Molecular Cloning and Expression of a Novel Human $\beta$-Gal-3-$O$-Sulfotransferase that Acts Preferentially on $N$-Acetyllactosamine in $N$- and $O$-Glycans*

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Running title: A novel galactose $3'$-$O$-sulfotransferase acting on $N$-acetyllactosamine
Summary

A novel cDNA encoding galactose 3-\(O\)-sulfotransferase was cloned by screening the EST database using the previously cloned cDNA encoding a galactosyl ceramide 3-\(O\)-sulfotransferase, which we term Gal3ST-1. The newly isolated cDNA encodes a novel 3-\(O\)-sulfotransferase, termed Gal3ST-3, that acts exclusively on \(N\)-acetyllactosamine present in \(N\)-glycans and core2 branched \(O\)-glycans. These conclusions were confirmed by analyzing CD43 chimeric proteins in CHO cells expressing core2 \(\beta1,6\)-\(N\)-acytethylglucosaminyltransferase. The acceptor specificity of Gal3ST-3 contrasts with that of the recently cloned galactose 3-\(O\)-sulfotransferase, which we term Gal3ST-2 in the present study (Honke K, Tsuda M, Koyota S, Wada Y, Iida-Tanaka N, Ishizuka I, Nakayama J, Taniguchi N. *J. Biol. Chem.* **276**, 267-274, 2001), since the latter enzyme can also act on core1 \(O\)-glycan and type 1 oligosaccharides, \(\text{Gal}\beta1\rightarrow3\text{GlcNAc}\). Moreover, Gal3ST-3 but not Gal3ST-2 can act on \(\text{Gal}\beta1\rightarrow4(\text{sulfo}\rightarrow6)\text{GlcNAc}\), indicating that disulfated sulfo\(\rightarrow3\text{Gal}\beta1\rightarrow4(\text{sulfo}\rightarrow6)\text{GlcNAc}\rightarrow\text{R}\) may be formed by Gal3ST-3 in combination with GlcNAc 6-\(O\)-sulfotransferase. Although both Gal3ST-2 and Gal3ST-3 do not act on galactosyl ceramide, Gal3ST-3 is only moderately more homologous to Gal3ST-2 (40.1\%) than to Gal3ST-1 (38.0\%) at the amino acid level. Northern blot analysis demonstrated that transcripts for Gal3ST-3 are predominantly expressed in the brain, kidney, and thyroid where the presence of 3’-sulfation of \(N\)-acetyllactosamine has been reported. These results indicate that the newly cloned Gal3ST-3 plays a critical role in 3’-sulfation of \(N\)-acetyllactosamine in both \(O\)- and \(N\)-glycans.
**Introduction**

Sulfate groups in carbohydrates play important roles in conferring highly specific functions on glycoproteins, glycolipids, and proteoglycans (1-3). One of these sulfated glycans is 3′-sulfo galactose attached to a type 2 oligosaccharide (N-acetyllactosamine), sulfo→3Galβ1→4GlcNAc→R, or attached to a type 1 oligosaccharide, sulfo→3Galβ1→3GlcNAc→R. Feizi and her colleagues demonstrated that 3′-sulfo galactose in a type 1 or type 2, fucosylated oligosaccharide functions as an E-selectin ligand (4). When oligosaccharides were released from ovarian cystadenoma glycoprotein and conjugated to lipids, sulfo→3Galβ1→3/4(Fucα1→4/3)GlcNAcβ1→3Gal was found to bind to Chinese hamster ovary (CHO)1 cells expressing E-selectin (4). On the other hand, sulfo→3Galβ1→4(Fucα1→3)GlcNAc acted as a P-selectin ligand when a synthetic oligosaccharide with this structure was transferred to cell surface glycoproteins through a fucose residue by α1,3-fucosyltransferase III (5). These studies suggest that 3′-sulfo galactose on the cell surface plays a role in carbohydrate-protein interactions, including those involved with selectin.

Recently, a comparison of the amino acid sequences of cloned sulfotransferases demonstrated that there is a weak but discernible homologous sequence motif among Golgi-associated sulfotransferases (6-9). In particular, the amino acid sequences that are responsible for binding 5′-phosphosulfate and 3′-phosphate groups of the donor substrate, 3′- phosphoadenosine 5′-phosphosulfate (PAPS), are well conserved and are often highly homologous to each other among those that share the same acceptor specificity (10-15). Previously, galactosyl ceramide 3′-
sulfotransferase that forms a sulfatide, sulfo→3Gal→ceramide, has been cloned based on the amino acid sequence of purified protein (16). Since this enzyme, which we now term Gal3ST-1, is thought not to add a sulfate to glycoproteins (17), galactose 3-ß-sulfotransferase was molecularly cloned by searching for an enzyme homologous to Gal3ST-1. This reported enzyme, which we now term Gal3ST-2, has the unique property of adding a sulfate on both type 1 and type 2 oligosaccharides and core1 ß-glycans, Galß1→3GalNAcα1→R (18). On the other hand, the structures of ß-linked oligosaccharides containing 3′-sulfo galactose reported to date show that 3′-sulfo galactose is present in N-acetyllactosamine in core2 ß-glycans, sulfo→3Galß1→4GlcNAcβ1→6(Galß1→3)GalNAcα1→R (19); core3 ß-glycans, sulfo→3Galß1→4GlcNAcβ1→3GalNAc (20); and core1 extended structures, sulfo→3Galß1→4GlcNAcβ1→3Galfß1→3GalNAc (21). Moreover, no 3′-sulfo galactose in core1 ß-glycans such as sulfo→3Galß1→3GalNAcα1→R has been previously reported.

Similarly, galactose 3-ß-sulfotransferase in human respiratory mucosa was found to act exclusively on N-acetyllactosamine in core2 branched ß-glycans and not on core1 ß-glycans (22). The presence of 3′-sulfo galactose in ß-glycans has been extensively studied in human, bovine, and porcine thyroglobulins and these studies showed that 3′-sulfo galactose is present in N-acetyllactosamine in complex ß-glycans (23, 24). Notably, galactose 3-ß-sulfotransferase present in the thyroid was found to act only on N-acetyllactosamine but not on type 1 oligosaccharides, which differs from the properties of Gal3ST-2 (25). Although no glycoprotein acceptor was tested for Gal3ST-2, these results suggested that there is another galactose 3-ß-sulfotransferase (Gal3ST) yet to be identified.
In the present study, we first identified a novel cDNA by screening the EST database for cDNAs related to human Gal3ST-1 (16). The expression of a full-length cDNA revealed that this cDNA encodes a novel galactose 3-O-sulfotransferase, termed Gal3ST-3, that adds a sulfate exclusively in the 3′-position of galactose in N-acetyllactosamine in both N- and O-glycans, but not on a type 1 Galβ1→3GlcNAc or core1 Galβ1→3GalNAc structure. When CD43 (leukosialin) was tested as an acceptor, Gal3ST-3 preferentially acted on N-acetyllactosamine present in core2 branched O-glycans, while Gal3ST-2 acted on both core1 and core2 branched O-glycans. Moreover, we show that this novel enzyme is expressed almost exclusively in the thyroid, kidney and brain, in contrast to the previously reported ubiquitous expression of Gal3ST-2 (18).
Experimental Procedures

Isolation of cDNAs encoding galactose 3-\(O\)-sulfotransferase

Galactosyl ceramide 3′-sulfotransferase (Gal3ST-1) shares homologous sequences to the binding sites for 3′-phosphate and 5′-phosphosulfate with other Golgi-associated sulfotransferases. The amino acid sequences of residues 76-100 and 156-179, which includes the above motifs, were used as probes to search the dbEST database using the TBLASTN program. Initially two ESTs, AI860920 and AA010976, were identified that exhibited a significant homology to the 3′-phosphate binding site and 5′-phosphosulfate binding site of Gal3ST-1. After sequence analysis of both cDNAs obtained from Incyte Genomics (St. Louis, MO), a BLAST search of the working draft of the human genome with both cDNAs revealed that the ESTs encode a continuous portion of chromosome 11 linked at a NotI site. The cDNAs from both ESTs were excised with EcoRI and NotI and then cloned into the EcoRI site of pcDNA3/Neo (Invitrogen). After confirmation of the correct orientation by sequencing, pcDNA3-Gal3ST-3 was established. The sequence of the Gal3ST-3 transcript was confirmed by sequencing the 5′- RACE and 3′-RACE products obtained using human brain Marathon Ready cDNA (Clontech) as a template.

Human Gal3ST-1 cDNA was cloned by polymerase chain reaction using transcribed cDNAs from kidney (Clontech) as a template. The 5′- and 3′-primers for the PCR were 5′-CCACGCCTGGTGCTGTA-3′ (corresponding to nucleotides −18 to −2, where nucleotides 1-3 encode the initiation methionine) and 5′-CCCTTACTCTGTAGGTCT-3′ (corresponding to nucleotides 1751 to 1769)(16). PCR was carried out using Taq polymerase and the resultant PCR products were cloned into a pCR2.1-TOPO TA cloning vector (Invitrogen). Correctly amplified
cDNAs were confirmed by sequencing, excised from the *Eco*RI sites of the pCR2.1-TOPO vector, and ligated into the *Eco*RI site of pcDNA3/Neo, resulting in pcDNA3-Gal3ST-1. Gal3ST-2 cDNA was cloned in a similar manner using primers 5′-
CCAGAGGCCAAGATGATGTC-3′ (corresponding to nucleotides −12 to 3) and 5′-
CGGAGAGAGGAGCTGGTGT-3′ (corresponding to nucleotides 1361 to 1380) (18) and reverse transcribed cDNAs from human colon (Clontech) as a template.

**Construction of vectors encoding soluble forms of the enzymes tagged with 6×His peptide**

To create a soluble form of the enzymes, cDNAs encoding the transmembrane portions of the enzymes were removed and the resultant cDNA fragments were cloned into a pcDNA3.1/HSH vector. This pcDNA3.1/HSH vector harbors cDNAs encoding a signal peptide for the pcDNA3.1•A vector (26) and 6×His peptide, which had been constructed as described previously (27). pcDNA3-Gal3ST-3 was digested with *Bpl*I and *Eco*RI, blunt ended, and cloned into the blunted *Bam*HI site of pcDNA3.1/HSH, resulting in pcDNA3.1/HSH-Gal3ST-3 (encoding amino acid residues 41 to 431 of Gal3ST-3). pcDNA-Gal3ST-1 cDNA was similarly digested with *Bgl*II and *Bam*HI, blunt ended, and cloned into the blunted *Bam*HI site of the pcDNA3.1/HSH vector, resulting in pcDNA3.1/HSH-Gal3ST-1 (encoding amino acid residues 40 to 423 of Gal3ST-1). cDNA encoding a soluble form of Gal3ST-2 was prepared by PCR using 5′-
AAGGATCCAGGATTCTGCAC**T**CCG**A**CGT**T**-3′ (*Bam*HI site is underlined and nucleotides 217 to 236 are doubly underlined) and 5′-**A**ATCTAGAC**G**AGAGAG**A**GCTGGGTG-3′ (*Xba*I site is underlined and nucleotides 1362 to 1380 are doubly underlined). PCR products were digested with *Bam*HI and *Xba*I and cloned into the same sites of the pcDNA3.1/HSH

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vector, resulting in pcDNA3.1/HSH-Gal3ST-2 (encoding amino acid residues 29 to 398 of Gal3-ST-2).

Oligosaccharide acceptors

\[ \text{Gal}^\beta_1 \rightarrow 3 \text{GlcNAc}^\beta_1 \rightarrow \text{octyl}, \text{Gal}^\beta_1 \rightarrow 4 \text{GlcNAc}^\beta_1 \rightarrow \text{octyl}, \]
\[ \text{Gal}^\beta_1 \rightarrow 4 \text{GlcNAc}^\beta_1 \rightarrow 6(\text{Gal}^\beta_1 \rightarrow 3) \text{GalNAc}^\alpha_1 \rightarrow \text{octyl}, \text{and} \]
\[ \text{GlcNAc}^\beta_1 \rightarrow 6(\text{Gal}^\beta_1 \rightarrow 3) \text{GalNAc}^\alpha_1 \rightarrow \text{octyl} \]
were synthesized as described previously (28, 29).

\[ \text{NeuNAc}^\alpha_2 \rightarrow 3 \text{Gal}^\beta_1 \rightarrow 3 \text{GalNAc}^\alpha_1 \rightarrow \text{octyl} \]
was synthesized using \( \alpha_2,3 \)-sialyltransferase (Calbiochem) and \( \text{Gal}^\beta_1 \rightarrow 3 \text{GalNAc}^\alpha_1 \rightarrow \text{octyl} \) as a precursor.

\[ \text{Gal}^\beta_1 \rightarrow 4(\text{Fuc}^\alpha_1 \rightarrow 3) \text{GlcNAc}^\beta_1 \rightarrow \text{octyl}, \text{Gal}^\beta_1 \rightarrow 4(\text{sulfo} \rightarrow 6) \text{GlcNAc}^\beta_1 \rightarrow \text{octyl}, \]
\[ \text{NeuNAc}^\alpha_2 \rightarrow 3 \text{Gal}^\beta_1 \rightarrow 4 \text{GlcNAc}^\beta_1 \rightarrow \text{octyl} \]
and \( \text{NeuNAc}^\alpha_2 \rightarrow 3 \text{Gal}^\beta_1 \rightarrow 3 \text{GlcNAc}^\beta_1 \rightarrow \text{octyl} \)
were chemically synthesized as described previously (30, 31).

\[ \text{Gal}^\beta_1 \rightarrow 4 \text{GlcNAc}^\beta_1 \rightarrow 2 \text{Man}^\alpha_1 \rightarrow 6 \text{Man}^\beta_1 \rightarrow \text{octyl}, \]
\[ \text{Gal}^\beta_1 \rightarrow 4 \text{GlcNAc}^\beta_1 \rightarrow 6 \text{Man}^\alpha_1 \rightarrow 6 \text{Man}^\beta_1 \rightarrow \text{octyl}, \]
\[ \text{Gal}^\beta_1 \rightarrow 4 \text{GlcNAc}^\beta_1 \rightarrow 3 \text{Gal}^\beta_1 \rightarrow 4 \text{GlcNAc}^\beta_1 \rightarrow 6 \text{Man}^\alpha_1 \rightarrow 6 \text{Man}^\beta_1 \rightarrow \text{octyl} \]
and
\[ (\text{Gal}^\beta_1 \rightarrow 4 \text{GlcNAc}^\beta_1 \rightarrow 3)_2 \text{Gal}^\beta_1 \rightarrow 4 \text{GlcNAc}^\beta_1 \rightarrow 6 \text{Man}^\alpha_1 \rightarrow 6 \text{Man}^\beta_1 \rightarrow \text{octyl} \]
were synthesized as described previously (32, 33). \( \text{Gal}^\beta_1 \rightarrow 3 \text{GalNAc}^\alpha_1 \rightarrow p \)-nitrophenol and

\[ \text{Gal}^\beta_1 \rightarrow 3(\text{Fuc}^\alpha_1 \rightarrow 3) \text{GlcNAc}^\beta_1 \rightarrow 3 \text{Gal}^\beta_1 \rightarrow 4 \text{Glc}^\beta_1 \rightarrow 2 \)-aminopyridine were purchased from Toronto Research Chemicals and Takara Biochemicals, respectively.

Sulfotransferase assays

CHO cells were transfected with pcDNA3.1/HSH-Gal3ST-1, pcDNA3.1/HSH-Gal3ST-2, pcDNA3.1/HSH-Gal3ST-3, or mock pcDNA3.1/HSH using Lipofect AMINE PLUS (Life
Technologies) as described previously (34). Twenty-four h after transfection, the medium was changed to serum-free OptiMEM medium (Life Technologies). After an additional 48 h of culture, the supernatant was filtered and concentrated with Microcon 30 (Millipore), and used as the enzyme source as described previously (35).

Sulfotransferase activities of Gal3ST-3 were assayed as described previously with modification (16). Briefly, the reaction mixture (20 µl) contained 50 mM MES (2-(N-morpholino)ethane sulfonic acid) buffer (pH 7.0), 10 mM MgCl₂, 0.1% Triton X-100, 10 mM NaF, 2 mM ATP, 500 µM acceptor, 0.08 nmol ³⁵S-PAPS, and 10 µl enzyme solution. After incubation at 37°C for 2 h, the reaction was terminated by boiling for 2 min. The reaction products were then adjusted to 0.25 M ammonium formate, pH 4.0, and applied to C18 reverse phase columns (Alltech) as described previously (6). After washing the column with the same solution, the product was eluted with 30% acetonitrile. Radioactivity was measured by scintillation counting. Gal3ST-2 activity was assayed according to the previously described reaction mixture (16).

**Northern blot analysis**

Northern blots of multiple human tissues or human multiple tissue expression array (both from Clontech) were hybridized with cDNA fragments isolated from pcDNA3-Gal3ST-3 after ³²P-labeling using a nick translation kit (Prime-It RmT; Stratagene).

**Transient transfection and metabolic cell labeling**

cDNA encoding a soluble form of CD43 or NCAM was ligated to human IgG hinge plus constant region as described previously (36, 37). These cDNAs were cloned into pcDM8 and
pIG vector, respectively resulting in pcDM8-CD43•IgG and pIG-NCAM•IgG. CHO cells, CHO mutant Lec1 (38), Lec2 (39), and Lec8 (40) cells were transiently transfected with pcDNA3.1-Gal3ST-1, pcDNA3.1-Gal3ST-2, or pcDNA3.1-Gal3ST-3 together with pcDM8-CD43•IgG or pIG-NCAM•IgG as described previously (11). In some of the experiments, pcDNA1-C2GnT-1 (41) was transfected into cells, together with the above cDNA vectors. Twenty-four h after transfection, the medium was replaced with sulfate-free medium S-MEM (Gibco BRL) containing 10% dialyzed fetal bovine serum plus 0.1 mM MEM non-essential amino acids solutions (Gibco BRL), supplemented with sodium $[^{35}S]$sulfate (100 $\mu$Ci/ml, NEN Life Science Products). After an additional 48 h of culture, CD43•IgG or NCAM•IgG in the culture medium was purified by protein A-Sepharose as described previously (37).

**Structural analysis of oligosaccharides attached to CD43•IgG**

To elucidate the structures of mucin-type $O$-glycans attached to CD43•IgG, CD43•IgG was isolated from CHO cells, as described above, after cells were metabolically labeled with $[^3H]$glucosamine (20 $\mu$Ci/ml) or $[^3H]$galactose (20 $\mu$Ci/ml), together with sodium $[^{35}S]$sulfate (100 $\mu$Ci/ml) in the sulfate-free medium, similarly described above.

Purified CD43•IgG was first digested with pronase and subjected to Sephadex G-50 gel filtration. Glycopeptides that eluted close to the void volume contained mucin-type $O$-glycans and were subjected to alkaline borohydride treatment (42). The released $O$-glycans were recovered after Sephadex G-50 gel filtration.
Sialylated sulfated oligosaccharides were then applied to a column of QAE-Sephadex A-25. QAE-Sephadex A-25 column chromatography was carried out in 10 mM pyridine-acetate buffer, pH 5.5, and stepwisely eluted with increasing concentrations of NaCl (11). By using standard oligosaccharides, we found that monosialosyl, disialosyl, and trisialosyl oligosaccharides elute with 70 mM, 120 mM, and 140 mM NaCl, respectively, and 6-sulfo Gal (and 6-sulfo GlcNH₂) elutes with 70 mM NaCl. Bio-Gel P-4 gel filtration was carried out in 0.1 M ammonium acetate buffer, pH 6.7 as described previously (11). To remove sulfate groups, samples were treated with solvolysis in 90% dimethylsulfoxide/10% methanol at 80°C for 5 h as described previously (11).

To identify the core2 oligosaccharide Galβ1→4GlcNAcβ1→6(Galβ1→3)GalNAcOH, HPLC was carried out using a column (4.6×300mm) of NH₂-bonded silica (Varian Micropak AX-5) equipped in Gilson 306 as described previously with slight modification (41). The AX-5 column was equilibrated with 3.6 mM KH₂PO₄ at room temperature, and eluted with a solution linearly increased to 4.2 mM KH₂PO₄ in the first 5 min, then again linearly increased to 9 mM KH₂PO₄ in the next 60 min at a flow rate of 0.8 ml/min. Oligosaccharides were sequentially digested with α2,3-specific sialidase (NANaseI; Glyko) and jack bean β-galactosidase (Sigma) and β-N-acetylhexosaminidase A, and then subjected to Bio-Gel P-4 gel filtration as described previously (11). β-N-Acetylhexosaminidase A can cleave both non-sulfated and sulfated N-acetyllactosamines.

Analysis of sulfated products

To determine the product sulfated by Gal3ST-3, Galβ1→4GlcNAcβ1→p-nitrophenol was incubated with ³⁵S-PAPS and a soluble form of Gal3ST-3 as prepared above, and the product
was purified by Sep-Pak cartridge column chromatography. The purified product was partially hydrolyzed in 40 ml HCl at 100°C for 2 h (15). The acid hydrolysate was then purified by Bio-Gel P-4 gel filtration and analyzed by HPLC using a Whatman Partisil SAX-10 column (4.6 × 250 mm) (Whatman, Clifton, NJ) equilibrated with 10 mM KH₂PO₄ at room temperature. The column was eluted with an isocratic elution of 10 mM KH₂PO₄ in the first 40 min, then linearly increased to 40 mM KH₂PO₄ in the next 20 min. After elution for an additional 20 min at 40 mM KH₂PO₄, the column was then re-equilibrated at 10 mM KH₂PO₄. The flow rate was 0.5 ml/min and each fraction contained 0.25 ml. The position of the standard sulfo→6Gal (Sigma) was determined by boiling aliquots of the eluate in 4% orcinol (w/v) and 80% H₂SO₄ (16.1 M) for 1 min and measuring OD₅₂₀. Sulfo→6GlcNAc and sulfo→3GlcNAc (both from Sigma) were detected at OD₂₁₄ and used as internal standards. Sulfo→6Gal and sulfo→6GlcNAc eluted at the same position, while sulfo→3Gal eluted slightly earlier than sulfo→6Gal under these conditions.
Results

Isolation of cDNA encoding novel galactose 3-O-sulfotransferase (Gal3ST)

By searching the EST database for a novel cDNA related to Gal3ST-1, two novel cDNAs were found to have homology to Gal3ST-1. The cDNAs (AA010976 and AI860920) thus represent the 5′-portion and 3′-portion of the novel cDNA separated by a NotI site. The full-length cDNA obtained by ligation of the two cDNAs encodes an open reading frame of 1293 base pairs, predicting a protein of 431 amino acid residues (MW 48,878 Da), which we subsequently termed Gal3ST-3 (Fig.1). Although two additional methionine residues are present at residues 14 and 15, the nucleotide sequence surrounding the first methionine is more consistent with Kozak’s consensus sequence for translation initiation (43), suggesting that the first methionine is likely the initiation methionine. The cDNA encoding Gal3ST-3 was cloned into pcDNA3/Neo, resulting in pcDNA3-Gal3ST-3. Although we searched the new human genome database for the Gal3ST-3 sequence, the 3′-region including the 3′-untranslated sequence has not been deposited yet. We thus still have limited knowledge on the genomic structure of Gal3ST-3 other than the fact that it is located on chromosome 11.

The comparison of the amino acid sequences of Gal3ST-3 with those of Gal3ST-1 and Gal3ST-2 reveals the following points (Fig. 2). The sequences corresponding to the binding sites for the 5′-phosphosulfate and 3′-phosphate groups are highly homologous among these enzymes. Moreover, these binding sites are much closer to the transmembrane/anchoring domains in the Gal3ST gene family than in all of the other sulfotransferases cloned to date; the transmembrane/anchoring domain and 5′-phosphosulfate binding site are separated by only 21 to
38 residues, while in the majority of Golgi-associated sulfotransferases, the distance is more than 50 residues. On the other hand, the size of the entire amino acid sequence of the Gal3ST gene family is relatively large among Golgi-associated sulfotransferases, indicating that the size of the polypeptide from the 3′-phosphate binding site to the COOH-terminal is larger than that of the other Golgi-associated sulfotransferases cloned to date. Overall, the amino acid sequence of Gal3ST-3 is slightly more homologous to that of Gal3ST-2 (40.1%) than that of Gal3ST-1 (38.0%). None of the other amino acid sequences in the database showed significant homology to these three sulfotransferases.

**Acceptor specificity of Gal3ST-3**

To determine the acceptor specificity of Gal3ST-3, a soluble form of Gal3ST-3 was incubated with ^35^S-PAPS and various acceptor oligosaccharides. For comparison, a soluble form of Gal3ST-2 was prepared in the same manner. Gal3ST-3 was assayed at pH 7.0 as the enzymatic activity was found to be optimal between pH 7.0 and 8.0 in MES buffer (data not shown). The enzyme activity was 1.7 fold higher in 10 mM Mg\(^{2+}\) than Mn\(^{2+}\), and thus 10 mM MgCl\(_2\) was added to the assay solution (data not shown).

As shown in Fig. 3, Gal3ST-3 exhibits significant activity toward core2 branched acceptors:

\[ \text{Galβ1} \rightarrow 4\text{GlcNAc(Galβ1} \rightarrow 3)\text{GalNAcα1} \rightarrow \text{octyl}, \text{ N-acetyllactosamine}, \]

\[ \text{Galβ1} \rightarrow 4\text{GlcNAcβ1} \rightarrow \text{octyl}, \text{ and those mimicking N-glycan structures such as} \]

\[ \text{Galβ1} \rightarrow 4\text{GlcNAcβ1} \rightarrow 6\text{Manα1} \rightarrow 6\text{Manβ1} \rightarrow \text{octyl}, \]

\[ \text{Galβ1} \rightarrow 4\text{GlcNAcβ1} \rightarrow 3\text{Galβ1} \rightarrow 4\text{GlcNAcβ1} \rightarrow 6\text{Manα1} \rightarrow 6\text{Manβ1} \rightarrow \text{octyl}, \text{ and} \]

\[ (\text{Galβ1} \rightarrow 4\text{GlcNAcβ1} \rightarrow 3)_{2}\text{Galβ1} \rightarrow 4\text{GlcNAcβ1} \rightarrow 6\text{Manα1} \rightarrow 6\text{Manβ1} \rightarrow \text{octyl}. \text{ Notably, the} \]

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enzyme also exhibited significant activity toward 6-sulfo N-acetyllactosamine, Galβ1→4(sulfo→6)GlcNAcβ1→octyl. On the other hand, the enzyme exhibited relatively weak activity toward Galβ1→4GlcNAcβ1→2Manα1→6Manβ1→octyl and no activity toward core1 O-glycan, Galβ1→3GalNAcα1→octyl, or type 1 oligosaccharide, Galβ1→3GlcNAcβ1→octyl. This acceptor specificity contrasts with that of Gal3ST-2, which can act on GlcNAcβ1→6(Galβ1→3)GalNAcα1→octyl and type 1 oligosaccharide but not on Galβ1→4(sulfo→6)GlcNAcβ1→octyl (Fig. 3). Moreover, Gal3ST-3 prefers a side chain derived from the 6-position of α-mannose over that derived from the 2-position of α-mannose, which is opposite to the preference by Gal3ST-2. Both enzymes did not act on Lewis x structures, Galβ1→4(Fucα1→3)GlcNAcβ1→octyl. These results demonstrate that Gal3ST-2 and Gal3ST-3 differ significantly in acceptor specificity.

Incorporation of $^{35}$S-sulfate to NCAM and CD43 (leukosialin) chimeric proteins by Gal3ST-3

The substrate specificity of Gal3ST-3 was examined using NCAM•IgG and CD-43•IgG chimeric proteins in the absence and presence of core2 β1,6-$N$-acetylglucosaminyltransferase (C2GnT-1). NCAM contains almost exclusively N-glycans while CD43 contains one N-glycan and approximately 70 O-glycans (37, 42). As the first of a series of experiments, CHO cells were transfected with vectors encoding NCAM•IgG or CD43•IgG chimeric protein with or without expression of full-length cDNA encoding Gal3ST-3. Since CHO cells lack C2GnT activity (41, 42), core2 O-glycans were absent when C2GnT was not transfected.

Fig. 4A illustrates that the incorporation of $^{35}$S-sulfate into CD43 chimeric protein was significantly increased by the expression of Gal3ST-3. The incorporated $^{35}$S-sulfate was mostly
removed by N-glycanase treatment. $^{35}$S-sulfate incorporation into NCAM was moderately increased by Gal3ST-3 and the majority of the incorporated radioactivity was removed by N-glycanase treatment. These results indicate that $^{35}$S-sulfate was incorporated by Gal3ST-3 mainly into N-glycans in the absence of C2GnT. In contrast, there was only a slight increase in $^{35}$S-sulfate incorporation into NCAM by Gal3ST-2. Moreover, $^{35}$S-sulfate incorporated by Gal3ST-2 into CD43 was less susceptible to N-glycanase treatment (Fig. 4A).

To further evaluate the acceptor specificity of Gal3ST-3, CD43•IgG, Gal3ST-3, and C2GnT-1 were expressed in CHO cells. As shown in Fig. 4B, $^{35}$S-sulfate incorporated into CD43 in the presence of C2GnT-1 was only slightly removed by N-glycanase, while the radioactivity incorporated in the absence of C2GnT-1 was significantly removed by the same treatment. These results indicate that $^{35}$S-sulfate incorporation shifted from N-glycan sulfation to O-glycan sulfation with core2 branch formation.

To further examine the dependency of C2GnT on Gal3ST-3 and Gal3ST-2, Lec2 cells which are defective in Golgi-sialylation were used as recipient cells. In Lec2 cells, it was expected that $^{35}$S-sulfate incorporation would be enhanced, since there is no competition between 3’-sulfation and sialylation. The results shown in Fig. 5A illustrate that $^{35}$S-sulfate incorporation into CD43 by Gal3ST-3 was significantly increased in the presence of C2GnT-1 and that the majority of the incorporated radioactivity was resistant to N-glycanase treatment. On the other hand, $^{35}$S-sulfate incorporation by Gal3ST-2 was significantly increased even in the absence of C2GnT-1 and the incorporated label was only slightly removed by N-glycanase treatment. These results indicate that Gal3ST-2 acts on CD43 O-glycans in the absence of core2 branched O-glycans, while
Gal3ST-3 preferentially acts on core2 branched O-glycans. These results are consistent with the above results that Gal3ST-2 but not Gal3ST-3 can act on a GlcNAcβ1→6(Galβ1→3)GalNAc structure (Fig. 3)

To further corroborate these findings, CD43 chimeric protein together with Gal3ST-3 were expressed in Lec1 and Lec8 cells, respectively. The results shown in Fig. 5B indicate that 35S-incorporation by Gal3ST-3 is entirely dependent on the presence of core2 branched O-glycans when the recipient cells lack complex N-glycans as do Lec1 cells. Furthermore, sulfation is entirely dependent on galactose residues since no incorporation by Gal3ST-3 was observed in Lec8 cells, which lack Golgi-galactosylation (Fig. 5C).

**Structural analysis of O-glycans sulfated by Gal3ST-3**

To determine the structure of sulfated O-glycans synthesized by Gal3ST-3, CD43 chimeric protein was produced in CHO cells that express Gal3ST-3 and C2GnT-1. These transfected cells were metabolically labeled with 3H-glucosamine or 3H-galactose and 35S-sulfate, and the CD43 chimeric protein released into the medium was collected. Sephadex G-50 gel filtration of glycopeptides obtained by pronase digestion of Gal3ST-3-labeled CD43 chimeric protein showed that the majority of glycopeptides eluted close to the void volume, but a minority of glycopeptides eluted at fractions 43-60 which contain N-glycans (Fig. 6A). In contrast, Gal3ST-2-labeled CD43 produced only glycopeptides that eluted at the void volume, which contain multiple O-glycans in a peptide (Fig. 6B). These glycopeptides containing multiple O-glycans in each peptide, which were derived from Gal3ST-3 labeled cells, were then subjected to alkaline borohydride treatment to release O-glycans and then subjected to the same Sephadex G-50 gel
filtration (Fig. 6C). After QAE-Sephadex column chromatography, the majority of the isolated O-glycans eluted as those containing two anionic charges (Fig. 6D), which were converted to a mono-sulfated form after desialylation (Fig. 6E). Upon Bio-Gel P-4 gel filtration, this oligosaccharide eluted at the elution position for core2 branched glycans, Galβ1→4GlcNAcβ1→6(Galβ1→3)GalNAcOH (Fig. 6F). Previous studies demonstrated that a monosulfated form of core2 branched O-glycans elutes at almost the same position as non-sulfated O-glycans (11). Almost identical results were obtained for O-glycans derived from Gal3ST-2 labeled CD43 in the presence of C2GnT-1 (data not shown).

After desulfation by solvolysis, the obtained O-glycans eluted at the same position where Galβ1→4GlcNAcβ1→6(Galβ1→3)GalNAcOH eluted upon HPLC analysis (Fig. 7A). Sulfate was judged to be attached to galactose in an N-acetyllactosaminyl core2 branch since the oligosaccharide was resistant to β-galactosidase treatment (data not shown). If the sulfate had been attached to a core1 structure such as Galβ1→4GlcNAcβ1→6(sulfo→3Galβ1→3)GalNAcOH, one galactose would have been released after β-galactosidase treatment. To confirm that a sulfate group was attached to the C-3 position of galactose, sulfated monosaccharides were released by acid hydrolysis and subjected to SAX-10 column chromatography. Monosulfated galactose eluted at the same position as sulfo→3galactose released from sulfo→3Galβ1→4GlcNAcβ1→p-nitrophenol prepared by Gal3ST-2 (Fig. 7B and C), demonstrating that Gal3ST-3 adds a sulfate to the C-3 position of galactose in N-acetyllactosamine. These results indicate that the majority of sulfated O-glycans conferred by Gal3ST-3 on CD43 is a sialylated and sulfated core2 O-glycan, sulfo→3Galβ1→4GlcNAcβ1→6(NeuNAcα2→3Galβ1→3)GalNAc.
Expression of Gal3ST-3 is highly restricted to brain, kidney, and thyroid

Northern blot analysis showed a highly specific Gal3ST-3 transcript of ~2.4 kilobases expressed in fetal brain and kidney, and adult brain, kidney, and thyroid (Fig. 8). Multiple tissue expression array analysis showed almost identical results and further demonstrated that expression is highly pronounced in the putamen, caudate nucleus, and pituitary gland of the brain (Fig. 9). These results contrast with the reported expression pattern of Gal3ST-2 showing ubiquitous expression in almost all tissues tested (18).
**Discussion**

The present study describes the isolation of a novel cDNA encoding galactose 3-\(O\)-sulfotransferase by searching the EST database for cDNAs homologous to the human galactosyl ceramide 3-\(O\)-sulfotransferase, Gal3ST-1 (16). Gal3ST-1 adds a sulfate to a \(\beta\)-galactose residue linked to ceramide, while Gal3ST-3 adds a sulfate to a \(\beta\)-galactose linked to \(N\)-acetylglucosamine through a 1,4-linkage. Previously, Gal3ST-2 was also cloned based on its similarity to Gal3ST-1, but the acceptor specificities of Gal3ST-2 and Gal3ST-3 differ substantially. While Gal3ST-3 acts exclusively on \(N\)-acetyllactosamine, Gal3ST-2 can act also on type 1 oligosaccharide, \(\text{Gal}\beta_1\rightarrow3\text{GlcNAc}\), and core1 oligosaccharide, \(\text{Gal}\beta_1\rightarrow3(\text{GlcNAc}\beta_1\rightarrow6)\text{GlcNAc}\). On the other hand, Gal3ST-3 can act on \(\text{Gal}\beta_1\rightarrow4(\text{sulfo}\rightarrow6)\text{GlcNAc}\beta_1\rightarrow R\), but Gal3ST-2 cannot act on the same acceptor (Fig. 3).

Interestingly, the best acceptor for Gal3ST-3 is poly-\(N\)-acetyllactosamine attached to a side chain derived from C-6 of \(\alpha\)-mannose, while the best acceptor for Gal3ST-2 is \(N\)-acetyllactosamine attached to a side chain derived from C-2 of \(\alpha\)-mannose (Fig. 3). Moreover, Gal3ST-3 preferentially acts on \(N\)-acetyllactosamine attached to an \(N\)-glycan mannose core, such as \(\text{Gal}\beta_1\rightarrow4\text{GlcNAc}\beta_1\rightarrow6\text{Man}\alpha_1\rightarrow6\text{Man}\beta_1\rightarrow\text{octyl}\), than on \(N\)-acetyllactosamine itself, while Gal3ST-2 does not possess this preference (Fig. 3). These results indicate that Gal3ST-2 and Gal3ST-3 act differentially on various acceptor glycoproteins.

In the present study, the transcripts of Gal3ST-3 were found to be expressed selectively in the brain, kidney, and thyroid. Previously, it was reported that human thyroglobulin contains a sulfo\(\rightarrow3\text{Gal}\beta_1\rightarrow4\text{GlcNAc}\beta_1\rightarrow R\) structure in the majority of \(N\)-glycans (23). This 3'-\(O\)-sulfated
N-acetyllactosamine side chain was shown to exist in both bi-antennary and highly branched tri- and tetra-antennary \(N\)-glycans. In porcine thyroglobulin, the majority of sulfo\(\rightarrow\)3Gal\(\beta\)1\(\rightarrow\)4GlcNAc\(\beta\)1\(\rightarrow\)R are present in side chains derived from the 6-position of \(\alpha\)-mannose. Moreover, the same glycoprotein contains a Gal\(\beta\)1\(\rightarrow\)4(sulfo\(\rightarrow\)6)GlcNAc side chain in a portion of the \(N\)-glycans (24). As shown previously, sulfo\(\rightarrow\)6GlcNAc\(\rightarrow\)R is first formed from a GlcNAc\(\rightarrow\)R structure and then converted to Gal\(\beta\)1\(\rightarrow\)4(sulfo\(\rightarrow\)6)GlcNAc\(\rightarrow\)R (11, 12). Gal\(\beta\)1\(\rightarrow\)4(sulfo\(\rightarrow\)6)GlcNAc\(\rightarrow\)R can then be converted by Gal3ST-3 to sulfo\(\rightarrow\)3Gal\(\beta\)1\(\rightarrow\)4(sulfo\(\rightarrow\)6)GlcNAc\(\rightarrow\)R, considering that Gal3ST-3 can act on Gal\(\beta\)1\(\rightarrow\)4(sulfo\(\rightarrow\)6)GlcNAc\(\rightarrow\)R. In the structural studies described above, no multi-sulfated \(N\)-glycans were analyzed (24). It is possible that sulfo\(\rightarrow\)3Gal\(\beta\)1\(\rightarrow\)4(sulfo\(\rightarrow\)6)GlcNAc\(\rightarrow\)R may be found with further analysis of highly sulfated \(N\)-glycans in thyroglobulin.

It has been reported that a major glycoprotein in calf thyroid contains core2 branched \(O\)-glycans (44), and that core2 branches in the thyroid are likely synthesized by C2GnT-1 and C2GnT-2 (45, 46). Core2 branched oligosaccharides from calf thyroid apparently lack 3'\(^{-}\)-sulfate galactose. In contrast to human thyroglobulin, the presence of 3'\(^{-}\)-sulfate galactose in calf thyroglobulin is minimal, presumably due to the competition with the strong \(\alpha\)1,3-galactosyltransferase activity in calf thyroid (44). It has also been reported that galactose 3\(-\)-O-sulfotransferase in the thyroid acts only on \(N\)-acetyllactosamine as does Gal3ST-3 (25). These results indicate that Gal3ST-3 is most likely responsible for the formation of sulfo\(\rightarrow\)3Gal\(\beta\)1\(\rightarrow\)4GlcNAc attached to both \(N\)-glycans and core2 branched \(O\)-glycans synthesized in the thyroid.
Although it was reported that the transcripts of Gal3ST-2 are expressed in various tissues, the amount of transcripts was relatively low in brain and kidney when estimated by reverse transcription-PCR (18). Since the transcripts of Gal3ST-3, on the other hand, are highly expressed in brain and kidney, it is likely that GalST-3 plays a major role in the brain and kidney in addition to the thyroid. Previously, the existence of two different galactosyl 3-O-sulfotransferases has been reported: one (A) acts on core1 O-glycans, while the other (B) acts on N-acetyllactosamine (47, 48). Although it is not straightforward to correlate these findings with the acceptor specificities of the cloned enzymes, it appears that A and B correspond to Gal3ST-2 and Gal3ST-3, respectively. On the other hand, the acceptor specificity of Gal3ST-3 appears to be identical to the enzyme described in human airways (17) while Gal3ST-2, which acts on type 1 oligosaccharides, is most likely responsible for the formation of sulfo→3Galβ1→4(Fucα1→3)GlcNAcβ1→R (4, 49).

It has been reported that the sulfo→3Galβ1→4(Fucα1→3)GlcNAc→R structure serves as an E-selectin ligand (4, 50). The same oligosaccharide was, on the other hand, shown to be a ligand for P-selectin but not for E-selectin when a synthetic oligosaccharide attached to GDP-fucose was transferred to the cell surface (5). Recently, it has been shown that 6-sulfo sialyl Lewis x, NeuNAcα2→3Galβ1→4[Fucα1→3(sulfo→6)]GlcNAcβ1→R, is a potent physiological ligand for L-selectin (11, 12). It will be of significance to determine if a 3′-sulfate analogue, sulfo→3Galβ1→4[Fucα1→3(sulfo→6)]GlcNAcβ1→R, also serves as an L-selectin ligand.

Considering the specificity of Gal3ST-3, sulfation should occur first on N-acetylglucosamine and then on galactose, forming sulfo→3Galβ1→4(sulfo→6)GlcNAcβ1→R. This product would then be converted to sulfo→3Galβ1→4[Fucα1→3(sulfo→6)]GlcNAc by α1,3-fucosyltransferase. It
is anticipated that all of these different oligosaccharides containing 3′-sulfo galactose can be synthesized in cells with the necessary cDNAs including those encoding Gal3ST-3.

In this context, it is noteworthy that Gal3ST-3 has a relatively narrow acceptor specificity compared to Gal3ST-2. Due to this distinct acceptor specificity, it is likely that the expression of Gal3ST-3 results in the formation of sulfo→3Galβ1→4GlcNAc→R and related structures in a well-defined set of carbohydrates attached to glycoproteins. It is thus expected that the cDNA encoding Gal3ST-3, cloned in the present study, will be a powerful tool to determine the structure/function of sulfo→3Galβ1→4GlcNAc→R and related structures.

Acknowledgements

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Footnotes

For page 1:

* This work was supported by the grant CA48737 awarded by the National Cancer Institute, National Institutes of Health.

For page 3:

1. Abbreviations used are: CHO, Chinese hamster ovary; PAPS, 3′-phosphoadenosine 5′-phosphate; Gal3ST, galactose 3-O-sulfotransferase; EST, expressed sequence tag; PCR, polymerase chain reaction; MES, 2-(N-morpholino)ethane sulfonic acid; C2GnT-1, core2 β1,6-N-acetylglicosaminyltransferase-1; HPLC, high performance liquid chromatography.

For page 13:

2. Gal3ST-3 cDNA was deposited in GenBank with the accession number AY026481.
Figure legends

Fig. 1. Nucleotide and translated amino acid sequences of Gal3ST-3. The signal membrane anchoring domain is denoted by a bold underline and N-glycosylation sites are marked by closed circles.

Fig. 2. Comparison of amino acid sequences of Gal3ST-1, Gal3ST-2, and Gal3ST-3 using the Clustal W program. Introduced gaps are shown by hyphens and identical aligned residues are boxed (black for all sequences, dark gray for two sequences). The transmembrane anchoring domains are underlined, and putative binding sites for 5′-phosphosulfate (5′-PSB) and 3′-phosphate (3′-PB) are denoted.

Fig. 3. Incorporation of [35S]sulfate into various synthetic acceptors by Gal3ST-2 and Gal3ST-3. Soluble forms of Gal3ST-2 and Gal3ST-3 were transiently expressed in CHO cells, and concentrated cell medium from the transfected CHO cells were used as an enzyme source. Products were purified by Sep-Pak cartridge column chromatography. Relative activities are shown in parentheses. Complete structures of the acceptors are described in “Experimental Procedures.” pNP, p-nitrophenol; PA, 2-aminopyridine.

Fig. 4. Incorporation of 35S-sulfate into NCAM•IgG and CD43•IgG chimeric proteins by Gal3ST-3 and Gal3ST-2. A. CHO cells were transiently transfected with NCAM•IgG or CD43•IgG cDNA together with Gal3ST-2 or Gal3ST-3 cDNA and metabolically labeled with 35S-sulfate. CHO cells apparently contain a sulfotransferase that can act on the N-glycans of
NCAM. B. CHO cells were transiently transfected with CD43•IgG and Gal3ST-3 in the presence of C2GnT-1 and metabolically labeled with $^{35}$S-sulfate. In all of these transfection studies, the total amount of plasmid was maintained constant. After isolation of the chimeric proteins with protein A-Sepharose, a portion of the sample was digested with N-glycanase. The amount of NCAM•IgG and CD43•IgG chimeric protein was estimated by Western blot analysis using anti-human IgG antibodies and an equivalent amount of the chimeric proteins were subjected to SDS-polyacrylamide gel electrophoresis followed by fluorography. The migration positions of standard molecular weight markers are shown at left.

Fig. 5. $^{35}$S-sulfate incorporation into CD43•IgG chimeric protein in CHO mutant, Lec2, Lec1, and Lec8 cells. A. Lec2 cells were transfected with plasmid vectors encoding CD43•IgG and Gal3ST-3 or Gal3ST-2 in the presence or absence of C2GnT-1 cDNA. B. Lec1 cells were transiently transfected with CD43•IgG and Gal3ST-3 in the presence or absence of C2GnT-1. C. Lec8 cells were transiently transfected with CD43•IgG and Gal3ST-3. The transfected cells were metabolically labeled with $^{35}$S-sulfate and the released CD43•IgG chimeric protein was analyzed in the same manner as Fig. 4.

Fig. 6. Analysis of O-linked oligosaccharides of CD43 labeled by Gal3ST-3 and Gal3ST-2. A, B. $^{35}$S-sulfate and $^3$H-galactose-labeled CD43•IgG chimeric protein in the presence of C2GnT-1 and Gal3ST-3 (A) or Gal3ST-2 (B) was subjected to pronase digestion and applied to a Sephadex G-50 column. C. Glycopeptides derived from Gal3ST-3 labeled CD43, shown as a bar in A, were pooled and subjected to alkaline borohydride treatment and the same Sephadex G-50
gel filtration. D, E. QAE-Sephadex column chromatography of O-glycans shown in C, before (D) and after (E) desialylation. Arrows indicate the elution positions with 70 mM (a), 120 mM (b), 140 mM (c), 170 mM (d), 250 mM (e), 500mM (f), and 1 M NaCl (g). F. Bio-Gel P-4 gel filtration of O-glycans shown in E. The arrow indicates the elution position of Galβ1→4GlcNAcβ1→6(Galβ1→3)GalNAcOH. In each chromatograph, fractions indicated by a horizontal bar were pooled and subjected to subsequent analysis. ○, 35S-sulfate; ●, 3H-galactose.

**Fig. 7. HPLC separation of O-glycans and 35S-labeled galactose obtained by Gal3ST-3.** A. O-glycans obtained after Bio-Gel P-4 gel filtration, shown in Fig. 6F, were desulfated and subjected to HPLC using an amino-bonded column (AX-5). Arrows denote the elution positions of core1 disaccharide (core1) and core2 tetrasaccharide (core2). B, C. Monosulfated galactose from Gal3ST-2 (B) and Gal3ST-3 (C) labeled N-acetyllactosamine was subjected to Partisial SAX-10 column chromatography. The elution positions of sulfo→6GlcNAc (arrow 3), sulfo→3GlcNAc (arrow 1), and sulfo→3Gal (arrow 2) are shown. Sulfo→6Gal elutes at the identical position as sulfo→6GlcNAc. Dotted lines denote KH2PO4 concentration.

**Fig. 8. Northern blot analysis of Gal3ST-3 transcripts.** Each lane contained 2 µg of poly(A)+ RNA. The blots were hybridized with 32P-labeled Gal3ST-3 cDNA. The migration positions of molecular markers are shown at left. The positions of the transcripts are indicated by arrowheads.

**Fig. 9. Dot blot analysis of Gal3ST-3 transcripts.** Human Multiple Tissue Expression (MTE™) Array shown at the left was hybridized with 32P-labeled Gal3ST-3 cDNA.
Suzuki et al. Figure 2
Suzuki et al. Figure 3
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Suzuki et al. Figure 5
Suzuki et al. Figure 7
Suzuki et al. Figure 8
Gal3ST-3

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Suzuki et al. Figure 9
Molecular cloning and expression of a novel human b-Gal-3-O-sulfotransferase that acts preferentially on N-acetyllactosamine in N- and O-glycans
Atsushi Suzuki, Nobuyoshi Hiraoka, Masami Suzuki, Kiyohiko Angata, Anup K. Misra, Joseph McAuliffe, Ole Hindsgaul and Minoru Fukuda

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