Tumor Necrosis Factor-α Up-regulates the Expression of β1,4-Galactosyltransferase I in Primary Human Endothelial Cells by mRNA Stabilization*

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During the course of an inflammatory response, the pro-inflammatory cytokine tumor necrosis factor-α (TNFα) triggers endothelial cells to increase the expression levels of adhesion molecules that are pivotal for the rolling, adhesion, and transmigration of leukocytes over the endothelial cell wall. Here we show that TNFα, in addition, has a regulatory function in the biosynthesis of proper carbohydrate molecules on endothelial cells that constitute ligands for adhesion molecules on leukocytes. Our data show that TNFα induced an increase in the expression of β1,4-galactosyltransferase-1 (β4GalT-1) in primary human umbilical vein endothelial cells in a time- and concentration-dependent manner. The β4GalT-1 mRNA up-regulation correlated with an increase in the Golgi expression and catalytic activity of the enzyme. Furthermore, an enhanced incorporation of galactose was observed in newly synthesized glycoproteins. Analysis of the molecular mechanism behind the up-regulation of β4GalT-1 showed that the increase in mRNA levels is due to an enhanced stability of the transcripts. These data strongly demonstrate that TNFα modulates the glycosylation of endothelial cells by a mechanism that directly enhances the stability of β4GalT-1 mRNA transcripts.

The cytokine TNFα1 is released during the inflammatory response by activated macrophages (acute inflammation) or lymphocytes (chronic inflammation) situated at the site of injury. It activates endothelial cells to expose adhesion molecules that are necessary for the rolling, adhesion, and transmigration of leukocytes through the endothelial cell wall (1). Glycans play important roles in the recruitment of leukocytes because they are involved in many of the leukocyte-endothelial interactions. It has been reported previously that the glycan biosynthesis changes during an immune response (2). Accordingly, in recent preliminary data, we observed that TNFα induces changes in the expression levels of several glycosyltransferase genes2 in primary HUVECs, of which up-regulation of the mRNA of β4GalT-1 was the most prominent one.

To date, β4GalT-1 (E.C. 2.4.1.22/38) is one of the best-studied glycosyltransferases. Although several aspects of its regulation and function have been extensively studied (3–5), there are still many uncharacterized features. β4GalT-1 is constitutively expressed in all tissues, with the exception of the brain (6), as a Golgi-resident protein (7). It catalyzes the transfer of galactose from the activated sugar donor, UDP-galactose, to oligosaccharides carrying terminal N-acetyl-glucosamine. This reaction results in the formation of β4-N-acetylglactosamine (Galβ1,4-GlcNAc) or poly-β4-N-acetyllactosamine sequences, also known as type 2 chains, that are abundantly present in N- and O-linked glycans as well as in glycolipids (8). Thus, β4GalT-1 plays an important role in the synthesis of the backbone structure of important carbohydrate epitopes involved in leukocyte-endothelial cell interactions, such as selectin ligands, and also galectin and siglec ligands (9). Additionally, in the lactating mammary gland, α-lactalbumin converts β4GalT-1 to lactose synthase. Interaction of β4GalT-1 with this cofactor greatly lowers the $K_m$ of the enzyme for glucose, so that glucose can be effectively utilized as acceptor substrate at physiological concentrations (10, 11).

Although β4GalT-1 is ubiquitously expressed in human tissues, the levels of expression vary and are subject to different stimuli, as in lactation (12, 13), T-lymphocyte activation and differentiation (14, 15), and activation of monocytes (16). In the case of the lactating mammary gland, the regulatory mechanism involves two steps: an initial ~10-fold increase in mRNA levels is achieved by transcriptional regulation (12, 17), and an additional ~5-fold increase is obtained by translational control (13). During mid- and late pregnancy, the expression of lactating gland-specific transcription factors induces a change in the transcription initiation site, leading to the expression of a shorter transcript. These shorter mRNAs contain less stable secondary structures in the 5′-UTR and, as a result, are translated more efficiently than the longer and highly structured transcript of the non-lactating gland. However, the regulatory mechanisms in tissues other than the lactating mammary gland differ completely and are not yet fully elucidated. The regulatory mechanism in activated HL60 cells, in contrast with lymphocytes, seems to involve post-transcriptional control at the level of mRNA stability (14, 16). A further indication that the expression of β4GalT-1 might be regulated at the post-transcriptional level is found in the short half-life of ~80 min of

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY864848.
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1 The abbreviations used are: TNFα, tumor necrosis factor-α; UTR, untranslated region; ARE, AU-rich element; β4GalT-1, β1,4-galactosyltransferase I; HUVEC, human umbilical vein endothelial cell; PBS, phosphate-buffered saline; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
2 J. J. García-Vallejo, W. van Dijk, B. van het Hof, I. van Die, M. A. Engelse, V. W. M. van Hinsbergh, and S. I. Gringhuis, submitted for publication.
its mRNA in mice (18), similar to other labile mRNAs that are also regulated at the level of mRNA stability (19).

In the present study, we set out to investigate the mechanism involved in the TNFα-mediated up-regulation of β4GalT-1 in primary HUVECs and to explore the effect on the activity and subcellular localization of the enzyme. Our data show that β4GalT-1 is up-regulated in a time- and concentration-dependent manner in response to TNFα stimulation due to an increase in the stability of the mRNA transcript. Furthermore, the β4GalT-1 mRNA up-regulation correlated with an increase in the Golgi-restricted expression of the enzyme and an augmented in vitro β4GalT-1 activity and resulted in an enhanced galactose incorporation in newly synthesized glycoproteins.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures and Reagents**—HUVECs were isolated from five healthy donors by a modification of the method of Jaffe et al. (20). The cells were cultured in M199 supplemented with 100 units/ml penicillin-streptomycin, 10% human serum, 10% newborn calf serum, 5 units/ml heparin (Leo Pharmaceutical Products), and 10 ng/ml basic fibroblast growth factor (Sigma) on 1% gelatin (Fluka)-coated 6-well plates (Costar). When confluence was reached, cells were trypsinized (0.18% trypsin-EDTA), and plated again to one-third of their density. All experiments were performed 2 passages after isolation. Cells were serum-starved for 2 h and exposed to 100 units/ml TNFα (Stratagene) for 6 h or otherwise indicated in the text.

The human cervical epithelial carcinoma cell line HeLa Tet-Off (Clontech), which is stably transfected to continuously express the tetracycline-controlled transactivator, was cultured in high-glucose Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal calf serum (Clontech) supplemented with 2 mM t-glutamine, 4.5 g/liter d-glucose, 100 units/ml penicillin, and 100 units/ml streptomycin. Cells were serum-starved for 2 h prior to experiments.

**Plasmids**—The 3′-UTR of the β4GalT-1 gene (GenBank™ NM_001497, bases 1269–end) was cloned into the BglIII site of the pTetBBB plasmid (a kind gift from Dr. A. Shyu, Houston, TX) (21), which contains the rabbit β-globin gene, to generate pTetBBB/β4GalT1-3′UTR plasmid. This plasmid was transfected into cell lines expressing the tetracycline-controlled transactivator, such as the HeLa Tet-Off cell line, then a chimeric mRNA is continuously transcribed. The four ATTTA sequences within the β4GalT-1 3′-UTR (AUUUA in mRNA) were changed to ACGTA using the QuickChange XL site-directed mutagenesis kit (Stratagene) as indicated in Fig. 5A, thus generating pTetBBB/β4GalT1-3′UTR(mAU1), pTetBBB/β4GalT1-3′UTR(mAU2), pTetBBB/β4GalT1-3′UTR(mAU3), and pTetBBB/β4GalT1-3′UTR(mAU4).

**Transfection**—HeLa Tet-Off cells were grown to 90% confluence before transfection. The transfection with CLONfectin (Clontech) was performed as recommended by the manufacturer, using 2 μg of reporter plasmid and 1 μg of CLONfectin per 2.5 × 10⁶ cells. Cells were used for experiments 24 h after transfection.

**mRNA Half-life Determinations**—HUVECs were stimulated as previously indicated by 10 μg/ml actinomycin D (Sigma) and 1 μg/ml doxcycline (Clontech) to block transcription. After stimulation of transfected HeLa Tet-Off, 1 μg/ml doxycycline (Clontech) was added to block tetracycline-controlled transactivator-dependent transcription of chimeric mRNA. Lysates for mRNA isolation were taken 0, 30, 60, 120, 180, and 240 min after the addition of either actinomycin D or doxycycline.

**Isolation of RNA and cDNA Synthesis**—mRNA was specifically isolated by capturing of poly(A) RNA in streptavidin-coated tubes with a mRNA Capture kit (Roche Applied Science), and cDNA was synthesized with the reverse transcription system kit (Promega) following the manufacturer's guidelines. Cells (2 × 10⁵ cells/well) were washed twice with ice-cold PBS and harvested with 200 μl of lysis buffer. Lysates were incubated with biotin-labeled oligo(dT)₅₀ for 5 min at 37 °C, and then 50 μl of the mix were transferred to streptavidin-coated tubes and incubated for 5 min at 37 °C. After washing three times with 250 μl of washing buffer, 30 μl of the reverse transcription mix (5 mM MgCl₂, 1 × reverse transcription buffer, 1 μM deoxynucleotide triphosphate, 0.4 unit of recombinant RNasin ribonuclease inhibitor, 0.4 unit of avian myeloblastosis virus reverse transcriptase, and 0.5 μM of random hexamers in 30 μl of nuclelease-free water) were added to the tubes and incubated for 10 min at room temperature followed by a 45-min incubation at 42 °C. To inactivate the avian myeloblastosis virus reverse transcriptase and separate mRNA from the streptavidin-biotin complex, samples were heated at 99 °C for 5 min, transferred to microcentrifuge tubes, and incubated in ice for 5 min. Then, they were diluted 1:2 in nuclease-free water and stored at −20 °C until analysis.

**Real-time PCR—Oligonucleotides** (Table I) have been designed by using Primer Express 2.0 computer software (Applied Biosystems). All oligonucleotides were provided by Invitrogen. Oligonucleotide specificity was computer tested (BLAST; National Center for Biotechnology Information) by homology search with the human genome and specifically with all the known galactosyltransferases (CLUSTALW; European Molecular Biology Laboratory), and later confirmed by dissociation curve analysis and resolving the PCR products in agarose electrophoresis. The efficiency (22) of the oligonucleotides was determined using the computer program LinReg (23) and resulted in an average of 90%. PCRs were performed in an ABI 7900HT sequence detection system (Applied Biosystems) using SYBR Green Master Mix (Applied Biosystems) according to the manufacturer's guidelines.

The fluorescence monitoring occurred at the end of each cycle. Additionally, dissociation curve analysis was performed at the end of every run, showing in all cases one single peak at the Tm (melting temperature of the amplicon) predicted by the Primer Express 2.0 software. The Ct (cycle at the threshold) value is defined as the fluorescence during the first 15 cycles and typically corresponds to 0.2 relative fluorescence unit. This threshold is set constant throughout the study and corresponds to the log linear range of the
amplification curve. The normalized amount of target (24) reflects the relative amount of target transcripts with respect to the expression of the endogenous reference gene. In this study, the endogenous reference gene chosen was glyceraldehyde 3-phosphate dehydrogenase, based on previous results (25).

Immunofluorescence Microscopy—HUVECs were seeded in glass-bottom Petri dishes (Nalge Nunc Inc.) and cultured to confluence. Subsequently, cells were serum-starved for 2 h and incubated in the presence or absence of 100 IU/ml TNFα. After 8 h, cells were washed with PBS and fixed with 3% paraformaldehyde/PBS. Immediately, slides were permeabilized in PBS containing 0.075% saponin and 0.2% bovine serum albumin (permeabilizing buffer). Subsequently, the preparations were incubated for 45 min with a primary antibody solution containing GT2/36/118 (a gift of Dr. E. Berger, Zurich, Switzerland) (26) and phallolidin-rhodamine (a kind gift of Dr. H. de Vries, Amsterdam, The Netherlands) in the permeabilizing buffer, washed in the same buffer, and incubated for 45 min with goat anti-mouse Alexa 488 (Molecular Probes) and Hoechst (Molecular Probes). Finally, preparations were mounted in 15% polyvinyl alcohol/33% glycerol in PBS and analyzed under a Nikon Eclipse E800 microscope (Nikon Europe bv) and a Leica TCS SP2 confocal laser-scanning fluorescence microscope (Leica).

Flow Cytometry—Cells were washed twice in cold PBS and resuspended in PBS. Subsequently, cells were fixed with 3% paraformaldehyde/PBS (10 min, room temperature), centrifuged, and treated with 20 mM glycine/PBS (10 min, room temperature) in order to quench free aldehyde groups. Subsequently, cells were permeabilized with 0.1% saponin/PBS for 30 min, incubated for 30 min at room temperature with 25 μl of the primary antibody (GT2/36/118) diluted in 0.1% saponin/1% bovine serum albumin/PBS, washed twice with 0.1% saponin/PBS, and incubated for 30 min at room temperature with secondary antibody (PE-rabbit anti-mouse) diluted in 0.1% saponin/1% bovine serum albumin/PBS. After the second incubation, cells were washed twice with 0.1% saponin/PBS and resuspended in a final volume of 100 μl of 0.1% saponin/1% bovine serum albumin/PBS for analysis in the FACS Calibur (BD Biosciences). Cells were analyzed for immunofluorescence by collecting data for 104 cells/histogram. Corresponding negative controls were performed by using a secondary antibody alone.

Galactosyltransferase Assay—Galactosyltransferase assays were performed as described previously (27) by incubating cell lysates in 100 mM sodium cacodylate buffer, pH 7.2, 20 mM MnCl2, 4 mM ATP, 1% Triton X-100, 0.5 mM UDP-[3H]galactose (specific activity, 10 Ci/mmol), and 30 mM N-acetyl-glucosamine. After incubation at 37 °C, the product was isolated by ion-exchange using Dowex 1×200 resin (Sigma) and separated in HPAEC-PAD (high pH anion-exchange chromatography with pulsed amperometric detection), using Gal-β1,3-GlcNAc and Gal-β1,4-GlcNAc as standards. Radioactivity incorporated into the product was determined in a 1900TR liquid scintillation analyzer (Packard). Metabolic Labeling and Autoradiography—HUVECs were grown to confluence in T175 flasks, serum-starved for 2 h, and incubated for 10 h with 5 μCi/ml [3H]galactose (American Radiolabeled Chemicals) in the presence or absence of 100 units/ml TNFα (Stratagene Biotech). Cells were washed and resuspended in PBS and lysed in Laemmli sample buffer. The cell lysate was subjected to SDS-PAGE in a 10% polyacrylamide gel according to Laemmli (28) and blotted onto a polyvinylidene difluoride membrane (Millipore). The membrane was exposed for autoradiography in a tritium screen (Amersham Biosciences) and analyzed with a STORM™ 860 system (Amersham Biosciences).

Statistics—Results are shown as the average ± S.D. of five independent measurements. Statistical significance was evaluated using the Kruskal-Wallis test in the SPSS 11.0 software (license number AZVU-7061649). Computer analysis of the β4GalT-1 gene and mRNA sequences was performed using TRANSFAC (29) and the CpGplot application at European Molecular Biology Laboratory-European Bioinformatics Institute (www.ebi.ac.uk/Tools/sequence.html).

RESULTS

TNFα Up-regulates β4GalT-1 mRNA Expression in HUVECs in a Time- and Concentration-dependent Manner—One of the functions of TNFα during the inflammatory response is to activate endothelial cells and to prompt them to produce adhesion molecules, which are necessary for the recruitment of leukocytes to the injured tissue. Preliminary observations showed that TNFα modulates the expression of several glycosylation-related genes. Among the genes up-regulated by TNFα in endothelial cells were β4GalT-1, -5, and -6, whereas no effect was apparent in other β3- or β4-galactosyltransferases. The expression of β4GalT-1 was considerably higher than that of other β4-galactosyltransferases. Expression of β3-galactosyltransferases was more than 50-fold less abundant than β4GalT-1 expression. Hence, we decided to focus on β4GalT-1. To establish the kinetics of the TNFα-mediated up-regulation of β4GalT-1 mRNA levels, confluent monolayers of primary HUVECs were incubated in the presence of increasing concentrations of TNFα and for different times. Prior to this study, glyceraldehyde 3-phosphate dehydrogenase was determined as the optimal endogenous reference gene for normalization in the experimental conditions used in the present work (25). The expression of E-selectin was determined as a positive control for the activation of HUVECs. Low concentrations of TNFα (20 units/ml) were ineffective in up-regulating both E-selectin and β4GalT-1, whereas concentrations between 50 and
VECs results in a 2-fold increase in the 
*in vitro* (27). TNFα/H9252 HUVECs than in control cells (Fig. 2, Fig. 3). These data indicate that TNFα/H9251 by quantitative real-time PCR. The activation of HUVECs by enzyme activity remained unchanged (0.06/11006 C were permeabilized; stained with GT2/36/118, phalloidin, and Hoechst; and analyzed for immunofluorescence in a Nikon Eclipse E800 microscope. C, HUVECs were grown to confluence in T25 flasks, serum-starved for 2 h; incubated for 8 h in the presence or absence of 100 units/ml TNFα; mechanically detached and subsequently fixed, permeabilized, and stained with GT2/36/118; and analyzed for immunofluorescence using a FACSCalibur. Results are representative of three separate experiments.

250 units/ml produced an almost linear increase in the expression, reaching plateau values at higher concentrations (Fig. 1A). For analysis of the time dependence of this effect, HUVECs were incubated with culture medium alone or with medium containing 100 units/ml TNFα, and the relative abundance of E-selectin and β4GalT-1 transcripts was determined by quantitative real-time PCR. The activation of HUVECs by TNFα resulted in a 2-fold increase in the expression of β4GalT-1 at 6 h after the addition of the cytokine (Fig. 1B) and a >500-fold increase in the E-selectin mRNA. After this time point, mRNA levels slowly decreased, returning to the basal levels, although a small degree of up-regulation was still visible after 12 h of incubation.

β4GalT-1 Is Exclusively Localized in the Golgi System and Enhanced in Response to TNFα—Several functions have been assigned to β4GalT-1, some of which depend on the localization of the enzyme. Both the expression and localization of β4GalT-1 in control and TNFα-treated HUVECs were examined by immunocytochemistry after staining with the monoclonal antibody GT2/36/118, raised against the stalk region of β4GalT-1 (26). Phalloidin-rhodamine staining was employed to define the limits of the cells and control for the activation of HUVECs, as seen by the formation of stress fibers (Fig. 2B), whereas Hoechst was used to stain the nucleus. The staining patterns correspond to a juxtanuclear localization, which is compatible with an exclusive Golgi localization (Fig. 2, A and B). No extracellular membrane staining could be detected. The intracellular staining was more intense in TNFα-treated HUVECs than in control cells (Fig. 2, A and B). Fluorescence-activated cell-sorting analysis with the monoclonal antibody GT2/36/118 confirmed the 2-fold increase in intracellular β4GalT-1 expression after TNFα treatment (Fig. 2C). These data indicate that the increase in β4GalT-1 mRNA results in an increase in the amount of the enzyme in the Golgi compartment.

**The Up-regulation of β4GalT-1 mRNA Correlates with an Increase in β4GalT Enzyme Activity**—In order to determine whether the increase in the intracellular expression of β4GalT-1 coincided with an increase in enzyme activity, cell lysates of unstimulated and TNFα-stimulated HUVECs were assayed for specific β1,4-galactosyltransferase activity using N-acetyl-glucosamine and UDP-[3H]galactose as substrates (27). TNFα induced a 2-fold increase in the intracellular β4-galactosyltransferase activity (from 0.18 ± 0.02 to 0.36 ± 0.03 nmol/μg protein, p < 0.05), whereas the β3-galactosyltransferase activity remained unchanged (0.06 ± 0.01 nmol/μg protein). In addition, autoradiography after SDS-PAGE of lysates of cells grown in the presence of [3H]galactose revealed that several bands in the molecular mass range between 45 and 80 kDa were more strongly radiolabeled after TNFα treatment (Fig. 3). These data indicate that TNFα stimulation of HUVECs results in a 2-fold increase in the *in vitro* measured β4-galactosyltransferase activity and in the incorporation of galactose into newly synthesized glycoproteins. Our data strongly suggest that the observed increase in β4-galactosylation can be attributed to an increase in β4GalT-1 enzyme levels. The contribution of the up-regulation of β4GalT-5 and -6β to the increase in β4-galactosylation is expected to be low because it has been shown that the enzymatic activity of β4GalT-5 is several orders of magnitude lower than that of β4GalT-1 (30), whereas β4GalT-6 prefers glycolipids as acceptors (31).

**TNFα-mediated Increase of β4GalT-1 Expression Is Regulated by mRNA Stabilization**—In order to identify potential regulatory sequences responsible for the up-regulation of β4GalT-1, the 2000 bp upstream of the transcription initiation site were analyzed using the computer program TRANSFAC and the European Molecular Biology Laboratory application CpG Plot (European Molecular Biology Laboratory-European Bioinformatics Institute). TRANSFAC was employed to identify potential binding sites for transcription factors in the 500 bp upstream of the transcription initiation site, whereas the CpG Plot application predicts the presence of CpG islands. The analysis revealed the existence of a relatively long CpG island close to the site of initiation of transcription (−679 to −57), which correlated with a high density of binding site with the transcription factor Sp1. Importantly, no binding sites for transcription factors activated by the TNFα signaling pathway were found within the 5'-flanking region of the β4GalT-1 gene.

In the lactating mammary gland, there is a change in the transcription initiation site that results in β4GalT-1 transcripts with a lower GC content in the 5'-UTR. The resulting
The up-regulation of β4GalT-1 by TNFα is a post-transcriptional phenomenon involving mRNA stabilization. A. HUVECs were grown to confluence in 24-well plates, serum-starved for 2 h, and incubated for 6 h in the presence or absence of 100 units/ml TNFα. Actinomycin D (10 μg/ml) was added to the cell cultures, and cells were lysed for mRNA isolation as described under “Experimental Procedures” at the selected time points (0, 20, 40, 60, 90, 120, and 240 min). Results are shown as the average ± S.D. of the remaining β4GalT-1 transcripts in five independent experiments. B. HUVECs were grown to confluence in 24-well plates, serum-starved for 2 h, and incubated for 0, 10, 30, 60, or 360 min in the presence of 100 units/ml TNFα. After the time points mentioned, actinomycin D (10 μg/ml) was added to the cell cultures, and cells were lysed for mRNA isolation 0, 20, 40, 60, 90, 120, and 240 min after actinomycin D addition. Subsequently, β4GalT-1 half-life was determined as described in A. Results are shown as the average ± S.D. of the β4GalT-1 half-life in five independent experiments.

DISCUSSION

Although β4GalT-1 has long been considered a housekeeping gene, evidence is accumulating showing that its expression is regulated and involved in important functions (3). In this report, we provide new evidence showing that TNFα up-regulates the expression of β4GalT-1 in endothelial cells by a post-transcriptional mechanism involving the control of mRNA stability. Furthermore, the time- and concentration-dependent increase in mRNA levels is accompanied by an increase in the Golgi localization of the enzyme and the enzyme activity, suggesting a role for β4GalT-1 in the changes occurring in the glycosylation of endothelial cells during inflammation.

β4GalT-1 mRNA expression is regulated by TNFα in a time- and concentration-dependent manner, indicative of a direct effect of TNFα signaling. The involvement of delayed regulatory systems, such as the synthesis of intermediate regulatory molecules or the effect of secondary cytokines secreted by HUVECs under the influence of TNFα (32), is excluded by the short time needed to reach a maximum effect as well as by the observation that the activation by TNFα appears to be saturable.

Computational analysis of the 5′-flanking region of the β4GalT-1 gene revealed the presence of a CpG island and a high frequency of binding sites for the transcription factor Sp1. CpG islands have been associated with a low degree of methylation and, consequently, with an “active” state of transcription (33), whereas Sp1 is a transcription factor generally associated with the transcription of so-called “housekeeping genes” (34). Because no binding sites for transcription factors related to TNFα signaling pathways were found in the 5′-flanking region of the β4GalT-1 gene, we hypothesized that the transcription of β4GalT-1 is set at a basal level, and consequently, it is likely that other mechanisms are involved in the regulation of gene expression of β4GalT-1 by TNFα.

Observations that increased the half-life of β4GalT-1 mRNA and TNFα stimulation induced an increase in the half-life of 4GalT-1 were obtained when the chimeric βGal-β4GalT1–3′-UTR construct was transfected into HEK19 cells. The expression of this construct was increased by TNFα stimulation and subsequently mutated in the βGlobin-β4GalT1–3′-UTR construct (Fig. 5A). The elimination of binding sites for AU-binding proteins by individually mutating the AUUUA sequences of AU1 through AU4 to ACGUA only affected the half-life of the chimeric mRNA when AU2 was mutated (Fig. 5B). Chimeric mRNA harboring the mutated AU2 (βGlobin-β4GalT1-mAU2) showed an enhanced half-life (257 ± 55 min, p < 0.01), similar to endogenous β4GalT-1 mRNA in TNFα-stimulated HUVECs (Fig. 4A). Furthermore, TNFα did not induce an additional stabilization of chimeric βGlobin-β4GalT1-mAU2 mRNA (Fig. 5C). These results indicate that the stability of the β4GalT-1 mRNA is modulated solely through one ARE within its 3′-UTR (AU2). Finally, these results suggest that in resting cells a destabilizing factor is bound to AU2.
Although ectopic localization of apparatus and are in agreement with previous reports (41). A juxtanuclear pattern compatible with localization in the Golgi coproteins. Immunostainings of and the incorporation of galactose into newly synthesized glycan structures of the enzyme in the absence of the sugar donor UDP-galactose, we could not demonstrate any plasma membrane localization after sequential analysis of consecutive time points mentioned, actinomycin D (10 µg/ml) was added to the cell cultures, and cells were lysed for mRNA isolation as described under “Experimental Procedures” at the selected time points (0, 20, 40, 60, 90, 120, and 240 min). Results are shown as the average ± S.D. of the remaining β4GalT-1 transcripts in five independent experiments. B, HUVECs were grown to confluence in 24-well plates, serum-starved for 2 h, and incubated for 0, 10, 30, 60, or 240 min in the presence of 100 units/ml TNFα. After the time points mentioned, actinomycin D (10 µg/ml) was added to the cell cultures, and cells were lysed for mRNA isolation 0, 20, 40, 60, 90, 120, and 240 min after actinomycin D addition. C, subsequently, β4GalT-1 half-life was determined as described in A. Results are shown as the average ± S. D. of the β4GalT-1 half-life in five independent experiments.

FIG. 5. The TNFα-induced increase in mRNA stabilization involves AU2. A, HUVECs were grown to confluence in 24-well plates, serum-starved for 2 h, and incubated for 6 h in the presence or absence of 100 units/ml TNFα. Actinomycin D (10 µg/ml) was added to the cell cultures, and cells were lysed for mRNA isolation as described under “Experimental Procedures” at the selected time points (0, 20, 40, 60, 90, 120, and 240 min). Results are shown as the average ± S.D. of the remaining β4GalT-1 transcripts in five independent experiments. B, HUVECs were grown to confluence in 24-well plates, serum-starved for 2 h, and incubated for 0, 10, 30, 60, or 360 min in the presence of 100 units/ml TNFα. After the time points mentioned, actinomycin D (10 µg/ml) was added to the cell cultures, and cells were lysed for mRNA isolation 0, 20, 40, 60, 90, 120, and 240 min after actinomycin D addition. C, subsequently, β4GalT-1 half-life was determined as described in A. Results are shown as the average ± S. D. of the β4GalT-1 half-life in five independent experiments.

phatidylinositol 3-kinase (36) signaling pathways, which are also activated by TNFα (37). Furthermore, tristetraproline has long been known as a destabilizing factor (38). The identities of the β4GalT-1 ARE-binding proteins and how they are affected by TNFα signaling pathways are currently under investigation. It is still unclear why only AU2 is involved in the stabilization of the β4GalT-1 mRNA. It has previously been suggested that the function of AU-binding proteins might be dependent on the density of ARE in a particular sequence and the formation of mRNA secondary structures (39, 40).

The TNFα-induced stabilization of the β4GalT-1 transcripts in HUVECs results in an increase in the expression of the enzyme in the Golgi, the in vitro enzyme activity in cell lysates, and the incorporation of galactose into newly synthesized glycoproteins. Immunostainings of β4GalT-1 in HUVECs showed a juxtanuclear pattern compatible with localization in the Golgi apparatus and are in agreement with previous reports (41). Although ectopic localization of β4GalT-1 has been described previously (3, 42, 43) and associated with adhesive, lectin-like properties of the enzyme in the absence of the sugar donor UDP-galactose, we could not demonstrate any plasma membrane localization after sequential analysis of consecutive planes ranging from the basolateral to the apical membrane (data not shown). We conclude from these data that the up-regulation of β4GalT-1 transcripts results in a functional increase in β4-galactosylation mediated by the increase in the Golgi expression of the enzyme. Indeed, TNFα-treated HUVECs demonstrated an increase in the incorporation of galactose in proteins with a molecular mass between 45 and 100 kDa, which coincides with the molecular mass of numerous glycoforms with a molecular mass between 45 and 100 kDa, which coincides with the molecular mass of numerous integral and adhesion molecules (intercellular adhesion molecule-1, intercellular adhesion molecule-2, and vascular cell adhesion molecule-1) that are expected to act as scaffolds for the presentation of carbohydrate ligands for selectins, galectins, or siglecs. Interestingly, it has been shown that α6-sialyllectosamine, the ligand for the B-cell molecule CD22, is up-regulated in TNFα-treated HUVECs, which has been attributed to an increase in the expression of ST6Gal I (44, 45). However, the TNFα-induced up-regulation of β4GalT-1 described in the present study can be expected to contribute to the change in glycan synthesis. This might be of special importance in the migration of B cells to the inflamed joints in rheumatoid arthritis, where B cells are found in high numbers and partly contribute to the progression of the disease (46). Accordingly, TNFα blockade therapy reduces the numbers of synovial CD22+ B cells in rheumatoid arthritis patients (47). This may involve blocking of the TNFα-dependent up-regulation of β4GalT-1 and ST6Gal I. The TNFα-dependent up-regulation of β4GalT-1 might also be critical in the expression of 6′sulfated α3′sialyl-α2′fucosyl-lactosamine, the L-selectin ligand, in activated endothelial cells (48). The up-regulation of L-selectin ligands is the main mechanism for the migration of monocytes and lymphocytes in chronic inflammation, as has been demonstrated in several models, such as contact hypersensitivity (49), Arthus reaction (50), Sjögren’s syndrome (51), or ulcerative colitis (52). The further dissection of the mechanisms involved in regulating the expression of β4GalT-1 is important to increase our understanding of the biosynthesis of complex-type glycans that constitute ligands for adhesion molecules during inflammation, which will enable the design of more specific therapies to fight chronic inflammatory diseases.

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