The ELAM ligand fucosyltransferase, ELFT, directs E-selectin binding to a secreted scaffold protein: a method to produce and purify large quantities of specific carbohydrate structures

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Many biological processes depend on cell surface recognition of receptor–ligand pairs. Some receptors, such as the selectins, recognize specific carbohydrate structures as part of their ligands. The ability to synthesize such ligands for use in the study of cell adhesion mechanisms or as inhibitors of a variety of pathological conditions would be extremely useful. However, the chemical or enzymic *in vitro* synthesis of carbohydrate-based ligands has thus far been difficult and costly. We have used E-selectin and its carbohydrate ligand as a model system to test if it is possible to express specific carbohydrate structures on a secreted, glycosylated and easily purified scaffold protein and to use this newly modified protein as a functional adhesion molecule. We co-expressed a fucosyltransferase (ELFT) and a secreted immunoglobulin-LFA3 fusion protein (LFA3IG) in the same

cell to modify the carbohydrate structures on the secreted LFA3IG scaffold protein (we refer to this novel protein as X-LFA3IG). Using glycosidase digestion, lectin binding, carbohydrate composition analysis and antibody-binding assays, we show that approximately 50 % of the potential N-linked carbohydrate sites on X-LFA3IG are, indeed, modified and that the modification is the addition of fucose. Furthermore, we show that X-LFA3IG contains epitopes recognized by anti-Sle^x antibodies, and, using an E-selectin-specific adhesion assay, we demonstrate that X-LFA3IG is a functional ligand for E-selectin. This *in vivo* approach for generating specific carbohydrate structures could be generalized to produce and purify large quantities of other biologically important carbohydrate structures.

INTRODUCTION

Over the past few years it has become increasingly apparent that carbohydrates play a critical role in many biological systems. Processes in which cell surface (and thus perhaps carbohydrate) recognition is important, such as development, metastasis and certain immune responses, depend on receptor–ligand interactions. These recognition events occur in a number of ways, including soluble molecules interacting with cells, cells interacting with substrates such as basement membranes and cells interacting with other cells, and occur in many settings both normal and pathological. One of the settings that has recently gained a great deal of attention is inflammation. An early step in inflammation is the binding of leucocytes to the endothelial lining of blood vessels. These bound leucocytes eventually extravasate into the extracellular space, setting in motion the rest of the inflammatory cascade [1–4]. Many laboratories have been interested in inhibiting the initial leucocyte–endothelial cell adhesion events with the goal of blocking the damaging downstream part of the inflammatory process.

Several families of molecules have been identified as cell adhesion proteins, including the integrins [5–7] and the selectins [8–12]. Whereas integrin-mediated adhesion appears to depend on protein–protein interactions, selectin-mediated adhesion apparently requires protein–carbohydrate interactions. One of the adhesion pairs in which we have had a particular interest is E-selectin and its ligand [13]. E-selectin (ELAM1) is a 110 kDa protein found on the surface of tumour necrosis factor- or interleukin 1-activated endothelial cells [14]. Ligands for E-selectin have thus far been found on neutrophils, eosinophils, monocytes, some T-cells and a number of cell lines ([15] and reviewed in [16]). E-selectin, like the other selectins, has an N-

terminal lectin domain related to mammalian lectins that bind sugars. *In vitro* experiments have shown that some carbohydrate structures containing an α -2,3-linked terminal sialic acid and a fucose on the ultimate *N*-acetylglucosamine (e.g. Sle^x) are capable of binding E-selectin-bearing cells [17–21]. The other selectins appear to bind to related structures [22–26]. To thoroughly study cell–cell adhesion events, a source of these types of structure would be very useful. Unfortunately, their synthesis either chemically or enzymically in large quantity is difficult.

An alternative method for synthesizing such carbohydrate structures is to design a cell that attaches Sle^x or related structures to a soluble, easily purified, scaffold protein (Figure 1), which can then be used to study and, potentially, to inhibit E-selectin-mediated binding. We and others have shown that transfection of certain glycosyltransferase genes into mammalian cells creates carbohydrate structures on the cell surface which can be recognized by E-selectin and by antibodies to Sle^x [17,20]. We chose the fucosyltransferase, ELFT, a 405-amino acid polypeptide expressed in a number of cell types including neutrophils and HL60 cells [20], as the model glycosyltransferase. Although there has been some controversy as to whether ELFT can direct the expression of E-selectin or Sle^x ligands [27–29], recent studies by our group and others have confirmed that ELFT-expressing cells do bear structures that are recognized by anti-Sle^x antibodies and that can function as E-selectin ligands (P. Stanley and S. Goelz, unpublished work). In the selection of the scaffold protein, primary consideration was given to the number of potential N-linked glycosylation sites available and the ease with which the protein could be purified. Our model scaffold protein, LFA3IG, consists of the first 92 amino acids of LFA3 [30] fused to the human IgG1 hinge, heavy-chain constant domain two (C_H2) and three (C_H3). This results in a recombinant fusion protein which

Figure 1 Scheme for production of a recombinant E-selectin ligand

Transfecting a plasmid coding for the scaffold protein, LFA3IG, into a CHO cell already expressing the recombinant gene coding for the fucosyltransferase, ELFT, results in the secretion of X-LFA3IG displaying a new carbohydrate structure which functions as an E-selectin ligand.

is secreted as a dimer and contains a total of eight potential N-linked glycosylation sites, six in the LFA3 portion and two in the IgG portion. In this report, we describe the use of the fucosyltransferase, ELFT, to create a modified LFA3IG (designated X-LFA3IG) containing carbohydrate structures that are recognized by anti-Sle^a antibodies and that can function as ligands for E-selectin. This *in vivo* approach could be generalized to make different carbohydrate structures, furnishing tools to study and potentially inhibit other kinds of adhesion events.

EXPERIMENTAL

Construction of LFA3IG fusion proteins

The human IgG1 heavy-chain constant domain containing the hinge region, the C_H2 and the C_H3 sequences, were isolated with heavy-chain-specific primers by PCR amplification of cDNA from RNA of COS-7 cells that had been transfected with plasmids containing 3 kb genomic DNA inserts. The genomic DNA inserts had been isolated by digestion of the partial *Sau*3a human fibroblast genomic library EMBL/5X (a gift from Mark Pasek, Biogen, Inc.). Heavy-chain-specific oligonucleotide primers were designed so that the resulting C_H2 and C_H3 IgG1 PCR-amplified sequences were flanked by a 5' *Sal*I and a 3' *Not*I restriction recognition site. The N-terminal 92-amino acid sequence of mature LFA3, preceded by the LFA3 signal sequence, was isolated by PCR amplification of pHT16-6 cDNA using one primer containing a 5' *Not*I site and one with a 3' *Sal*I site. Amplified DNA fragments of the LFA3 and the IgG1 sequences were ligated and inserted into the expression vector pSAB132 (a derivative of pJOD-S) [31]. The resulting plasmid, pSAB152, contains cDNA sequences coding for the signal sequence and the first 92 amino acids of LFA3, ten amino acids of the hinge region and the C_H2 and C_H3 constant regions of IgG1, all flanked by *Not*I restriction sites. Plasmids pSAB152 (coding for LFA3IG)

and pFASV NEO (coding for G418 resistance) [32] were linearized with 0.18 unit of *Pvu*I and 1 unit of *Aat*II/ μ g of DNA respectively following the conditions suggested by the manufacturer (New England Biolabs, Beverly, MA, U.S.A.). The linearized plasmids (57 μ g of pSAB152 and 2.5 μ g of pFasv NEO) were electroporated at 280 V (960 μ F) into 10⁷ CHO/ELFT cells which had been previously transfected with the fucosyltransferase gene, ELFT [20]. The cells were selected in 0.7 mg/ml G418 (Sigma), and surviving colonies (1 colony/10⁶ transfected cells) were picked, expanded and their supernatants assayed by an e.l.i.s.a. specific for LFA3 [33]. The highest-producing clone, secreting 0.5 mg/l, was selected for production and purification of X-LFA3IG.

Purification of LFA3IG fusion proteins

The cells were grown on collagen microspheres in sCCM3 (Verax Corporation, Lebanon, NH, U.S.A.) supplemented with 5% (v/v) fetal bovine serum (FBS), 200 nM methotrexate, 150 μ g/ml streptomycin, 50 μ g/ml gentamicin and 4 mM L-glutamine in roller bottles at 37 °C. After 72 h, conditioned medium was collected, filtered to remove the cell debris and concentrated 20-fold by ultrafiltration in a S1Y30 spiral cartridge (Amicon). Typically, concentrated protein from 10 litres of culture supernatant was bound to a 2 ml Protein A-Sepharose column (Pharmacia). The column was washed with about 10 column vol. of PBS (14.4 mM Na₂HPO₄, 5.6 mM NaH₂PO₄, 150 mM NaCl, pH 7.2), and X-LFA3IG was eluted with 50 mM glycine plus 250 mM NaCl, pH 3.0. The fractions were collected into 0.1 fraction vol. of 1 M Hepes, pH 7.5. The eluted material was diluted with 1 vol. of 1.5 M glycine plus 3 M NaCl, pH 8.9, and again loaded on to a Protein A-Sepharose column. The second Protein A-Sepharose column was eluted with a linear decreasing pH and salt gradient of 1.5 M glycine plus 3 M NaCl, pH 8.9, to 0.1 M glycine, pH 3.0 (20 column vol. total gradient volume), and the fractions were collected into 0.1 vol. of 1 M Hepes, pH 7.5. The tubes containing pure X-LFA3IG were identified by SDS/PAGE. Peak fractions were pooled and concentrated by ultrafiltration using a YM30 filter in a stirred cell (Amicon). The concentrate was subjected to Superose 6 (Pharmacia) gel-filtration chromatography in PBS at 20 ml/h. Fractions (0.5 ml) were collected and monitored for A₂₈₀. Peak fractions were pooled and stored at -70 °C in portions. The final pool (0.9 A₂₈₀/ml or 0.58 mg/ml) was more than 95% pure as estimated by SDS/PAGE. The control protein, LFA3IG, was purified by a combination of Protein A-Sepharose and gel filtration as described [31].

E-selectin-binding assay

X-LFA3IG (50 μ l diluted to 6.4 μ g/ml in 50 mM NaHCO₃, pH 9.2) was added to each well of a 96-well flat-bottomed plate (no. 76-231-05, Linbro, Titertek; Flow Labs, McLean, VA, U.S.A.) and incubated overnight at 4 °C. The wells were washed with 100 μ l of PBS and subsequently blocked with 100 μ l of 1% (w/v) BSA in PBS plus 0.02% sodium azide for 1 h at room temperature. R1.1 ELAM [20] cells (R1.1/E-selectin), a suspension murine cell line expressing recombinant membrane-associated E-selectin [5 $\times$ 10⁶ cells/ml in RPMI 1640 plus 2% (v/v) FBS, pH 7.4] were fluorescein-labelled by adding 2',7'-bis-(2-carboxyethyl)-5-(and -6)-carboxyfluorescein acetoxymethyl ester (no. B-1150; Molecular Probes Inc., Eugene, OR, U.S.A. to a final concentration of 2 μ M and incubated for 25 min at 37 °C. The cells were pelleted by centrifugation and washed twice with RPMI 1640 plus 2% (v/v) FBS, each wash being followed by a

centrifugation step. The final cell pellet was resuspended in RPMI 1640 plus 2% (v/v) FBS to a final concentration of 2×10^6 cells/ml. This cell suspension (50 μ l) was added to each well, and cell binding was allowed to occur for 10 min at room temperature. The unbound cells were removed and the wells were carefully washed twice with 100 μ l of RPMI 1640, plus 1% (v/v) FBS. In some assays, the plates were inverted and briefly centrifuged. Bound cells were quantified by treating the wells with 100 μ l of 1% (v/v) Nonidet P40 in water and reading fluorescence intensity in a fluorescence scanner (Titertek Fluoroskan II; Flow Labs) using 485 nm excitation and 530 nm emission wavelengths. Fluorescence per cell was calculated by measuring the fluorescence generated from a known number of cells. To test if the binding was E-selectin-specific, R1.1/E-selectin cells were incubated with the anti-(E-selectin) monoclonal antibody (IgG2b) BB11 [34] or a mouse IgG2b isotype control at 1 μ g/ml for 30 min at room temperature before their addition to the binding assay.

Carbohydrate composition analysis

The carbohydrate composition analysis was performed at the University of Georgia, Complex Carbohydrate Research Center, Athens, GA, U.S.A. Samples were prepared and analysed as previously described [35]. Briefly, methyl glycosides were made by methanolysis in 1 M HCl in methanol, followed by *N*-acetylation with pyridine and acetic anhydride (for the detection of amino sugars). The sample was then treated with Tri-Sil to generate trimethylsilyl glucosides. G.c. analysis of the trimethylsilyl glycosides was performed on an HP 5890 gas chromatograph using a Supelco DB1 fused silica capillary column. G.c./m.s. analysis was performed using an HP 5890 gas chromatograph coupled to an HP 5970 mass selective detector.

Digestion of X-LFA3IG and LFA3IG with neuraminidase

Sialic acid residues were removed from 100 μ g of protein with 0.015 unit of insoluble neuraminidase (*Clostridium perfringens*, type X-A from Sigma) attached to agarose beads in 50 mM sodium acetate, pH 5.0. After an overnight incubation at room temperature, the beads were removed by centrifugation and the digests were analysed by SDS/PAGE on a 4–20% gradient gel under non-reducing conditions. The digested material was subjected to Superose 6 gel-filtration chromatography in PBS before being analysed in the binding assay. As a control for the neuraminidase treatment, parallel samples were subjected to the same treatment in the absence of the enzyme.

Lentil lectin chromatography

Protein A–Sepharose-purified X-LFA3IG or LFA3IG was diluted with PBS to approximately 0.5 mg/ml and loaded on a lentil lectin–Sepharose (Pharmacia) column at 1 mg of protein/ml of resin at a flow rate of 1 column vol./h by gravity at 4 °C. The column was washed with 4 column vol. of PBS and subsequently treated with PBS containing 0.5 M *N*-acetylglucosamine and 0.5 M methyl α -D-glucopyranoside (both from Sigma). Fractions (0.5 column vol.) were collected and analysed for protein by measuring A_{280} . Fractions containing protein from the load, flowthrough and elution were pooled separately, quantified by A_{280} and analysed by SDS/PAGE stained with Coomassie Brilliant Blue.

Anti-Sle^x e.l.i.s.a.

LFA3IG, X-LFA3IG and BSA were adsorbed on to plastic (TiterTek 96-well plates) by incubating 50 μ l of protein (10 μ g/ml

in 50 mM NaHCO₃/Na₂CO₃, pH 9.2) per well overnight at 4 °C. The wells were blocked with 100 μ l of PBS containing 1% (v/v) BSA [blocking buffer (BB)] for at least 2 h. After removal of BB, 50 μ l of primary antibody (0.2 μ g/ml in BB; 50 μ l/well) was added to each well and incubated for 1.5–2 h at room temperature. (The anti-Sle^x antibody, CSlex1, was purchased from P. Terazaki at UCLA, Los Angeles, CA, U.S.A. and the anti-LFA3 antibody, 7A6, was a gift from P. Chisholm, Biogen Inc.) The wells were then washed six times with 100 μ l of BB. The secondary antibody [50 μ l of horseradish peroxidase-conjugated goat anti-mouse IgG(H&L) (Jackson ImmunoResearch Laboratories) diluted 1:5000 in BB] was added to each well and allowed to incubate for 30 min at room temperature. The wells were then washed five times with BB, once with PBS and developed as follows: 50 μ l of substrate buffer [5 ml of 100 mM sodium acetate, pH 4.9, with citric acid; 50 μ l of 42 mM 3,3',5,5'-tetramethylbenzidine (TMB) in dimethyl sulphoxide; 0.75 μ l of hydrogen peroxide] was added to each well, colour was allowed to develop for 2–5 min and then 50 μ l of 0.5 M H₂SO₄ was added to stop the reaction. The plate was read at 450 nm on a Molecular Devices microplate reader.

RESULTS AND DISCUSSION

Characterization of X-LFA3IG

With the goal of producing an easily purified secreted glycoprotein expressing sialylated fucosylated carbohydrate structures of the type known to be ligands for E-selectin, we supertransfected the plasmid pSAB152 coding for the recombinant fusion protein LFA3IG into CHO cells expressing the recombinant membrane-associated fucosyltransferase ELFT. Since methotrexate has been used to generate the stable ELFT cell line, we chose to use G418/neomycin for LFA3IG selection. The ELFT-expressing CHO cells were cotransfected with plasmids pFASV NEO and pSAB152 and selected for transfected cells with G418. Surviving cells were picked as single colonies and assayed for the production of X-LFA3IG using an LFA3-specific e.l.i.s.a. The highest-producing clone, which secreted approximately 0.5 mg/l X-LFA3IG, was picked for large-scale culture. The secreted X-LFA3IG was purified by Protein A–Sepharose affinity chromatography followed by gel filtration, which yielded product more than 95% pure. As a control, we used LFA3IG secreted from CHO cells and purified in a similar fashion.

In order to determine if LFA3IG was being modified by ELFT, we performed the following experiments. Figure 2(a) shows both purified X-LFA3IG (lanes 1 and 4) and LFA3IG (lanes 2 and 5) run under reducing and non-reducing conditions. X-LFA3IG appeared larger than the control protein LFA3IG in this gel system. *N*-Glycanase treatment of both proteins gave core polypeptides of the same size (results not shown). However, X-LFA3IG still appeared larger than LFA3IG after both preparations were treated with neuraminidase, indicating that the difference in mobility was caused by a modification in the N-linked carbohydrate structures unrelated to the extent of sialylation (results not shown). Since the control protein, LFA3IG, binds well to lentil lectin, we examined the behaviour of X-LFA3IG on this resin. We diluted the Protein A–Sepharose elution pools containing either X-LFA3IG or LFA3IG to approx. 0.5 mg/ml with PBS and passed the solutions through the lentil lectin–Sepharose column. The bound protein was then washed with PBS and subsequently eluted with a mixture of methyl glucoside and *N*-acetylglucosamine. We analysed the load, flowthrough and elution pools of both preparations by u.v.

Figure 2 Analysis of purified X-LFA3IG and LFA3IG by SDS/PAGE and lentil lectin chromatography

(a) Purified X-LFA3IG (lanes 1 and 4) and LFA3IG (lanes 2 and 5) run under reducing (lanes 1 and 2) and non-reducing (lanes 4 and 5) conditions on a 4–20% gradient SDS/PAGE. Lane 3 contains high-molecular-mass prestained molecular-mass markers. The bands were visualized with Coomassie Blue stain. (b) Load (lanes 1 and 3) and flowthrough (lanes 2 and 4) fractions of X-LFA3IG and the control protein, LFA3IG, chromatographed on lentil lectin–Sephacrose as described in the Experimental section.

Table 1 Carbohydrate composition of X-LFA3IG and LFA3IG (nmol of monosaccharide/nmol of protein)

The values are expressed as nmol of monosaccharide/nmol of protein and are the mean of two determinations. The last column contains the composition data obtained for X-LFA3IG expressed as a fraction of the levels measured for LFA3IG.

Monosaccharide	LFA3IG	X-LFA3IG	Ratio
Sialic acid	15.3 ± 3.7	14.3*	0.94
<i>N</i> -Acetylglucosamine	27.1 ± 2.2	22.7 ± 0.6	0.84
Galactose	20.1 ± 1.4	20.2 ± 0.6	1.05
Mannose	22.0 ± 3.3	19.7 ± 1.6	0.90
Fucose	5.3 ± 0.6	11.4 ± 1.9	2.15

* For sialic acid determination, only a single data point was obtained. In the other experiment, the sialic acid had degraded before composition analysis.

absorption and SDS/PAGE and found that, in contrast with the control protein LFA3IG, for which 65% of the loaded protein bound and could be eluted, only 8% of X-LFA3IG bound and could be recovered from the lectin column (Figure 2b). The majority (> 90%) of X-LFA3IG was in the flowthrough fractions. It is likely that the difference in binding between the two forms is due to a modification in carbohydrate structure caused by ELFT.

To determine the extent to which X-LFA3IG was modified with fucose, both LFA3IG and X-LFA3IG were subjected to carbohydrate composition analysis. To facilitate the interpretation of the carbohydrate composition data, we converted the raw data obtained in the analysis into nmol of monosaccharide/nmol of protein. As shown in Table 1, approx. 5 additional mol of fucose are added per mol of protein, suggesting that at least 50% of the eight potential N-linked glycosylation sites on each LFA3IG molecule are modified with an extra fucose residue. This large increase in fucose is consistent with the data in Figure 2(a) showing that most, if not all, of the X-LFA3IG appears to be modified. Whereas we measured minor changes (2–20%) in the amounts of sialic acid, glucosamine, galactose and mannose obtained in the composition analysis of X-LFA3IG, the most striking difference (> 100%) was obtained in the amount of fucose released from the protein. A number of different interpretations of this twofold increase in fucose on X-

LFA3IG are possible, the simplest being that ELFT adds fucose on to ultimate *N*-acetylglucosamine residues of the carbohydrate trees on the scaffold protein to create a structure like Sle^x (e.g. starting with NeuNAc α 2,3-Gal β 1,4-GlcNAc β 1,3-R and

going to NeuNAc α 2,3-Gal β 1,4-GlcNAc β 1,3-R). This interpretation is strengthened by the observation, that, in an e.l.i.s.a. format, an anti-Sle^x antibody (CSlex1) preferentially recognized X-LFA3IG over the control protein LFA3IG (Table 2). An anti-LFA3 antibody was used as a positive control for both LFA3-containing proteins. Thus the change in mobility on SDS/PAGE, the difference in lectin binding between X-LFA3IG and the control protein, the increased level of fucose associated with X-LFA3IG and the appearance of the epitope for CSlex1 are all entirely consistent with ELFT-induced modifications of the carbohydrate structure of the chosen scaffold protein. Furthermore, we find that these modifications do not impair secretion or purification of the scaffold protein.

Binding of E-Selectin to X-LFA3IG

To test if an ELFT-modified scaffold protein can be recognized by E-selectin, we immobilized both X-LFA3IG and the control protein LFA3IG on plastic and asked if anchorage-independent R1.1 cells transfected with full-length membrane-associated E-selectin (R1.1/E-selectin) would bind to the immobilized protein. The E-selectin-expressing cells bound well (approx. 20% of input cells) to the wells coated with X-LFA3IG, whereas only minimal binding (< 0.5% of input cells) was observed in the control wells coated with LFA3IG. We tested coating concentrations of X-LFA3IG of 0.6, 3.2, 6.4, 25.6 and 64.2 μ g/ml and found that binding reached a plateau when the protein was plated at 6.4 μ g/ml. Lower coating concentrations resulted in a reduction in cell binding, and increasing the coating concentration did not improve the number of cells bound to the wells (results not shown).

To test whether the binding we observed is E-selectin-specific, we performed several experiments. First, we compared the binding of the E-selectin-expressing R1.1 cells with the parental R1.1 line. Whereas good binding was again observed with the R1.1/E-selectin cells, the parental R1.1 cells failed to show any significant binding to the X-LFA3IG-coated wells (Table 3). Second, when we preincubated R1.1/E-selectin cells with the E-selectin-specific blocking monoclonal antibody BB11 or a control IgG2b, BB11 completely inhibited R1.1/E-selectin binding to X-LFA3IG, whereas the subclass-specific control monoclonal antibody had only a minimal effect on cell binding. Third, we tested if E-selectin binding to X-LFA3IG requires the presence of sialic acids as had previously been shown [18–21]. We treated a preparation of X-LFA3IG with neuraminidase and subsequently assayed it for R1.1/E-selectin cell binding and found that neuraminidase treatment of X-LFA3IG significantly reduces E-selectin-mediated cell binding (> 80%). Treating the control protein, LFA3IG, with neuraminidase has no effect on its ability to inhibit sheep erythrocyte rosetting, suggesting that the enzyme treatment does not significantly alter the structure of the scaffold protein (results not shown). Thus, as is true for other E-selectin ligands described, X-LFA3IG also requires sialic acid to function as an E-selectin ligand. These data strongly suggest that the fucosyltransferase, ELFT, is capable of creating E-selectin-specific carbohydrate-binding structures on a scaffold protein which can then be secreted and purified.

Once the E-selectin binding to X-LFA3IG was clearly established, we tested whether preincubation of R1.1/E-selectin

Table 2 Anti-Sle^x binding of LFA3IG fusion proteins

First, 96-well plates were coated with 10 µg/ml X-LFA3IG, LFA3IG or BSA. After the wells had been blocked, CSlex1 (anti-Sle^x monoclonal antibody) or 7A6 (anti-LFA3 monoclonal antibody) was added. The bound antibody was detected with a goat-anti-(mouse horseradish peroxidase) conjugate and visualized with TMB. The values are the mean of two determinations and are given in absorbance units at 450 nm.

Sample	C-Slex1	7A6
X-LFA3IG	0.775 ± 0.003	1.37 ± 0.02
LFA3IG	0.147 ± 0.001	1.39 ± 0.01
BSA	< 0.02	< 0.02

Table 3 Direct binding of E-selectin-transfected cells to X-LFA3IG

First, 96-well plates were coated with 10 µg/ml LFA3IG, X-LFA3IG or X-LFA3IG pretreated with neuraminidase. R1.1 or R1.1/E-Selectin cells (10⁵ cells/well), preincubated with no addition, a control antibody (IgG2b) or an E-selectin-specific antibody (BB11) were added and incubated for 10 min at room temperature. Cells bound were quantified as described in the Experimental section. Typically, 20% of the input R1.1/E-selectin cells bound to the wells coated with X-LFA3IG and were used as maximum number of cells bound. The relative number of R1.1 or R1.1/E-selectin cells bound to the wells is expressed as a percentage of maximum bound. N.D., not determined.

	R1.1 (%)	R1.1/E-selectin (%)
LFA3IG	< 0.1	2.2
X-LFA3IG	< 0.1	100
X-LFA3IG + neuraminidase	N.D.	15.5
X-LFA3IG (IgG2b control)	N.D.	88.9
X-LFA3IG (BB11 control)	N.D.	< 0.1

cells with X-LFA3IG could inhibit binding to plates coated with X-LFA3IG. We saw no significant inhibition of binding at concentrations of up to 320 µg/ml (approximately 3 µM) X-LFA3IG (results not shown). To determine if the soluble form of X-LFA3IG binds to R1.1/E-selectin cells, we incubated the cells with X-LFA3IG followed by a phycoerythrin-labelled goat anti-human IgG second antibody and then analysed them by fluorescence-activated cell sorting. Whereas we observed no staining in the X-LFA3IG sample, the R1.1/E-selectin cells incubated with BB11 and then stained with anti-murine IgG were positive. Although many explanations for the lack of X-LFA3IG binding to E-selectin-expressing cells are possible, we believe that the number of potential E-selectin-binding sites (e.g. valency) and possibly the type of E-selectin ligand displayed on a single X-LFA3IG molecule is not optimal for binding. It has been previously shown that valency can be directly related to the ability of a soluble receptor to block binding. Using the receptor/ligand pair LFA3 and CD2 present on sheep erythrocytes and Jurkat cells respectively, we and others [34,36] have shown that the potency of soluble LFA3 to inhibit erythrocyte rosetting with Jurkat cells is directly related to the number of LFA3 monomers present in the soluble multimer. In this assay, the dimers, trimers and tetramers exhibit a 15-, 150- and 430-fold increase in inhibition activity over monomeric LFA3. An increase in E-selectin ligand valency displayed on the soluble inhibitor might well allow it to compete better with the ligands immobilized on plastic or displayed on the surface of cells. Furthermore, if LFA3IG favours monolactosamine structures, this would lead to Sle^x when modified by ELFT, and,

if other related structures such as Sdile^x are better ligands for E-selectin than Sle^x, then perhaps X-LFA3IG does not display the optimal carbohydrate structure to be an effective inhibitor. We are currently examining alternative more highly glycosylated scaffold proteins with a high propensity to be modified with polylactosamine structures as well as multimeric forms of X-LFA3IG to make functional inhibitors.

In summary, we have shown that coexpression of a scaffold protein and a glycosyltransferase can result in a secreted readily purified reagent which can function as the ligand for an adhesion molecule. In this case the ligand was formed by using the fucosyltransferase, ELFT, to modify the carbohydrate moieties of LFA3IG with fucose to form a functional ligand for E-selectin.

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