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Transcriptional activation of pig Galβ1,3GalNAc α2,3-sialyltransferase (pST3Gal I) gene by TGF-β1 in porcine kidney PK-15 cells

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ABSTRACT

In this study we investigated for the first time the transcriptional regulation of pig Galβ1,3GalNAc α2,3-sialyltransferase (pST3Gal I) in response to TGF-β1 in porcine kidney PK-15 cells. The pST3Gal I gene was found to span about 90 kb and to be composed of 8 exons including 2 exons in the 5′-untranslated region. RT-PCR analysis indicated that the induction of pST3Gal I by TGF-β1 is regulated at the transcriptional level. Functional analysis of the 5′-flanking region of the pST3Gal I gene revealed the −1257 to −976 region functions as the TGF-β1-inducible promoter and that the Smad-binding site at −1020 is crucial for TGF-β1-induced expression of pST3Gal I in PK-15 cells. In addition, the transcriptional activity of pST3Gal I induced by TGF-β1 in PK-15 cells was strongly inhibited by SIS3, which is a specific Smad-3 inhibitor. In summary, our results identified the core promoter region in the pST3Gal I promoter and demonstrated that Smad-3 binding to the Samd-3 binding site at −1020 is essential for transcriptional activation of pST3Gal I in TGF-β1-induced PK-15 cells.

1. Introduction

Sialic acid (NeuAc or Sia) residues present at the terminal positions of the carbohydrate groups of glycoproteins and glycosphingolipids and play an important role in a variety of biological processes, including cell–cell communication, cell-matrix interaction, cell differentiation, cell adhesion, virus infections, clearance of asialo glycoproteins from circulation, and protein targeting [1,2]. Three linkage patterns, (NeuAcα2,3Gal, NeuAcα2,6Gal, and NeuAcα2,6GalNAc), are commonly found in mammalian glycoproteins, and two, (NeuAcα2,3Gal and NeuAcα2,8NeuAc), frequently occur in glycosphingolipids. These linkages are formed by specific sialyltransferases which catalyze the transfer of NeuAc from CMP-NeuAc to glycoconjugates [3].

To date, twenty members of the sialyltransferase gene family have been cloned and characterized. These enzymes are divided into four groups on the basis of the type of carbohydrate linkages they synthesize: β-galactoside α2,3-sialyltransferase (ST3Gal I–VI), β-galactoside α2,6-sialyltransferase (ST6Gal I and II), GalNAc α2,6-sialyltransferase (ST6GalNAc I–VI), and α2,8-sialyltransferase (ST8Gal I–VII) [4]. These enzymes exhibit acceptor substrate specificities for glycoproteins and glycolipids and show remarkable tissue-specific expression, which are correlated with the existence of cell type-specific carbohydrate structure. In general their expression appears to be regulated at the transcriptional level. Precise molecular knowledge of sialyltransferases including their gene expression is vital for understanding the regulatory mechanism for sialylation of glycoconjugates.

Numerous studies have shown that cell surface sialylation as well as the expressions of sialylated glycoproteins and sialyltransferases can be altered by cytokines and growth factors [5–9]. Stimulation of human endothelial cells by tumor necrosis factor-α (TNF-α) and interleukin-1α (IL-1α) increased ST6Gal I mRNA levels and activity, which enhances the expression of endothelial glycoproteins with α2,6-linked sialic acids, such as E-selectin, ICAM-1, and VCAM-1 [5]. The upregulation of ST6Gal I expression and consequent increment of α2,6-linked sialic acids in serum proteins were induced in response to IL-1β and TNF-α [6]. In addition, biphasic increases in expression of ST3Gal I, ST3Gal IV, and ST6GalNAc III mRNAs were observed in human colon adenocarcinoma HT-29 cells treated with TNF-α [7]. More recently, it has been reported that IL-1β induces increased expression of ST3Gal I during maturation of human monocytes [8] and ST3Gal I expression is markedly upregulated by treatments of IL-1β and TNF-α in primary human chondrocytes [9].

Until now, cytokine effects on sialyltransferase expression have usually been investigated in human cells, but not reported in other

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mammalian cells. In the present study, we examined the effects of various cytokines and growth factors on sialyltransferase expression in pig kidney cells. We have shown for the first time that the expression of pig Galβ1,3GalNAc α2,3-sialyltransferase (pST3Gal I) was markedly induced by the anti-inflammatory cytokine transforming growth factor-β1 (TGF-β1) in pig kidney PK-15 cells. Also, to investigate the molecular basis of pST3Gal I gene expression induced by TGF-β1, the promoter region to direct up-regulation of reporter gene transcription in PK-15 cells in response to TGF-β1 was functionally characterized.

2. Materials and methods

2.1. Materials

Recombinant IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-12, IFN-γ, TNF-α, TGF-β1, EGF and VEGF were obtained from R&D Systems (Minneapolis, MN, USA). Specific smad-3 inhibitor (SIS3) was purchased from EMD Chemicals (Gibbstown, NJ, USA). Dual-Luciferase Reporter Assay System was purchased from Promega (Madison, WI, USA). Fetal bovine serum (FBS) and other cell culture reagents were purchased from WelGENE Co. (Daegu, South Korea). Smad-3 antibody and IgG were purchased from Santa Cruz Biotechnology (CA, USA).

2.2. Cell cultures

The pig kidney cell line PK-15 was obtained from American Type Culture Collection (Rockville, MD, USA) and cultured in Dulbecco's modified Eagle medium (DMEM, WelGENE Co., Daegu, South Korea) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C under 5% CO_2.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from PK-15 cells treated with or without various cytokines and growth factors using Trizol reagent (Invitrogen, USA). One micrograms of RNA was subjected to reverse transcription with random nonamers utilizing Takara RNA PCR kit (Takara Bio, Shiga, Japan) according to the manufacturer's protocol. The cDNA was amplified by PCR with pST3Gal I-S and pST3Gal-A for pST3Gal I (781 bp), and β-actin-S and β-actin-A for β-actin (427 bp) (Supplementary Table 1). PCR products were analyzed by 1% agarose gel electrophoresis and visualized by treatment with ethidium bromide. The intensity of the bands obtained from the RT-PCR product was estimated with a Scion Image Instrument (Scion Corp., Frederick, MD, USA). The values were calculated as a percent of the control and are expressed as means ± SD.

2.4. 5′-Rapid amplification of cDNA ends (RACE) PCR

Amplification of the 5′-end of pST3Gal I was performed with the 5′-RACE kit (Invitrogene, USA) according to the manufacturer's instructions, using 5 μg of mRNAs from PK-15 cells. The gene-specific primer GSP-RT was used for initial reverse transcription. After synthesis of the first strand cDNA, a GeneRacer 5′ primer provided by the company and the gene-specific primer GSP1 were used in the first PCR. The second PCR was performed with a GeneRacer 5′ nested primer and the gene-specific primer GSP2. Primer sequences used in 5′-RACE are shown in Supplementary Table 1. The PCR products were subcloned into pGEM-T Easy vector (Promega, Madison, WI, USA) and sequenced.

2.5. Cloning of the 5′-flanking region of the pST3Gal I gene

Using the sequence information of *Sus scrofa* chromosome 4 clone CH242-235M5 (GenBank accession number CU 466547.2) of the National Center for Biotechnology Information (NCBI), An 1531 bp fragment of the 5′-flanking sequence of the pST3Gal I gene were obtained by long and accurate PCR (LA-PCR) amplification with LA-*Taq* polymerase (Takara Bio, Japan). LA-PCR was performed with a sense primer P-1531S containing a *Kpn*I site and an antisense primer P + 1A containing a *Hind*III site (Supplementary Table 1), and pig genomic DNA isolated from PK-15 cells as template. The reaction was performed using the following conditions: 94 °C for 1 min, then 30 cycles of 98 °C for 20 s and 68 °C for 3 min, with a final elongation of 72 °C for 10 min. PCR products were subcloned into pGEM-T Easy vector (Promega) to give pGF1. PCR products were sequenced in both directions by cloning convenient restriction fragments into pUC119 or using primers designed from a known sequence.

2.6. Construction of reporter plasmids and mutagenesis

To identify the TGF-β1-induced minimal promoter sequence in the 5′-flanking region of the pST3Gal I gene, pST3Gal I promoter fragments containing varying lengths of 5′-flanking sequence, such as pGL3-1531 including its derivatives (pGL3-304 to pGL3-1257), and pGL3-641/-304 including its derivatives (pGL3-1531/-1257, pGL3-1257/-976, pGL3-976/-641), were generated by LA-PCR with sense and antisense primers containing *Kpn*I and *Hind*III sites, respectively (Supplementary Table 1), using pGF1 described above as template. The PCR fragments were subcloned into pGEM-T Easy vector (Promega) and sequenced. Each fragment obtained by digestion with *Kpn*I and *Hind*III was inserted into the corresponding sites of the pGL3-Basic vector, which was used as a negative control. Mutations with base substitution at the NKX32, MESP-1, Twist, Smad-3 binding sites were constructed using a QuikChange® II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol using the following oligonucleotide primers, as shown in Supplementary Table 1. The presence of mutation was verified by sequence analysis.

2.7. Transfection and luciferase assay

For the reporter analysis of pST3Gal I promoter in response to TGF-β1 treatment, PK-15 cells (3.0×10^5 cells/well) were seeded in each well of a 24-well tissue culture plate and allowed to grow to 50% confluence. Cells in each well were transiently co-transfected with 0.5 μg of each reporter plasmid and 50 ng of the control renilla-luciferase vector pRL-TK (Promega) with 1 μl lipofectamine 2000 reagent and grown in serum-free medium without TGF-β1 for 24 h. The medium was changed to serum-free medium containing TGF-β1 (2 ng/ml) and then incubated for 24 h. Cells were collected and treated with passive lysis buffer (Promega). The firefly and renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System according to the manufacturer's instructions with a GloMax™ 20/20 luminometer (Promega). Firefly-luciferase activity of the reporter plasmid was normalized to renilla-luciferase activity and expressed as a fold induction over pGL3-Basic used as a negative control. Independent triplicate experiments were performed for each plasmid.

2.8. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed using the ChIP kit (Upstate Biotechnology, NY, USA) following the manufacturer's protocol. Briefly, after treatment with TGF-β1 (2 ng/ml) for 24 h, PK-15 cells (1×10^7 cells for one assay) were cross-linked in 1% formaldehyde at room temperature for 10 min to cross-link the proteins and DNAs, followed

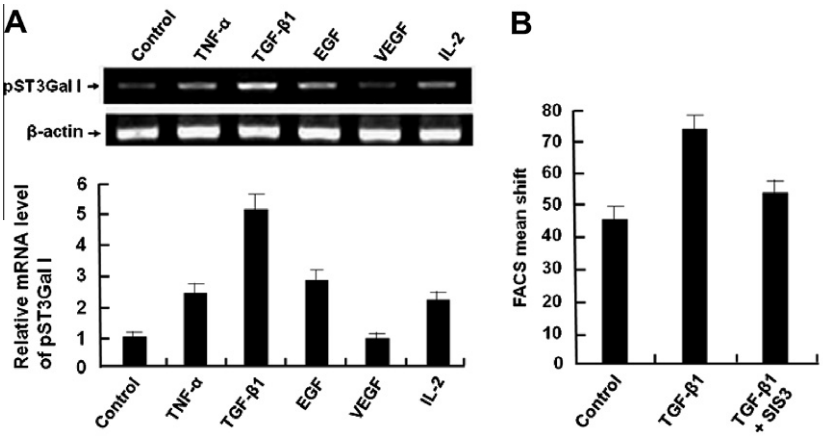

Fig. 1. RT-PCR analysis of pST3Gal I mRNA expression levels by cytokines in PK-15 cells and analysis of α(2,3)-linked sialic acid in TGF-β1-treated PK-15 cells. (A) Total RNA from PK-15 cells was isolated after 24 h of TNF-α (10 ng/ml), TGF-β1 (10 ng/ml), EGF (0.2 μg/ml), VEGF (10 ng/ml), and IL-2 (10 ng/ml) treatments. pST3Gal I mRNA was detected by RT-PCR using gene specific primers as described in Section 2. The internal control for the RT-PCR reaction was performed by running parallel reaction mixtures with the housekeeping gene β-actin. (B) PK-15 cells were grown for 24 h in the presence or absence of 20 μM SIS3 pretreatment for 1 h before the stimulation with TGF-β1. MAA was used as a lectin for α(2,3)-linked sialic acid. The mean fluorescence intensities of the cells are also shown as a histogram.

by sonication to shear the DNAs to an average size of 200–1000 bp. Immunoprecipitation was carried out using 10 μg of Smad-3-specific antibody and IgG. After reversal of cross-linking, the DNA fragments were purified by phenol extraction and ethanol precipitation, followed by PCR analysis using primers flanking the Smad-3 binding sites in the pST3Gal I promoter: 5′-GGACGTTCCAGCCTAACTCACTT-3′ (forward) and 5′-AAGAGGCACAGCTTGGCCTA-3′ (reverse).

3. Results

3.1. Expression of pST3Gal I gene and analysis of the sialylated structure in TGF-β1-induced PK-15 cells

Initial experiments were designed to determine whether various cytokines and growth factors modulate the expression of pig sialyltransferase genes responsible for the biosynthesis of sialylated oligosaccharides in PK-15 cells. After cells were treated with nine kinds of cytokines (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-12, IFN-γ and TNF-α), and three kinds of growth factors (TGF-β1, EGF and VEGF), we examined the gene expression of fourteen members of the pig sialyltransferase gene by RT-PCR and did not find marked changes of gene expression patterns, except for pST3Gal I (data not shown). RT-PCR results showed that mRNA levels of pST3Gal I were significantly increased in PK-15 cells induced by only TGF-β1 and the increased level was about 2.5-fold in the cells treated with 10 ng/ml TGF-β1 as compared with untreated cells (Fig. 1A). To investigate whether or not pST3Gal I mRNA induction by TGF-β1 increases the cellular expression level of NeuAcα2-3Galβ1-3GalNAc residue synthesized by pST3Gal I, we performed lectin binding assay by flow cytometry (FACS) using biotinylated-MAA lectin and FITC-conjugated streptavidin as the secondary antibody. As shown in Fig. 1B, the level of terminal monosialylated trisaccharide NeuAcα2-3Galβ1-3GalNAc residue was increased in PK-15 cells induced by TGF-β1 compared with TGF-β1-uninduced PK-15 cells. The enhanced level of MAA binding was reduced by a Smad-3 inhibitor (SIS3), suggesting that the biosynthesis of NeuAcα2-3Galβ1-3GalNAc residue by pST3Gal I may be regulated through the Smad signaling pathway in TGF-β1-induced PK-15 cells.

3.2. Determination of the transcription start site and genomic structure of the pST3Gal I gene

To determine the pST3Gal I transcript in TGF-β1-induced PK-15 cells, we analyzed the transcription start site of the pST3Gal I gene in TGF-β1-induced PK-15 cells using 5′-RACE analysis. When the secondary nested PCR was performed according to the manufacturer's instructions, a PCR product of about 1.2 kb fragment was obtained (Fig. 2B). From the sequence analysis of this product, the transcription start site of the pST3Gal I in TGF-β1-induced PK-15 T cells was found at 1053 bp upstream of the ATG codon (Fig. 2C). This sequence included a 205 bp segment at their 3′-ends that exactly matched the sequence in the 5′-untranslated region (UTR) of the previously described pST3Gal I cDNA [10] and the extended 963 bp further in the 5′ direction. This result confirms that the 5′-RACE product obtained from TGF-β1-induced PK-15 T cells represents the 5′-flanking region of the pST3Gal I gene. By comparison of the nucleotide sequences of the *S. scrofa* chromosome 4 clone CH242-235M5 (GenBank accession number CU 466547.2) with the cDNA sequence including 1053 bp of 5′-UTR, most of the exon–intron organization was defined including the exons coding for the entire open reading frame (ORF), and 5′- and 3′-UTRs. The pST3Gal I gene consisted of 8 exons, which spanned

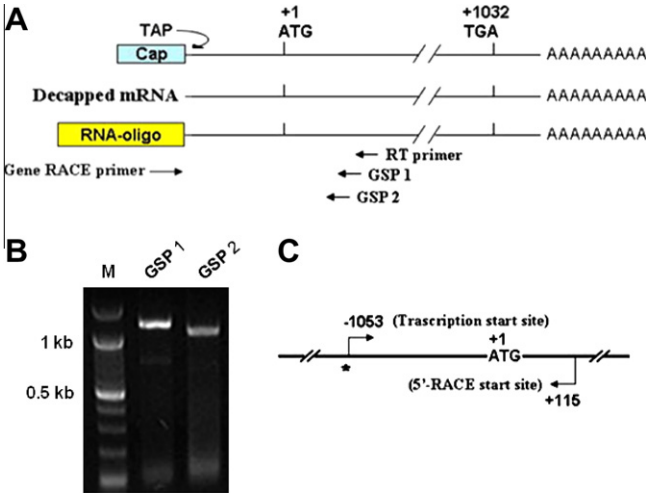

Fig. 2. Identification of the transcription start site in the 5′-flanking region of TGF-β1-induced pST3Gal I gene by RACE-PCR. (A) mRNA from PK-15 cells was prepared after 24 h of TGF-β1 treatment. Reverse transcriptase reaction was performed using RT primer and PCR was performed with Gene RACE primer and Gene specific primers (GSP1 and GSP2) with 5′-RACE strategy. (B) Sequencing and agarose gel (1%) analysis of the 5′-RACE nested PCR product. (C) Transcription initiation sites of TGF-β1-induced pST3Gal I gene. The star indication represents transcription start site as resulted in 5′-RACE.

approximately 90 kb, with exons ranging in size from 46 to 905 bp (Supplementary Table 2). The coding sequence was divided into six exons (exons 3–8), and the 5′-UTR of the cDNA was contained in exons 1–2 (Supplementary Fig. 1). Exon 3 contained a part of the 5′-UTR, the cytoplasmic domain, the transmembrane domain, and a part of the catalytic domain of the pST3Gal I. A part of exon 3 and exons 4–8 encoded the catalytic domain of pST3Gal I. The last exon 8 of 174 bp also contained the stop codon (TGA) and the 3′-UTR. All exon–intron boundaries followed the GT-AG rule that introns begin with GT and end with AG [11] (Supplementary Table 2).

3.3. Cloning and sequence analysis of the 5′-flanking region of pST3Gal I gene

We identified the nucleotide sequences of the *S. scrofa* chromosome 4 clone CH242-235M5 (GenBank accession number CU 466547.2) including the pST3Gal I cDNA sequence. The genomic sequence completely matched the coding region of the pST3Gal I gene and 5′-UTR obtained from 5′-RACE. To determine whether this sequence included the promoter region of the pST3Gal I, a 1531 bp fragment of the 5′-flanking sequence of the pST3Gal I gene was obtained by LA-PCR and sequenced. Analysis of the 5′-flanking region

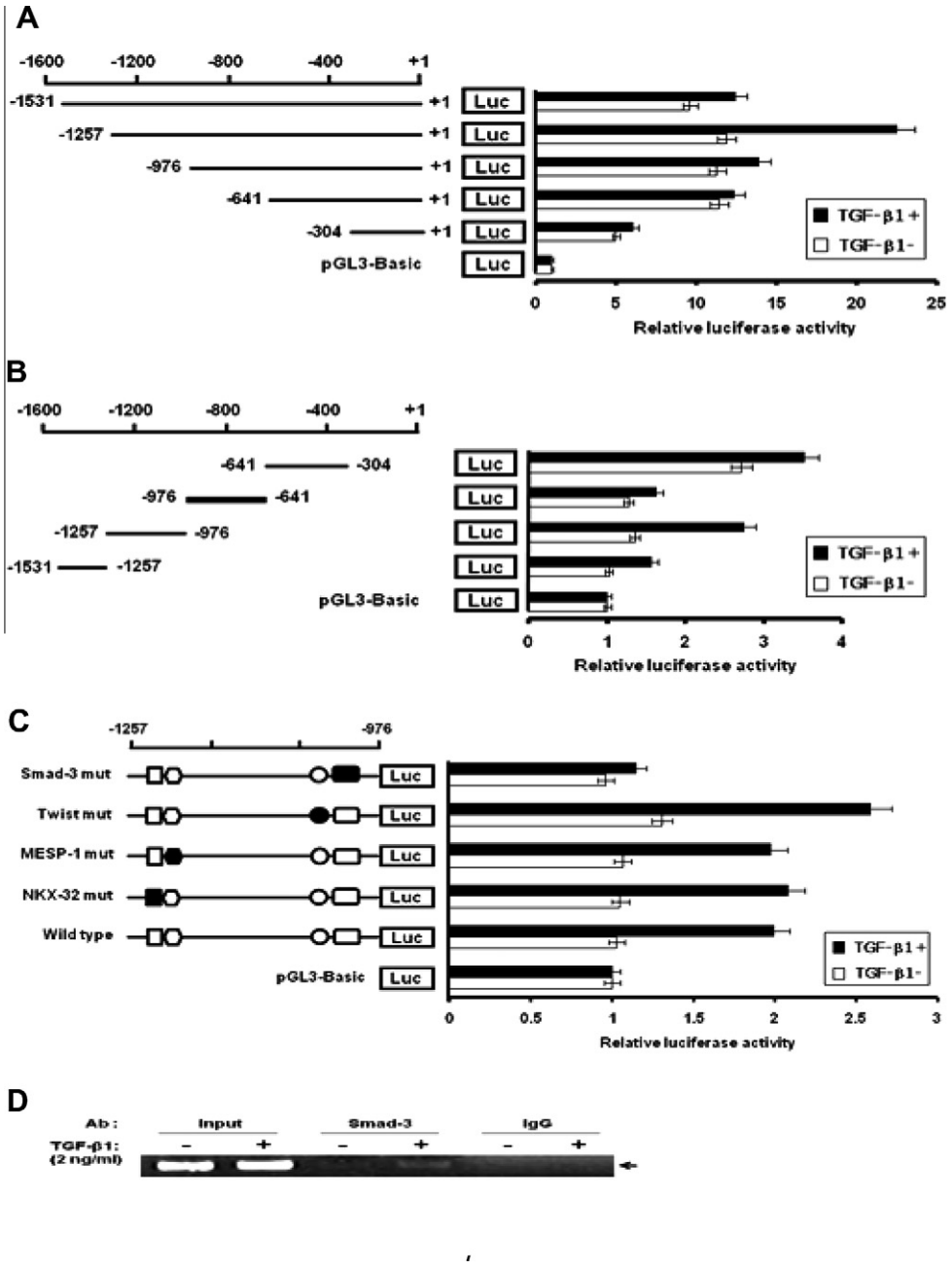

Fig. 3. Analysis of pST3Gal I promoter activity in PK-15 cells and ChIP assay. The schematic diagrams represent DNA constructs containing various lengths of the wild-type pST3Gal I promoter (A and B), or a promoter with mutant sequences in the 5′-flanking region (C), upstream of a luciferase reporter gene; the transcription start site is designated +1. The pGL3-Basic without any promoter and enhancer was used as a negative control. Each construct was co-transfected into PK-15 cells with pRL-TK as the internal control. The transfected cells were incubated in the presence (solid bar) and absence (open bar) of TGF-β1 (2 ng/ml) for 24 h. Relative luciferase activity was measured using the Dual-Luciferase Reporter Assay System normalized with renilla-luciferase activity derived from pRL-TK. The values represent the mean ± SD of three independent experiments with triplicate measurements. (C) PCR amplification from the −1142 and −860 region of the pST3Gal I promoter on immunoprecipitated chromatin obtained from PK-15 cells treated or not treated with TGF-β1. The input (10-fold diluted) represents the positive control.

by Genomatix promoter inspector software (http://www.genomatix.de/) revealed that this region lacks canonical TATA and CAAT boxes, but contains several putative transcription factor binding sites such as SP1, MZF1, GATA-1, NF-κB, Smad-3, NKX-32, and AML-1a (Supplementary Fig. 2).

3.4. Functional analysis of the 5′-flanking region of the pST3Gal I gene in TGF-β1-induced PK-15 cells

To determine whether or not the 5′-flanking sequence of the pST3Gal I gene contains a TGF-β1-responsive promoter, we constructed five kinds of reporter plasmids (pGL3-1531, pGL3-1257, pGL3-976, pGL3-641, pGL3-304) containing each 5′-flanking region of the pST3Gal I gene fused to the promoterless and enhancerless luciferase gene in pGL3-Basic. The constructed reporter plasmids and pGL3-Basic plasmid as a negative control were transfected into the TGF-β1-uninduced PK-15 cells and regulation of the pST3Gal I promoter activity by TGF-β1 was examined. As shown in Fig. 3A, the pGL-1257 showed a remarkable increase of promoter activity in TGF-β1-induced PK-15 cells, which was about 2-fold higher than in TGF-β1-uninduced PK-15 cells. However, this significant response to TGF-β1 was not observed in the other constructs, pGL3-1531, pGL3-976, pGL3-641, and pGL3-304, although they exhibited a high level of promoter activity as compared with that of the pGL3-Basic vector in TGF-β1-uninduced PK-15 cells. These results clearly suggest that the region between nucleotides −1257 and −976 is important for the expression of pST3Gal I gene in TGF-β1-induced PK-15 cells.

Based on this finding, to further determine whether the region from nucleotides −1257 to −976 has TGF-β1-responsive elements in PK-15 cells, we prepared four additional reporter plasmids containing simultaneous deletions from both 5′ and 3′ ends of the pST3Gal I gene promoters, transfected them into TGF-β1 -uninduced PK-15 cells, and then promoter activities by TGF-β1 stimulation were measured. As shown in Fig. 3B, a remarkable increase of promoter activity was obtained with pGL3-1257/-976 and reached to 2-fold higher than in TGF-β1-uninduced PK-15 cells. These results show that the region between nucleotides −1257 and −976 functions as the TGF-β1-inducible promoter in PK-15 cells. These results also suggest that promoter elements located between nucleotide positions −1257 and −976 are dominantly working for enhanced expression of the pST3Gal I gene by TGF-β1 in PK-15 cells.

3.5. Determination of binding sites for transcriptional regulation of pST3Gal I gene in TGF-β1-induced PK-15 cells

As shown in Supplementary Fig. 2, the region from −1257 and −976 contains putative binding sites such as Smad-3, Twist, NKX-32 and MESP-1. To determine whether these binding sites contribute to transcriptional regulation of pST3Gal I gene in response to TGF-β1 in PK-15 cells, four mutants (pGL3-1257/-976mtSmad-3, mtTwist, mtMESP-1 and mtNKX-32) were prepared, which contained the exact same construct as wild-type pGL3-1257/-976 except that combined nucleotides within these binding sites had been changed. A series of substituted mutations of luciferase constructs were transfected into TGF-β1-uninduced PK-15 cells, and the promoter activities by TGF-β1 induction were analyzed. The activity of each construct was compared with that of pGL3-1257/-976wt in response to TGF-β1 stimulation. In TGF-β1-induced PK-15 cells, pGL3-1257/-976mtSmad-3 of four constructed mutations markedly reduced transcriptional activity to 52% of pGL3-1257/-976wt, whereas the activities of the pGL3-1257/-976mtTwist, mtMESP-1 and mtNKX-32 constructs were not decreased (Fig. 3C). We also performed ChIP assay to confirm the binding of Smad-3 to pST3Gal I promoter in PK-15 cells. An amplification of the pST3Gal I promoter regions was obtained in the presence of Smad-3 specific antibody and IgG. As shown Fig. 3D, only Smad-3 had the specific amplification and DNA–protein complex observed in PK-15 cells treated with TGF-β1 to regulate the expression of the pST3Gal I gene. There was no detectable binding in a control assay with TGF-β1 non-treatment or IgG. These results indicate that pST3Gal I gene expression was modulated by interaction between the nuclear protein, Smad-3 and Smad-3 elements at nucleotide positions −1020 and −1012.

3.6. Transcriptional activation of pST3Gal I via Smad pathway in PK-15 cells induced by TGF-β1

We also investigated whether TGF-β1-induced transcriptional activity of a pGL3-1257/-976 containing Smad-3 binding site was stimulated via the Smad signal pathway. As shown in Fig. 4A, RT-PCR showed that expression of pST3Gal I V mRNA was increased in TGF-β1-treated PK-15 cells, compared to untreated PK-15 cells. However, Smad-3 inhibitor (SIS3) produced a decrease of pST3Gal I expression in the TGF-β1-stimulated PK-15 cells, compared to PK-15 cells induced by TGF-β1 in absence of Smad-3 inhibitor, as evidenced by the luciferase promoter assay. The activity of pGL3-1257/-976 was increased in TGF-β1-stimulated PK-15 cells, compared to untreated PK-15 cells. However, the promoter activity of pGL3-1257/-976 in PK-15 cells stimulated by TGF-β1 was significantly inhibited by Smad-3 inhibitor (SIS3), compared to PK-15 cells induced by TGF-β1 in absence of Smad-3 inhibitor (Fig. 4B), as evidenced by RT-PCR. These results indicate that promoter activity and mRNA transcription of the pST3Gal I gene were regulated by the Smad signaling pathway in TGF-β1-induced PK-15 cells.

4. Discussion

A previous study has revealed that the expression of human ST3Gal I (hST3Gal I) mRNA is up-regulated by TNF-α in human co-

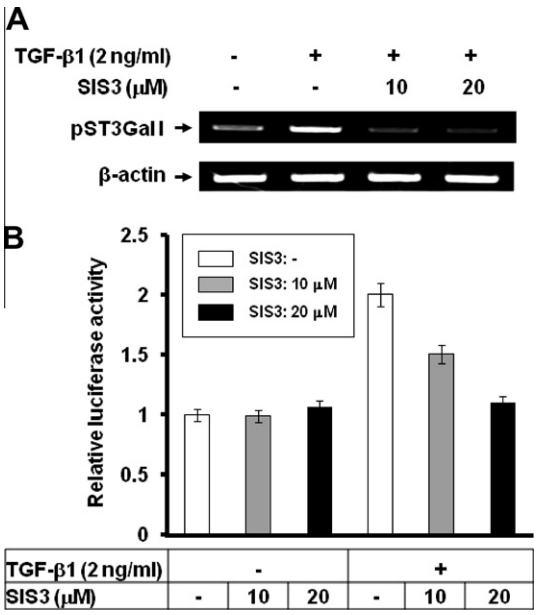

Fig. 4. Transcriptional activation of pST3Gal I through Smad pathway in TGF-β1-treated PK-15 cells. (A) PK-15 cells were treated with 10 μM or 20 μM of SIS3 inhibitor in the absence or presence of TGF-β1 (2 ng/ml) for 24 h in serum-free DMEM medium. Total RNA from these cells was isolated and pST3Gal I mRNA was detected by RT-PCR analysis. Beta-actin was included as an internal control. (B) The pGL3-1257 was co-transfected into PK-15 cells with pRL-TK as the internal control. The transfected cells were incubated in the presence and absence of TGF-β1 (2 ng/ml) with SIS3 inhibitor for 24 h. All firefly activity was normalized to the *Renilla* luciferase activity derived from pRL-TK. The values represent the mean ± SD for three independent experiments with triplicate measurements.

lon cancer HT-29 cells and that NF-κB binding site in the promoter region of hST3Gal I is crucial for the TNF-α-induced expression of the hST3Gal I gene [7]. In the present study, we have shown for the first time that the expression of pST3Gal I mRNA is up-regulated by TGF-β1 in pig kidney PK-15 cells. Furthermore, we have demonstrated in this study that TGF-β1 treatment increases the level of NeuAcα2-3Galβ1-3GalNAc glycan structure synthesized by the pST3Gal I in PK-15 cells, as shown by FACS analysis. These results suggest that the elevation of this glycan structure appears in close temporal relation to the TGF-β1-induced increase in the pST3Gal I gene expression, and that TGF-β1-responsive element(s) exists in the promoter region of the pST3Gal I gene. This also supports that the induction of the pST3Gal I gene expression would finally direct the formation of NeuAcα2-3Galβ1-3GalNAc glycan structure in response to TGF-β1 in PK-15 cells.

Although pST3Gal I cDNA sequence was previously reported [10], its genomic structure and transcriptional mechanism remain unknown. In this study, we determined the genomic organization of the pST3Gal I gene in which we found two exons in the 5′-UTR by 5′-RACE and characterized its promoter region by luciferase assays. The genomic structure of the coding region of the pST3Gal I, composed of six exons, was similar to that of mouse ST3Gal I (mST3Gal I) reported previously [4]. In addition, the coding sequences for sialylmotifs L and S were separated into two exons in pST3Gal I and mST3Gal I, respectively.

In this study, we focused on transcriptional regulation of the pST3Gal I gene in response to TGF-β1 stimulation. To investigate TGF-β1-responsive elements involved in the increased expression of the pST3Gal I gene in PK-15 cells, our first step was to identify the promoter region important for expression of the pST3Gal I gene induced by TGF-β1. Reporter gene assays using transient expression systems clarified that the 5'-flanking region of the pST3Gal I exhibited TGF-β1-inducible promoter activity. Deletion analysis of the 5′-flanking region suggested that the region between −1257 and −976 functions as the core promoter essential for transcriptional activation of the pST3Gal I in TGF-β1-induced PK-15 cells. This region contains putative functional binding sites such as Smad-3, Twist, NKX-32 and MESP-1. Of particular interest, in this region we found the consensus motif (5′-GTCTG-3′) for transcription factor Smad-3 which is well known to be activated in TGF-β1-stimulated cells [12,13]. Our data obtained by site-directed mutagenesis and ChIP analysis indicated that binding to this Smad-3 element mediated TGF-β1-dependent up-regulation of the pST3Gal I gene expression in PK-15 cells. Furthermore, promoter activity of the region between −1257 and −976 was significantly inhibited by a specific inhibitor of Smad-3 (SIS3). These results indicate that Smad-binding element (SBE) at nucleotide positions −1020 to −1012 are crucial for the transcription of the pST3Gal I gene in TGF-β1-induced PK-15 cells.

The Smad family of transcription factors functions as the intracellular mediators of signal transduction in response to TGF-β and related cytokines. TGF-β triggers phosphorylation of Smad-2 and Smad-3 at their C termini and consequently induces the stable formation of heteromeric complexes with Smad-4. These complexes are then translocated into the nucleus, where they function as transcription factors to regulate the expression of TGF-β target genes [12,13]. Smad-3 and Smad-4 directly bind to SBEs, which have the core sequence motif 5′-CAGAC-3′ or its reverse complement 5′-GTCTG-3′, through a conserved β-hairpin loop in the MH1 domain [13,14]. This SBEs motif has been identified as the targeted TGF-β-responsive element in the promoter region of various genes [15]. In line with these findings, our present result suggests that this SBE element mediates TGF-β1-dependent up-regulation of pST3Gal I gene expression in PK-15 cells and that the Smad-3 signaling pathway contributes to transcriptional activity of pST3Gal I in response to TGF-β1 in PK-15 cells.

Although the precise mechanisms involved in the TGF-β1 -dependent activation of Smad-3 leading to a transcriptional upregulation of the pST3Gal I gene in PK-15 cells are unknown, we have demonstrated here for the first time that the Smad-3 activation regulates the expression of pST3Gal I in TGF-β1-stimulated PK-15 cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.09.043.

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