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Significance of NF-κB/GATA Axis in Tumor Necrosis Factor-α-induced Expression of 6-Sulfated Cell Recognition Glycans in Human T-lymphocytes*

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Sulfated glycans play critical roles in various cell recognition events among leukocytes. The 6-sulfated lactosamine glycans in particular have been widely noted for their importance because they are involved in cell recognition events mediated by cell-adhesion molecules such as selectins and sialic acid-recognizing molecules such as siglecs and also in the activation of CD44 in binding to extracellular matrix hyaluronate. A pro-inflammatory cytokine, tumor necrosis factor-α, induces expression of 6-sulfated glycans on human leukocytes. Here we report that the transcription of the GlcNAc6ST-1 gene, the gene encoding a sulfotransferase for 6-sulfated glycan synthesis, is induced in human T-lymphoid cells through tandem NF-κB and GATA motifs in its 5′-regulatory region. Results of our reporter assays, immunoprecipitation, and chromatin immunoprecipitation analyses indicated that GATA-3 and/or GATA-2, but not GATA-1, associates with NF-κB in a transcription factor complex on the 5′-regulatory region of the gene and acts synergistically with NF-κB in triggering GlcNAc6ST-1 transcription. Recently, a skin-homing subset of helper memory T cells exhibiting the Th2 marker CCR4 was shown to specifically express 6-sulfated glycans. The transactivation mechanism described here suggested that GlcNAc6ST-1 transcription is coordinated with the NF-κB/GATA-3 axis, which is known to figure heavily in Th2 cell differentiation. In line with this, *in vitro* differentiation of human T cells to Th2 cells was found to significantly induce GlcNAc6ST-1 transcription and 6-sulfated glycan expression.

Sulfation of cell surface glycoconjugates is implicated in various cell-to-cell interactions. The sulfation of lactosamine and polylactosamines in cell surface glycoproteins has especially attracted the attention of researchers (1–3). Sulfation of polylactosamine occurs at its GlcNAc and galactose residues. As sulfation at the C-6′ position of the galactose residue in lactosamine is postulated to require prior 6-sulfation at the GlcNAc residue (4), the sulfation of the C-6 position of GlcNAc is considered to be a *de facto* rate-limiting step in polylactosamine sulfation. Sulfation at the C-6 position of GlcNAc is involved in several important biological recognition phenomena including selectin-mediated cell adhesion (5–10), cell-to-cell interaction through siglecs (11–15), activation of CD44-mediated cellular interaction (16–18), and dendritic cell function (19–21).

Sulfation at the C-6 position of GlcNAc in glycoproteins in leukocytes is catalyzed by enzymes in the GlcNAcβ:6-*O*-sulfotransferase family, and the first gene cloned for this enzyme family was GlcNAc6ST-1 (22). GlcNAc6ST-1 is known to be involved in the synthesis of L-selectin ligands (6, 9, 23) and induced by inflammatory cytokines such as IL-1β[2] in endothelial cells (6). GlcNAc6ST-1 is proposed also to play a major role in the activation of CD44 in TNF-α-activated monocytes (16). The enzyme synthesizes 6-sulfated lactosamine in *N*-glycans carried by CD44 upon TNF-α stimulation, and this carbohydrate modification of CD44 activates its hyaluronate binding activity (16, 17). Inhibition of sulfation abrogates TNF-α-induced CD44 activation (18). Transcriptional regulation of sulfation at the C-6 position of GlcNAc, however, is not well studied to date. In view of our recent finding that a subset of T cells exhibiting a Th2 marker express 6-sulfated glycans (10), we attempted to clarify transcriptional regulation of GlcNAc6ST-1, which is involved in the induction of 6-sulfated glycan expression in human T-lymphocytes.

EXPERIMENTAL PROCEDURES

Cells, Antibodies, and Flow-cytometric Analysis—Human cell lines (Jurkat and HL-60) were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal calf serum. Monoclonal anti-6-sulfo-*N*-acetyllactosamine (LacNAc) antibody (AG107, murine IgM) and anti-sialyl-6-sulfo-LacNAc antibody (G72, murine IgM) were prepared as described previously (5, 22). AG107 is reactive to 6-sulfo-LacNAc, but not to non-sulfated LacNAc. G72 is specific to sialyl-6-sulfo-LacNAc and does not react to non-sulfated sialyl LacNAc. Anti-GATA-1 (H-200, sc-13053), anti-GATA-2 (H-116, sc-9008), anti-GATA-3 (H-48, sc-9009), anti-p65 (A, sc-109), and anti-p50 (H-119, sc-7178) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

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[2] The abbreviations used are: IL, interleukin; TNF-α, tumor necrosis factor-α; LacNAc, *N*-acetyllactosamine; siRNA, small interfering RNA.

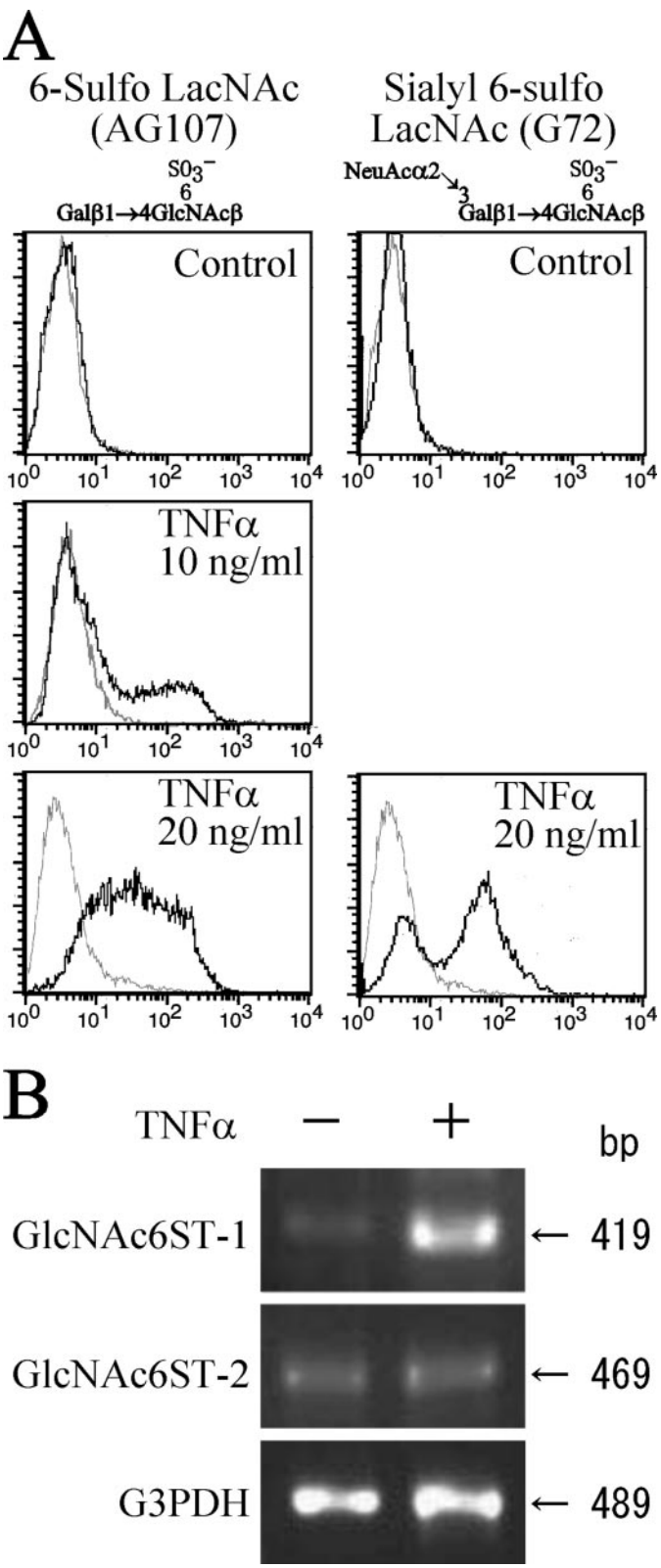

FIGURE 1. **Expression of 6-sulfated glycans on cultured T-lymphoid and Jurkat cells, induced by TNF-α.** *A*, TNF-α induced expression of the 6-sulfated glycans on Jurkat cells as ascertained by flow-cytometric analysis using AG107 and G72 antibodies. Structures of glycans defined by the AG107 and G72 antibodies are also shown. AG107 recognized 6-sulfo-LacNAc, and G72 recognized sialylated 6-sulfo-LacNAc. *Gray lines* indicate the results obtained with isotype-matched control IgM. *B*, reverse transcription-PCR analysis of the expression of the two major GlcNAcβ:6-*O*-sulfotransferases in Jurkat cells upon TNF-α stimulation is shown. *G3PDH*, glyceraldehyde-3-phosphate dehydrogenase.

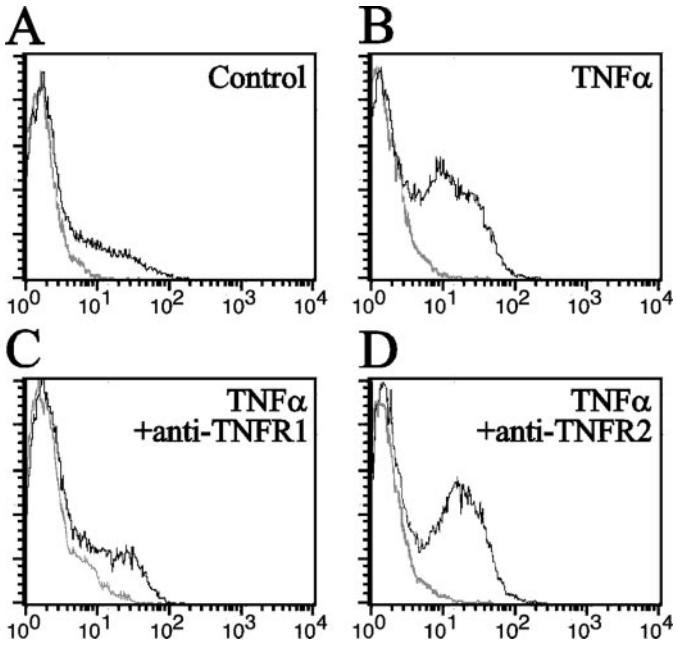

FIGURE 2. **Roles of TNF-α receptors in induction of surface 6-sulfated glycan expression on Jurkat cells after TNF-α stimulation as ascertained by flow-cytometric analysis using AG107 antibody.** *A*, no treatment (negative control); *B*, Jurkat cells stimulated with TNF-α (positive control); *C*, Jurkat cells stimulated with TNF-α in the presence of neutralizing antibody to TNFR1; *D*, Jurkat cells stimulated with TNF-α in the presence of neutralizing antibody to TNFR2. *Gray lines* indicate the results obtained with isotype-matched control IgM.

For flow-cytometric analysis, cells were harvested at a semiconfluent stage and stained with the respective antibody using purified antibody at 1 μg/ml or culture supernatant at a dilution of 1:5. The cells were then stained with a 1:100 dilution of fluorescein isothiocyanate-conjugated second antibody and analyzed with FACSCalibur (BD Biosciences). Control murine IgM was obtained from BD Biosciences. In some experiments, the Jurkat cells were stimulated with recombinant human TNF-α (10~20 ng/ml; R&D Systems, Minneapolis, MN) for 48 h and harvested for luciferase report assay or flow-cytometric analysis. The sialidase treatments (Roche Applied Bioscience, catalog no. 11080725001, used at 1:10 dilution) of the cells, when indicated, were performed at 37 °C for 2 h before staining.

Primary Culture of T-lymphocytes and Th1/Th2 Polarization—Human T-lymphocyte were isolated from peripheral blood mononuclear cells obtained by Ficoll-Paque^{TM} PLUS centrifugation (Amersham Biosciences) of buffy coats prepared from healthy adult blood donors, who provided informed consent according to the Declaration of Helsinki. The T-lymphocytes were cultured at a cell concentration of 10^6 cells/ml in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum. The cells were cultured with or without recombinant TNF-α at 10 ng/ml for 36 h. Naive $CD4^+$ T cells were isolated by negative selection with magnetic beads (naive $CD4^+$ T cell isolation kit, Miltenyi Biotec), when indicated. For induction of Th1 and Th2 differentiation, the naive $CD4^+$ T cells were treated as previously reported with modification (24). Briefly, the cells were stimulated with immobilized anti-CD3 (1 μg/ml; R&D Systems) and soluble anti-CD28 (10 μg/ml; BD Biosciences) antibodies during the first 2 days. The cells were

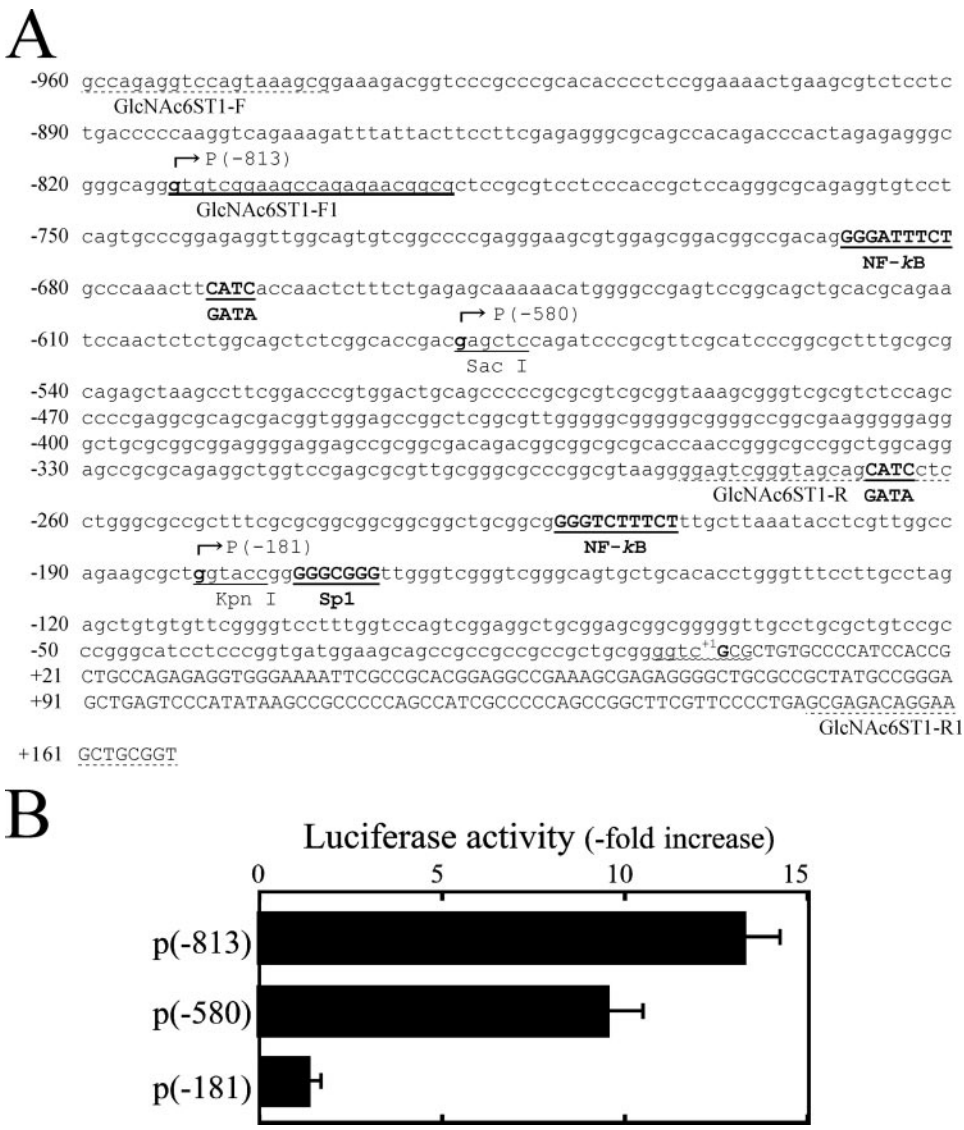

FIGURE 3. **Sequence of GlcNAc6ST-1 promoter and results of reporter assays.** *A*, genomic sequence of the 5′-untranslated region of the GlcNAc6ST-1 gene. The numbers to the left indicate the nucleotide location of the first letter in each lane with respect to the +1 site, which corresponds to the principal transcription start site. The initiator sequence regarding this transcription start site is underlined with a *wavy line*. *Uppercase letters* indicate exon sequences, and *lowercase letters* indicate untranscribed sequences. The NF-κB, GATA, and Sp1 elements are *underlined*. *B*, results of reporter assays using deleted constructs of the GlcNAc6ST-1 promoter.

cultured for 6 days with IL-12 (10 ng/ml; R&D Systems), anti-IL-4 antibody (10 μg/ml; R&D Systems), and IL-2 (20 ng/ml; R&D Systems) for Th1 differentiation or with IL-4 (30 ng/ml; R&D Systems), anti-IL-12 antibody (10 μg/ml; R&D Systems), and IL-2 (20 ng/ml) for Th2 differentiation, respectively.

RNA Extraction and Reverse Transcription-PCR Analysis—Cultured cells were collected, and total cellular RNA was isolated according to the acid guanidinium thiocyanate-chloroform extraction method using an Isogen kit (Nippon-Gene, Tokyo, Japan). First-strand cDNA was prepared using 300 ng of total cellular RNA. Synthesis of cDNA was carried out in a 20-μl reaction volume using the Superscript Pre-amplification System (Invitrogen) according to the manufacturer's protocol with oligo(dT) as initiation primer. The primers used in reverse transcription-PCR analysis for human GlcNAc6ST-1 reported by Uchimura *et al.* (25) were 5′-ATGCA-ATGTTCCTGGAAGGC-3′ and 5′-TTGATGTAGTTCTCCAGG-AAG-3′, which give a 419-bp product; those for HEC-GlcNAc6ST (GlcNAc6ST-2) reported by Bistrup *et al.* (26) were 5′-GCAGCATGA-GCAGAAACTCAAG-3′ and 5′-TCCAGGTAGACAGAAGATC-CAG-3′, which give a 469-bp product. The primers for GATA-2 were 5′-TGTTGTGCAAATTGT-CAGACG-3′ and 5′-CACAGGT-GCCATGTGTCCAGC-3′, which give a 279-bp product, and those for GATA-3 were 5′-AAGGCATCC-AGACCAGAAACCG-3′ and 5′-AGCATCGAGCAGGGCTCTA-ACC-3′, which give a 273-bp product. The primers for T-bet were 5′-GATCATCACCAAGCAGGG-ACG-3′ and 5′-TCCACACTGCAC-CCACTTGC-3′, which give a 154-bp product. Those for glyceraldehyde-3-phosphate dehydrogenase were 5′-TGAAGGTCGGAGTCAACG-GATTTGGT-3′ and 5′-CATGT-GGGCCATGAGGTCCACCAC-3′, which give a 983-bp product.

Plasmid Constructs—The GlcNAc6ST-1 luciferase report plasmid constructs were prepared from genomic DNA from Jurkat cells. The following primers were used to amplify the regulatory region: GlcNAc6ST-1-F1, 5′-GGAAGCTTGTGTCGGAAGCCAGA-GAACGGCG-3′ and GlcNAc6ST1-R1; 5′-GGAAGCTTACCGCAGC-TTCCTGTCTCGC-3′, where the underlined sequence is a synthetic HindIII site. The PCR fragment was subcloned into pGEM-T easy vector (Promega), and then the sequences were confirmed by the ABI 377 sequencer. For generation of the luciferase reporter plasmids, the HindIII fragments were subcloned into the corresponding sites of promoter-less luciferase reporter plasmid pGL3 (Promega), to yield p(−813). p(−580) and p(−181) were derived from p(−813): a SacI fragment was cut out from p(−813) and the remaining DNA was then self-ligated to yield p(−580); a KpnI fragment was cut out from p(−813), and the remaining DNA was then self-ligated to yield p(−181). GATA-1, -2, and -3 expression constructs were prepared as described previously (27). Standard molecular cloning techniques described by Maniatis *et al.* (28) were used for preparing these constructs. All constructs were verified by restriction enzyme digestion and DNA sequencing.

Transfection and Luciferase Assay—Transfection and luciferase assay were performed as described previously (29). The

luciferase reporter plasmids (1 μg) and pRL/CMV plasmid (50 ng), used as an internal standard for normalization of the transfection efficiency, were co-transfected. After 40 h, cell extracts were prepared and used for luciferase assay (Dual-Luciferase™ Reporter Assay System, Promega). All experiments were performed in triplicate.

Small Interfering RNA (siRNA) Transfection—For siRNA experiments, GATA-1 siRNA (human) (sc-29330), GATA-2 (human) (sc-37228), GATA-3 (human) (sc-29331), and control siRNA-A (sc-37007) were obtained from Santa Cruz Biotechnology. The siRNA was electroporated into Jurkat cells with SiPORT electroporation buffer (8990G; Ambion, Austin, TX). After 72 h, the cells were collected for flow-cytometric analyses.

In Vitro Neutralization of Soluble TNF-receptors p55 and p75—Neutralization of TNF-receptors p55 and p75 was done by adding the neutralizing anti-soluble TNFR1 (10 μg/ml; R&D Systems MAB225) or anti-TNFR2 (10 μg/ml, R&D Systems MAB226) into cultured Jurkat cells. The cells were collected for flow-cytometric analysis after 24 h.

Immunoprecipitations and Western Blot Analysis—Whole Jurkat cell lysates were prepared in buffer containing 0.5% Nonidet P-40, 50 mM Tris (pH 7.2), 0.1 mM EDTA, 150 mM NaCl, and protease inhibitors (1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). An equal amount of the extract was incubated with 4 μg of the respective antibody with gentle shaking overnight at 4 °C. Samples were then mixed with 60 μl of protein A-conjugated agarose beads (Upstate Biotechnology Inc., Lake Placid, NY) for 2 h at 4 °C, and immunoprecipitates were washed four times with phosphate-buffered saline and re-suspended in SDS sample buffer for Western blot analysis. Proteins in the immunoprecipitates were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The concentration of running gel was 10% unless otherwise specified. After blocking, the blots were incubated with respective primary antibody (1:1000 dilution). Following incubation with the second antibody (horseradish peroxidase-conjugated goat anti-rat IgG, rabbit anti-goat IgG, or goat anti-mouse IgG) (1:5000 dilution), the signal was detected with an ECL kit (Amersham Biosciences).

Chromatin Immunoprecipitation Assay—Analysis was performed following a kit protocol (Upstate). Chromatin samples were isolated from Jurkat cells as described previously (29). The antibodies used for chromatin immunoprecipitation were anti-GATA-1, anti-GATA-2, anti-GATA-3, anti-p65, and anti-p50. The primers used for detection were GlcNAc6ST1-F (5′-GCCAGAGGTCCAGTAAAGCG-3′) and GlcNAc6ST1-R (5′-GAGGATGCTGCTACCCGTCTCC-3′).

RESULTS

Induction of Cell Surface 6-Sulfated Glycan Expression by TNF-α—To determine whether the expression of 6-sulfated glycan is induced in response to TNF-α, human cultured T-lymphoid Jurkat cells were treated with TNF-α and tested for reactivity to monoclonal antibody AG107, which recognizes 6-sulfated LacNAc, and monoclonal antibody G72, which recognizes sialylated 6-sulfated LacNAc, by flow cytometry. The structures of glycans recognized by these antibodies are shown in Fig. 1*A*.

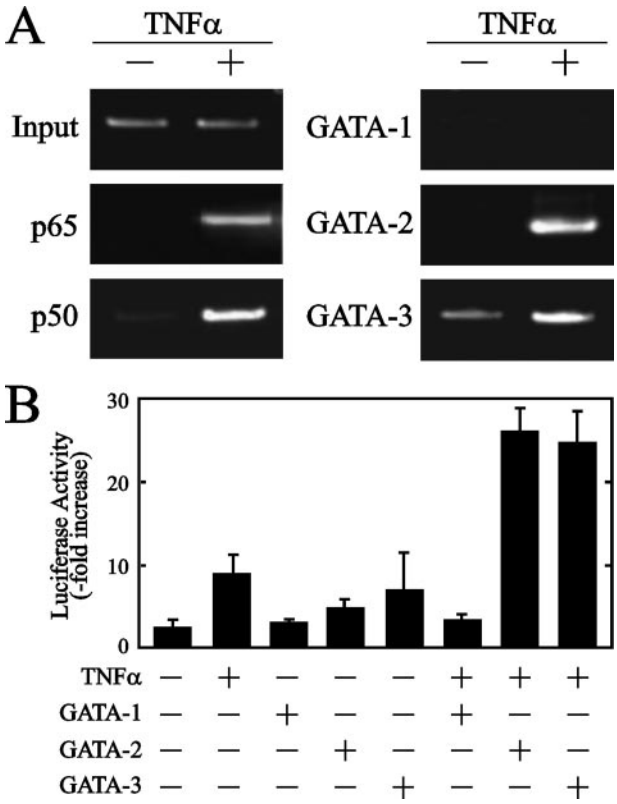

FIGURE 4. **Synergic effect of NF-κB and GATA transcription factors in induction of GlcNAc6ST-1 transcription by TNF-α.** *A*, chromatin immunoprecipitation analysis showing binding of GATA-2, GATA-3, and NF-κB p65/p50 on the GlcNAc6ST-1 promoter region in Jurkat cells. *B*, results of reporter assays indicating enhanced GlcNAc6ST-1 transcription by transfection of GATA-2 or GATA-3 expression vector in Jurkat cells. Jurkat cells were transfected with GATA constructs and incubated for 40 h in the absence or presence of TNF-α prior to luciferase assays. *Closed bars* indicate the results using the p(−580) GlcNAc6ST-1 reporter construct; *open bars* indicate those with the p(−181) construct.

The results of flow cytometry demonstrated that unstimulated Jurkat cells expressed low levels of the 6-sulfated and sialylated 6-sulfated lactosamine glycans and that their expression was significantly increased after TNF-α stimulation (Fig. 1*A*). Similar results were also obtained with HL-60 cells (data not shown). This indicated that TNF-α can induce expression of 6-sulfated LacNAc glycans in a wide range of human leukemic cells as had been observed previously with SR91 cells (17).

To determine whether the TNF-α-induced 6-sulfated glycan expression is accompanied by transcriptional induction of genes for specific sulfotransferases, we studied the mRNA levels of two major known GlcNAcβ:6-*O*-sulfotransferases by semi-quantitative reverse transcription-PCR in Jurkat cells (Fig. 1*B*). A weak expression of HEC-GlcNAc6ST mRNA was detected in unstimulated Jurkat cells, and its level did not change upon TNF-α stimulation. mRNAs for other known 6-*O*-sulfotransferases, including I-GlcNAc6ST, GlcNAC6ST-4, and C-GlcNAc6ST, were hardly detectable (data not shown). In contrast, GlcNAc6ST-1 was not readily detectable in unstimulated Jurkat cells, but was induced significantly upon TNF-α stimulation.

TNFR1 Mainly Mediates Induction of 6-Sulfated Glycan Expression by TNF-α—TNF-α elicits responses through its binding to the receptors 55-kDa TNFR1 and 75 kDa TNFR2. Both TNFR1 and

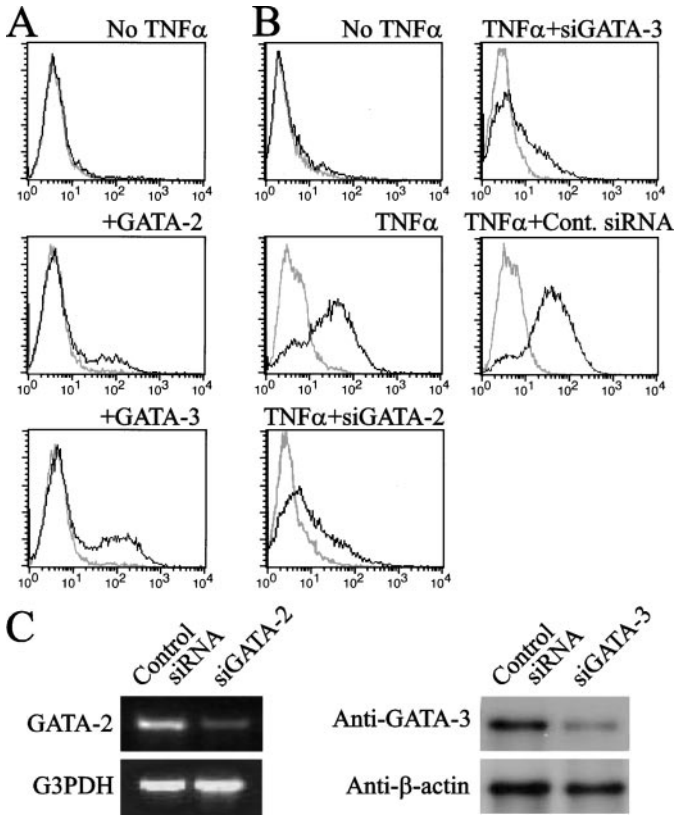

FIGURE 5. **Modulation of cell surface expression of 6-sulfated glycans by GATA-2 or GATA-3.** *A*, flow-cytometric analysis of 6-sulfated glycans defined by AG107 antibody on Jurkat cells transfected with expression vectors for GATA-2 or GATA-3. *B*, effect of siRNA for GATA-2 or GATA-3 or control siRNA on TNF-α-induced expression of 6-sulfated glycans on Jurkat cells as ascertained by flow-cytometric analysis using AG107 antibody. *Gray lines* indicate the results obtained with isotype-matched control IgM. *C*, effect of siRNA on expression of GATA-2 and GATA-3 as ascertained by RT-PCR for GATA-2 (*left*), and by Western blotting for GATA-3 (*right*). *G3PDH*, glyceraldehyde-3-phosphate dehydrogenase.

TNFR2 contain a similar extracellular domain but are different in their cytoplasmic domains. TNFR1 has a so-called death domain region, whereas TNFR2 does not. To test which receptor mediates the TNF-α function in Jurkat cells, we tried to inhibit the TNF-receptor p55 or p75 by adding the neutralizing anti-TNFR1 or anti-TNFR2 antibodies into the culture medium of Jurkat cells when stimulated with TNF-α. Flow-cytometric analysis showed that TNF-α-induced 6-sulfated glycan expression was abrogated by anti-TNFR1, but not efficiently by anti-TNFR2 (Fig. 2), indicating that TNFR1 plays a predominant role in TNF-α-induced 6-sulfated glycan expression in Jurkat cells.

Importance of NF-κB and GATA Binding Sites for GlcNAc6ST-1 Transcription—To elucidate the mechanism of the increased transcription of GlcNAc6ST-1 upon TNF-α stimulation, we cloned the GlcNAc6ST-1 promoter region from Jurkat cells, the sequence of which is shown in Fig. 3*A*. There was no conventional TATA box upstream of the principal transcription start site, but an initiator element was juxtaposed with an Sp1 consensus site for the accurate initiation of RNA transcription. This situation was similar to the regulatory region of fucosyltransferase 7 (*FUT7*), a gene for another glycosyltransferase involved in synthesis of glycans involved in cell-cell adhesion (29, 30).

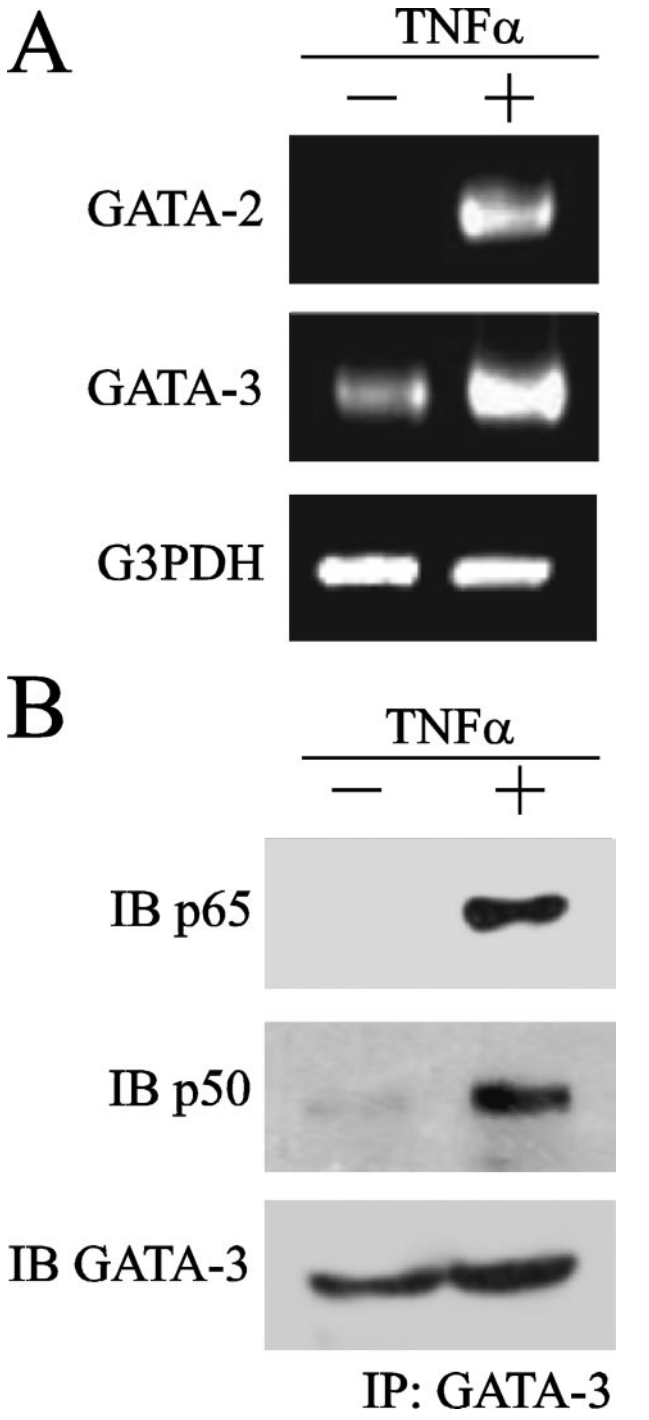

FIGURE 6. **Induction of NF-κB/GATA interaction by TNF-α stimulation.** *A*, reverse transcription-PCR analysis of enhanced expression of GATA-2 and GATA-3 in Jurkat cells upon TNF-α stimulation. *B*, immunoprecipitation analysis indicating interaction of endogenous NF-κB p65/p50 with GATA-3 in Jurkat cells, which was significantly enhanced upon TNF-α stimulation. Jurkat cells were immunoprecipitated (*IP*) with anti-GATA-3 antibody and immunoblotted (*IB*) with anti-p65, anti-p50, or anti-GATA-3 antibody. *G3PDH*, glyceraldehyde-3-phosphate dehydrogenase.

We subcloned the fragment into the luciferase reporter plasmid and prepared several deletion and mutated constructs. Stimulation of the promoter construct p(−580) and p(−813) with TNF-α resulted in a remarkable transcriptional activation compared with that of the promoter construct p(−181) (Fig. 3*B*). As the reporter constructs p(−580)

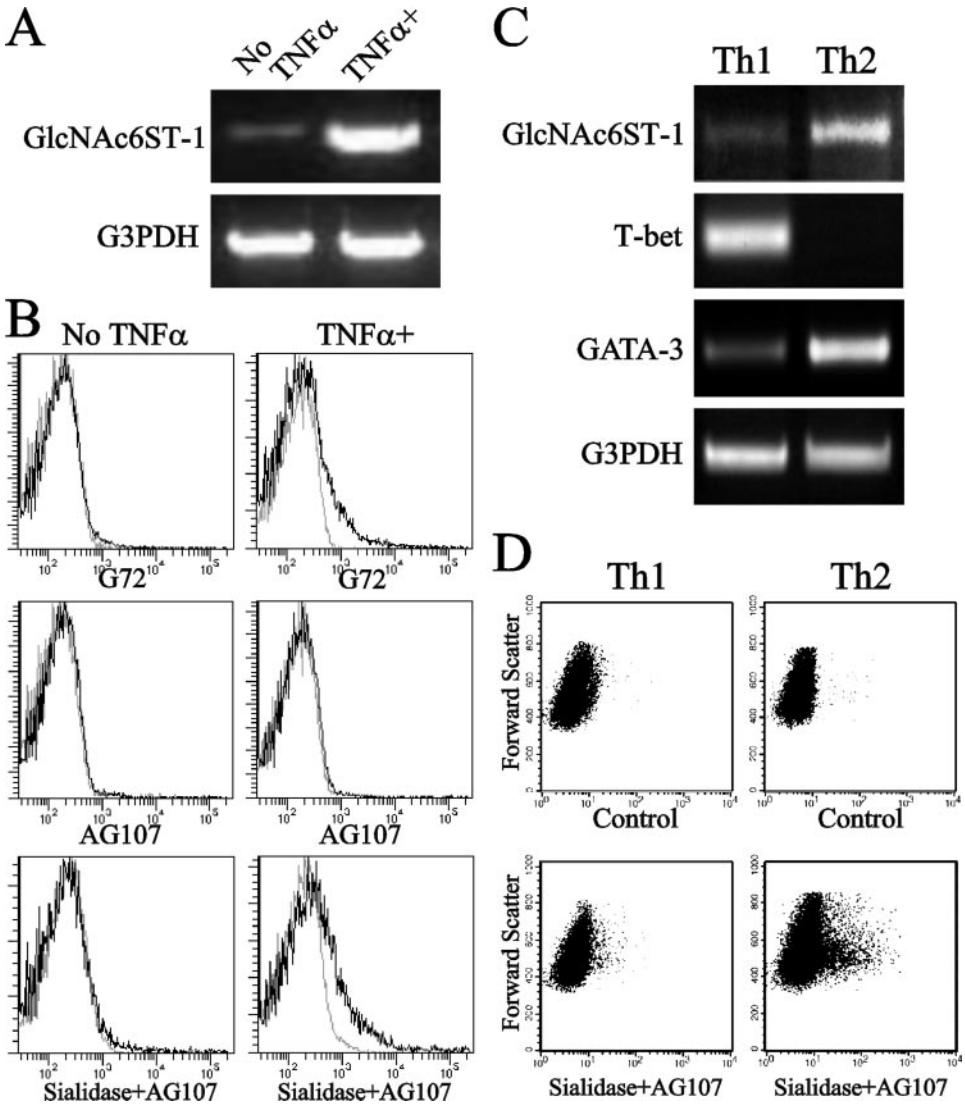

FIGURE 7. **Induction of GlcNAc6ST-1 transcription in TNF-α stimulated primary T cells and Th2-differentiated cells.** *A*, reverse transcription-PCR analysis of TNF-α-stimulated human peripheral T cells for GlcNAc6ST-1. Human peripheral T cells were stimulated with TNF-α for 36 h. *B*, flow-cytometric analyses of TNF-α-stimulated human peripheral T cells using G72 and AG107 antibodies. G72 recognized sialylated 6-sulfo-LacNAc, and AG107 recognized 6-sulfo-LacNAc. *Gray lines* indicate the results obtained with isotype-matched control IgM. *C*, reverse transcription-PCR analysis of human naive helper T cells after Th1/Th2 polarization. See "Materials and Methods" for conditions for Th1 and Th2 differentiation. *D*, flow-cytometric analyses of human Th1 and Th2 cells using AG107 antibody after sialidase treatment. *G3PDH*, glyceraldehyde-3-phosphate dehydrogenase.

through p(−181) is activated by TNF-α, the TNF-α-responsive element should reside between nucleotides −181 and −580. In this region, we found the sequence GGGTCTTTCT, which contains just two different nucleotides with the NF-κB motif GGGACTTTCC, and a GATA binding site, CATC.

To test whether these transcription factors directly bind to the GlcNAc6ST-1 promoter region *in situ*, we performed a chromatin immunoprecipitation assay (Fig. 4*A*). The results indicated a significant binding of p65 and p50 (Fig. 4*A*), as well as that of GATA-2 and GATA-3, to the promoter region of GlcNAc6ST-1 (Fig. 4*A*). The results above showed that GATA-2/3 and NF-κB p65/p50 directly bind to the GlcNAc6ST-1 promoter. To confirm the role of the NF-κB and GATA transcription factors in TNF-α-mediated transactivation of the GlcNAc6ST-1 promoter, reporter assays were performed with co-transfection of GATA expression vectors (Fig. 4*B*). GATA-2 and GATA-3, but not GATA-1, were found to preferentially activate transcription of the reporter construct. Further enhancement of promoter activity was observed when the cells transfected with GATA-2 or GATA-3 constructs were stimulated with TNF-α (Fig. 4*B*). These results indicated that both the NF-κB and GATA binding sites are important for the transcription of GlcNAc6ST-1 in Jurkat T cells.

Modulation of Cell-Surface Expression of 6-Sulfated Glycans by GATA-2 and GATA-3—As GATA-2 or GATA-3 was found to figure heavily in transcriptional induction of GlcNAc6ST-1, we next tested whether GATA-2 or GATA-3 indeed affects cell surface expression of 6-sulfated glycans by TNF-α. The Jurkat cells, which do not express 6-sulfated glycans under the usual culture conditions, showed weak but significant expression of the glycans after introduction of GATA-2 or GATA-3 expression vector (Fig. 5*A*), whereas siRNA for GATA-2 or GATA-3 blocked the TNF-α-induced expression of 6-sulfated glycans (Fig. 5, *B* and *C*). This indicated that the presence of GATA-2 and/or GATA-3 is required to attain maximum expression of cell surface 6-sulfated glycans upon TNF-α stimulation.

Enhanced Interaction between NF-κB p65/p50 and GATA-3 upon TNF-α Stimulation—TNF-α stimulation was found to induce transcription of GATA-2 and increase transcription of GATA-3 in Jurkat cells (Fig. 6*A*), and this may be at least partly involved in TNF-α-induced expression of 6-sulfated glycans. It is well known that NF-κB activation induces transcription of GATA-3 during T cell activation toward Th2 differentiation, and this phenomenon is designated as NF-κB/GATA-3 axis for Th2 differentiation (31, 32). Immunoprecipitation of nuclear extracts of Jurkat cells with anti-GATA3 antibodies followed by Western blotting with anti-p65 and anti-p50 antibodies indicated that endogenous GATA-3 binds to p65 and p50 subunits of NF-κB in TNF-α-stimulated Jurkat cells (Fig. 6*B*), suggesting that both transcription factors participate in the formation of transcription factor complex upon TNF-α stimulation.

Induction of GlcNAc6ST-1 Transcription in TNF-α-stimulated Primary T Cells and Th2-differentiated Cells—We next tested if TNF-α stimulation of human peripheral primary T cells induces GlcNAc6ST-1 transcription. The results indicated a marked induction of GlcNAc6ST-1 transcription (Fig. 7*A*), accompanied with 6-glycan expression. In the primary T cells, only the sialylated form of 6-sulfated glycan was induced as detected by the G72 antibody, and the non-sialylated form defined by the AG107 antibody was not efficiently induced (Fig. 7*B*). This was confirmed by the appearance of the non-sialylated form of 6-sulfated glycans after sialidase treatment of the TNF-α-treated cells (Fig. 7*B*).

Experiments on Th1- and Th2-differentiated T cells showed that the induction of GlcNAc6ST-1 transcription occurred specifically in Th2-differentiated cells, but was not detectable in the Th1-differentiated cells (Fig. 7*C*). This was compatible with the finding that GATA-3 was induced only in Th2-differentiated cells but not in Th1-differentiated cells (Fig. 7*C*). Instead, the latter cells showed a significant induction of T-bet, the Th1-specific transcription factor. The GlcNAc6ST-1 transcription in Th2-differentiated cells was accompanied with the cell surface expression of 6-sulfated glycans, which was ascertained by staining with the specific antibody AG107 after sialidase treatment (Fig. 7*D*).

DISCUSSION

The biological significance of 6-sulfated lactosamine glycan was perhaps first noted when it was identified as a specific ligand for L-selectin in homing of naive T-lymphocytes. The endothelial cells of the high endothelial venules in human peripheral lymph nodes express several products of 6-sulfotransferase including α2–3 sialylated 6-sulfo Lewis X, which are known to serve as a specific ligand for L-selectin (5, 6, 9). Both GlcNAc6ST-1 and HEC-GlcNAc6ST are known to be involved in its synthesis (9). More recently, endothelial cells have been known to also express α2–6 sialylated 6-sulfo-LacNAc, which is suggested to serve as a preferred ligand for CD22/Siglec-2 (14). Several genes are known to be regulated by tandem NF-κB and GATA motifs in endothelial cells, such as erythropoietin (33) and VCAM-1 (34, 35). The mechanism for transcriptional regulation of the GlcNAc6ST-1 gene, however, is unique and is not similar to that for the erythropoietin or VCAM-1 genes. Transcription of the erythropoietin gene is quite different from that for the GlcNAc6ST-1 gene in that both NF-κB and GATA-2 strongly inhibit erythropoietin transcription. Transcription of VCAM-1 is induced by GATA-2 but not by GATA-3. This is different from that of GlcNAc6ST-1, which is induced by either GATA-2 or GATA-3.

Induction of the GlcNAc6ST-1 gene transcription by inflammatory cytokines was first noted in endothelial cells with IL-1β (6) and later in monocytes with TNF-α (16). Carbohydrate 6-sulfation is involved in activation of CD44 in the monocyte/macrophage lineage of cells (16–18, 36). A non-sialylated 6-sulfated lactosamine glycan is known as a specific marker of a distinct subset of dendritic cell precursors in peripheral blood monocytes (19). The population of this dendritic cell precursor subset is highly migratory (37), exhibits readily activated characteristics, and secretes TNF-α and other inflammatory cytokines (21). Among T-lymphocytes, a subpopulation of skin-homing helper memory T-lymphocytes bearing Th2-specific chemokine receptor CCR4 was recently shown to express carbohydrate determinant bearing the 6-sulfated lactosamine structure (10). In the skin-homing helper memory T-lymphocytes, the 6-sulfated lactosamine glycans are further modified with fucose and sialic acid, leading to sialyl 6-sulfated Lewis X determinant, which serves as an essential ligand for vascular E- and P-selectins, which mediate skin homing of the lymphocyte population.

We have demonstrated in this study that TNF-α-induced carbohydrate 6-sulfation on cell adhesion molecules requires synergistic interaction of transcription factors through tandem NF-κB and GATA motifs in the 6-sulfotransferase GlcNAc6ST-1 gene. In T-lymphocytes, the so-called NF-κB/GATA-3 axis is known to be essential for differentiation to the Th2 phenotype (31, 32, 38). Th2-predisposed T cells are known to have dominant expression of GATA-3, and this favors a role for GlcNAc6ST-1 in synthesis of the 6-sulfated glycans in Th2 cells. In good agreement with this, our results on *in vitro* differentiated Th2 cells showed significant induction of GlcNAc6ST-1 transcription and 6-sulfated glycan expression.

Five isoenzymes are known to date in humans for 6-sulfotransferases catalyzing sulfation at the C-6 position of GlcNAc in glycoproteins (39). Among them, two isoenzymes, GlcNAc6ST-1 and HEC-GlcNAc6ST, figure heavily in leukocytes and endothelial cells. It is not always clear at present which 6-sulfotransferase is involved in the expression of 6-sulfated glycans in the diverse biological phenomena described above. The mechanism for regulation of the HEC-GlcNAc6ST transcription is entirely open for future study. Judging from the finding that inflammatory cytokines such as TNF-α induce GlcNAc6ST-1 transcription but not that of HEC-GlcNAc6ST both in monocytes (16) and lymphocytes (this work), GlcNAc6ST-1 is more deeply involved in the enhanced expression of 6-sulfated glycans in the pro-inflammatory response.

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