, Chemical Laboratory, The University, Canterbury, Kent, UK), and examined by immunofluorescence microscopy (Leitz DM RB microscope).

Sections to be stained with the monoclonal antibody MECA-79 (48) were fixed on ice for 20 min in 2% paraformaldehyde in phosphate-buffered saline, washed at room temperature with phosphate-buffered saline, and then incubated for 20 min at room temperature with 50 μg/ml of MECA-79 in phosphate-buffered saline. The slides were then rinsed briefly in water, permeabilized with 100% methanol for 20 min on ice, rehy-
in phosphate-buffered saline, and then incubated overnight at room temperature with blocking solution A (phosphate-buffered saline containing 2% goat serum, 0.05% Triton X-100, 0.05% Tween 20). The blocking solution was then aspirated, and the sections were incubated for 1 h at 7°C with MECA-79 at a concentration of 5 μg/ml in blocking solution A. Sections were then washed extensively with phosphate-buffered saline at room temperature and then washed for 1 h at 7°C with a tetramethyl rhodamine iso-thiocyanate-conjugated goat anti-rat IgM reagent (Jackson ImmunoResearch) used at a dilution 1:200 in blocking solution A. The slides were then washed three times at room temperature with phosphate-buffered saline, mounted with ditrifluor, and examined.

Sections to be stained with the L-selectin/gm chimera were fixed in 1% paraformaldehyde and 0.1% sodium cacodylate, pH 7.1, for 20 min on ice, then washed with Tris-buffered saline, pH 7.4. The L-selectin/gm chimera was applied to the sections at a concentration of 60 μg/ml in blocking solution B (Tris-buffered saline, pH 7.4, containing 2% goat serum) supplemented with either 3 mM CaCl2 or with 5 mM EDTA, and were allowed to incubate overnight at 7°C. Sections were then washed extensively with ice-cold Tris-buffered saline supplemented with 3 mM CaCl2. Sections were then incubated for 1 h at 7°C with a biotinylated goat anti-human IgM reagent (Sigma), diluted 1:200 in blocking solution B, and supplemented either with 3 mM CaCl2 or with 5 mM EDTA. The sections were then washed with ice-cold Tris-buffered saline supplemented with 3 mM CaCl2 and were incubated for 1 h at 7°C with a FITC-conjugated biotin-avidin reagent (Vector Labs, Burlingame, CA) diluted 1:200 in blocking solution B supplemented with 3 mM CaCl2. The slides were washed with ice-cold Tris-buffered saline supplemented with 3 mM CaCl2, mounted with ditrifluor, and examined by immunofluorescence microscopy (Leitz DM RB microscope).

In Situ Hybridization Procedures—In situ hybridization procedures were completed using a modification of published procedures (59). Fresh murine axillary lymph nodes, mesenteric lymph nodes, and Peyers patches were embedded in OCT medium (Tissue-Tek, MILES, Elkhart, IN) and quick-frozen in isopentane on liquid nitrogen. Cryostat sections (10 μm) were collected on Superfrost Plus microscope slides (Fisher) fixed in freshly prepared 4% paraformaldehyde in PBS for 30 min on ice, washed twice in PBS, and digested for 5 min at room temperature with 1 μg/ml pronase K in 50 mM Tris-HCl, pH 7.5, and 5 mM EDTA. The slides were then washed in PBS, fixed again in 4% paraformaldehyde, rinsed in water, and treated with 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0, for 10 min at room temperature. Acetylation was followed by room temperature washes in PBS and then in 0.85% NaCl. The slides were then dehydrated in a graded series of solutions of ethanol in water (30, 50, 80, 95, and 100%). Air-dried sections were overlaid with a hybridization solution containing 35S-labeled RNA in sense or antisense orientation. RNA probes were derived by in vitro transcription procedures, using recombinant T7 or Sp6 RNA polymerases, initiating on the T7 or Sp6 promoter sequences flanking a DNA segment derived from the coding region of the mouse Fuc-TIII gene (base pairs 2197–2494; see Fig. 2) as subcloned into the vector pCDNA1 (Invitrogen). The hybridization solution contained 50% deionized formamide, 0.3 M NaCl, 20 mM Tris-HCl, pH 8.0, 5 mM EDTA, 10 mM phosphate buffer, pH 8.0, 10% dextan sulfate, 1 × Denhardt’s solution (49), 0.5 μg/ml yeast tRNA, 10 mM dithiothreitol, and 100 μM/ml of radiolabeled probe. The hybridization solution was sealed over the sections with a coverslip and DPX mounting media (BDH Lab Supplies, Poole, UK). Hybridization was carried out for 16 h at 55°C in a sealed container humidified with 5 × SSC. After hybridization, the DPX mounting media seal was removed, and slides were washed at 55°C for 30 min in 5 × SSC and 10 min dithiothreitol and then at 65°C for 30 min in formamide wash buffer (50% formamide, 2 × SSC, 20 μM dithiothreitol). The slides were then washed 4 times at 37°C in 0.5 M NaCl, 10 mM Tris-HCl, pH 7.5, 5 mM EDTA. Slides were then digested with RNaseA (1 μg/ml) for 30 min at 37°C and were washed in formamide buffer, then in 2 × SSC, and then in 0.1 × SSC. Slides were dehydrated in a graded series of solutions of ethanol in 0.3 M ammonium acetate (30, 50, 80, 95, and 100% ethanol), air-dried, and coated with NTB2 liquid emulsion (Kodak). Following a 2–3-week exposure period, the slides were developed using standard procedures suggested by the manufacturer. Sections were then stained with hematoxylin and eosin and examined and photographed with bright field and dark field modalities, using a Leitz DM RB microscope.

RESULTS
A Hybridization Screen Identifies a Novel Murine a(1,3)Fucosyltransferase Locus—In an effort to isolate novel murine a(1,3)fucosyltransferase genes, a murine genomic DNA phage library was screened with a probe corresponding to the catalytic domain of the human Lewis a(1,3,4) fucosyltransferase (Fuc-TIII) (21) using low stringency hybridization conditions (see “Experimental Procedures”). One phage was isolated that contained an insert with a translational reading frame sharing approximately 40% amino acid sequence similarity with the amino acid sequences encoded by four previously cloned members of the human a(1,3)fucosyltransferase family (Fuc-TIII-VI) (21–26).

To identify transcripts corresponding to this genomic sequence, a segment of the phage insert representative of the open reading frame was used to probe Northern blots prepared from mouse cell lines and tissues. Transcripts corresponding to this probe were identified in the murine cytotoxic T-cell line 14-Td (42, 43). A cDNA library constructed from this cell line (32) was screened by hybridization with a segment of the phage insert, yielding 16 hybridization positive colonies. The sequences of all 16 cDNA clones were determined, as was the sequence of the corresponding genomic DNA (Fig. 1A). Analysis of this sequence data indicates that this locus yields multiple structurally distinct transcripts derived from alternative splicing events and possibly also from alternative transcription initiation events. Five classes of cDNAs were identified (Fig. 1). Analysis of these cDNA sequences identifies three methionine codons that may function to initiate translation of an open reading frame with amino acid sequence similarity to human Fuc-TIII, Fuc-TIV, Fuc-TV, and Fuc-TVI (Fig. 2). The positions of these methionine codons predicts the synthesis of a(1,3)fucosyltransferases with different cytosolic domains (encoded by exons 2 and/or 3) but with identical Golgi-localized catalytic domains (encoded by exon 4). One relatively abundant class of cDNAs (represented by cDNA 14) maintains an open reading frame initiating at the methionine codon at nucleotide 1947. This reading frame predicts a 342-residue, 39,424 Da type II transmembrane protein with a hydrophobic transmembrane segment derived from amino acids 9–31 (Fig. 2). An in-frame methionine codon at nucleotide 2126 predicts a 318-residue, 36,836 Da polyprotein that initiates within the hydrophobic transmembrane segment of the polyprotein predicted by the longer reading frame initiated at nucleotide 1947. A similar structural arrangement is found in two other cDNA classes, represented by cDNAs 6 and 10. However, these two cDNAs differ from cDNA 14 in that they contain an additional upstream exon with a methionine codon corresponding to nucleotide 996. The translational reading frame initiated by this methionine codon is truncated by a termination codon in exon 2 at a position proximal to the methionine codon at nucleotide 1947 and thus cannot generate a polyprotein that shares similarity to the human a(1,3)fucosyltransferases. However, in cDNA 5, the absence of exon 2 allows the translational reading frame generated by the methionine codon at nucleotide 996 to continue in frame with sequence in exon 4. This arrangement predicts the synthesis of a 389-residue, 44,492 Da type II transmembrane protein with the same putative transmembrane segment defined for the protein predicted by cDNA 14 (Fig. 2). Finally, cDNA 3 is representative of a relatively abundant class of cDNAs that each initiate between the splice acceptor site of exon 4 and the methionine codon at nucleotide 2126. This class of cDNAs predicts a 318-residue, 36,836 Da polyprotein that initiates within the transmembrane segment predicted for the proteins corresponding to the other cDNA classes.

Because the polyprotein predicted by these murine cDNAs share primary sequence similarity to the four human a(1,3)fucosyltransferases known at the time (Fuc-TIII, IV, V, and VI), we anticipated that one or more of them would function as an
However, because the murine peptide sequence shares approximately equivalent sequence similarity to each of these human enzymes, we expected that it did not represent the murine homologue of any of them and consequently named it Fuc-TVII. This appellation has been justified by subsequent work in which this murine gene has been used to isolate cDNAs encoding the human Fuc-TVII (27). None of the three putative initiation codons are embedded in the putative murine enzyme sequences.
likely that individually or together, each directs the expression of identical cell surface antigen profiles in COS-7 cells, it seems isomers. Because all four of these cDNAs direct qualitatively identical surface-localized sLex expression (35.1, 21.8, and 16.5%, respectively, above a 2% background) when introduced into COS-7 cells by transfection. cDNA 5 also directs cell surface sLex expression in COS-7 cells but at a level (9% positive cells) that is lower than the sLex expression levels determined by purified rabbit polyclonal antibody generated against a recombinant form of the predicted polypeptide (Fig. 1).

In contrast to the results obtained with cDNAs 5, 6, 10, and 14, cDNA 3 does not direct detectable sLex expression. This result suggests that the methionine codon at nucleotide 2126 in cDNA 3, as well as all other cDNAs; Fig. 1), is informative for expression of the Lewis x and sLex determinants but indicate that the activity does not efficiently utilize neutral type II oligosaccharide Lewis x precursors nor to neutral type II precursors.

In order to confirm that the sLex expression efficiency characteristic of each cDNA correlates with the level of expression of the corresponding protein, cell extracts of the transfected COS-7 cell line were subjected to assays to (i) identify cDNA-determined cell surface-localized fucosylated oligosaccharide antigens, (ii) identify and quantitate the polypeptides encoded by cDNAs, and (iii) identify and partially characterize cDNA-determined polypeptides. One protein is predicted to initiate at the methionine codon localized to nucleotide positions 2126–2128 (318 residues, 36,836 Da; cDNA 3, as well as all other cDNAs; Fig. 1). A second protein is predicted to initiate at the methionine codon localized to nucleotide positions 1947–1949 (342 residues, 39,424 Da; cDNA 6, 10, and 14; Fig. 1). The third protein is predicted to initiate at the methionine codon localized to nucleotide positions 996–998 (389 residues, 44,492 Da; cDNA 5; Fig. 1). A fourth protein is predicted to initiate at the methionine codon localized to nucleotide positions 1477–1479 (324 residues, 36,912 Da; cDNA 14, Fig. 1) each determines relatively high levels of cell surface-localized sLex expression (35.1, 21.8, and 16.5%, respectively, above a 2% background) when introduced into COS-7 cells by transfection. cDNA 5 also directs cell surface sLex expression in COS-7 cells but at a level (9% positive cells) that is lower than the sLex expression levels determined by purified rabbit polyclonal antibody generated against a recombinant form of the predicted polypeptide (Fig. 1).

Fig. 2. Nucleotide and deduced amino acid sequence of the isolated mouse Fuc-TVII gene. The DNA sequence was derived from a phage containing the murine Fuc-TVII locus. The DNA sequence present in cDNAs (Fig. 1) is shown in lowercase letters, whereas the DNA sequence corresponding to intronic positions is displayed in uppercase letters. Amino acid sequences predicted by the cDNA sequences are shown in single-letter code. As discussed in detail in the text, alternative splicing events yield different cDNAs that may in turn encode the different polypeptides. One protein is predicted to initiate at the methionine codon localized to nucleotide positions 996–998 (389 residues, 44,492 Da; cDNA 3, as well as all other cDNAs; Fig. 1).

To confirm that the sLex expression efficiency characteristic of each cDNA correlates with the level of expression of the corresponding protein, cell extracts of the transfected COS-7 cells were subjected to Western blot analysis using an affinity purified rabbit polyclonal antibody generated against a recombinant form of the predicted polypeptide (Fig. 1B). Cells transfected with cDNAs 6, 10, and 14 express two major forms of the polypeptides that individually or together maintain essentially identical acceptor substrate specificities (at least for the four antigens examined). In contrast to the results obtained with cDNAs 5, 6, 10, and 14, cDNA 3 does not direct detectable sLe expression. This result suggests that the methionine codon at nucleotide 2126 in this cDNA does not efficiently promote initiation of translation of the cognate mRNA and thus does not encode functionally significant levels of enzyme activity. Alternatively, this cDNA may encode a polypeptide without α(1,3)fucosyltransferase activity.

Qualitatively identical results were obtained when these five cDNAs were expressed in another cell line (CHO-Tag cells) (32) informative for expression of the Lewis x and sLe determinants (data not shown). Unlike COS-7 cells, this cell line is also capable of forming the internally fucosylated VIM-2 determinant (NeuAc2,3Galα1,4GlcNAcβ1,3Galα1,4(Fucα1,3) GlcNAc-R) (22). We found that none of the cDNAs directs expression of the VIM-2 epitope when expressed in the CHO-Tag cells (data not shown). Considered together, these results indicate that some, though not all, of the cDNAs can encode an α(1,3)fucosyltransferase activity that can catalyze α(1,3)fucosylation of the N-acetylgalactosamine moiety on a terminal α(2,3) sialylated lactosamine unit but not to internal N-acetylgalactosamine moieties on α(2,3)sialylated polyglycosamine precursors nor to neutral type II precursors.

To confirm that the sLe expression efficiency characteristic of each cDNA correlates with the level of expression of the corresponding protein, cell extracts of the transfected COS-7 cells were subjected to Western blot analysis using an affinity purified rabbit polyclonal antibody generated against a recombinant form of the predicted polypeptide (Fig. 1B). Cells transfected with cDNAs 6, 10, and 14 express two major forms of the polypeptides that individually or together maintain essentially identical acceptor substrate specificities (at least for the four antigens examined).
proteins, with molecular masses of 35 and 37 kDa. Smaller amounts of several other proteins are also evident in these cells. The amount of immunoreactive protein generated by these three cDNAs correlates with the level of Slε expression directed by each. This observation indicates that the relative Slε expression level directed by each is a function of the efficiency with which each corresponding mRNA is translated and thus the relative intracellular accumulation of the cognate polypeptide.

Cells transfected with cDNA 5 cells also contain multiple immunoreactive polypeptides (Fig. 1B). The most abundant pair of these proteins migrate more rapidly than do the proteins detected in cells transfected with cDNAs 6, 10, and 14 yet are approximately similar in quantity to the immunoreactive protein directed by cDNAs 6 and 10. Because cDNA 5 directs lower levels of cell surface Slε expression than these two cDNAs, it is therefore possible that the lower M, immunoreactive polypeptides found in cDNA 5-transfected cells maintain substantially lower specific enzyme activity than do the proteins encoded by cDNAs 6, 10, and 14 or are otherwise less able to direct Slε expression in COS-7 cells. Finally, cells transfected with cDNA 3 do not contain any detectable immunoreactive proteins. This implies that the putative initiator codon at base pair 2126 in this cDNA does not initiate translation of an active protein. This suggests that the putative initiator codon at base pair 2126 in this cDNA does not initiate translation of an active protein.

Considered together, these results suggest that differential splicing and/or transcriptional initiation events can control the level of Slε fucosyltransferase activity and thus cell surface Slε expression level through mechanisms that depend on the efficiency with which each transcript is translated.

Transcription of the Mouse Fuc-TVII Locus Is Restricted to Cells Found in the Bone Marrow and the Lung—Northern blot analysis indicates that transcripts corresponding to the Fuc-TVII locus accumulate to detectable levels in only a few tissues in the adult mouse (Fig. 3). Abundant transcript accumulation is only observed in the lung and in the bone marrow, with very small amounts evident in the spleen, salivary gland, and skeletal muscle. Northern blot analysis of cultured murine blood cell-type cell lines indicates that the Fuc-TVII transcript is relatively abundant in the mouse cytotoxic T lymphocyte line 14-fd (used to clone the Fuc-TVII cDNAs) and in the mouse T cell line EL4 (34). Less abundant transcript accumulation is evident in the murine macrophage-derived cell line RAW (39, 40) and P388 (41). Fuc-TVII transcripts are not evident in the murine erythroleukemia cell line MEL (37, 38) nor in three murine B lymphocyte lineage cell lines (S107 (33); TH2.54.63 (35); 180.1 (36)).

Both the marrow and lung maintain several differently sized transcripts, including two abundant transcripts of approximately 1.6 and 2.2 kilobase pairs in size and a fainter transcript at approximately 3.0 kilobase pairs. These three transcripts are similar in size to the three most abundant transcripts observed in the murine 14-7fd cytotoxic T cell line. These observations suggest that cells in the bone marrow and lung yield alternatively spliced transcripts similar in structure to those characterized by cDNA cloning studies in the 14-7fd cells. These data also suggest that in the marrow, the Fuc-TVII locus is transcribed in cells assigned to the myeloid and T lymphoid lineages but not in B lymphoid lineage cell types and suggest that expression of this fucosyltransferase correlates with selectin ligand expression on myeloid and T lymphocyte lineage cell types.

Fuc-TVII Is Expressed in Endothelial Cells Lining the High Endothelial Venules in Peripheral Lymph Nodes, Mesenteric...
Lymph Nodes, and Peyer’s Patches—The identity of the cell types in the lung responsible for the Northern blot signal in that organ was disclosed by in situ hybridization analyses. These studies identified Fuc-TVII transcripts in paratracheal lymph nodes within the extirpated lung but not in any other cell type (data not shown). The pattern of expression in these nodes suggested that the Fuc-TVII transcripts were localized to the high endothelial venules within these nodes (data not shown). When considered together with recent observations suggesting that a sulfated derivative of the sialyl Lewis x determinant represents a terminal oligosaccharide moiety found on HEV-specific L-selectin ligands (17–19), detection of Fuc-TVII transcripts in these HEV suggests a possible role for this locus in the synthesis of the fucosylated oligosaccharide and in controlling lymphocyte homing. To further characterize the HEV-specific expression of Fuc-TVII, this was systematically evaluated using in situ hybridization analysis of HEV in peripheral lymph nodes, in mesenteric lymph nodes, and in Peyer’s patches, where L-selectin ligand expression has been well characterized (6). These analyses (Fig. 4) indicate that Fuc-TVII transcripts accumulate to easily detectable levels in the HEV of all three types of lymphoid aggregates. An in situ hybridization signal is also obtained with the antisense Fuc-TVII probe in a population of cells that line the gut luminal surface overlying the Peyer’s patches. Although these are presumed to be epithelial cells, and may represent so-called M cells (61), their precise identity remains unknown.

As noted above, not all Fuc-TVII-derived transcripts yield a protein product. Immunohistochemical analyses were therefore used to confirm that the Fuc-TVII transcripts detected in HEV are accompanied by Fuc-TVII polypeptide expression and to confirm that such expression co-localizes with L-selectin ligand expression. A rabbit polyclonal antibody raised against the Fuc-TVII peptide yields an intracellular staining pattern in the endothelial cells within HEV in all three lymphoid aggregates (Fig. 5). The perinuclear intracellular staining pattern seen with the anti-Fuc-TVII antibody is consistent with the notion that this enzyme is localized to the Golgi apparatus, where it may participate in the synthesis of fucosylated oligosaccharides with L-selectin ligand activity. In each of the three types of lymphoid aggregate, expression of immunoreactive Fuc-TVII co-localizes with expression of epitopes recognized by the MECA-79 antibody shown previously to stain HEV and to interfere with L-selectin binding to HEV (48). Fuc-TVII expression also co-localizes with expression of L-selectin ligands on HEV, as detected with a recombinant mouse L-selectin/human IgM chimeric protein. These observations imply that Fuc-TVII may participate in the synthesis of the sialylated, sulfated, and α(1,3)fucosylated candidate oligosaccharide components of HEV-derived L-selectin ligands.

DISCUSSION

In an effort to understand the functions of cell surface fucosylated oligosaccharides in animals, we have established a program to isolate murine α(1,3)fucosyltransferase genes to be used initially as reagents to characterize tissue-specific expression patterns of the loci that control expression of cell surface fucosylated oligosaccharides. These reagents and the information gathered from their application will be used eventually with transgenic approaches to uncover functions of their cognate cell surface fucosylated oligosaccharides by perturbing their expression patterns.

A cross-hybridization approach outlined here yielded a novel genomic sequence that cross-hybrizes with segments derived from the conserved portions of the human Fuc-T’s III, V, and VI genes, in a position corresponding to their catalytic domains. Following the isolation of this murine genomic locus, functional analyses indicated that it encoded an α(1,3)fucosyltransferase,
termed Fuc-TVII, with structural features and catalytic activities that were, at the time of its isolation, unique to the \((1,3)\)fucosyltransferase family. In particular, this locus was the only \((1,3)\)fucosyltransferase known to maintain a coding region distributed over more than one exon and the first fucosyltransferase with multiple distinct initiation codons with the potential to yield structurally distinct polypeptides characterized by different cytoplasmic domains but with essentially identical catalytic activities. The catalytic activity of each Fuc-TVII isoenzyme is characterized by an ability to utilize an \((2,3)\)sialylated type II \(N\)-acetyllactosamine precursors without the ability to utilize neutral type II, neutral type I, or \((1,3)\)fucosylated \(N\)-acetyllactosamine substrates. Similar observations have been made for the human homologue of Fuc-TVII isolated subsequently by our group (27) and by others (28). This catalytic specificity and the leukocyte-specific expression pattern of this gene strongly suggest that it plays a pivotal role in the biosynthetic scheme that yields the \((2,3)\)sialylated and \((1,3)\)fucosylated lactosaminoglycans essential to E- and P-selectin ligand activity.

Other observations made in the work described here suggest a role for Fuc-TVII in directing synthesis of the oligosaccharide components of the ligands for L-selectin. As Rosen and colleagues have shown, L-selectin ligands on HEV correspond to \(O\)-linked carbohydrate determinants displayed by the mucin-type glycoproteins GlyCAM-1, CD34, and MADCAM-1 (13–15). Their earlier biochemical analyses indicate that the oligosaccharides relevant to L-selectin ligand activity are sialylated, sulfated, and possibly fucosylated (16). More recent structural analyses from the Rosen group are consistent with the hypothesis that the capping groups on such oligosaccharides correspond to sulfated versions of the sialyl \(Le^\alpha\) moiety, with sulfate attached via the 6-hydroxyl of the terminal galactose moiety, \([\text{NeuN}Ac\alpha2,3(\text{SO}_4\text{)Gal}\beta1,4(\text{Fuc}\alpha1,3)\text{GlcNAc-R}]\), the 6-hydroxyl of the subterminal \(N\)-acyethylglucosamine moiety, \([\text{NeuN}Ac\alpha2,3\text{Gal}\beta1,4(\text{SO}_4\text{)Fuc}\alpha1,3)\text{GlcNAc-R}]\), or both (17–19). Nonfucosylated forms of these structures were also identified, however, and the evidence that fucose is required for activity of physiological L-selectin ligands remains circumstantial.
The identification of such nonfucosylated structures suggests the possibility that these sialylated and sulfated molecules represent acceptor substrates for α(1,3)fucosyltransferases expressed in HEV endothelial cells. Our observation that expression of the Fuc-TVII locus co-localizes with L-selectin ligand expression in such cells suggests that Fuc-TVII may operate in this context. The notion that sulfated and sialylated lactosamine moieties represent acceptor substrates for enzymes like Fuc-TVII is supported by studies suggesting that Fuc-TVII (62) and Fuc-TV (63) can utilize sialylated, sulfated lactosamine-type acceptors. Indirect evidence derived from studies on the biosynthesis of GlyCAM-1 are also consistent with this hypothesis (17, 64). However, our results indicate that the nonfucosylated entity NeuNAcα2,3Galβ1,4GlcNAc is used in vivo and in vivo by Fuc-TVII. It therefore remains to be determined if the two sulfated forms of this substrate are also utilized by Fuc-TVIII or if alternatively in HEV endothelial cells, sialylation and then fucosylation in turn precede sulfation. Thus, the biosynthetic scheme for such molecules remains to be defined, especially in the context of the possible role for Fuc-TVII in this pathway, and the relative order(s) of addition of sialic acid, sulfate, and fucose, on such molecules. This work is currently in progress in this laboratory.

There is evidence to suggest that L-selectin expressed by granulocytes and other leukocytes mediates adhesion of these cells to activated vascular endothelium through as yet unde-
Expression of the (1,3)Fucosyltransferase Fuc-TVII in Lymphoid Aggregate High Endothelial Venules Correlates with Expression of L-Selectin Ligands

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