The β1,3-Galactosyltransferase β3GalT-V Is a Stage-specific Embryonic Antigen-3 (SSEA-3) Synthase*

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We have previously reported the molecular cloning of β1,3-galactosyltransferase-V (β3GalT-V), which catalyzes the transfer of Gal to GlcNAc-based acceptors with a preference for the core3 O-linked glycan GlcNAc(β1,3)-GalNAc structure. Further characterization indicated that the recombinant β3GalT-V enzyme expressed in Sf9 insect cells also utilized the glycolipid Lac3Cer as an efficient acceptor. Surprisingly, we also found that β3GalT-V catalyzes the transfer of Gal to the terminal GalNAc unit of the globoside Gb4, thereby synthesizing the glycolipid Gb5, also known as the stage-specific embryonic antigen-3 (SSEA-3). The SSEA-3 synthase activity of β3GalT-V was confirmed in vivo by stable expression of the human β3GalT-V gene in F9 mouse teratocarcinoma cells, as detected with the monoclonal antibody MC-631, by flow cytometry analysis and immunostaining of extracted glycolipids. The biological relation between SSEA-3 formation and β3GalT-V was further documented by showing that F9 cells treated with the differentiation-inducing agent retinoic acid induced the expression of both the SSEA-3 epitope and the endogenous mouse β3GalT-V gene. This study represents the first example of a glycosyltransferase, which utilizes two kinds of sugar acceptor substrates without requiring any additional modifier molecule.

Glycosyltransferase enzymes mediate the synthesis of glycosylated structures by catalyzing the transfer of sugar units to various acceptor molecules utilizing nucleotide-activated sugars as donor substrates. In most cases, a single glycosyltransferase enables the transfer of one type of carbohydrate acceptor, although some exceptions have been reported. The IgT8 glycosyltransferase from Neisseria meningitidis represents such an exception, as it can transfer two donors, GlcNAc and GalNAc, to Gal-based acceptors (1). Another notable exception is represented by the β1,4-galactosyltransferase-I enzyme, which is able to change its acceptor substrate specificity from GlcNAc to Gal by interacting with α-lactalbumin (2, 3).

Previous work from this group and other laboratories has revealed a family of structurally related β1,3-galactosyltransferase enzymes (4–10). During the course of our investigation, we have found that one member of this family, the β1,3-galactosyltransferase (β3GalT)-V enzyme that normally acts on GlcNAc-based acceptors, can catalyze the transfer of galactose to GalNAc presented in the context of the Gb4 globoside structure. The resulting product, the Gb5 globoside, is also known as the stage-specific embryonic antigen (SSEA)-3 (11), which is found in the 4–8 cell stage mouse embryo as well as some adult tissues. We have established the SSEA-3 synthase activity of β3GalT-V both in vitro and in vivo in F9 mouse teratocarcinoma cells as a model system.

EXPERIMENTAL PROCEDURES

Galactosyltransferase Assays—The human β3GalT-V enzyme was expressed in Sf9 insect cells as a recombinant baculovirus as described previously (10). Enzyme assays with glycolipid acceptors were performed with 10 μl of baculovirus-infected cell lysate in a 50-μl reaction volume containing 100 mM sodium cacodylate (pH 7.0), 10 mM MnCl2, 10 mM CDP-choline, 0.4% Triton CF-54, 0.2 mM UDP-[14C]galactose (1 × 106 cpm/nmol) and either 20 μg of Gb4 (Matreyre, Pleasant Gap, PA) or 5 μg of Lac3Cer. Incubations were performed for 4 h at 37 °C, then stopped by the addition of 0.1 mM potassium chloride/methanol:H2O:chloroform (48:47:3), and lipid products purified by reverse phase extraction on Bond-Elut C18 columns (Varian). Lipid residues dried under N2 revealed a family of structurally related glycans GlcNAc(β1,3)-GalNAc. The resulting product, the Gb5 globoside, is also known as the stage-specific embryonic antigen (SSEA)-3 (11), which is found in the 4–8 cell stage mouse embryo as well as some adult tissues. We have established the SSEA-3 synthase activity of β3GalT-V both in vitro and in vivo in F9 mouse teratocarcinoma cells as a model system.

Glycolipid Analysis—Glycolipids were extracted from NCCIT cells as described previously (13). Lipids were separated in a FACSscan flow cytometer (Becton Dickinson).

Flow Cytometry—The monoclonal antibody MC-631 (12) was purchased from the Developmental Studies Hybridoma Bank (Iowa City, IA). G418-resistant cells (5 × 105) were first incubated with MC-631 (1:500) for 15 min on ice. A fluorescein isothiocyanate-labeled rabbit anti-rat IgM antibody (Zymed Laboratories Inc., San Francisco, CA) diluted at 1:500 was used as secondary reagent. Labeled cells were analyzed in a FACScan flow cytometer (Becton Dickinson).

The abbreviations used are: β3GalT, β1,3-galactosyltransferase; SSEA, stage-specific embryonic antigen; RT, reverse transcription; PCR, polymerase chain reaction; Gb4, GalNAc(β1,3GalNAc(β1,4Gal-β1,3)Glc-ceramide; Gb5, Gal(β1,3)GalNAc(β1,3GalNAc(β1,4Gal-β1,3)Glc-ceramide; Lac3Cer, GlcNAc(β1,3Galβ1,4Glc-ceramide; FTT, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; bp, base pair(s).
species by fractionation on DEAE-Sephadex A-25 (Sigma). Immuno-

staining of extracted glycolipids was performed essentially as described previously (14). Briefly, glycolipid species were chromatographed on aluminum high-performance TLC plates (E. Merck) in a solvent system of chloroform:methanol:0.25% CaCl2. Plates were fixed with 0.05% poly-
(isobutyl methacrylate) in n-hexane and blocked with 5% bovine

serum albumin prior to 4°C incubation with 1:5 diluted MC-631 hybrid-
doma supernatant. After washing and incubation with 1:400 diluted

peroxidase-conjugated goat anti-mouse IgM (Roche Molecular Bio-

chemicals), reactivity was visualized with 4-chloronaphthol substrate

(Sigma).

**Retinoic Acid Treatment**—F9 cells were seeded at low density (5,000
cells/80-cm² dish) and treated with 10⁻⁷ m all-trans-retinoic acid
(Sigma) for 10 days. Cells were analyzed for SSEA-3 expression by
flow cytometry as described above.

**Cloning and Expression of the Mouse β3GalT-V Gene**—A 129/SvJ
mouse genomic A-library (Stratagene) was screened as described previ-
ously (4) using the human β3GalT-V gene as a probe. The genomic
region, including the sequence encoding the catalytic domain of the
β3GalT-V enzyme, was amplified by PCR with the primers 5'-CAAG-

CGATGCTCTCAGAAGAATC-3' and 5'-CCCAAGGATTCGTGATATC-3'
and subcloned into the pFLAG-CMV-1 (vector). The medium of COS-7 cells transiently transfected with the β3GalT-V vector was incubated with 20 μl of anti-FLAG M2-agarose beads (Sigma) for 2 h at 4 °C. After washing three times with phosphate-

buffered saline, the beads were directly used for galactosyltransferase
assays. For metabolic labeling, transfected cells were starved for 2 h in
methionine-free Dulbecco’s modified Eagle’s medium, then incubated
in the presence of 0.1 mCi of [³⁵S]methionine for 1 h. After replacement
with complete medium, cells were further incubated for 4 h at 37 °C.
After this time, the cell supernatant was collected and incubated with
anti-FLAG M2-beads as described above.

**Reverse Transcription (RT)-PCR**—Total RNA was extracted from F9
cells using the procedure of Chomczynski and Sacchi (15). RNA samples
were digested with RNase-free DNase-I (Sigma) before RT-PCR. The
mouse β3GalT-V RNA (5 μg) was reverse-transcribed using 1 unit of
enhanced avian myeloblastosis virus reverse transcriptase (Sigma) and
the primer 5'-AGTCCTGTTTCTTCAGAAG-3' and 5'-GAAAGGATTTAGACTGTACATGC-3'. Samples in a final
volume of 20 μl were incubated for 2 h at 42 °C. PCR amplification was
performed using 4 units of Taq polymerase (Sigma) with 10 μl of the
RT reaction product and the primers 5'-CAAGGATTCCTCACTTCAGC-

CCTG-3', 5'-CCCAAGGATTCGTGATATC-3' and 5'-CCCAAGGATTCGT-

GATATC-3' and 5'-GAAAGGATTTAGACTGTACATGC-3' and 5'-GTGA-

TCTCTTCTTCCTCTG-3' using the same conditions as described above.

**RESULTS**

During our investigation of the donor and acceptor substrate
specificity of the β3GalT-V enzyme, we have found that this
enzyme exhibited a preference for GlcNAc-based acceptors (10).
However, when assaying glycolipid acceptors, the β3GalT-V
enzyme showed activity toward the lacto-series glycolipid
LacCer and toward the globoside Gb4 (Fig. 1). The latter activity
was surprising as the β3GalT-V enzyme displayed no activity

**Fig. 1.** SSEA-3 synthase activity of β3GalT-V in vitro. Lysate from S9 cells infected with recombinant β3GalT-V baculovirus or with a mock baculovirus were incubated with or without Gb4 glycolipid acceptors. The left panel shows the orcinol staining of the reaction products resolved on a thin-layer chromatography plate. The right panel shows an autoradiogram of the same plate. The migration of the standard glycolipids LacCer and Gb4 is indicated on the left.

**Fig. 2.** Purification of the soluble tagged β3GalT-V enzyme. The supernatants of COS-7 cells transfected with a mock vector or with a FLAG-tagged soluble β3GalT-V construct were analyzed after 1-h labeling with [³⁵S]methionine. Cell supernatant proteins bound to anti-

FLAG M2-agarose beads were separated on SD8-polyacrylamide gel
electrophoresis and autoradiographed. The results from two transfec-
tions (1) and (2) are shown. The arrow at the right indicates the expected size of the tagged soluble β3GalT-V protein.

but lacks the Gb5 synthase activity (16). F9 cells stably ex-
expressing the human β3GalT-V gene were first analyzed for the
presence of the Gb5 product by flow cytometry. As shown in
Fig. 3A, F9 cells expressing the human β3GalT-V gene showed
increased levels of the SSEA-3 epitope at their surface when
compared with F9 cells transfected with a mock vector. For a
comparison, the human teratocarcinoma cell line NCCIT
expressing the human β3GalT-V gene was first analyzed for the
expression of the Gb5 product by flow cytometry (Fig. 3B).

**Fig. 3.** Effects of retinoic acid on SSEA-3 expression in F9 and NCCIT cells. (A) Effect of retinoic acid on SSEA-3 expression in F9 cells. NCCIT and F9 cells were treated with 1 μM retinoic acid for 24 h. SSEA-3 expression was analyzed by flow cytometry using an anti-MC-631 antibody. (B) SSEA-3 expression in NCCIT cells. NCCIT cells were treated with 1 μM retinoic acid for 48 h. SSEA-3 expression was analyzed by flow cytometry using an anti-MC-631 antibody. 

The relation between β3GalT-V and the induction of SSEA-3 was
further investigated in wild-type F9 cells. Treatment of F9
teratocarcinoma cells with retinoic acid is known to promote
cell differentiation and to induce the expression of the SSEA-3
epitope (17) (Fig. 4A). To determine whether the increase in
SSEA-3 levels is paralleled by the induction of β3GalT-V gene
transcription, we first cloned and expressed the mouse β3GalT-
V (GenBank™ accession number AF254738). The mouse pro-

tein is 308 amino acid long and shows 71% identity to the
human β3GalT-V at the amino acid level. The mouse β3GalT-V enzyme exhibited a galactosyltransferase activity when transiently expressed in COS-7 cells (data not shown). The presence of β3GalT-V mRNA in F9 cells was analyzed by RT-PCR. As shown in Fig. 4B, F9 cells treated with retinoic acid for 10 days were positive for β3GalT-V transcript, whereas untreated F9 cells remained negative. As a comparison, human β3GalT-V mRNA was also detected in NCCIT cells, which constitutively express SSEA-3 at their surface (see Fig. 3). This experiment demonstrated that the expression of the β3GalT-V gene correlates with the induction of the SSEA-3 epitope.

**DISCUSSION**

In the present study, we report a dual acceptor specificity for the β3GalT-V enzyme by establishing its ability to transfer Gal to the terminal GalNAc residue of Gb4 in addition to its activity toward GlcNAc-based acceptors (9, 10). This represents one of the few glycosyltransferases reported to date, which are capable of transferring a donor sugar molecule to different terminal monosaccharide acceptors. The best documented case is the β1,4-galactosyltransferase-I enzyme, which is able to use both GlcNAc and Glc as substrates (2, 3). However, whereas the β1,4-galactosyltransferase-I enzyme must interact with another modifier molecule, α-lactalbumin, to switch the substrate specificity from GlcNAc to Glc, the β3GalT-V enzyme does not require any additional modifier to switch from one acceptor substrate to the other.

β3GalT-V is the first member of the recently cloned β3GalT family that utilizes Gb4 as an acceptor. While the tissue distribution pattern of the SSEA-3 epitope and its biochemical nature have been characterized for many years, a distinct β3GalT enzyme with SSEA-3 synthase activity has yet to be cloned. It remains unclear whether β3GalT-V is the only enzyme that can perform this role in vivo. Also, it must be established whether the SSEA-3 synthase activities from human teratocarcinoma cells (18) and from mouse kidney (19) represent orthologous proteins.

The human and mouse β3GalT-V genes displayed differences in their tissue expression patterns. The human β3GalT-V gene was mainly expressed in small intestine, pancreas, and testis (10), and mouse β3GalT-V transcripts were mainly detected in brain and kidney but not in testis (data not shown). Noteworthy, expression of SSEA-3 has been reported to be confined to the kidneys in adult mice (20). However, it is difficult to relate the pattern of expression of the β3GalT-V gene with the distribution of SSEA-3 among tissues, since additional factors, such as the availability of the Gb4 acceptor, will also affect the presence or absence of the antigen on specific cell types.

The parallel induction of SSEA-3 and β3GalT-V expressions in F9 cells treated with retinoic acid supports the concept of a direct relation between both events. Nevertheless, we cannot exclude the existence of other β3GalT enzymes, which may participate to the formation of SSEA-3 in vivo. On the other hand, it is also possible that the SSEA-3 synthase activity indeed represents a second activity of the type-1 chain synthase β3GalT-V enzyme. In this case, the enzyme may function either way depending on the levels of the respective acceptor available in cells. The targeted inactivation of the mouse β3GalT-V gene will provide answers to this question and may represent a valuable model to address the biological functions associated to SSEA-3 during embryonic development.
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