The evolution of galactose α 2,3-sialyltransferase: *Ciona intestinalis* ST3GAL I/II and
Takifugu rubripes ST3GAL II sialylate Gal β 1,3GalNAc structures on glycoproteins but
not glycolipids

Friederike Lehmann¹, Sørge Kelm², Frank Dietz², Mark von Itzstein¹, Joe Tiralongo^{1*}

¹ Institute for Glycomics, Gold Coast Campus, Griffith University QLD 4222 Australia

² Centre for Biomolecular Interactions Bremen, Department of Biology and Chemistry,
University Bremen, 28334 Bremen, Germany

*Corresponding Author: Dr Joe Tiralongo
Institute for Glycomics, Gold Coast Campus
Griffith University QLD 4222 Australia
Tel.: +61 7 5552 7029
Fax: +61 7 5552 8098
email: j.tiralongo@griffith.edu.au

Keywords: sialic acids, sialyltransferase, *Ciona intestinalis*, *Takifugu rubripes*, evolution

Abstract

Sialyltransferases are a family of enzymes catalyzing the transfer of sialic acid residues to terminal non-reducing positions of oligosaccharide chains of glycoproteins and glycolipids. Although expression of sialic acid is well documented in animals of the deuterostomian lineage, sialyltransferases have been predominantly described for relatively recent vertebrate lineages such as birds and mammals. This study outlines the characterization of the only sialyltransferase gene found in the tunicate *Ciona intestinalis*, the first such report of a non-vertebrate deuterostomian sialyltransferase, which has been discussed as a possible orthologue of the common ancestor of galactose α 2,3-sialyltransferases. We also report for the first time the characterization of a ST3Gal II gene from the bony fish *Takifugu rubripes*. We demonstrate that both genes encode functional α 2,3-sialyltransferases that are structurally and functionally related to the ST3Gal family of mammalian sialyltransferases. However, characterization of the recombinant, purified forms of both enzymes reveal novel acceptor substrate specificities, with sialylation of the disaccharide Gal β 1,3GalNAc and asialofetuin, but not GM1 or GD1b observed. This is in contrast to the mammalian ST3Gal II that predominantly sialylates gangliosides. Taken together the ceramide binding/recognition site previously proposed for the mouse ST3Gal II might represent a unique feature of mammalian ST3Gal II that is missing in the evolutionary more distant fish and tunicate species reported here. This suggests that during the evolution of the ST3Gal II, probably following the separation of the teleosts, a significant shift in substrate specificity enabling the sialylation of gangliosides took place.

Introduction

Sialic acids (Sia) are a family of 9-carbon α -keto acids found predominantly at distal positions of oligosaccharide chains of glycoproteins and glycolipids. They are glycosidically linked to either the 3- or 6-hydroxyl group of galactose (Gal) residues or to the 6-hydroxyl group of *N*-acetylglucosamine (GlcNAc) or *N*-acetylgalactosamine (GalNAc) residues, and can form oligo- to polysialic acid chains through either their 8-hydroxyl or 9-hydroxyl groups. The complexity of sialylated glycans is further enhanced through various substitutions of Sia - approximately 50 naturally occurring Sia derivatives have been identified [1, 2]. Because of their terminal position and unique physicochemical properties, sialylated oligosaccharide sequences are considered key determinants in a variety of complex biological regulatory and signaling events. Not only do Sia act as ligands in their own right, modulating a variety of recognition processes between cells and molecules mediated through Sia-specific lectins, but they also mask underlying structures, thus preventing binding of other lectins [2-4].

The transfer of Sia to glycoconjugates is catalyzed by a family of enzymes termed sialyltransferases (STs). These enzymes use CMP-Sia as the activated sugar donor, transferring Sia residues to the oligosaccharide chains of glycoproteins and glycolipids. Thus far, about 20 different STs have been cloned in mammals, each exhibiting a typical acceptor substrate specificity, forming one of the glycosidic linkages mentioned above [5-7]. Based on the type of glycosidic linkage formed, STs have been classified into four major subfamilies ST6Gal, ST6GalNAc, ST3Gal and ST8Sia, which synthesize Sia α 2,6Gal, Sia α 2,6GalNAc, Sia α 2,3Gal, and Sia α 2,8Sia, respectively [8]. It should be noted that the ST responsible for synthesizing Sia α 2,6GlcNAc is yet to be identified or cloned. Within each of the four major subgroups the acceptor substrate specificities are quite complex with large to almost complete overlap for some enzymes [5, 9], leading to partial redundancy.

All known vertebrate STs are type II transmembrane proteins with a short cytoplasmic domain and a signal anchor at their N-termini that is responsible for retention of the protein in the Golgi apparatus. This is followed by a stem region and a large C-terminal catalytic domain that faces the Golgi lumen. A comparison of peptide sequences strongly indicates that the length and amino acid composition of catalytic domains are relatively well conserved between STs and variations in protein sizes are generally attributable to differences in the length of the stem region [10]. All eukaryotic STs exhibit conserved peptide regions in their catalytic domain referred to as L (long)-, S (short)- and VS (very short) sialylmotifs, suggesting their common ancestry [11, 12]. In addition, another sialylmotif located between motifs S and VS, that seems to be involved in acceptor recognition, has been described [6]. Although expression of Sia is well documented in animals of the deuterostomian lineage (mainly in echinoderms and vertebrates), the evolution of STs still remains unclear. This is primarily because these enzymes have been predominantly described for relatively recent vertebrate lineages such as birds and mammals. However, phylogenetic analyses of different metazoan genomes suggests that ST subfamilies evolved by gene duplication following the protostomes-deuterostomes split, with the presence of ST subfamilies being a unique feature of deuterostomian species [13].

To provide further insights into the processes leading to the evolution of recent ST families it is necessary to determine the function of individual proteins and compare those with homologues in other deuterostomian species. In the present study, we report for the first time the characterization of the ST3Gal gene product identified in the tunicate *C. intestinalis* that has been discussed as a possible precursor to the ST3Gal subfamily [13], and the ST3Gal II gene from the bony fish *T. rubripes*. We demonstrate that these genes encode functional Gal- α 2,3-STs that are structurally and functionally related to the ST3Gal subfamily of mammalian STs, but have distinct acceptor substrate specificities. The characterisation of the *C.*

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4 *intestinalis* and *T. rubripes* STs described here therefore represents a significant advancement
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6 towards understanding the evolution of the animal ST3Gal family.
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10 **Material and Methods**

11 *Materials*

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15 *T. rubripes* brain RNA was obtained from MRC Gene Service (Cambridge, UK). Adult *C.*
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17 *intestinalis* were obtained from the Alfred-Wegener-Institut für Polar- und Meeresforschung,
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19 Helgoland, Germany. Tissue culture media were purchased from Invitrogen (Carlsbad, CA,
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21 USA). Unless otherwise stated all reagents were purchased from Sigma-Aldrich (St Louis, MI,
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23 USA). The carbohydrate compounds (TF, Cat. No. D11; Le^c, Cat. No. D8; Lac, Cat. No. D9;
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25 LacNac, Cat. No. D10; LacdiNac, Cat. No. D21 and Globoside, Cat. No. Te272) were
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27 provided by the Glycan Synthesis Core (D) of the Consortium for Functional Glycomics.
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32 33 34 *Homology searches for members of the ST3Gal subfamily in fish and tunicate genomes and* 35 36 *analysis of the identified sequences* 37

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39 The sequences of the human and murine Gal- α 2,3-STs were used as templates in homology
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41 searches of the *T. rubripes* whole genome shotgun assembly version 4.0 and the *C.*
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43 *intestinalis* whole genome shotgun assembly version 2.0 at the DOE Joint Genome Institute
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45 (JGI) Web site [14], and the Ensembl Zebrafish, Fugu and Tetraodon Genome Browsers at the
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47 Sanger Institute. Scaffolds showing e-values below 10^{-5} were assembled using VectorNTI and
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49 analyzed for putative protein-coding sequences using the GENSCAN Web Server at the MIT
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51 Department of Biology [15]. The multiple protein sequence alignment was constructed by
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53 VectorNTI using ClustalW algorithm [16]. Protein sequences of putative ST3Gal II
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55 orthologues were analyzed using the ExPASy Molecular Biology Server, the DAS-
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57 Transmembrane Prediction server [17], and the prediction servers available at the CBS
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(Center for Biological Sequence Analysis) home page. Genomic structures were deduced from comparison of cDNAs with genomic DNA sequences. The phylogenetic tree was constructed by a minimum evolution analysis (CN1 search level 2, gapped sites were pairwise deleted) using MEGA 3.0 software [18].

Construction of pPAXaHis

Using the vector pPROTA [19] as a template for PCR, the coding sequence of the signal peptide transin fused downstream in-frame to the IgG-binding domain of *S. aureus* Protein A was amplified with primers 5'-GCGGTACCATGAAAGGGCTCCCAG-3' and 5'-GCGGATCCGTGATGGTGATGGTGATGCGATCCTCTCCTTCCTCGATACCAGATCGTCTTTAAGGC-3' incorporating the *KpnI* and *BamHI* sites, and the coding sequence of endoprotease Xa and a 6xHis-tag expressed downstream of Protein A. The amplified PCR product was subsequently cloned into *KpnI/BamHI* digested pcDNA3 (Invitrogen, Carlsbad, CA, USA.) generating pPAXaHis. The integrity of the pPAXaHis was verified by DNA sequencing.

Isolation of putative ciona ST3Gal I/II and putative figu ST3Gal II genes and construction of expression vectors

Total RNA was prepared from *C. intestinalis* tissue using TriFast reagent (Peqlab, Erlangen, Germany) as described by the manufacturers. *C. intestinalis* heart and *T. rubripes* brain cDNA were generated using RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, Burlington, Canada). To verify the 3'-ends of the cDNA, nested RACE-PCR was performed using the GeneRacer Kit (Invitrogen) and the gene specific primers 5'-CGGACATGATTTTGTTCATTCG-3' (PCR 1) and 5'-CCTACCTATAACTATG-3' (PCR 2)

for *C. intestinalis*, and 5'-GCTGTTGTGGGCAACTCTGGGAACC-3' (PCR 1 and 2) for *T. rubripes*.

cDNA fragments encoding truncated forms of ciona and fugu ST3Gal lacking the *N*-terminal and transmembrane domain were prepared for expression by nested PCR using the primer sets 5'-CAACTTGTAATTAAGTCGTGTGATTGC-3' and 5'-GAGTAGGGCTAATTGTTTGGAAACTATG-3' (PCR 1), and 5'-GCGATATCCAAAAACAAAGCCGGCCAAG-3' and 5'-CGTCTAGACTATGATGATTGTTCCGACCTGTG-3' (PCR 2) for ciona. The primer sets 5'-CTGGAGGCGCCGGGGTGGGA -3' and 5'-CTCTAGATTACTTCCCAGGGAACACGGTGAT-3' (PCR 1), and 5'-GCGATATCCCGCGTGAAGCTGGTGCCCAG3' and 5'-GCTCTAGATTACTTCCCAGGGAACACGGTGAT-3' (PCR 2) were used for fugu. PCR was performed using cDNA generated from ciona heart and fugu brain RNA as template under the following conditions: 94 °C for 2 min, 25 cycles of 94 °C for 30 s, 55 °C for 45 s, 72 °C for 90 s, and 72 °C for 2 min. The resulting fragments were digested with *EcoRV* and *XbaI*, ligated into *EcoRV/XbaI* cut pPAXaHis, and the integrity of plasmids confirmed by sequencing.

Stable expression of ST3Gal/protein A-constructs

To establish stable cell lines expressing truncated forms of the putative ciona and fugu ST3Gal, CHO-K1 cells were transfected with the appropriate expression vectors using ExGen 500 *in vitro* transfection reagent (Fermentas). After 14 days of selection with G418 sulfate (Merck, Darmstadt, Germany) expression of recombinant protein A-His-tagged-ST3Gal was analyzed by Western blot using a His-tag (27E8) monoclonal antibody (Cell Signaling Technology, Beverly, MA, USA). Cells expressing the required protein were subjected to

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4 limiting dilution to obtain single transformant clones. Ten single transformant clones for each
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6 construct were established and again analyzed for recombinant ST3Gal expression. The
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8 clones showing highest expression were selected and cultivated in Ex-Cell CD CHO serum-
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10 free medium (14361C, Sigma-Aldrich).
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12 13 14 *Preparation of asialo-glycoproteins*

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16 Bovine submandibular mucin (BSM) and α_1 -acid glycoprotein (10 mg/mL) were incubated
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18 for 1 h in 0.05 M sulfuric acid at 80°C. The asialo-products were then neutralized, dialyzed
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20 and lyophilized prior to use.
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23 24 25 26 *Purification of ST3Gal/protein A-constructs and sialyltransferase assays*

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28 For purification of recombinant protein A-His-tagged-ST3Gal, medium from stably
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30 expressing CHO cell lines was collected after 72 h of cultivation and incubated with IgG-
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32 Sepharose beads (Sigma-Aldrich) on a rotator at 4°C overnight. Subsequently, the beads were
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34 washed with phosphate-buffered saline (PBS) and ST assay buffer (50 mM MOPS buffer (pH
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36 6.5), 20 mM MnCl_2) and suspended in 5 μL of ST assay buffer. The amount of purified
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38 protein was quantified by direct comparison of IgG beads-protein A-His-tagged-ST3Gal and
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40 His-tagged protein standards (Qiagen, Hilden, Germany) by Western blot using a monoclonal
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42 His-tag (27E8) antibody. Accurate quantitation of purified protein was confirmed in parallel
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44 using the monoclonal anti-Protein A antibody (clone SPA-27, Sigma-Aldrich).
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48 Enzyme assays were performed, with modification, as previously described [20]. Briefly, 5
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50 μL IgG beads-protein A-His-tagged-ST3Gal were combined with 5 μM CMP-[^{14}C]Neu5Ac
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52 (0.5 kBq) (GE Healthcare, Buckinghamshire, UK) and an appropriate acceptor substrate in ST
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54 assay buffer at 25°C with shaking for 16h. Acceptor substrates were used at final
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56 concentrations of 5 mg/mL glycoproteins, 1 mg/mL glycolipids, and 10 mM oligosaccharides
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4 in a volume of 10 μ L. Optimal acceptor substrate concentrations were identified by
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6 performing assays at various concentrations of substrates (1 - 5 mg/ml glycoproteins, 0.1 - 3
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8 mg/ml glycolipid and 0.5 - 10 mM oligosaccharide). When glycolipids were used as acceptor
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10 substrates, additional assays were performed in the presence of 0.5% Triton-X 100. As
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12 positive control instead of loaded IgG-beads 2 mU of either rat ST3Gal II (α 2,3-OST, Merck)
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14 or rat ST6Gal I (α 2,6-NST, Merck) were used and assays were carried out at a temperature of
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16 37°C.
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20 Reaction products were separated from CMP-[14 C]Neu5Ac depending on the acceptor
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22 substrate utilised. For glycoproteins, the reaction was terminated by the addition of SDS-
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24 PAGE loading buffer, and directly subjected to SDS-PAGE. For glycolipids, the reaction
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26 mixture was applied to a Phenomenex StrataTM X column (Phenomenex, Torrance, CA, USA)
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28 and purified glycolipids were analyzed on HPTLC silica gel 60 plates (Merck) using the
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30 mobile phase chloroform/methanol/0.02% CaCl_2 (50:40:10). Reaction mixture containing
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32 oligosaccharides were spotted directly onto HPTLC silica gel 60 plates and developed in 1-
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34 propyl alcohol/aqueous ammonia/water (6:1:2.5) as the mobile phase. The radioactive
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36 materials were visualized and quantified using radio-imaging.
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43 *Linkage Analysis*

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45 Sia linkage analysis was performed by initially radiolabelling asialofetuin with [14 C]Neu5Ac
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47 using either the ciona ST3Gal I/II, fugu ST3Gal II, rat ST3Gal II or rat ST6Gal I as described
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49 above, except that the reaction was stopped by the addition of 10% trichloroacetic acid. The
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51 resulting precipitate was suspended in an appropriate buffer as specified by the manufacturers
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53 and treated with *Salmonella typhimurium* α 2,3-sialidase (New England Biolabs, Beverly, MA,
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55 USA) and *Vibrio cholerae* α 2,3/6/8-sialidase (GE Healthcare) at 37°C for 3 h with shaking.
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59 Neo-sialylated asialofetuin for all ST investigated also underwent mock treatment in the
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4 absence of sialidase. The digested materials were then analysed by SDS-PAGE and radio-
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6 imaging.
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10 *Expression analysis via semiquantitative PCR*

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12 For semi-quantitative PCR, cDNA was synthesized from RNA of carp (*C. carpio*) tissue
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14 using RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas), and relative
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16 expression levels of carp ST3Gal II mRNAs were estimated using nested primers 5'-
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18 TGGTGGGTGATGTTGCAGCC-3' and 5'-ATCTTGCCAGCCTTGGCCAG-3' (PCR 1),
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20 and 5'-CATGTACCCAGAGAGTGCC-AAAAACC-3' and 5'-
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22 GATCTTGCCAGCCTTGGCCAG-3' (PCR 2). As a control, glyceraldehydes 3-phosphate
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24 dehydrogenase (GAPDH) gene expression was measured using GAPDH-specific primers 5'-
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26 GGTTTACATGTTCAAGTATGACTCC-3' and 5'-
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28 GGAATGACTTTGCCCACAGCCTTGGC-3'. PCR were performed as follows: 94°C for 3
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30 min, 25 cycles of 94°C for 30 s, 55°C for 30 s, 72 °C for 1 min and 72 °C for 10 min. PCR
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32 products were visualized following separation on 1% agarose gel.
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35 The analysis of ciona ST3Gal I/II gene expression was performed using total RNA extracted
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37 from *C. intestinalis* tissue using TriFast reagent (Peqlab) and cDNA was synthesized using
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39 RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas). Relative expression levels
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41 of ciona ST3Gal I/II mRNA were estimated by semi-quantitative PCR using nested primers
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43 5'-CTCATGACAGAGAGGCTGGC-3' and 5'-TTCCCCGCTCTGTAGCTCC-3' (PCR 1),
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45 and 5'- TGTGCTGTGGTTGGAAACTC-3' and 5'- TGCTGAGTCCATCGGTCATG-3'
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47 (PCR 2). As a control, actin gene expression was also measured using actin-specific primers
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49 5'- GTGTCGCACCAGAAGAGCAC-3' and 5'-TAACACGCAGCCTCGATTGG-3'. PCR
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51 were performed as follows: 94°C for 2 min, 25 cycles of 94°C for 30 s, 54 °C for 30 s, and 72
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°C for 1 min for the ST3Gal I/II gene, and 30 cycles for the actin gene; and 72 °C for 10 min. PCR products were visualized following separation on 1% agarose gel.

Results

Identification of putative ST3Gal genes in the genomes of Takifugu rubripes and Ciona intestinalis

By searching the complete *C. intestinalis* (ciona) genome database, we were able to identify a single putative mammalian-type ST gene (accession number AJ703817) showing a high sequence similarity to mammalian members of the ST3Gal family. A single putative ST (accession number AJ703818) with 70% amino acid sequence identity to the *C. intestinalis* putative ST gene was also identified in the *Ciona savignyi* genome. Further searches of the ciona genome failed to reveal any additional genes encoding proteins with significant similarity to vertebrate STs. Thus, the identified sequence appears to be the sole mammalian-like ST gene in *C. intestinalis*.

In contrast, the search of the *T. rubripes* (fugu) nucleotide sequence database revealed putative orthologues for almost all members of the ST3Gal subfamily, including a putative ST3Gal II (accession number AJ705069) with approximately 69% identity to the human ST3Gal II at the amino acid level. In addition, putative ST3Gal II orthologues can also be found in the genomes of other teleost fish species like *Danio rerio* (accession number AJ705062), *Tetraodon nigrovidis* (accession number AJ864514) and *Oryzias latipes* (accession number AJ871409) showing 87%, 90% and 86% identity at the amino acid level to the putative fugu ST3Gal II, respectively. Like mammalian STs, analysis of the primary sequence of both the putative fugu ST3Gal II and the putative ciona ST revealed a type II transmembrane protein containing a prominent N-terminal hydrophobic region corresponding to a putative signal peptide/anchoring domain. Moreover, their primary sequences contain all

sialylmotifs, including all amino acids known to be critical for enzymatic activity, as well as a high conservation in the linkage-specific motifs recently defined by Patel *et al.* (Fig. 1A) [6, 21, 22].

While the fugu sequence was easily assigned as a putative ST3Gal II, an unambiguous assignment of the ciona sequence was not possible. Although comparison of the ciona amino acid sequence with the mammalian ST3Gal subfamily revealed an affiliation with the ST3Gal I/II subgroup of mammalian ST3Gal, the sequence could not be ascribed to either, with approximately 39% sequence identity to both the human ST3Gal I and ST3Gal II. This is in good agreement with the phylogenetic analysis that placed the ciona ST as a single branch at the origin of the ST3Gal I/II clade suggesting that the branching of this putative ST occurred before the duplication event leading to the recent ST3Gal I and ST3Gal II. Based on this, the putative ciona ST sequence was named ciona ST3Gal I/II. In contrast, the fugu sequence is clearly placed within the ST3Gal II clade revealing its affiliation to mammalian ST3Gal II representatives (Fig. 1B).

These findings are further supported by the genomic organization of these genes (Fig. 2).

Although the start codon of the putative fugu ST3Gal II has not been identified, the genomic organization clearly shows its affiliation to the ST3Gal I and II members of mammalian Gal- α 2,3-STs. Interestingly, exon 2 of the *T. rubripes* and the closely related *T. nigroviridis* ST genes are split (Fig. 2), representing what may be a unique characteristic of ST genes from pufferfish species. In contrast, the gene of the putative ciona ST3Gal I/II shows a unique organization, completely different from those known from mammalian α 2,3-STs, further supporting its distinctive position within the ST3Gal subfamily.

Sialyltransferase activity and substrate specificity of fugu ST3Gal II and ciona ST3Gal I/II

To determine and characterise the enzymatic function of the newly identified putative ST genes, plasmids were constructed allowing the expression of truncated forms of the enzymes lacking the transmembrane domain as a secretable protein fused with the IgG-binding domain of *Staphylococcus aureus* protein A. These protein A-fused ST were then stably expressed in CHO-K1 cells, with secreted protein absorbed to IgG-Sepharose resin and subsequently used in a series of ST assays. In initial experiments, both enzymes were found to transfer radiolabelled Neu5Ac to the glycoprotein acceptor asialofetuin (Fig. 3A), with activity being optimal at 25°C. Therefore all subsequent assays were performed at this temperature. To further examine the substrate specificity, a panel of oligosaccharide, glycoprotein and glycolipid acceptors were assayed.

All enzymes of the ST3Gal subfamily described so far transfer Neu5Ac residues in an α 2,3-linkage to terminal galactose residues found in glycoproteins and glycolipids. Whereas mammalian ST3Gal I and II use exclusively the type III oligosaccharide structure Gal β 1-3GalNAc-R; ST3Gal III, IV, V, and VI transfer Neu5Ac to the oligosaccharide isomers Gal β 1-3/4Glc(NAc)-R [5, 23]. Among the oligosaccharides investigated, both the fugu ST3Gal II and ciona ST3Gal I/II showed high activity towards the disaccharide Gal β 1,3GalNAc (type III) (Fig 3B and Table 1), clearly demonstrating their affiliation to the mammalian ST3Gal I and II subgroup. As shown in Table I, significant activity was also observed using Gal β 1,3GlcNAc (type I). However, no activity was detected with the oligosaccharides Gal β 1,4GlcNAc (type II or LacNAc), Gal β 1,4Glc (Lac), GalNAc β 1,4GlcNAc (LacdiNAc), or GalNAc β 1,3Gal α 1,4Gal β 1,4Glc (Globo-N-tetraose). Notably, both the fugu ST3Gal II and ciona ST3Gal I/II exhibited the highest activity towards the GM1 oligosaccharide Gal β 1,3GalNAc β 1,4[Neu5Ac α 2,3]Gal β 1,4Glc β 1, but could not utilise either of the gangliosides GM1 or GM1b as acceptor substrates under the experiment conditions employed (Fig. 4B and Table 1). The addition of 0.5% Triton X-100 had no effect

on fugu ST3Gal II and ciona ST3Gal I/II activity against the ganglioside tested. The lack of ST activity against ganglioside acceptors is in stark contrast to that observed for rat liver ST3Gal II that sialylated the gangliosides GD1b and GM1 (Fig. 4B and Table 1), as well as asialofetuin (Fig. 4A and Table 1). This is in good agreement with previous reports describing gangliosides, particularly GM1 as the predominant acceptor for rat ST3Gal II [24]. Among the glycoproteins tested, only asialofetuin was utilised by the fugu ST3Gal II and ciona ST3Gal I/II as an acceptor substrate. No activity was detected against fetuin, α_1 -acid glycoprotein and bovine submaxillary gland mucin, or their asialo-forms (Table 1). The fact that no signal was detected using asialo-BSM as acceptor substrate may be explained by the fact that only 5% of the total carbohydrate chains of asialo-BSM contain the Gal β 1,3GalNAc sequence [25]. Remarkably, in comparison to the ciona ST3Gal I/II, the fugu ST3Gal II exhibited an extremely low *in vitro* ST activity, with a relative activity of 8% and 1.6% against Gal β 1,3GalNAc and asialofetuin, respectively (Fig. 3C).

Linkage analysis by sialidase treatment

To determine the nature of the incorporated Sia linkage, asialofetuin was initially labelled with [14 C]Neu5Ac using either fugu ST3Gal II or ciona ST3Gal I/II. The resulting sialylated product was then subjected to treatment with *S. typhimurium* α 2,3-sialidase. Neo-sialylated asialofetuin labelled using the fugu ST3Gal II (Fig. 5A), the ciona ST3Gal I/II (Fig. 5B) and the positive control rat ST3Gal II (Fig. 5C) were α 2,3-sialidase-sensitive, while asialofetuin labelled using the rat ST6Gal I (Fig. 5D) was α 2,3-sialidase-insensitive. In all cases, the labelled Neu5Ac transferred to asialofetuin was sensitive to *V. cholerae* α 2,3/6/8-sialidase treatment. These results clearly illustrate that the putative fugu ST3Gal II and putative ciona ST3Gal I/II transfer Sia to asialofetuin to form an α 2,3-linkage, and therefore can be classified as members of the ST3Gal subfamily.

Expression of fish ST3Gal II and ciona ST3Gal I/II genes in different tissues

The expression of fish ST3Gal II was examined using RNA isolated from carp (*Cyprinus carpio*) tissue due to the scarcity of fugu tissue, and the ability to isolate sufficient quantities of RNA for analysis. Therefore, an authentic probe for the putative carp ST3Gal II (accession number AM409320) that possesses 83% sequence identity to fugu ST3Gal II, was synthesized and sequenced. ST3Gal II expression was detected using RT-PCR in all tissue investigated, with the highest expression in the brain, followed by the heart, liver and pronephros, and only very low expression in the spinal cord, muscle and kidney (Fig. 6A).

The expression of ciona ST3Gal I/II mRNA was examined using semi-quantitative nested RT-PCR. ST3Gal I/II gene was significantly detected in the heart and gonads, with no expression observed in the body-wall muscle and unfertilized eggs (Fig. 6B).

Discussion

Traditionally, the animal ST superfamily has been divided into four subfamilies: ST3Gal, ST6Gal, ST6GalNAc, and ST8Sia. Within each of these subfamilies amino acid sequences and genomic organization are highly conserved and the acceptor substrate specificities are quite complex with large to almost complete overlap for some enzymes [5, 23]. Recent phylogenetic studies on the ST superfamily have suggested a common genetic origin of these subfamilies from one ancestral gene followed by gene duplication and divergent evolution [13].

Although bony fish genomes contain putative orthologues for almost all known mammalian STs, only single putative representatives of the four main subfamilies have been detected in non-vertebrate deuterostomes, with none being direct orthologues of a mammalian ST [13].

Therefore, it has been suggested that the duplication events leading to the complex ST

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4 superfamily found in mammals occurred early in the vertebrate lineage, with some of these
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6 single invertebrate ST being ancestral orthologues of the four vertebrate subfamilies.
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8 However, a complete understanding of the origin of the gene lineage leading to the evolution
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10 of mammalian Gal- α 2,3-STs is greatly hampered by the lack of functional and substrate
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12 specificity data for any of the invertebrate STs. In order to elucidate the origin of the ST3Gal
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14 gene lineage, we have studied the only ST-like gene present in the genome of the sea squirt *C.*
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16 *intestinalis* since this gene exhibits the highest similarity to the mammalian ST3Gal I and
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18 ST3Gal II and may represent an orthologue of the common ancestor of the ST3Gal subfamily.
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20 To further track the evolution of the ST3Gal subfamily in early vertebrates, the putative
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22 orthologue of the mammalian ST3Gal II in the pufferfish *T. rubripes* was also characterized.
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24 Both the fugu ST3Gal II and ciona ST3Gal I/II possess protein topologies typical of STs, as
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26 well as ST-distinctive sialyl motifs and strictly conserved Cys, Tyr, Gly and His residues
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28 within these motifs (Fig. 1). Both the ciona ST3Gal I/II and fugu ST3Gal II genes encode
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30 functional Gal- α 2,3-STs, with enzymatic characterisation of the purified enzymes revealing
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32 that the transfer of Sia occurs, as seen in mammalian ST3Gal's, via an α 2,3-linkage to
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34 terminal Gal residues. Like the mammalian ST3Gal I and ST3Gal II members [20, 24], the
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36 non-mammalian Gal- α 2,3-STs described here almost exclusively utilise the type III
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38 oligosaccharide structure Gal β 1,3GalNAc-R as an acceptor substrate, showing low activity
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40 towards the type I oligosaccharide Gal β 1,3GlcNAc-R and no activity towards
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42 Gal β 1,4GlcNAc-R (type II), which is the predominant acceptor substrate for mammalian
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44 ST3Gal III to VI. These findings clearly show their affiliation to the ST3Gal I and II subgroup
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46 of the ST3Gal subfamily confirming the results of the phylogenetic analyses that place the
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48 fugu ST within the ST3Gal II clade and the ciona sequence at the origin of the ST3Gal I/II
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50 clade. However, our study has revealed a distinct substrate specificity difference between the
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52 ciona ST3Gal I/II and fugu ST3Gal II and previously described mammalian ST3Gal I and
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ST3Gal II. Whereas mammalian ST3Gal I and ST3Gal II sialylate glycolipids with gangliosides being the predominant acceptor for mammalian ST3Gal II [26], the ciona ST3Gal I/II and fugu ST3Gal II were found not to utilise glycolipids as acceptor substrates. The ability of the ciona ST3Gal I/II and fugu ST3Gal II to sialylate asialofetuin to a similar extent as the rat ST3Gal II positive control (Fig. 4A), but not GM1 and GM1b (Fig. 4B), even though the same quantity of enzyme were used in both the asialofetuin and ganglioside assays, clearly substantiates the difference in specificity. Also, we do not believe that the observed lack of activity against gangliosides reflects the use of soluble truncated forms of the enzymes fused to protein A as shown for some fucosyltransferases [27], since several studies utilising protein A-fused soluble mammalian ST3Gal I and ST3Gal II, non protein A-fused constructs, as well as purified native enzyme did not reveal any major differences in substrate specificity [28-31]. However, it should be noted that N-terminal truncation of the human ST6Gal I and ST6Gal II has been shown to impact on acceptor substrate specificity [10, 32].

Unfortunately, a prediction of the fine specificity of the non-mammalian STs reported here is greatly hampered by the lack of knowledge regarding the respective glycomes, with structural and functional studies mostly focused on very specific forms of glycosylation. Even though, like the mouse ST3Gal II, the carp ST3Gal II shows strongest expression in brain, the fact that fugu ST3Gal II does not have any detectable *in vitro* activity towards glycolipids suggests a function for the fish ST3Gal II that is related more to the sialylation of glycoproteins or oligosaccharides than to the sialylation of gangliosides. Our data clearly shows that the fugu ST3Gal II is unlikely to represent the orthologue of the sialyltransferase-IV isolated from cichlid fish brain that catalyzes the synthesis of gangliosides GM1b from GA1, G1a from GM1, and GT1b from GD1b, as well as GQ1c from GT1c [33]. Notably, the genomes of *T. rubripes*, and other fish species like *D. rerio* and *T. nigroviridis*, contain two homologues of the ST3Gal I gene that may be responsible for the synthesis of the above mentioned

gangliosides that are enriched particularly in the fish nervous system. Instead the fish ST3Gal II might be involved in the synthesis of sialylated core 1 *O*-glycans carrying a $\text{Fuca}\alpha 1,3\text{GalNAc}\beta 1,4(\text{Neu5Gc/Neu5Ac}\alpha 2,3)\text{Gal}\beta 1,3\text{GalNAc}$ sequence that have recently been identified as the predominant *O*-glycans in all developmental stages of the zebrafish *D. rerio* [34]. Moreover, the fish ST3Gal II may also participate in the synthesis of *O*-glycans of mucin-type glycoproteins found in the vitelline envelope of salmonide fish species containing either KDN or Neu5Gc $\alpha 2,3$ -linked to $\text{Gal}\beta 1,3\text{GalNAc}$ [35]. Interestingly, compared to the ciona ST3Gal I/II and rat ST3Gal II, the fugu ST3Gal II exhibited very low *in vitro* activity towards all substrates tested, a fact that has also been described for polySTs in rainbow trout [36]. This raises the possibility that the fugu ST3Gal II might prefer CMP-KDN or CMP-Neu5Gc as the donor substrate instead of CMP-Neu5Ac. In this context it is important to note that at least in the zebrafish glycome, Neu5Ac and Neu5Gc are not evenly distributed, suggesting that some form of ST donor substrate selectivity may occur in fish [34]. Further studies are currently underway to examine the donor substrate specificity of the fugu ST3Gal II.

The cellular function of the ciona ST3Gal I/II is particularly difficult to predict, mainly because in contrast to other deuterostomes, very little is known about the presence and function of Sia in urochordates. Early studies failed to detect Sia in the eggs, larvae or adult forms of different urochordate species [37, 38]. However, the expression of a ST in different tissues of adult *C. intestinalis* (Fig. 6B), and the presence of a gene encoding a highly conserved putative orthologue of the vertebrate CMP-Sia synthetase (accession number AM262834) suggests that *C. intestinalis* may express sialylated glycoconjugates. However, extensive searches of the *C. intestinalis* genome have failed to identify putative orthologues of other key proteins involved in Sia biosynthesis including UPD-GlcNAc-2-epimerase/ManNAc kinase, Neu5Ac-9-phosphate synthase, the CMP-Sia transporter and

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4 CMP-Sia hydroxylase. This together with the fact that tunicate genomes contain only one ST
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6 gene suggests that the sialylation apparatus in general and, in particular the diversity of
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8 sialylated glycans, might have undergone a secondary reduction in urochordate species.
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10 Interestingly, Kojima *et al.* showed that asialoGM1 is a non-competitive inhibitor of the
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12 mouse ST3Gal II when Gal β 1,3GalNAc or asialofetuin was used as the acceptor substrate.
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14 This suggests that either the mammalian ST3Gal II recognize gangliosides and glycoproteins
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16 as distinct acceptors with distinct binding sites, or that a second recognition/binding site
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18 accommodating the ceramide moiety in addition to the Gal β 1,3GalNAc moiety exists [26]. In
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20 this context, the fact that under the experimental conditions used the ciona and the fugu α 2,3-
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22 STs are able to utilize the GM1 oligosaccharide
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24 Gal β 1,3GalNAc β 1,4[Neu5Ac α 2,3]Gal β 1,4Glc β 1 as an acceptor but not GM1 or GM1b
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26 suggests that the ceramide portion may prevent binding of the oligosaccharide moiety thus
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28 influencing enzyme activity. Therefore, the ceramide binding/recognition site proposed for
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30 the mouse ST3Gal II [26] may represent a unique feature of mammalian ST3Gal II that is
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32 missing in evolutionary more distant species like fish and tunicates. Taken together, our
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34 results suggest that the first Gal- α 2,3-ST specifically sialylated glycoproteins, with substrate
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36 specificities similar to that demonstrated here for the ciona ST3Gal I/II. Evolving from this
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38 ancestor and subsequent to the separation of the teleosts, vertebrate ST3Gal II broadened their
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40 substrate specificities to enable sialylation of gangliosides, possibly through the attainment of
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42 a ceramide-binding site. This has obvious implications for ganglioside evolution, particularly
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44 given the phylogenetic differences in brain ganglioside content, composition and variability
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46 seen in deuterostomes [39, 40].
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Acknowledgements

FL gratefully acknowledges the Deutsche Forschungsgemeinschaft (DFG) for the awarding of a Research Fellowship. SK acknowledges the funding by a Sir Allan Sewell Fellowship. MvI thanks the Australian Research Council (ARC) for the award of a Federation Fellowship and the Alexander von Humboldt Stiftung for the award of a von Humboldt Forschungspreis. JT gratefully acknowledges the ARC for the awarding of an Australian Postdoctoral Fellowship. The authors also wish to thank Dr. Milton Kiefel for providing helpful advice. We also wish to acknowledge the Consortium for Functional Glycomics Grant number GM62116 for providing the oligosaccharides used in this study.

Legends to figures

Fig. 1. Fugu ST3Gal II and ciona ST3Gal I/II are structurally related to Gal- α 2,3-STs. A. Amino acid sequence alignment. Peptide sequences of the sialylmotif regions and linkage-specific motifs as defined by Patel *et al.* 2006 [22] of human (h) and murine (m) members of the ST3Gal subfamily have been aligned with corresponding sequence regions of *T. rubripes* (f) ST3Gal II and *C. intestinalis* (c) ST3Gal I/II using VectorNTI based on the ClustalW algorithm as described under Experimental Procedures. Amino acid residues known to be essential for enzyme activity are indicated with asterisks. B. Dendrogram of human (h) and murine (m) members of the ST3Gal subfamily together with homologues from *T. rubripes* (f) and *C. intestinalis* (c). Complete protein sequences were aligned and the phylogenetic tree was constructed by a minimum evolution analysis using MEGA 3.0 software. The scale bar shows relative units of evolutionary distances.

Fig. 2. Genomic organization of Gal- α 2,3-STs. Schematic diagram showing the genomic structures of ciona ST3Gal I/II and fish representatives of ST3Gal II, along with mammalian members of the ST3Gal subfamily. Exons are boxed with the corresponding lengths indicated in base pairs within. The length of the first exon was calculated from the start codon and the length of the last exon includes the stop codon. Unknown starts codons are indicated by dashed lines. Sialylmotifs are indicated by black bars. Accession numbers are given on the right side. *Has*, *Homo sapiens*; *Mmu*, *Mus musculus*; *Cin*, *Ciona intestinalis*; *Dre*, *Danio rerio*; *Fru*, *Takifugu rubripes* and *Tni*, *Tetraodon nigrovidis*. Figure is not drawn to scale.

Fig. 3. Comparison of the ciona ST3Gal I/II and fugu ST3Gal II enzyme activities. Ciona ST3Gal I/II and fugu ST3Gal II were tested for their activity using asialofetuin (A) and

Gal β 1,3GalNAc (B). The radioactive products were visualized and quantified by radio-imaging (I.). The amount of recombinant ST was quantified by Western-blot analysis using His-tag antibody (II.). Proteins were incubated for 16 h at 25°C in the presence of 5 mg/ml asialofetuin or 10 mM Gal β 1,3GalNAc. Both substrates also underwent mock treatment in the absence of enzyme. For asialofetuin, the reaction was terminated by the addition of SDS-PAGE loading buffer, and directly subjected to SDS-PAGE. When using Gal β 1,3GalNAc, the reaction mixture was spotted directly on HPTLC plates and developed in 1-propyl alcohol/aqueous ammonia/water (6:1:2.5). Sialylated Gal β 1,3GalNAc in panel B (I.) is indicated with an arrow. Panel C shows enzyme activities (two independent experiments performed in duplicate) for the ciona ST3Gal I/II (set at 100%) and fugu ST3Gal II. Enzyme activities were determined by normalizing the formation of product (I.) against the amount of recombinant ST quantified by Western-blot analysis using His-tag antibody (II.).

Fig. 4. Substrate specificity differences between the fugu ST3Gal II, ciona ST3Gal I/II and rat ST3Gal II. Fugu ST3Gal II, ciona ST3Gal I/II and rat ST3Gal II were assayed using asialofetuin (A) and gangliosides GD1b (lane 1) and GM1 (lane 2) (B) as acceptors. Enzyme was incubated for 16 h at 25°C (fugu and ciona) or 37°C (rat) in the presence of 5 mg/ml asialofetuin or 1 mg/ml ganglioside. Two mU of rat ST3Gal II, and equal quantities of fugu ST3Gal II and ciona ST3Gal I/II, as determined by Western-blot analysis using His-tag antibody, were used to evaluate ST activity against both asialofetuin and gangliosides. For asialofetuin, the reaction was terminated by the addition of SDS-PAGE loading buffer, and directly subjected to SDS-PAGE. For glycolipids, the reaction mixture was applied to a Phenomenex StrataTM X column and the purified glycolipids were analyzed on HPTLC plates using the mobile phase chloroform/methanol/0.02% CaCl₂ (50:40:10). The radioactive

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10 **Fig. 5.** Analysis of ciona ST3Gal I/II and fugu ST3Gal II linkage specificity. [¹⁴C]Neu5Ac-
11 labelled asialofetuin produced using fugu ST3Gal II (A), ciona ST3Gal I/II (B), rat ST3Gal II
12 (C) and rat ST6Gal I (D) were subjected to sialidase treatment either with *Salmonella*
13 *typhimurium* α 2,3-sialidase or *Vibrio cholerae* α 2,3/6/8-sialidase, with the resulting products
14 separated on SDS/PAGE and detected by radio-imaging.
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24 **Fig. 6.** Expression analysis of fish ST3Gal II and ciona ST3Gal I/II genes. A. RT-PCR
25 analysis of ST3Gal II expression in carp (*C. carpio*) tissue by semi-quantitative PCR. Lane 1,
26 brain; lane 2, spinal cord; lane 3, muscle; lane 4, heart; lane 5, liver; lane 6, kidney; lane 7,
27 pronephros. B. RT-PCR analysis of ST3Gal I/II expression in *C. intestinalis* tissue by semi-
28 quantitative PCR. Lane 1, heart; lane 2, gonads; lane 3, body-wall muscle; lane 4, unfertilized
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Table 1. Comparison of the substrate specificity of the ciona ST3Gal I/II and fugu ST3Gal II with rat ST3Gal II. The table shows ST activities against various putative acceptor substrates for each enzyme relative to the incorporation of [¹⁴C]Neu5Ac into asialofetuin, GM1 and Galb1,3GalNAc disaccharide. Substrates were used at the final concentration of 5 mg/ml glycoproteins, 1 mg/ml glycolipids and 10 mM oligosaccharides. n.d., not determined; * shown to be an acceptor substrate by Lee *et al.* [24]; # shown to be an acceptor substrate by Toivonen *et al.* [41].

	Structure(s) ^a	cST3Gal I/II	fST3Gal II	rST3Gal II
Glycoproteins				
Fetuin	Neu5Acα2,3Galβ1,3GalNAcβ1-O-Ser/Thr Neu5Acα2,3Galβ1,3[Neu5Acα2,6]GalNAc-O-Ser/Thr Neu5Acα2,3Galβ1,4GlcNAc-R	0	0	n.d.
Asialofetuin	Galβ1,3GalNAcβ1-O-Ser/Thr Galβ1,4GlcNAcβ1-R	+++	+++	+++*
α ₁ -Acid glycoprotein	Neu5Acα2,6Galβ1,4GlcNAcβ-R	0	0	n.d.
Asialo-α ₁ -Acid glycoprotein	Galβ1,4GlcNAcβ-R	0	0	0
BSM	Neu5Acα2,6GalNAcβ1-O-Ser/Thr Galβ1,3[Neu5Acα2,6]GalNAc-O-Ser/Thr GlcNAcβ1,3[Neu5Acα2,6]GalNAc-O-Ser/Thr	0	0	n.d.*
Asialo-BSM	GalNAcβ1-O-Ser/Thr Galβ1,3GalNAc-O-Ser/Thr GlcNAcβ1,3GalNAc-O-Ser/Thr	0	0	n.d.
IgG	Neu5Acα2,6Galβ1,4GlcNAc-R Galβ1,4GlcNAc-R	0	0	n.d.
Gangliosides				
GM1	Galβ1,3GalNAcβ1,4[Neu5Acα2,3]Galβ1,4Glcβ1-Cer	0	0	+++*
GD1b	Galβ1,3GalNAcβ1,4[Neu5Acα2,8Neu5Acα2,3]Galβ1,4Glcβ1-Cer	0	0	+++*
Oligosaccharides				
TF	Galβ1,3GalNAcα-R ₁ ^b	+++	+++	n.d.*
Lec	Galβ1,3GlcNAcβ-R ₂ ^b	++	+	0
Lac	Galβ1,4Glcβ- R ₂ ^b	0	0	n.d.
LacNAc	Galβ1,4GlcNAcβ- R ₂ ^b	0	0	0
LacdiNAc	GalNAcβ1,4GlcNAcβ- R ₂ ^b	0	0	n.d.
Globoside (P-antigen)	GalNAcβ1,3Galα1,4Lacβ- R ₂ ^b	0	0	n.d.#
GM1	Galβ1,3GalNAcβ1,4[Neu5Acα2,3]Galβ1,4Glcβ1	+++	++++	n.d.

^a Data from Green *et al.* 1988 and Spiro and Bhoyroo 1974 for fetuin [42, 43]; Fournier *et al.* 2000 for α₁-acid glycoprotein [44]; Tsuji and Osawa 1986 for BSM (bovine submaxillary mucin) [25]; and Wormald *et al.* 1997 for IgG [45].

^b R₁ represents Thr(NAc)OMe and R₂ represent (-CH₂CH₂N₃)

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