Sialylated Complex-type N-Glycans Enhance the Signaling Activity of Soluble Intercellular Adhesion Molecule-1 in Mouse Astrocytes

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The best understood biological function of membrane-bound ICAM-1 is its involvement in leukocyte extravasation. ICAM-1 on the surface of vascular endothelial cells mediates firm adhesion and transendothelial migration of leukocytes by binding to the β2 integrins leukocyte function associated antigen-1 (LFA-1) and macrophage antigen-1 (Mac-1) (10, 11). Beyond its ability to provide adhesion sites for leukocytes, membrane-bound ICAM-1 behaves as a signal transducer, allowing brain endothelial cells and astrocytes to respond actively to leukocyte adhesion (12).

The biological functions of sICAM-1 are less well understood. Some studies (13–15) suggest that sICAM-1 is a competitive inhibitor of leukocyte adhesion to vascular endothelium, whereas others suggest that sICAM-1 has signaling functions. In this regard the following has been shown: (i) sICAM-1 enhances the production of the cytokines tumor necrosis factor-α, interferon-γ, and interleukin-6 during the mixed lymphocyte response (16), and (ii) mouse sICAM-1 induces the production of macrophage inflammatory protein-2 (MIP-2) in primary mouse astrocytes (9, 17).

Induction of MIP-2 production by sICAM-1 is likely to be involved in the inflammatory response to severe traumatic brain injury. The CXC chemokine MIP-2 is the functional analogue of human interleukin-8 (IL-8) in mice, and IL-8 and sICAM-1 are concomitantly elevated in CSF of patients with severe brain trauma (9). IL-8 appears to be produced within the brain, because its concentrations are higher in CSF than in serum. The elevated concentrations of IL-8 in CSF may be due to increased production of IL-8/MIP-2 by astrocytes exposed to sICAM-1. Increased IL-8 production after brain trauma may have dual consequences. IL-8 may regulate the extravasation of leukocytes into the injured brain and/or induce the production of nerve growth factor in astrocytes (18). Several lines of evidence indicate that nerve growth factor signaling facilitates the repair and reorganization of neural connections after neuronal injury (19). Taken together, induction of MIP-2/IL-8 production by sICAM-1 in astrocytes may play a direct role in the
Sialic Acid Contributes to Signaling by sICAM-1

regulation of leukocyte infiltration into the brain and an indirect role in promoting neuroregeneration after severe traumatic brain injury.

The signaling functions of sICAM-1 in astrocytes and in lymphocytes seem to be mediated by different receptors. Enhanced cytokine production during the mixed lymphocyte re- sponse is mediated by binding of sICAM-1 to LFA-1 (16). By contrast, signaling in mouse astrocytes seems to involve an as yet undefined sICAM-1 counter-receptor, because astrocytes do not express the known ICAM-1 ligands, LFA-1 and Mac-1 (20). Binding of human ICAM-1 to LFA-1 and Mac-1 was reported to be differentially affected by the glycosylation of ICAM-1 (21). Although binding to LFA-1 was not influenced by glycosylation, binding to Mac-1 was hindered if complex-type N-glycans were present on the third Ig domain of ICAM-1. The present study was aimed at elucidating how glycosylation may influence the LFA-1- and Mac-1-independent signaling function of sICAM-1 in mouse astrocytes.

For that purpose, several sICAM-1 glycosymes with specific alterations in glycosylation were expressed in different CHO cell lines differing in glycosylation, and their ability to induce MIP-2 production was assessed in astrocytes. Our results show that sICAM-1 from parental CHO cells carries mature, complex-type N-glycans and has potent signaling activity. By contrast, sICAM-1 deficient in either sialic acid, sialic acid and galactose, or containing only high mannose-type N-glycans had significantly less signaling activity in regard to MIP-2 production while retaining normal ability to bind LFA-1 on leukocytes. These results show that glycosylation of sICAM-1, and in particular sialylation and galactosylation of complex-type N-glycans, significantly enhances its ability to induce MIP-2 production in astrocytes.

EXPERIMENTAL PROCEDURES

Materials—The pcDNA3.1 (+) plasmid was from Invitrogen and Fu- GEINE 6 from Roche Applied Science. Geneticin (G 418) was obtained from Invitrogen, and kifunensine was from Toronto Research Chemi- cals Inc, North York, Ontario, Canada. Dulbecco’s modified Eagle’s medium and hypoxanthine/thymidine (HT supplement) were from Invitrogen; fetal bovine serum (FBS) was from Atlanta Biologicals, Norcross, GA; bovine fetuin (lyophilized, cell culture tested) and human fibroblasts (hFibROBs) were from Invitrogen, and MRC-5 were from ATCC, Manassas, VA. The SH-M-ICAM-1 cDNA was transferred into the pcDNA3.1 (+) plasmid containing the cDNA for tagged Form of Mouse ICAM-1—

Construction of an Expression Vector Encoding a Soluble, Epitope- tagged Form of Mouse ICAM-1—The plasmid containing the cDNA for mouse ICAM-1 (22, 23) was obtained from ATCC. The 12-amino acid HPC4 epitope tag (24) was inserted after the 5th Ig domain (G 468) of ICAM-1, immediately followed by a stop codon. The resulting construct encodes a soluble HPC4 epitope-tagged form of mouse ICAM-1 lacking the transmembrane domain and the cytoplasmic tail (SH-M-ICAM-1). The SH-M-ICAM-1 cDNA was transferred into the pcDNA3.1 (+) plasmid by using FuGENE 6, according to the manufacturer’s instructions, and cultured in Dulbecco’s modified Eagle’s me- dium containing 10% FBS (addition of 1/4 hypoxanthine/thymidine for the CHO cells) and 600 μg/ml genetricin to select for stably transfected cells. After 8 weeks of culturing in medium containing genetricin, the cells were cultured in the same medium without genetricin, and the culture medium was harvested every 3 days. One clone with particu- larly high protein expression was selected from the stably transfected HEK 293 cells. The stably transfected CHO cell line was also cultured in the presence of 20 μg kifunensine for 3 days, and then the culture medium was harvested. Conditioned culture media were immediately centrifuged at 2000 × g for 10 min to remove cellular debris and stored at 4 °C.

Purification of HPC4 Epitope-tagged sICAM-1—Conditioned culture medium containing HPC4 epitope-tagged sICAM-1 was incubated with a monoclonal antibody to the extracellular region of ICAM-1 coupled to Ultralink (5 mg of antibody/ml of beads, 2.5 μl of beads/ml of medium) overnight at 4 °C with slight agitation. The beads were collected by centrifugation at 1000 × g for 10 min, transferred to a chromatography column, and washed with 50 bed volumes of 20 mM Tris-HCl, pH 7.5, 0.1 m NaCl, 2 mM CaCl2. Bound protein was eluted with 5 mM EDTA in 20 mM Tris-HCl, pH 7.5, 0.1 m NaCl, and then dialyzed against 20 mM Tris-HCl, pH 7.5, 0.15 mM NaCl. sICAM-1 yields ranged from 0.1 to 1 mg/1 of conditioned culture medium.

Concentration Assessment—Absorbance of purified sICAM-1 from CHO cells was measured at 280 nm after concentrating the sample 10-fold with a Centricon 30 concentrator (Millipore, Billerica, MA). For each sample, the molar absorption coefficient (ε) was 42200 M–1 cm–1. Concentrations were calculated using the formula given in Ref. 25 for protein standards, broad range (Bio-Rad), were used as molecular weight markers. Blots were blocked with 5% nonfat dried milk in 20 mM Tris-HCl, pH 7.5, 0.3 m NaCl (TBS) for 90 min or overnight and then incubated with HPC4 antibody (10 μg/ml in TBS with 2 mM CaCl2 (TBS-CaCl2) and 1% bovine serum albumin). After washing three times with TBS-CaCl2, the blots were incubated with peroxidase-labeled secondary antibody (goat anti-mouse IgG, Kirkegaard & Perry Laboratories, Gaithersburg, MD) diluted 1:5000 in TBS-CaCl2 with 1% bovine serum albumin for 1 h at room temperature. After washing three times with TBS-CaCl2, immuno- noreactive bands were detected by enhanced chemiluminescence on BioMax film.

Isoelectric Focusing under Denaturing Conditions—Isoelectric focusing (IEF) was performed following the protocol given in Ref. 26 with some modifications. For the gradient, equal amounts of Pharmalyte pH 2.5–7.5 and Pharmalyte pH 3–10 were used, and the gels (10 × 7 cm × 1 mm) were cast immediately before use. As pH markers, amyloglucosi- dase (pI 4.0), carbonic anhydrase (pI 5.1), and bovine serum albumin (pI 5.6) were used (Sigma). The samples were denatured with 5% β-mercaptoethanol, 9.5 m urea, 2% Triton X-100 and focused at 100 V for 1 h, at 200 V for 1 h, and at 500 V overnight on ice. The gel was then washed five times in 50% methanol, 1% SDS, 5 mM Tris-HCl, pH 8.0, for 10 min. Subsequently, proteins were transferred to nitrocellulose and stained with Ponceau S to detect the positions of the pH markers and judge the quality of protein transfer, followed by HPC4 immunostaining as described above.

Glycanase Treatment—For PNGase F treatment, sICAM-1 samples were first denatured by boiling in 0.5% SDS and 1% β-mercaptoethanol for 10 min and then incubated with the enzyme (1000 units of enzyme/1 mg of sICAM-1) in 20 mM sodium phosphate, pH 7.5, 0.1% SDS, 0.1% Nonidet P-40 at 37 °C overnight. For Endo H treatment, the samples were denatured by boiling in 0.2% SDS and 4% β-mercaptoethanol for 10 min and treated with the enzyme (7 milliunits of enzyme/1 μg of sICAM-1) at 37 °C for 24 h. For neuraminidase treatment, the samples were incubated with the enzyme (10 milliunits of enzyme/1 μg of sICAM-1) in a reaction containing 50 mM sodium acetate, pH 5.5, and the neuraminidase mixture (complete mini EDTA-free, Roche Applied Science) for 1 h at 37 °C.

Carbohydrate Compositional Analysis—Recombinant sICAM-1 from CHO cells (14.4 μg of total protein) was divided into two equal fractions and dried in vacuo in 800-μl Reactival glass tubes. Each sample was reconstituted with 400 μl of water to which were added 0.8 μl of 50% trichlo- roacetic acid, 25% phosphotungstic acid reagent, a glycoprotein precipi- tant (27). The sICAM-1 was precipitated through incubation at −20 °C for 10 min and then the sample was centrifuged for 10 min at 4000 × g, 4 °C. The supernatant was discarded, and the pellet was washed
twice with cold acetone followed by re-centrifugation as above between each wash. The final sample was dried in vacuo, dissolved in fresh 2 M trifluoroacetic acid, and heated in a capped tube at 100 °C for 4 h. The hydrolyzed material was dried and resuspended in water for analysis by chromatography on a Dionex DX 60 600 HPAEC-PAD using a CarboPac PA-1 column eluted with 18 mM NaOH (28). Control analyses as above were conducted with 10 μg each of bovine fetuin (Sigma) or human apotransferrin (Calbiochem).

**Preparation of Primary Mouse Astrocyte Cultures**—Astrocytes were isolated from the brains of newborn C57BL/6 mice as described previously (9, 29) and cultured in polystyrene-coated tissue culture flasks (75 cm²) for 7 days. Astrocytes were then trypsinized, plated in 24-well tissue culture plates (1 × 10⁶ cells/well; Falcon, BD Biosciences), and cultured for another 6 days in Dulbecco’s modified Eagle’s medium (with high glucose and sodium pyruvate; Invitrogen) containing 10% low endotoxin FBS and 0.05% gentamycin (Invitrogen).

**Culture Stimulation with sICAM-1 and Quantification of MIP-2**—Sixteen hours before stimulation, the culture medium was replaced with medium containing 1% FBS. Astrocytes were then treated with either sICAM-1 or with the control glycoproteins bovine fetuin, hAGP, or hsVCAM-1 (concentrations indicated in the figure legends) in medium with 1% FBS. Astrocytes treated with medium alone were used as negative controls. Twenty four hours later, the culture supernatants were collected, centrifuged (2000 × g, 4 °C, 10 min), and stored at −20 °C until analysis. Mouse MIP-2 was quantified by enzyme-linked immunosorbent assay (standard curve, 0–500 pg/ml; lower detection limit, 1.5 pg/ml; R & D Systems).

**Ligand Coating of Fluorescent Beads**—Coupling of streptavidin to the fluorescent beads (carboxylate-modified TransFluoSpheres, 488/645 nm, 1.0 μm diameter; Molecular Probes, Eugene, OR) was performed as described (30). The HPC4 antibody was biotinylated using EZ-link NHS-LC-Biotin (Pierce) according to the instructions given by the manufacturer. For ligand coating, the streptavidin-coated beads (11 nl) were incubated with biotinylated HPC4 antibody (67 μg/ml) in 0.5 ml of coupling buffer (20 mM Tris-HCl, pH 7.4, 0.9% NaCl, 2 mM CaCl₂, 0.5% bovine serum albumin, 0.02% NaN₃) at 37 °C for 2 h. Subsequently, the beads were washed with coupling buffer and incubated either with different glycoforms of sICAM-1 (170 nM) in 0.55 ml of coupling buffer or with coupling buffer alone (negative control) at 4 °C overnight. The ligand-coated beads were then washed and stored in 100 μl of coupling buffer at 4 °C until use.

**Fluorescent Beads Adhesion Assay**—The fluorescent beads adhesion assay as used here to measure binding of mouse ICAM-1 glycoforms to human LFA-1 (31) was based on the assay described by Geijtenbeek et al. (30). The human lymphocyte cells HSB-2 (ATCC) were resuspended in TSA (20 mM Tris-HCl, pH 7.4, 0.9% NaCl, 2 mM CaCl₂, 2 mM MnCl₂, 0.5% bovine serum albumin; 2.5 × 10⁶ cells/ml). Aliquots of 125,000 cells were incubated with either TSA (vehicle control), the LFA-1-blocking antibody TS1/22 (40 μg/ml; Pierce), or an isotype-matched control antibody (clone 107.3, 40 μg/ml; BD Biosciences) at 37 °C for 30 min. The ligand-coated beads (20 beads/cell) were added, and the various cell bead suspensions were incubated at 37 °C for 30 min. Cells were washed and resuspended in 0.5 ml of ice-cold TSA. Adhesion of the beads to HSB-2 cells was assessed by flow cytometry using a FACSCalibur (BD Biosciences).

**RESULTS**

sICAM-1 Expressed in CHO Cells Is a More Potent Inducer of MIP-2 Production by Astrocytes than sICAM-1 Expressed in HEK 293 Cells—In previous studies on the ability of sICAM-1 to induce MIP-2 production, we used a commercially available mouse sICAM-1 (9, 17). However, to investigate how glycosylation may contribute to the signaling function of sICAM-1, a sICAM-1 whose glycosylation can be specifically modified was required. We therefore stably transfected CHO and HEK 293 cells with a recombinant plasmid encoding the extracellular part of mouse ICAM-1 with a C-terminal 12-amino acid HPC4 epitope tag (24). The recombinant glycoproteins in culture supernatants were immunoaffinity-purified by chromatography on immobilized HPC4 monoclonal antibody, and their ability to induce MIP-2 production was assessed in primary mouse astrocytes. As shown in Fig. 1, sICAM-1 expressed in CHO cells was significantly more active than sICAM-1 expressed in HEK 293 cells.

**Sialylation and Galactosylation of sICAM-1 Expressed in CHO Cells Greatly Enhance Its Ability to Induce MIP-2 Production**—Glycoproteins expressed in CHO and in HEK 293 cells have several differences in N-glycosylation, including the degree and linkage of sialylation and the synthesis of the lacto-N-acetyllactosamine (GalNAcβ1,3Galβ1,4GlcNAc-R and of sulfated N-glycans by HEK 293 cells but not CHO cells (32, 33). Therefore, we hypothesized that N-glycan structures may influence the signaling activity of sICAM-1.

To test this hypothesis, we generated various sICAM-1 glycoforms carrying truncated versions of the CHO cell-specific glycans, and we assessed their signaling activity in mouse astrocytes. sICAM-1 was expressed in stably transfected Lec2, Lec6, and Lec1 glycosylation mutants of CHO cells (Table I). The Lec2 mutant has a deletion mutation in the CMP-sialic acid transporter resulting in reduced targeting of the trans-
porter to the Golgi (34). This defect in CMP-sialic acid transport results in N- and O-glycans with a greater than 90% decrease in sialic acid content (35). The Lec8 mutant has a deletion mutation in the UDP-galactose transporter resulting in a truncated protein with a greatly reduced ability to translocate UDP-Gal into the lumen of the Golgi apparatus (36). Thus, Lec8 cells generate nongalactosylated and nonsialylated N- and O-glycans (37). The Lec1 mutant has a single insertion mutation that generates an inactive, truncated GlcNac transferase I and hence stops processing N-glycans at the Man$_5$GlcNAc$_2$-Asn stage (38, 39). Therefore, Lec1 cells lack sialylated and galactosylated N-glycans of the complex type, whereas their O-glycans have the normal amount of sialic acid and galactose. To control for potential clonal differences, sICAM-1 was generated by culturing the stably transfected CHO cell line in the presence of the -mannosidase I inhibitor kifunensine. Kifunensine stops the processing of N-glycans at the Man$_5$GlcNAc$_2$-Asn stage (40).

All five sICAM-1 glycoforms induced MIP-2 production in a dose-dependent fashion, but their signaling activity varied considerably (Fig. 2A). Fully glycosylated sICAM-1 from CHO cells was by far the most active glycoprotein. At the highest concentration used (37 nM), it increased MIP-2 production 150-fold whereas their isoelectric points of sICAM-1 from Lec2, and Lec8 cells were Endo H-resistant. These results indicate that the major N-glycans of sICAM-1 from CHO, Lec2, and Lec8 cells were Endo H resistant. These results indicate that the major N-glycans of sICAM-1 from CHO, Lec2, and Lec8 cells are of the complex type.

The Sialic Acid and Galactose Residues That Are Critical for MIP-2 Induction by sICAM-1 Expressed in CHO Cells Reside on Complex-type N-Glycans—To gain further insight into the glycan structures that enhance the signaling activity of sICAM-1, we analyzed the glycans of the five sICAM-1 glycoforms. The sICAM-1 sequence has nine predicted N-glycosylation sites and seven predicted O-glycosylation sites (45). In SDS-PAGE, the five sICAM-1 glycoforms displayed the expected size differences, with sizes ranging from ~80 to 115 kDa (Fig. 3, A and B). However, selective removal of N-glycans by PNGase F reduced all apparent molecular masses to ~55 kDa, which is close to the predicted molecular mass of the sICAM-1 polypeptide of 50 kDa (Fig. 3A). These data indicate that the major post-translational modification of sICAM-1 is N-glycosylation. To address whether the N-glycans are complex-type or high mannose/hybrid-type N-glycans, we treated the recombinant glycoproteins with Endo H, which cleaves high mannose/hybrid-type but not complex-type N-glycans. Endo H completely cleaved the N-glycans of sICAM-1 expressed in Lec1 cells and kifunensine-treated CHO cells, confirming that they are solely of the high mannose type (Fig. 3B), whereas sICAM-1 from other cells was unaffected. As seen in Fig. 3B, a minor low molecular weight fraction of sICAM-1 expressed in CHO, Lec2, and Lec8 cells was cleaved by Endo H as well, and this probably represents incompletely processed glycoproteins. However, the major high molecular weight fractions of the sICAM-1 glycoforms from CHO, Lec2, and Lec8 cells were Endo H-resistant. These results indicate that the major N-glycans of sICAM-1 from CHO, Lec2, and Lec8 cells are of the complex type.

The degree of sialylation and positioning of sialic acid on N- and/or O-glycans of sICAM-1 were examined by isoelectric focusing. sICAM-1 from Lec2, Lec8, Lec1, and kifunensine-treated CHO cells displayed an isoelectric point of about pH 5.9 (Fig. 3C). The similar isoelectric points of sICAM-1 from Lec2, Lec8, Lec1, and kifunensine-treated CHO cells suggest that in the absence of the sialic acid residues of complex-type N-glycans, there are relatively few, if any, charged groups resulting from post-translational modifications of sICAM-1. Carbohydrate compositional analysis showed that the most abundant monosaccharides present on sICAM-1 expressed in CHO cells are N-acetylgalactosamine, galactose, and mannose, confirming the predominance of complex-type N-glycans (Table II). The presence of 1 mol of fucose per 3 mol of mannose suggests that the N-glycans are probably core α6-fucosylated, as usually seen for CHO cell-derived glycoproteins. The least abundant monosaccharide detected was GalNAc, which is a common residue found in mucin-type O-glycans, where the GalNAc is directly linked to serine or threonine. CHO cells are unable to add GalNAc to complex-type N-glycans (32). Hence, the low amount of GalNAc in sICAM-1 from CHO cells suggests the presence of a small number of O-glycans (possibly 2–3 O-glycans/mol).
Although each of these putative O-glycans may contain sialic acid, their total contribution of charged groups appears to be too low to be detected by isoelectric focusing.

sICAM-1 glycoforms from CHO and sICAM-1 from HEK 293 cells had isoelectric points below pH 3.6 and were therefore not visible on the IEF gel (Fig. 3, C and D). A similarly low pI of 2.8–3.8 was reported previously (44) for hAGP, which is similar to sICAM-1 with regard to the pI predicted for the unglycosylated protein (hAGP, pI of 5.00; mouse sICAM-1, pI of 4.95 (ProtParam tool, www.expasy.org)) and the number and type of N-glycans. After neuraminidase treatment, sICAM-1 from CHO and HEK 293 cells assumed the same apparent pI as sICAM-1 from Lec2 cells, showing the following: (i) the acidity of sICAM-1 from CHO and HEK 293 cells is due to the presence of sialic acid, and (ii) with respect to net charge, sICAM-1 from Lec2 cells is identical to desialylated sICAM-1 from CHO cells (Fig. 3D). Taken together, these results show that the predominant post-translational modifications of sICAM-1 expressed in CHO cells are heavily sialylated complex-type N-glycans. Even though there is sialic acid may be located on O-glycans, this seems not to be functionally relevant, because sICAM-1 from Lec1 cells and kifunensine-treated CHO cells, which carry truncated N-glycans but would have normally glycosylated O-glycans (Table I), have the lowest MIP-2 inducing activity. Thus, we conclude that the sialic acid and galactose residues that are critical for MIP-2 induction reside on complex-type N-glycans.

The sICAM-1 Glycoforms Deficient in Sialic Acid and Galactose Retain Full Ability to Bind LFA-1 on Lymphocytes—The above results demonstrate that sICAM-1 expressed in CHO cells has strong signaling activity if the glycoprotein has sialylated and galactosylated complex-type N-glycans. However, to control for the possibility that the observed differences in activities of different sICAM-1 glycoforms may be due to protein conformational changes rather than to differences in glycosylation, we assessed the biological activity of the five sICAM-1 glycoforms with regard to binding to LFA-1 on lymphocytes. Binding of human ICAM-1 to LFA-1 is known to depend on conformation (46, 47) but not on glycosylation of ICAM-1 (21, 48). sICAM-1 from CHO, Lec2, Lec8, Lec1, and kifunensine-treated CHO cells was immobilized on fluorescent beads, and binding to activated LFA-1 on the T-lymphocyte cell line HSB-2 (49, 50) was measured by FACS analysis (Fig. 4). All five sICAM-1 glycoforms bound readily to HSB-2 cells, and binding was abolished by preincubation of the cells with a blocking anti-LFA-1 antibody. These data show that the sICAM-1 glycoforms deficient in sialic acid and galactose retain full ability to bind LFA-1 on lymphocytes.
coforms carrying truncated N-glycans retain the ability to bind LFA-1 and lack major changes of protein conformation. Therefore, incomplete glycosylation of mouse sICAM-1 specifically impairs its ability to induce MIP-2 production, while not affecting its interactions with LFA-1.

**DISCUSSION**

sICAM-1 is strongly elevated in the cerebrospinal fluid of patients with severe brain trauma (6), and recombinant mouse sICAM-1 induces production of the CXC chemokine MIP-2 in mouse astrocytes (9). Because neither of the two known integrin ligands of ICAM-1 (LFA-1, Mac-1) is expressed on astrocytes, this novel signaling function of sICAM-1 is likely to be mediated by an as yet unknown counter-receptor. Because complex-type N-glycans hinder binding of human ICAM-1 to Mac-1 while not affecting binding to LFA-1 (21), we considered whether glycosylation of mouse sICAM-1 could influence its signaling function in mouse astrocytes.

To explore this possibility, we first analyzed the activity of sICAM-1 expressed in two different mammalian cell lines differing in glycosylation profiles. sICAM-1 expressed in CHO cells was a much more potent inducer of MIP-2 production than sICAM-1 expressed in HEK 293 cells. This initial discovery supports the possibility of a glycosylation-dependent regulation...
of sICAM-1 function. Such glycosylation-dependent regulation has been seen for other glycoproteins derived from CHO versus HEK 293 cells. For example, human thyrotropin was six times more active if expressed in HEK 293 cells than if expressed in CHO-K1 cells, and this difference was attributed to an inhibitory effect of terminal sialic acid on CHO-K1-derived human thyrotropin (33). CHO cells synthesize more heavily sialylated glycans with sialic acid occurring in the α2,3-linkage only (51), whereas HEK 293 cells add sialic acid in both α2,3- and α2,6-linkages and also produce sulfated N-linked carbohydrates (33). Another study showed that laciDNaC-based glycans with the sequence GalNacβ4GlcNAc-R are essential for the biological function of human glycodelin and are only synthesized if the protein is expressed in HEK 293 cells (32). Most interestingly, we were unable to detect significant amounts of either sulfated glycans or glycans containing the laciDNaC epitope on sICAM-1 derived from HEK 293 cells. All significant changes on recombinant sICAM-1, as assessed by isoelectric focusing, were removed by neuraminidase treatment. Furthermore, we could not detect the laciDNaC epitope on desialylated sICAM-1 from CHO cells using Western blot and a specific monoclonal antibody (52) (data not shown). Thus, we hypothesize that differential sialylation of sICAM-1 from CHO cells versus HEK 293 cells is at least partly responsible for the differential signaling activity.

This interpretation is consistent with recent studies (53) on the site-specific glycosylation of human sICAM-1 expressed in either CHO or HEK 293 cells, as determined by electrospray ionization mass spectrometry. Both glycoforms of human sICAM-1 were found to carry mostly biantennary, triantennary, and tetraantennary complex-type N-glycans. Neither laciDNaC nor sulfate residues were found on human sICAM-1 from HEK 293 cells. The most prominent difference between human sICAM-1 from CHO and HEK 293 cells was a more complete sialylation of glycans on the CHO cell-derived glycoprotein (53). This seems also to hold true for the recombent mouse sICAM-1 described here, because its apparent molecular weight is higher if expressed in CHO cells, and this size difference disappears after neuraminidase treatment (Fig. 3D). However, CHO cells and HEK 293 cells differ in multiple ways (e.g. organ and organism of origin, protein expression level, and expression of glycosyltransferases), and hence alteration of differing biological activities to a specific difference in glycosylation is not possible.

To study the role of glycosylation of sICAM-1 for its signaling function more closely, we chose to modify specifically the glycosylation of sICAM-1 from CHO cells using the Lec2, Lec8, and Lec1 mutants of CHO cells, as well as the α-mannosidase I inhibitor kifunensine. Compared with fully glycosylated sICAM-1 from CHO cells, sialic acid-deficient sICAM-1 from Lec2 cells displayed a 3-fold reduced capacity to induce MIP-2 production in mouse astrocytes. Fully glycosylated sICAM-1 was found to be very acidic, displaying a pI below 3.6, but to assume the same pI as sICAM-1 from Lec2 cells after neuraminidase treatment. These data show that sialylation strongly enhances the ability of sICAM-1 to induce MIP-2 production.

There are only a few previous reports on sialylation being unfavorable to signaling activities of glycoconjugates. Most interestingly, one of these studies also regards a signaling process in primary mouse astrocytes. The polysialoganglioside GT1b was found to suppress the induction of major histocompatibility complex I molecules by interferon-γ in mouse astrocytes, and this suppressive action was critically dependent on the presence of sialic acid in the ganglioside (54). Another study (55) showed that proper sialylation of human chorion gonadotropin is necessary for optimal in vitro bioactivity.

Our results suggest that in addition to sialylation, galactosylation also promotes the ability of sICAM-1 to induce MIP-2. The activity of sICAM-1 from Lec2 cells was higher than that of sICAM-1 derived from either Lec8 or Lec1 cells. Galactose may directly contribute to the ability of sICAM-1 to induce MIP-2 production, based on what has been observed for other glycoproteins. For example, galactose residues are directly involved in Fringe-mediated inhibition of Jagged1-induced Notch signaling (56). Another possibility to explain the enhanced activity of sICAM-1 having terminal galactose over sICAM-1 having terminal GlcNAc or high mannose-type N-glycans could be the presence of a small but functionally significant degree of sialylation in Lec2 cells. These cells are slightly "leaky" in regard to sialylation and could add small levels of sialic acid to N-glycans of endogenous or recombinant glycoproteins (35). However, we could not detect a difference in pI between sICAM-1 from Lec2 and from Lec8 cells (Fig. 3C), and we did not detect a change in pI after neuraminidase treatment of sICAM-1 from Lec2 cells (data not shown). These results indicate that if some sialic acid is present in sICAM-1 derived from Lec2 cells, it is too low to be detected by isoelectric focusing. It is therefore more likely that the higher signaling activity of sICAM-1 from Lec2 cells compared with sICAM-1 from Lec8 and Lec1 cells is due to the presence of galactose than to some residual sialic acid.

The similar MIP-2 responses elicited by sICAM-1 from Lec8 and Lec1 cells suggest that neither terminal GlcNAc nor Man residues in N-glycans of sICAM-1 influence MIP-2 induction. The results also suggest that mature, sialylated O-glycans, which may be present on sICAM-1 from Lec1 cells but not on sICAM-1 from Lec8 cells, do not influence MIP-2 induction (Table I). Compared with sICAM-1 from Lec1 cells, signaling activity of sICAM-1 from kifunensine-treated CHO cells was further reduced. Even though both Lec1- and kifunensine-treated CHO cells solely produce high mannose-type N-glycans, it is known that the N-glycans from Lec1 cells are primarily Man₉GlcNAc₂Asn (39), whereas the glycans generated by kifunensine-treated cells are larger and primarily Man₉GlcNAc₂Asn (40). Hence, the present data suggest that mature complex-type N-glycans, rather than either large or small sized high mannose-type N-glycans, promote MIP-2 induction by sICAM-1.

Despite the large differences in signaling activity, the five sICAM-1 glycoforms were found to have similar abilities to bind to LFA-1 on lymphocytes. Because binding to LFA-1 critically depends on the conformation of ICAM-1, this finding shows that the five sICAM-1 glycoforms do not grossly differ in conformation. Furthermore, the results indicate that glycosylation has no major impact on binding of mouse ICAM-1 to LFA-1. This finding expands earlier data showing that treat-
ment with neuraminidase or the α-mannosidase I inhibitor deoxymannojirimycin did not alter binding of human ICAM-1 present on transfected L-cells to immobilized LFA-1 (21). In contrast to human ICAM-1, mouse ICAM-1 has a predicted N-glycosylation site in the first Ig domain (Asn-47), in close vicinity to the LFA-1-binding site. Whether the slightly reduced LFA-1 binding of fully glycosylated sICAM-1 from CHO cells compared with the sICAM-1 glycoforms with truncated N-glycans may be due to steric hindrance by sialylated N-glycans at Asn-47 cannot be decided from the present data, because we cannot exclude the possibility that the coating density of sICAM-1 from CHO cells may have been slightly lower.

Taken together, our results demonstrate that glycosylation of mouse sICAM-1, in particular sialylation and galactosylation of complex-type N-glycans, significantly enhances its ability to induce MIP-2 production in astrocytes but does not alter its binding to LFA-1 on lymphocytes. This raises the intriguing possibility that MIP-2 induction by sICAM-1 in vivo could be specifically regulated by subtle differences in glycosylation. Future studies should be directed to compare the glycosylation of sICAM-1 expressed in CHO cells to the glycosylation of sICAM-1 synthesized in different mouse tissues under different pathophysiological conditions. Glycosylation of sICAM-1 may be altered depending on the cell type expressing sICAM-1 and/or on the disease state. The signaling function of sICAM-1 may hence be favored in a specific cell type and/or under specific pathological conditions.

On the molecular level, sialylation and galactosylation of sICAM-1 may contribute to MIP-2 induction in various ways. A putative sICAM-1 counter-receptor could directly interact with the protein and glycan moieties of sICAM-1, similar to what has been shown for interactions of P-selectin with its ligand PSGL-1 (P-selectin glycoprotein ligand-1) (57, 58). Alternatively, the presence of sialic acid and galactose could stabilize a defined protein conformation that is favored by the receptor. Such a stabilizing effect by sialic acid on protein structure has been observed for bovine lactoferrin (59). Finally, the hydrophilic sialic acid-containing N-glycans may lead to exposure of hydrophobic protein domains thereby favoring the formation of sICAM-1 dimers through hydrophobic interactions. Binding of dimeric sICAM-1 to the unknown counter-receptor may occur with increased avidity and lead to receptor cross-linking and subsequent signal transduction. Future studies should be directed to define the sialylated glycan(s) required for sICAM-1 to signal and the mechanism by which sialylated glycans on sICAM-1 modulate MIP-2 induction in cultured astrocytes.

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Fig. 4. The sICAM-1 glycoforms deficient in sialic acid and galactose retain full ability to bind LFA-1 on lymphocytes. sICAM-1 from CHO, Lec2, Lec8, Lec1, and kifunensine-treated (+kif) CHO cells was immobilized on streptavidin coated fluorescent beads using biotylated HPC4 antibody. Beads coated with biotylated HPC4 antibody but no sICAM-1 were used as negative controls. The HSB-2 lymphocytes were either left untreated (vehicle control) or were preincubated with a blocking anti-LFA-1 monoclonal antibody (TS1/22) or an isotype-matched control antibody. The percentage of HSB-2 cells binding ICAM-1-coated fluorescent beads was measured by FACS analysis. The data are presented as average ± S.D. from three independent experiments.

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