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Secretion of α1,3-galactosyltransferase by cultured cells and presence of enzyme in animal sera

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Glycosyltransferases are normally synthesized as membrane-anchored proteins. However, we recently found that the murine enzyme UDP-Gal:Galβ1 → 4GLcNAc (Gal to Gal) α1,3 galactosyltransferase (α1,3GT) is secreted in a soluble form into media by mouse teratocarcinoma F9 cells (Cho SK, Yeh J-C, Cho M, Cummings RD (1996) *J Biol Chem* 271: 3238–46). To study the biosynthesis of this enzyme and whether secretion of the soluble enzyme is a general phenomenon, a solid-phase assay was developed for the α1,3GT activity. A recombinant and soluble form of the murine α1,3GT was produced in H293 cells (H293-α1,3GT) to aid in optimizing the assay. Desialylated orosomucoid was used as an immobilized acceptor in coated microtiter plates. The formation of product was detected by a biotinylated human-derived anti-α-Gal IgG and streptavidin conjugated to either alkaline phosphatase or the recombinant bioluminescent protein aequorin. Enzyme activity was dependent on the concentrations of asialoorosomucoid, UDP-Gal, α1,3GT and the time of incubation. The assay was also useful in monitoring α1,3GT activity during enzyme enrichment procedures. Using this assay, we found that α1,3GT activity was present in both cell extracts and culture media of several mammalian cell lines. Enzyme activity was also present in the sera from several mammals, but activity was absent in the sera from either humans or baboons. Our results demonstrate the development of a novel assay for the α1,3GT and provide evidence that secretion of the enzyme is a common biological phenomenon.

***Keywords*: glycosyltransferase, enzyme**

***Abbreviations*: LNnT, lacto-*N*-neotetraose (Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glc); PBS/NaN_3, phosphate-buffered saline containing sodium azide; α1,3GT, UDP-Gal:Galβ1 → 4GlcNAc (Gal to Gal) α1,3-galactosyltransferase; BSA, bovine serum albumin; RCA, *Ricinus communis* agglutinin; SNA, *Sambucus nigra* agglutinin; RA, all-*trans*-retinoic acid UDP, uridine diphosphate; ASOSM, asialoorosomucoid; ASF, asailofetuin; ASTransf, asailotransferrin**

Introduction

The enzyme UDP-Gal:Galβ1 → 4GlcNAc (Gal to Gal) α1,3-galactosyltransferase (α1,3GT) catalyzes the transfer of D-galactosyl units from the sugar nucleotide donor UDP-Gal to form an α1,3 linkage to the terminal β-linked galactosyl residue of *N*-acetyllactosamine units in glycoproteins and glycolipids [1]. α1,3GT exhibits an interesting species-specific distribution. The enzyme is expressed in a variety of mammalian species, but it is not expressed in Old World monkeys, apes, and humans [2, 3]. The gene encoding α1,3GT has been cloned from murine [4], bovine [5], porcine [6, 7], and New World monkey [8] cDNA libraries. Studies on the *α1,3GT* gene in humans demonstrated the presence of three pseudogenes [9–11] and no functional *α1,3GT* genes were identified. In mice, α1,3GT expression is developmentally-regulated [12], although a functional gene is not essential for mouse development [13]. The *α1,3GT* gene is transcriptionally regulated in mouse teratocarcinoma F9 cells induced to differentiate by treatment with all-*trans* retinoic acid [14]. In addition, a significant amount of the enzyme is secreted in a soluble form into the culture media by F9 and differentiated F9 cells [14]. This latter observation led to the inquiry as to whether secretion of the α1,3GT is a general biological phenomenon.

Most assays for the α1,3GT employ radiolabeled UDP-Gal and either glycoproteins or small oligosaccharide acceptors [1, 14, 15]. Although useful, these methods are complicated by the production of radioactive waste and the difficulty in defining the product of the reaction. In addition, these radioactive methods are not easily adaptable for processing large number of samples. To circumvent these problems, a solid-phase assay was developed and optimized that employs immobilized asialoorosomucoid as an acceptor and an anti-α-Gal antibody to detect the product. The

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anti-α-Gal Ab interacts specifically with the α-galactosyl epitope Galα1 → 3Galβ1 → 4GlcNAc → R [16, 17]. The anti-α-Gal Ab occurs naturally in the sera of animals lacking functional *α1,3GT* genes, but it is absent in the sera of New World monkeys and nonprimate mammals, which express functional *α1,3GT* genes [3]. This antibody was shown by LaTemple *et al.* [18] to be useful in detecting the product of the α1,3GT in a solid-phase format using a recombinant form of the marmoset enzyme and desialylated fetuin as an acceptor.

With this assay we addressed the question of whether secretion of the α1,3GT is a common process in cells expressing the enzyme. Most cells expressing the enzyme are capable of secreting it into the culture media. In addition, enzyme activity was detected in the sera of many mammals, but activity was absent in the sera of humans and baboons. These results support the hypothesis that secretion of soluble α1,3GT is a general biological process for those cells expressing the *α1,3GT* gene and raise questions about the functional significance of the soluble enzyme.

Materials and methods

Materials

Bovine fetuin, human transferrin, human orosomucoid (α_1-acid glycoprotein), Tween 20, uridine 5′-diphosphogalactose (UDP-Gal), cacodylic acid, *Arthrobacter ureafaciens* neuraminidase, melibiose-agarose, protein A-Sepharose, bovine serum albumin, α-methylgalactoside, α-methylglucoside, α-methylmannoside, lactose, phenylmethylsulfonyl fluoride, pepstatin, aprotinin, leupeptin and alkaline phosphatase substrate were purchased from Sigma Chemical Company (St Louis, MO). Streptavidin-alkaline phosphatase conjugate was purchased from Boehringer Mannheim (Indianapolis, IN). Sulfo-NHS biotin and BCA protein assay kit were obtained from Pierce Chemical Co. (Rockford, IL). Lacto-*N*-neotetraose (LNnT) (Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glc) was obtained from V-Labs, Inc. (Covington, LA). RCA-agarose was obtained from Vector Laboratories, Inc. (Burlingane, CA). Fetal bovine serum and Geneticin (G418) were obtained from Gibco BRL (Grand Island, NY). The streptavidin-aequorin conjugate (AquaLite™) was obtained from Sea-Lite Sciences, Inc. (Bogart, GA). Con A-Sepharose was purchased from Pharmacia LKB Biotechnology (Piscataway, NJ). LNnT-BSA was prepared as described previously [19] according to the methods of Gray [20]. Polystyrene microtiter plates Immulon 4 and Microlite 2 were purchased from Dynatech Laboratories (Chantilly, VA). Amicon Centriprep-10 concentrators were obtained from Amicon Inc. (Beverly, MA). Human sera were obtained from healthy blood donor at the Oklahoma Blood Institute. Animal sera were obtained from the animal facility at the University of Oklahoma Health Sciences Center.

Production of soluble murine α1,3GT in H293 cells

To produce a soluble form of murine α1,3 galactosyltransferase, a fusion protein containing the putative lumenal catalytic domain of the enzyme and the human transferrin signal peptide sequence was constructed in the mammalian expression vector RSV-PL4 (kindly provided by Dr Charles Esmon, Oklahoma Medical Research Foundation). Specifically, the lumenal domain of the α1,3GT was amplified by PCR using a 5′ primer, 5′-CCAACCCGGGAT TCCAGAGGTTGGTGAGAACAGATGGCAG-3′ with an *Sma*I site and a 3′ primer, 5′-CGGCTCTAGAGCCTTCAGACATTATTTCTAACCAAATT-3′ with an *Xba*I site. The plasmid pCDM7-αGT, which contains the cDNA of the murine α1,3GT [4], was used as the template for the PCR reaction. The PCR product was digested with *Xba*I and *Sma*I and then ligated into the *Xba*I/*Stu*I site of Rc/RSV vector, resulting in a fusion protein of the soluble α1,3GT in-frame to the transferrin signal sequence present in the vector. This construct was used to transfect into human 293 cells and clonal selection was carried out in the presence of 400 μg ml^{-1} Geneticin to obtain a stable transfectant (H293-α1,3GT). The culture media of H293-α1,3GT was concentrated 25-fold and used as the enzyme source.

Cell culture

Mouse teratocarcinoma F9 cells, Chinese hamster ovary (CHO) cells, Swiss mouse embryo 3T6 cells, bovine kidney MDBK cells, and canine kidney MDCK cells were obtained from the American Type Culture Collection (Rockville, MD). Embryonic African Green Monkey Kidney MA104 cells were obtained from BioWhittaker, Inc. (Walkersville, MD). Mouse teratocarcinoma F9 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 15% fetal calf serum on gelatin-coated tissue culture plates exactly as described by Strickland and Mahdavi [21]. For the induction of differentiation, cells were grown under identical conditions in media supplemented with 10^{-7} M retinoic acid (RA) for 3 days before being used for α1,3GT assays. These cells are designated as RA/F9 cells. Chinese hamster ovary (CHO) cells and 3T6 B1K CL.4 cells were maintained in α-minimal essential media (α-MEM) containing 10% FCS, penicillin (100 U ml^{-1}), and streptomycin (100 μg ml^{-1}). MA104 cells were cultured in α-MEM with Earle's BSS supplemented with 10% FCS and non-essential amino acids. MDBK cells and MDCK cells were cultured in α-MEM with Earle's BSS supplemented with 10% FCS.

Preparation of microsomes and culture media

Microsomes were prepared according to the procedure described [22] in the presence of protease inhibitors, phenylmethylsulfonyl fluoride (1 mM), pepstatin (1 μg ml^{-1}), aprotinin (10 μg ml^{-1}), and leupeptin (10 μg ml^{-1}). The membrane pellet was resuspended in 20 mM cacodylate

buffer, pH 7.0 containing 1% Triton X-100. The protein concentration was determined using a BCA protein assay kit with BSA as the reference standard. For the preparation of soluble enzyme from cell culture media, the media were collected and centrifuged at $100\,000 \times \mathbf{g}$ for 60 min. The media was concentrated using a centriprep-10 concentrator and then immediately used for α1,3GT assays.

Desialylation of intact glycoproteins

Bovine fetuin, human transferrin and human orosomucoid were dissolved in 100 mM sodium acetate (pH 4.8) to a concentration of 10 mg ml^{-1}. The glycoproteins were then treated overnight with 100 mU of *Arthrobacter ureafaciens* neuraminidase at 37 °C. The reaction was stopped by boiling for 10 min followed by dialysis against PBS/NaN_3 overnight. The completion of desialylation of glycoproteins was assessed in a solid-phase assay by reactivity of samples to biotinylated *Sambucus nigra* agglutinin (SNA). This lectin recognizes α2,6-linked sialic acid residues [23]. The bound biotinylated lectin was detected by the streptavidin-alkaline phosphatase conjugate. The glycoproteins after neuraminidase treatment showed no reactivity toward biotinylated SNA (data not shown).

Preparation of biotinylated anti-α-Gal antibodies

The natural anti-α-Gal immunoglobulin was purified from human sera according to the procedures described previously [24]. Purified human anti-α-Gal IgG was dialyzed in 50 mM bicarbonate buffer, pH 8.5 containing 200 mM α-methylgalactoside. To this was added a five-fold molar excess of sulfo-NHS biotin (prepared fresh as 1 mg ml^{-1} stock solution in water) and the mixture was incubated for 30 min at room temperature. The reaction mixture was then dialyzed against PBS/NaN_3 at 4 °C with several changes of buffer. The concentration of biotinylated antibody was measured by BCA protein assay.

Coating of microtiter plate wells

The wells of Immulon 4 or Microlite 2 polystyrene microtiter plates were coated overnight with 100 μl of bicarbonate coating buffer (150 mM Na_2CO_3, 348 mM $NaHCO_3$, 0.02% NaN_3, pH 9.6) containing varying amounts of glycoproteins at room temperature. The coated plates were washed three times with 300 μl of PBS/NaN_3 and blocked for 2 h with 250 μl of 5% BSA (prepared in PBS/NaN_3) at room temperature. The plates were washed three times with washing buffer (PBS/NaN_3/Tween: PBS/NaN_3 containing 0.05% Tween 20) immediately before use in the enzyme reactions. The standard assay for α1,3GT employing asialoorosomucoid used 10 μg ml^{-1} of the glycoprotein in coating buffer.

Assay of α1,3GT using the streptavidin-alkaline phosphatase method

The reaction mixture was added to the Immulon 4 wells coated with acceptor glycoproteins. The standard assay conditions were as follows: 100 mM cacodylate buffer, pH 7.0, 0.5 mM UDP-Gal, 5 mM ATP (to inhibit endogenous pyrophosphorylases), 20 mM $MnCl_2$, 50 mM D-galactono-1,4-lactone (to inhibit endogenous β-galactosidases), 10 mg ml^{-1} BSA and 10 μl enzyme source in a final volume of 100 μl. (Note: When animal sera were assayed, 60 μl of serum was added.) The reaction mixtures were incubated for 2 h at 37 °C. After removal of the reaction mixtures by aspiration, the wells were washed five times with the washing buffer. One hundred microliters of biotinylated anti-α-Gal IgG (10 μg ml^{-1}) in dilution buffer (PBS/NaN_3/0.05% Tween 20 containing 0.1% BSA) was then added to each well and incubated for 1 h at room temperature. The antibody solution was removed by aspiration and the wells were washed five times with washing buffer (PBS/NaN_3/0.05% Tween 20). One hundred microliters of the streptavidin-alkaline phosphatase conjugate (1:1000 in dilution buffer) was then added to each well and incubated for 1 h at room temperature. The wells were washed five times with washing buffer following by washing with deionized water three times. The wells were then treated with 100 μl of alkaline phosphatase substrate *p*-nitrophenylphosphate (1 mg ml^{-1}), which was freshly prepared in bicarbonate-coating buffer (pH 9.6) containing 1 mM $MgCl_2$. One hundred microliters of this substrate solution was added to each well and allowed to incubate for 1 h at 37 °C. The optical density at 405 nm of each well was recorded using a V-Max Kinetic Microplate Reader (Molecular Device Corp., Menlo Park, CA). Each assay was performed in triplicate and results are expressed as an average with standard error indicated by errors bars. The values from the assays in the absence of UDP-Gal were served as background and were subtracted from the values obtained from the standard assay.

Assay of α1,3GT using the streptavidin-aequorin method

Microlite 2 microtiter plates were used for assays employing the streptavidin-aequorin. The assay procedures were performed exactly as described above for the streptavidin-alkaline phosphatase method, except that following incubation with anti-α-Gal IgG, 20 mM EDTA was included in all ensuing buffers including the dilution and washing buffers. Briefly, 100 μl of streptavidin-aequorin (1:10 000 dilution) was added to each well and allowed to incubate for 1 h at room temperature. After washing, the wells were monitored for light activity using the Microlite ML 2250 Microplate Luminometer from Dynatech Laboratories. Light production was initiated in the luminometer by injection of 100 μl of triggering buffer (50 mM Tris, pH 7.5, containing 100 mM $CaCl_2$, 0.1% NaN_3) directly into the individual microtiter

wells. The α1,3GT activity in animal sera was measured as described above except that 60 µl of serum was used as the enzyme source. The reactions were carried out for 4 h at 37 °C and the streptavidin-aequorin was used to detect the products formed.

Enrichment of H293-α1,3GT using concanavalin A-Sepharose

Two ml of concanavalin A-Sepharose (Con A-Sepharose) was preequilibrated in 20 mM HEPES, 150 mM NaCl, 5 mM $MnCl_2$, pH 7.0 buffer at room temperature. About 0.4 ml of 25-fold concentrated H293-α1,3GT cell culture media was applied to the column and the flow was stopped for 15 min to allow equilibration. The unbound proteins were then eluted from the column by the same buffer and 10 fractions (1 ml each) were collected. The column was then eluted by 10 ml of 10 mM α-methylglucoside followed by 10 ml of 500 mM α-methylmannoside. Both hapten sugars were prepared in the same buffer mentioned above. The absorbance of each fraction at 280 nm was monitored. For assaying the enzyme activity, 60 µl of each fraction was added to the enzyme reaction mixture and incubated for 4 h at 37 °C. The products were detected using the streptavidin-aequorin method mentioned above. Control wells received no UDP-Gal and values from these wells were considered as background. These background values were subtracted from values obtained from the wells in which UDP-Gal was added.

Results

Assay of α1,3GT using different desialylated glycoprotein acceptors

The schematic representation of the assay for α1,3GT is shown in Figure 1. The α1,3GT utilizes terminal lactosaminyl Galβ1 → 4GlcNAc-R sequences as an acceptor and UDP-Gal as the donor to create the product Galα1 → 3Galβ1 → 4GlcNAc-R [1, 15]. We chose, therefore, to assess the acceptor activity of three commercially-available glycoproteins that contain the terminal lactosaminyl sequence; human orosomucoid (α_1-acid glycoprotein), which contains primarily complex-type tetraantennary N-glycans of mixed α2,6- and α2,3-linked sialic acid [25, 26]; bovine fetuin, which contains primarily complex-type N-glycans of mixed α2,6- and α2,3-linked sialic acid [27–31]; and human transferrin, which contains primarily complex-type diantennary N-glycans with only α2,6-linked sialic acid [32, 33]. The terminal β-galactosyl residues in the lactosaminyl units of these glycoproteins were quantitatively exposed upon treatment with neuraminidase, thus allowing the neuraminidase-treated proteins to be potential acceptors for the α1,3GT. For comparative purposes, we prepared the neoglycoprotein LNnT-BSA, which is a chemical conjugate

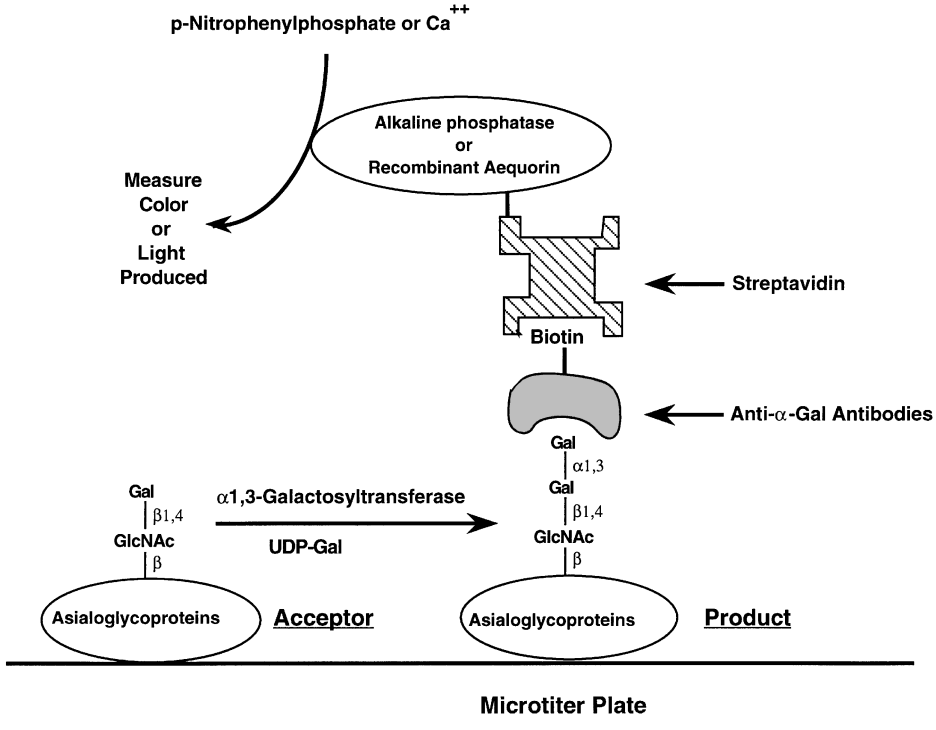

Figure 1. Schematic representation of the *a*1,3GT assay on a solid phase using desialylated glycoprotein as an acceptor. The product is detected by the binding of biotinylated Anti-*a*-Gal IgG, followed by the binding of the streptavidin conjugates of either alkaline phosphatase or the recombinant bioluminescent protein aequorin.

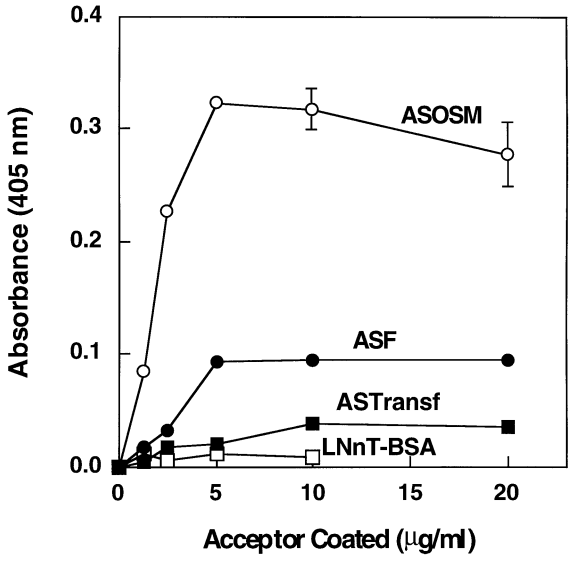

Figure 2. The α1,3GT activity toward different desialylated glycoproteins and neoglycoprotein acceptors. Desialylated glycoproteins asialoorosomucoid, asialofetuin, asialotransferrin, and LNneoT-BSA were coated on microtiter plates at increasing concentrations and the products were detected by the streptavidin-alkaline phosphatase method, as described under Materials and methods. Each assay was performed in triplicate and results are expressed as an average with standard error indicated by the errors bars.

of the human milk tetrasaccharide LNnT (Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glc) to BSA. A soluble form of recombinant α1,3GT produced by human H293 cells stably transfected with a murine *α1,3GT* gene (*H293-α1,3GT*), prepared as described in Materials and methods, was used as the enzyme source to optimize the assay protocol. The immobilized reaction products were detected by anti-α-Gal Ab purified from human serum, which specifically recognize Galα1 → 3Galβ1 → 4GlcNAc → R sequence [16]. The biotinylated anti-α-Gal Ab bound to product was detected by streptavidin conjugated to either alkaline phosphatase or to recombinant aequorin.

The α1,3GT demonstrated high reactivity with asialoorosomucoid, moderate activity with asialofetuin, and very weak activity with either asialotransferrin or LNnT-BSA (Figure 2). To ensure that this differential acceptor specificity was not due to differential coating of the glycoproteins or neoglycoprotein on the microtiter plates, we assessed their reactivity with the galactose-binding lectin biotinylated-RCA-I [27]. Each of the immobilized acceptors bound similar amounts of RCA-I (data not shown). These data indicate that the murine α1,3GT demonstrates branch specificity with glycoprotein acceptors and prefers the more highly branched N-glycans compared to diantennary N-glycans (asialotransferrin) or monoantennary glycans (LNnT-BSA). This is consistent with previous observations on the bovine and Erlich ascites tumor α1,3GTs [15, 34]. Based on these results immobilized asialoorosomucoid was used as the acceptor for all subsequent assays of the α1,3GT. The inability of the α1,3GT to utilize LNnT-BSA stands in contrast to results with several other glycosyltransferases. In previous studies, where we developed assays for the α1,3-fucosyltransferase capable of synthesizing the Le^x antigen, we found that LNnT-BSA was the preferred acceptor compared to asialofetuin and asialotransferrin [19].

Activity of α1,3GT toward immobilized asialoorosomucoid is dependent on time and UDP-Gal concentration

The formation of product by the H293-α1,3GT was dependent on the time of incubation (Figure 3A), the coating concentration of asialoorosomucoid (Figure 2), and the concentration of UDP-Gal (Figure 3B). As an additional method to improve the sensitivity of this assay, we substituted streptavidin-aequorin (Aqualite™) for streptavidin-alkaline phosphatase. Aequorin is a Ca^{2+}-sensitive photoprotein that emits a single photon of light per molecule within a few milliseconds after Ca^{2+} addition [35, 36]. In previous studies we have found that streptavidin-aequorin enhances the sensitivity and speed of solid-phase assays [19, 37, 38]. The assay with streptavidin-aequorin was highly sensitive and did not require the use of concentrated media for measuring enzyme activity (Figure 4B). With the streptavidin-aequorin assay we were able to detect the signal over the background with as low as 1 μl of non-concentrated H293-α1,3GT medium used as the enzyme source. In contrast, the streptavidin-alkaline phosphatase assay required to use of 1–2 μl of 25-fold concentrated media to obtain significant absorbance values.

Application of the solid-phase assay for measuring α1,3GT activity in column fractions

One potential advantage of the solid-phase assay of the α1,3GT is the ease of assaying activity in column fractions during purification and characterization of the enzyme. To demonstrate the utility of the solid-phase assay for determining enzyme activity during enzyme purification, and to address the question whether the recombinant α1,3GT is glycosylated, we examined the interaction of the recombinant α1,3GT with two plant lectins; *Ricinus communis* agglutinin-I (RCA-I), which interacts with highest affinity with complex-type-N- and O-glycans containing terminal β1,4-linked galactosyl residues [39, 40]; and concanavalin A (Con A), which interacts with high affinity with N-glycans of high mannose-/hybrid-type and with low affinity with N-glycans of biantennary complex-type [41–44]. Con A-Sepharose will not bind complex-type tri-/tetraantennary or bisected N-glycans and will not bind O-glycans. The cDNA sequence of murine α1,3GT predicts two potential N-linked glycosylation sites [4].

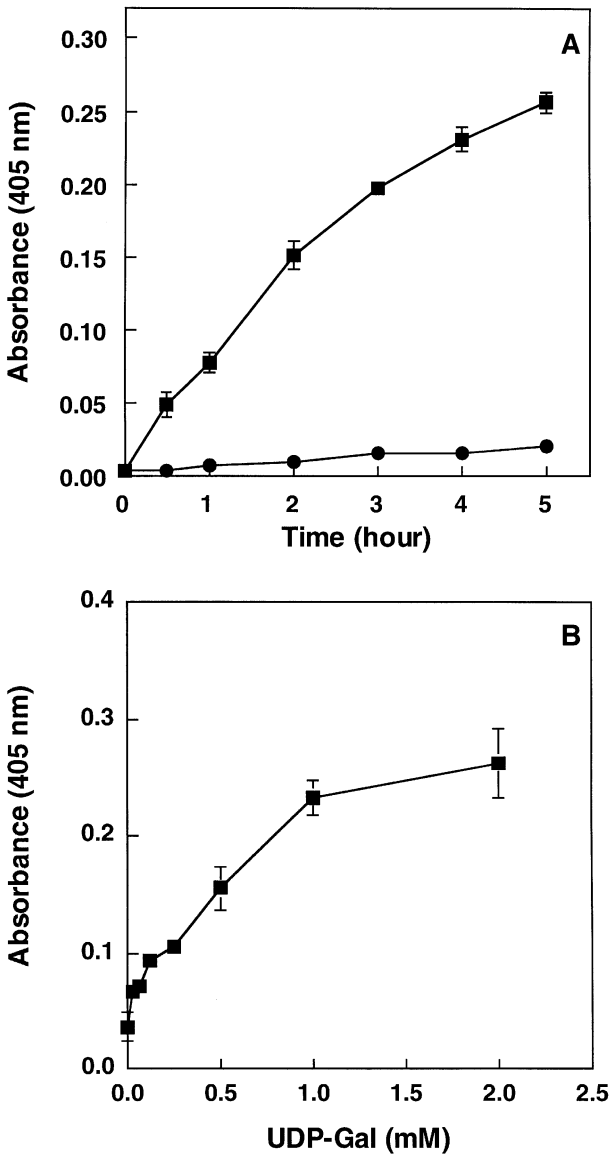

Figure 3. Dependence of *α*1,3GT activity on time and UDP-Gal. (A) Immulon 4 wells, coated with asialoorosomucoid, were incubated with either 500 μM UDP-Gal (square) or without UDP-Gal (circle) in the standard assay for the indicated times. The products were detected by the streptavidin-alkaline phosphatase method, as described under Materials and methods. (B) Wells coated with asialoorosomucoid were incubated with H293-*α*1,3GT culture media and indicated concentrations of UDP-Gal.

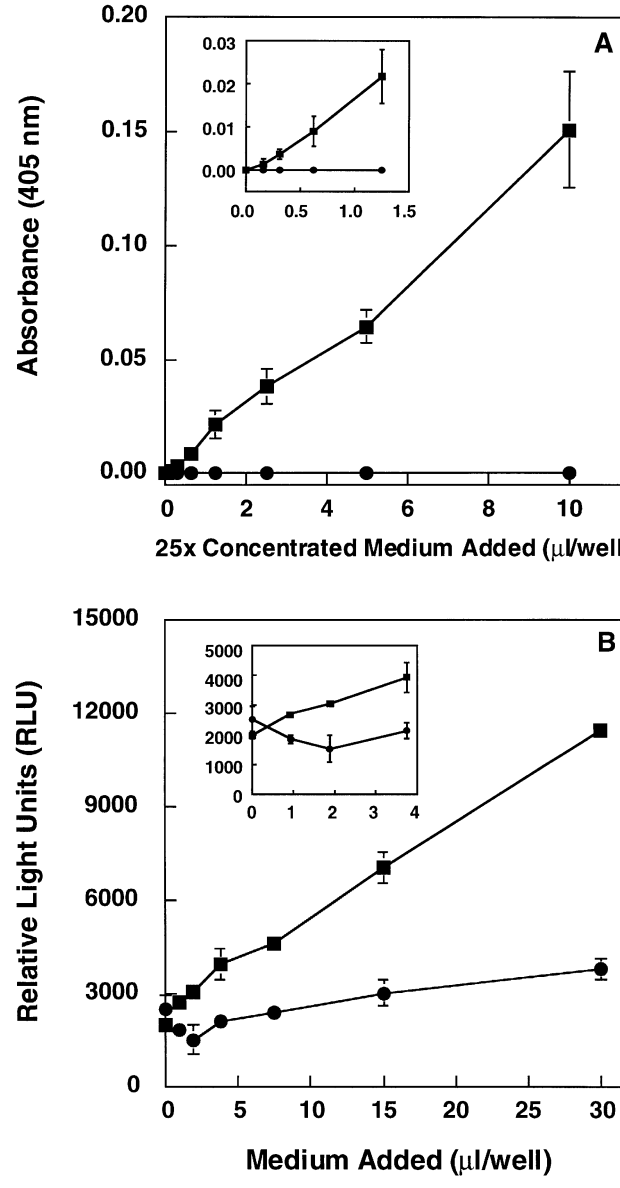

Figure 4. Comparison of *α*1,3GT assays using either the streptavidin-alkaline phosphatase or the streptavidin-aequorin. Either (A) concentrated or (B) non-concentrated H293-*α*1,3GT culture media was assayed for *α*1,3GT activity in the standard assay in the presence of 500 μM UDP-Gal (square) or absence of UDP-Gal (circle). Products were detected by either (A) the streptavidin-alkaline phosphatase or (B) the streptavidin-aequorin methods, as described under Materials and methods.

The concentrated H293-α1,3GT culture media was passed through Con A-Sepharose and the majority of α1,3GT activity was bound and eluted with 500 mM α-methylmannoside (Figure 5). The enzyme was enriched approximately 10-fold by this chromatography with >90% recovery of activity. These results suggests that the recombinant α1,3GT from H293 cells contain at least one biantennary complex-type/high-mannose-/hybrid-type N-glycans. It is likely that the N-glycans of the α1,3GT are high mannose-/hybrid-type, since none of the enzyme was bound by immobilized RCA-I (data not shown). These results demonstrate that this solid-phase assay for α1,3GT is useful for analyzing relatively large numbers of samples that are typically encountered in the purification and characterization of the enzyme.

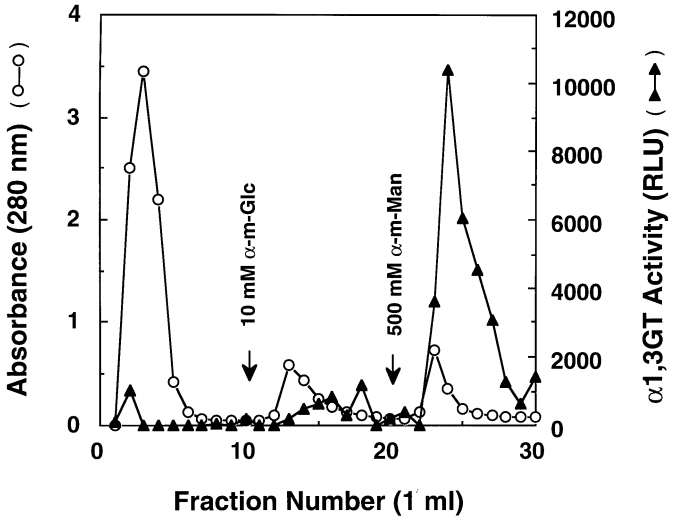

Figure 5. Enrichment of *a*1,3GT activity using con A-Sepharose. Twenty-five-fold concentrated H293-*a*1,3GT cell culture media (0.4 ml) was applied to a 2 ml column of con A-Sepharose. Bound glycoproteins were eluted from the column sequentially by 10 mM *a*-methylglucoside and 500 mM of *a*-methylmannoside. The fractions were monitored for protein amount by absorbance at 280 nm (open circle) and assayed for *a*1,3GT activity in the standard assay using the streptavidin-aequorin method (close triangle), as described under Materials and methods.

Detection of α1,3GT activity in F9 and RA/F9 cells

We previously demonstrated that there is an increase in α1,3GT activity accompanying the differentiation of the mouse teratocarcinoma cell line F9 induced by treatment with retinoic acid (RA) [45]. More recently, we found that RA transcriptionally regulates the α1,3GT expression and that much of the activity is secreted into culture media in a soluble form [14]. We sought to compare results with the new solid-phase assay with the previous radioisotope-based assays. Using this solid-phase assay we found that there is approximately four-fold higher activity in RA/F9 cells than that in F9 cells (Figure 6A). CHO cells were chosen as a negative control, since they do not contain this enzyme [46] and no activity was detected in the microsomes prepared from CHO cells. Using this assay, the α1,3GT activity was also detected in the culture media from F9 and RA/F9 cells, but not in media from CHO cells (Figure 6B). RA/F9 media showed about three-fold higher α1,3GT activity over media from F9 cells. These results are consistent with previous results using a radioactive assay for the α1,3GT [14].

Detection of α1,3GT activity in different cell culture media and animal sera

Soluble forms of glycosyltransferases have been found and purified from milk, sera, and other body fluids [47, 48]. The conversion of membrane-bound forms of glycosyltransferases into soluble enzymes by endogenous protease has been proposed to be responsible for this process [49–51, 48]. To test whether the secretion of α1,3GT into the culture media we observed in F9 cells is a common biological phenomenon, the enzyme activity in the microsomes and the culture media of several cell lines were examined using newly developed solid-phase assay. As shown in Figure 7,

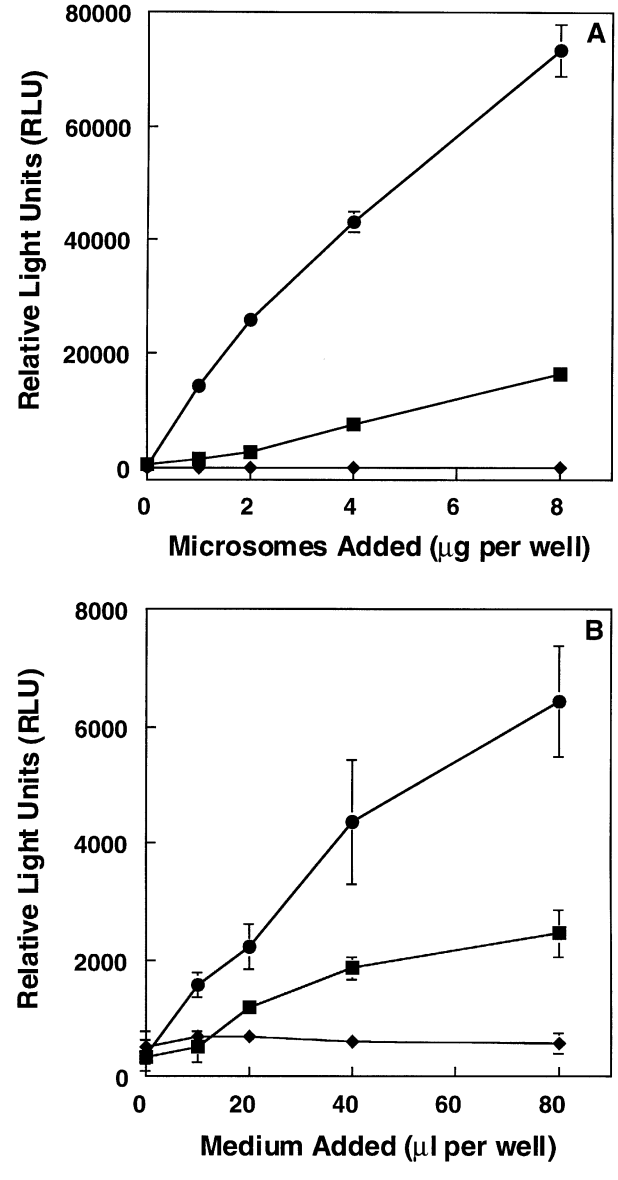

Figure 6. Measurement of *a*1,3GT activity in the microsomes and culture media of F9 and RA/F9 cells. Indicated amounts of (A) microsomes and (B) culture media prepared from F9 cells (square), RA/F9 cells (circle), and CHO cells (diamond) were assayed for *a*1,3GT in the standard assay using the streptavidin-aequorin method, as described in Materials and methods. Media was removed for assays from plates containing approximately equal numbers of F9 and RA/F9 cells and protein and no normalization factor is required to allow comparison of the amount of soluble enzyme in the media.

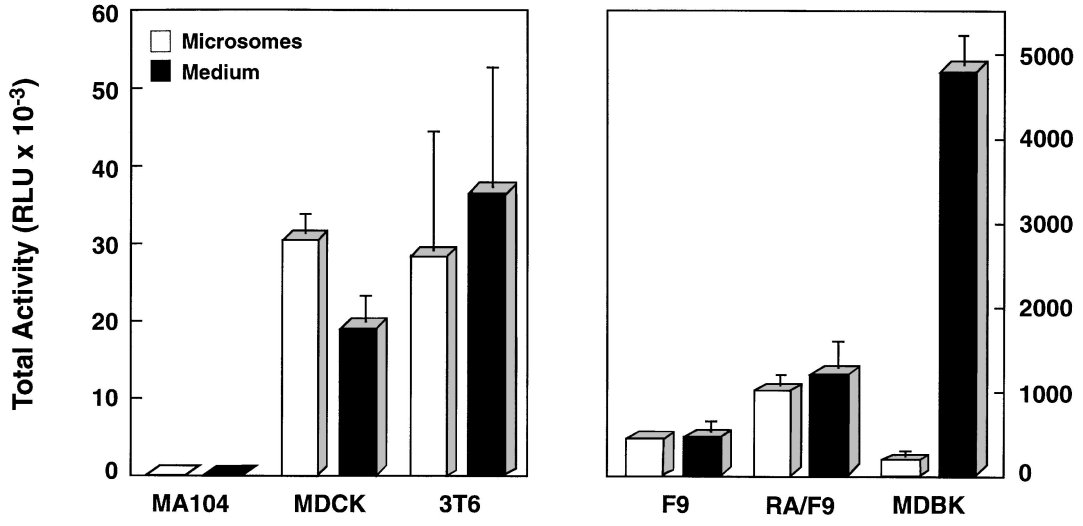

Figure 7. Measurement of *a*1,3GT activity in the microsomes and cell culture media from different cell lines. A portion of microsomes or culture media prepared from different cell lines was assayed for *a*1,3GT in the standard assay using the streptavidin-aequorin method, as described under Materials and methods. Based on the protein concentration of the microsomes and the volume of media, the total activity was calculated.

we were able to detect α1,3GT activity in microsomes of several cell lines, including mouse teratocarcinoma F9 cells, 3T6 mouse embryonic cells, Madine-Darby canine kidney (MDCK) cells, and Madine-Darby bovine kidney (MDBK) cells (Figure 7). No activity was detected in either the microsomes or media of the monkey cell line MA104. This is to be expected since these cells are derived from an Old World monkey. Interestingly, the amount of enzyme activity in the media relative to that in the microsomes differed between cell lines. This may reflect differential rates of cleavage and secretion of the enzyme.

α1,3GT has been shown to be expressed in non-primate mammals and in New World monkeys (monkeys of South America), but is absent in human, apes and Old World monkeys [3, 52, 53]. If the secretion of α1,3GT by cells is a common biological phenomenon, it is conceivable that the α1,3GT might be present in the sera of the animals expressing α1,3GT. To test this hypothesis, several animal sera were screened for the activity of α1,3GT using the solid-phase assay. α1,3GT activity was detected in the sera of mammals including cow, pig, dog, cat, guinea pig, rabbit and mouse; however, activity was absent in the sera of humans and baboons (Figure 8). These results demonstrate that animals expressing the functional *α1,3GT* gene express enzyme activity in their sera.

The possibility was considered that the standard assay format might not be reliable using animal sera that contains anti-α-Gal IgG. The autologous or endogenous antibody in the sample could potentially interfere with detection of the product formation, due to the binding of the autologous antibody to product prior to addition of biotinylated anti-α-Gal IgG in the standard assay format. To address this issue a modification of the standard assay method was developed. In this modification raffinose was tested for its ability to elute bound anti-α-Gal IgG prior to addition of the biotinylated anti-α-Gal IgG. Reactions were carried out with bovine sera, which contains α1,3GT activity, but lacks anti-α-Gal IgG, and human sera, which lacks the α1,3GT, but contains anti-α-Gal IgG (Figure 9). In the standard assay format (the first condition of Figure 9), α1,3GT activity was detected using bovine sera, but no activity was detected using human sera, as was also shown in Figure 8. In the second condition or version of the assay, the wells were incubated with serum samples as usual, but the wells were then washed with buffer containing raffinose *after* addition of the biotinylated anti-α-Gal IgG. No significant amount of product was detected with either serum sample, indicating that the raffinose wash effectively removed the biotinylated anti-α-Gal IgG bound to product. This demonstrates the feasibility of using raffinose to elute bound anti-α-Gal IgG. In the third assay condition, the wells were incubated with serum samples as usual, but were washed with buffer containing raffinose *prior* to addition of the biotinylated anti-α-Gal IgG. In this case, the raffinose had no significant effect on the amount of anti-α-Gal IgG subsequently bound. Enzyme product was detected using bovine sera, but no product was detected with human sera. These results demonstrate that a modification of the standard assay format, such as that used in the third assay condition, should be effective in removing potentially bound anti-α-Gal IgG arising from the sample. Thus, the absence of enzyme activity observed for human sera is not due to interference by autologous antibody in the enzyme source.

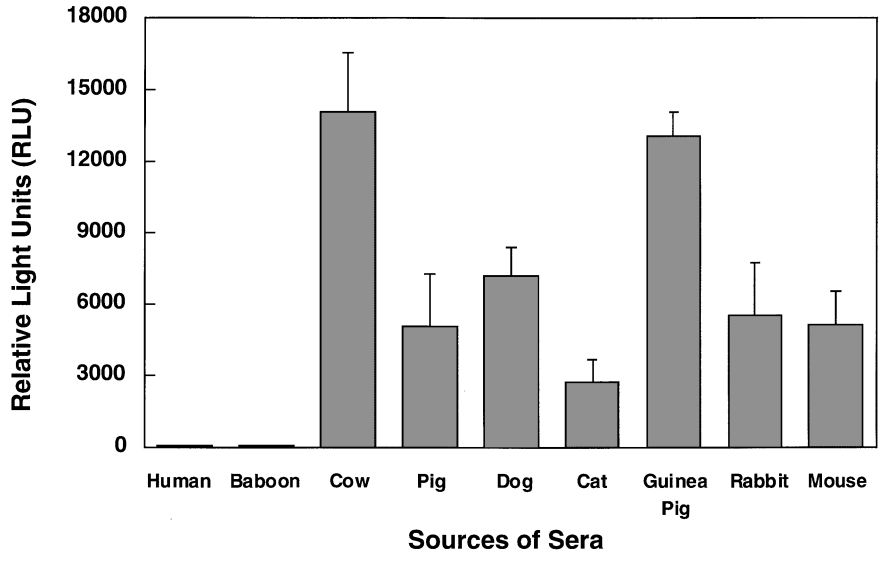

Figure 8. Measurement of *α*1,3GT activity in different animal sera. The assay was carried out at 37 °C for 4 h using 60 μl of animal serum in the standard assay using the streptavidin-aequorin method, as described under Materials and methods.

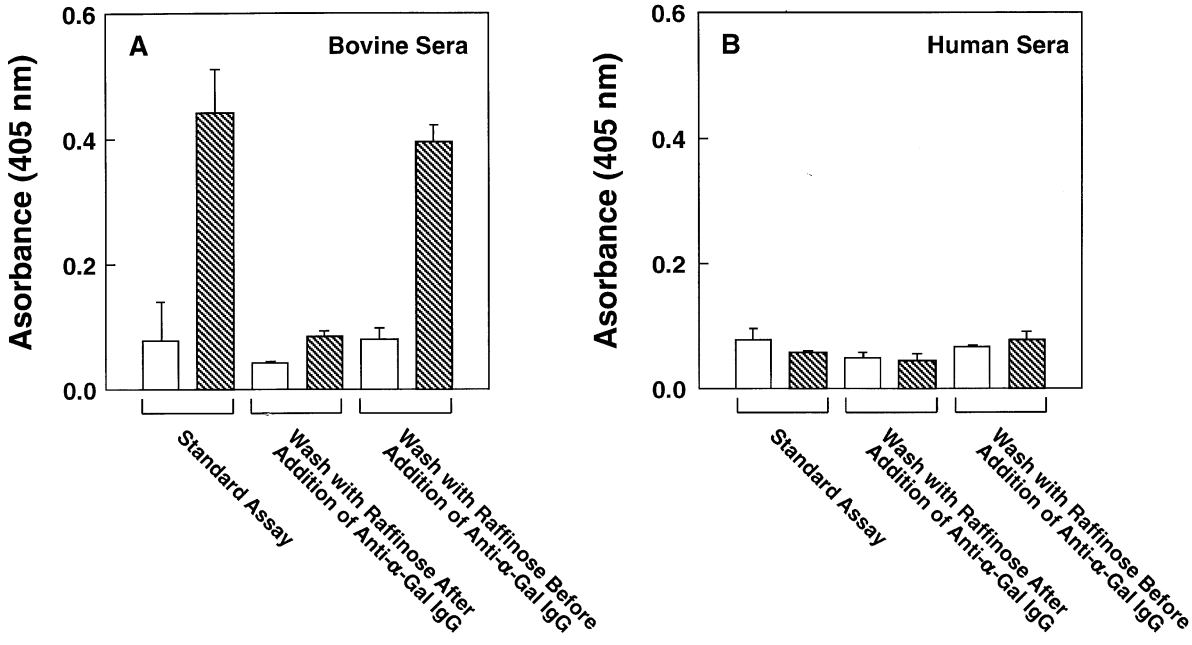

Figure 9. Measurement of *α*1,3GT in bovine and human sera using a modified assay format to determine potential interference of anti-*α*-Gal IgG in the assay sample. Either bovine sera (A) or human sera (B) (60 μl) were assayed under three different conditions, using the streptavidin/alkaline phosphatase method. The first condition was the standard assay format described in Materials and methods. In the second condition, the reaction components were removed from the wells by aspiration, and then washed five times as usual with standard washing buffer lacking raffinose. The wells were then incubated with biotinylated 1° anti-*α*-Gal IgG and then washed five times with standard washing buffer containing 200 mM raffinose, a hapten for the anti-*α*-Gal IgG. The wells were washed twice with standard washing buffer lacking raffinose before adding the streptavidin/alkaline phosphatase. In the third condition, the reaction components were removed from the wells by aspiration, but the wells were then washed five times with standard washing buffer containing 200 mM raffinose. The wells were washed twice with standard washing buffer lacking raffinose before the addition of the biotinylated 1° anti-*α*-Gal IgG and streptavidin/alkaline phosphatase. The open and hatched bars represent enzyme activity in the absence and presence of 500 μM UDP-Gal, respectively.

Discussion

The results presented demonstrate the development of a novel assay for the α1,3GT that is sensitive and specific. A related assay was recently developed by LaTemple *et al.* [18] using desialylated fetuin as an acceptor and peroxidase-based detection strategies. However, we found that asialoorosomucoid is several-fold better as an acceptor than asialofetuin. Furthermore, the assay described with asialoorosomucoid also uses aequorin and alkaline phosphatase and provides more sensitive detection methods. Using the new assay for the α1,3GT, we found that a soluble form of the enzyme is present in the culture media of all cells expressing the enzyme and in the sera of animals having a functional *α1,3GT* gene. These results complement previous studies in which we reported the presence of soluble α1,3GT activity in the culture media of mouse teratocarcinoma F9 cells and elevation of the activity by induced differentiation of the cells with all-trans retinoic acid treatment [4]. No previous studies have reported on the presence of the α1,3GT in the sera of animals, although enzyme activity was detectable in bovine colostrum [54].

The occurrence of soluble glycosyltransferases in body fluids may be a common phenomenon. Several glycosyltransferases such as fucosyltransferases, sialytransferases, β1,4galactosyltransferase, Sd(a+)β1,4GalNActransferase, and A, B, O(H)-blood group glycosyltransferases have been found in body secretions and fluids including milk, colostrum, and serum [55–64], and in the growth media from normal and transformed cell lines [65, 66].

The mechanism(s) of secretion of soluble and active glycosyltransferases is not clear. The amino acid sequences deduced from the cDNA sequences of cloned vertebrate glycosyltransferases have predicted that they share a common domain structure with a type 2 topology [67]. These enzymes all contain a short amino-terminal cytoplasmic tail, a single membrane anchoring domain, and an extended stem region which is followed by a large lumenal COOH-terminal catalytic domain [51]. The results from NH_2-terminal sequence analysis of soluble forms of the α2,6ST and β1,4GT suggest that proteolysis in the stem region releases the catalytic domain from the membrane [68, 69]. The release of the catalytic domain through the action of endogenous proteases in the Golgi apparatus or trans-Golgi network has been proposed to account for the appearance of the soluble secreted enzymes [59, 70]. The protease(s) responsible for the release of the enzymes are not defined, although evidence suggest that a cathepsin D-like protease may be responsible for the cleavage of α,2,6ST [71]. In some cases the levels of soluble enzymes appear to be affected by disease status and inflammation [71, 64].

The functional significance of soluble glycosyltransferases and their secretion is unclear, but there are several possibilities to consider. Secretion of the enzymes may be a necessary pathway for their turnover and their attached glycans may be recognized by specific receptors to facilitate their removal from the circulation by receptor-mediated endocytosis [72]. Because sugar nucleotides are generally thought to not occur in the extracellular milieu, it has been considered unlikely that the extracellular glycosyltransferase activities may be functionally important [73]. However, there appear to be some cases where extracellular sugar nucleotides and glycosyltransferases may glycosylate extracellular acceptors. There is evidence that CMP-sialic acid, the sugar nucleotide substrate for sialyltransferase, occurs in serum and is involved in the resistance of *Neisseria gonorrhoeae* to complement-mediated serum killing [74, 75]. The baculovirus genome encodes a secreted glucosyltransferase that can glucosylate ecdysteroids in the infected insect and prevent molting [76, 77], possibly by utilizing UDP-Glc present in the hemolymph of the insect. The extracellular β1,4GT may have access to UDP-Gal, thereby allowing the galactosylation of terminal GlcNAc residues in the glycans of laminin, an extracellular matrix glycoprotein [78]. Thus, under some situations the extracellular glycosyltransferases may function to remodel extracellular glycoconjugates. Finally, soluble enzymes could have a biosynthetic function within the Golgi. We have now shown that a soluble form of the α1,3GT can function within cells to galactosylate glycoproteins [79]. Of course, none of these possibilities are mutually exclusive and all may be operative.

The common secretion of glycosyltransferases by cells and the functionality of the soluble forms of the enzymes raise many questions about their biological importance. Our findings have led to ongoing studies to define the mechanism of secretion and potential functions of the soluble and secreted α1,3GT.

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References

1 Blanken WM, van den Eijnden DH (1985) *J Biol Chem* **260**: 12927–34.
2 Spiro RG, Bhoyroo VD (1984) *J Biol Chem* **259**: 9858–66.
3 Galili U, Shohet SB, Korbin E, Stults CLM, Macher BA (1988) *J Biol Chem* **263**: 17755–62.
4 Larsen RD, Rajan VP, Ruff MM, Kukowska-Latallo J, Cummings RD, Lowe JB (1989) *Proc Natl Acad Sci USA* **86**: 8227–31.
5 Joziasse DH, Shaper JH, van den Eijnden DH, van Tunen AJ, Shaper NL (1989) *J Biol Chem* **264**: 14290–97.
6 Sandrin MS, Dabkowski PL, Henning MM, Mouthouris E, McKenzie IFC (1995) *Xenotransplantation* **11**: 81–88.
7 Strahan KM, Gu F, Preece AF, Gustavsson I, Andersson L, Gustafsson K (1995) *Immunogenetics* **41**: 101–5.
8 Henion TR, Macher BA, Anaraki F, Galili U (1994) *Glycobiology* **4**: 192–201.
9 Joziasse DH, Shaper JH, Jabs EW, Shaper NL (1991) *J Biol Chem* **266**: 6991–98.

10 Larsen RD, Rivera-Marrero CA, Ernast LK, Cummings RD, Lowe JB (1990) *J Biol Chem* **265**: 7055–61.
11 Yamamoto F, McNeill PD, Hakomori S (1991) *Biochem Biophys Res Commun* **175**: 986–94.
12 Sheares BT, Mercurio AM (1987) *J Immunol* **139**: 3748–52.
13 Thall AD, Maly P, Lowe JB (1995) *J Biol Chem* **270**: 21437–40.
14 Cho SK, Yeh J-C, Cho M, Cummings RD (1996) *J Biol Chem* **271**: 3238–46.
15 Elices MJ, Blake DA, Goldstein IJ (1986) *J Biol Chem* **261**: 6064–72.
16 Galili U, Rachmilewitz EA, Peleg A, Flechner I (1984) *J Exp Med* **160**: 1519–31.
17 Galili U, Anaraki F, Thall A, Hill-Black C, Radic M (1993) *Blood* **82**: 2485–93.
18 LaTemple DC, Henion TR, Anaraki F, Galili U (1996) *Cancer Res* **56**: 3069–74.
19 Yan Y, Smith DF, Cummings RD (1994) *Anal Biochem* **223**: 111–18.
20 Gray GR (1974) *Arch Biochem Biophys* **163**: 426–28.
21 Strickland S, Mahdavi V (1978) *Cell* **15**: 393–403.
22 Cummings RD, Trowbridge IS, Kornfeld S (1982) *J Biol Chem* **257**: 13421–27.
23 Shibuya N, Goldstein IJ, Broekaert WF, Nsimba-Lubaki M, Peeters B, Peumans WJ (1987) *J Biol Chem* **262**: 1596–601.
24 Li S, Yeh J-C, Copper DKC, Cummings RD (1995) *Xenotransplantation* **2**: 279–88.
25 Bornstein I, Wagh PV, Winzler RJ (1969) *J Biol Chem* **244**: 658–65.
26 Treuheit MJ, Costello CE, Halsall HB (1992) *Biochem J* **283**: 105–12.
27 Baenziger JU, Fiete D (1979) *J Biol Chem* **254**: 789–95.
28 Berman E, Bendel P (1986) *FEBS Lett* **204**: 257–60.
29 Berman E (1986) *Carbohydr Res* **152**: 33–46.
30 Green ED, Adelt G, Baenziger JU, Wilson S, van Halbeek H (1988) *J Biol Chem* **263**: 18253–68.
31 Yet MG, Chin CC, Wold F (1988) *J Biol Chem* **263**: 111–17.
32 Marz L, Hatton MW, Berry LR, Regoeczi E (1982) *Can J Biochem* **60**: 624–30.
33 Yamashita K, Ideo H, Ohkura T, Fukushima K, Yuasa I, Ohno K, Takeshita K (1993) *J Biol Chem* **268**: 5783–89.
34 Seppo A, Penttila L, Leppanen A, Maaheimo H, Niemela R, Helin J, Wieruszeski JM, Renkonen O (1994) *Glycoconj J* **11**: 217–25.
35 Shimomura O, Johnson FH (1969) *Biochemistry* **8**: 3991–97.
36 Stults NL, Stocks NF, Rivera H, Gray J, McCann RO, O'Kane D, Cummings RD, Cormier MJ, Smith DF (1992) *Biochemistry* **31**: 1433–42.
37 Yeh J-C, Cummings RD (1996) *Anal Biochem* **236**: 126–30.
38 Zatta P, Nyame K, Cormier MJ, Mattox SA, Prieto PA, Smith DF, Cummings RD (1991) *Anal Biochem* **194**: 185–91.
39 Baenziger JU, Fiete D (1979) *J Biol Chem* **254**: 9795–99.
40 Rivera-Marrero CA, Cummings RD (1990) *Mol Biochem Parasitol* **43**: 59–68.
41 Kornfeld R, Ferris C (1975) *J Biol Chem* **250**: 2614–19.
42 Krusius T, Finne J, Rauvala H (1976) *FEBS Lett* **72**: 117–20.
43 Merkle RK, Cummings RD (1987) *Methods Enzymol* **138**: 232–59.
44 Ogata S, Muramatsu T, Kobata A (1975) *J Biochem* **78**: 687–96.
45 Cummings RD, Mattox SA (1988) *J Biol Chem* **263**: 511–19.
46 Smith DF, Larsen RD, Mattox S, Lowe JB, Cummings RD (1990) *J Biol Chem* **265**: 6225–34.
47 Beyer TA, Sadler JE, Rearick JI, Paulson JC, Hill RL (1981) *Adv Enzymol* **52**: 24–175.
48 Sadler JE (1984) In *Biology of Carbohydrates* (Ginsburg V, Robbins PW, eds) **2**: 199–288. New York: John Wiley and Sons.
49 Boegeman EE, Balaji PV, Sethi N, Masibay AS, Qasba PK (1993) *Protein Engineering* **6**: 779–85.
50 Kaplan HA, Woloski BMRNJ, Hellman M, Hamieson JC (1985) *J Biol Chem* **258**: 11505–9.
51 Paulson JC, Colley KJ (1989) *J Biol Chem* **264**: 17615–18.
52 Hendriks SP, He P, Stults CLM, Macher BA (1990) *J Biol Chem* **256**: 17621–26.
53 Ogiso M, Okingaga T, Komoto M, Nishiyama I, Hoshi M (1994) *Exp Eye Res* **59**: 653–64.
54 Hosomi O, Takeya A (1989) Nippon Juigaku Zasshi – *Japanese Journal of Veterinary Science* **51**: 961–68.
55 Badet J, Ropars C, Salmon C (1978) *J Immunogenetics* **5**: 221–31.
56 Berger EG, Kozdrowski I, Weiser MM, van den Eijnden DH, Schiphorst WE (1978) *Eur J Biochem* **90**: 213–22.
57 Fujita-Yamaguchi Y, Yoshida A (1981) *J Biol Chem* **256**: 2701–6.
58 Hudgin RL, Schachter H (1971) *Can J Biochem* **49**: 829–37.
59 Lammers G, Jamieson JC (1988) *Biochem J* **256**: 623–31.
60 Paulson JC, Beranek WE, Hill RL (1977) *J Biol Chem* **252**: 2356–62.
61 Piller F, Cartron JP (1983) *J Biol Chem* **258**: 12293–99.
62 Serafini-Cessi F, Malagolini N, Dall'Olio F (1988) *Arch Biochem Biophys* **266**: 573–82.
63 Strous GJ (1986) *CRC Critical Reviews in Biochemistry* **21**: 119–51.
64 Turco SJ, Heath EC (1976) *Arch Biochem Biophys* **176**: 352–57.
65 Klohs WD, Mastrangelo R, Weiser MM (1981) *Cancer Res* **41**: 2611–15.
66 Serafini-Cessi F, Malagolini N, Guerrini S, Turrini I (1995) *Glycoconjugate J* **12**: 773–79.
67 Narimatsu H (1994) *Microbiol Immunol* **38**: 489–504.
68 D'Agostaro G, Bendiak B, Tropak M (1989) *Eur J Biochem* **183**: 211–17.
69 Weinstein J, Lee EU, McEntee K, Lai P-H, Paulson JC (1987) *J Biol Chem* **263**: 17735–43.
70 Paulson JC, Weinstein J, Ujita EL, Riggs KJ, Lai P-H (1987) *Biochem Soc Trans* **15**: 618–20.
71 Lammers G, Jamieson JC (1989) *Biochem J* **261**: 389–93.
72 Weigel PH (1994) *Bioessays* **16**: 519–24.
73 Harder PG, Jamieson JC, Woloski BMRNJ (1990) *Int J Biochem* **22**: 11–14.
74 Parson NJ, Constantinidou C, Cole JA, Smith H (1994) *Microb Pathog* **16**: 413–21.
75 Nairn CA, Cole JA, Patel PV, Parsons NJ, Fox JE, Smith H (1988) *J Gen Microbiol* **134**: 3295–306.
76 O'Reilly DR, Miller LK (1989) *Science* **245**: 1110–12.
77 O'Reilly DR, Miller LK (1990) *J Virol* **64**: 1321–28.
78 Begovac PC, Shi YX, Mansfield D, Shur BD (1994) *J Biol Chem* **269**: 31793–99.
79 Cho SK, Cummings RD (1997) *J Biol Chem* **272**: 13622–28.

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