Remodeling of Mouse Milk Glycoconjugates by Transgenic Expression of a Human Glycosyltransferase*

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The mammary gland is a unique biosynthetic tissue that produces a variety of species-specific glycoconjugates, but the factors regulating the production of specific glycoconjugates are not well understood. To explore the underlying regulation, a fusion gene containing a cDNA encoding the human α1,2-fucosyltransferase (α1,2FT), which generates the H-blood group antigen, flanked by the murine whey acidic protein promoter and a polyadenylation signal, was introduced into mice. Milk samples from transgenic animals contained soluble forms of the α1,2FT, as revealed by Western blots of milk samples using an anti-α1,2FT antisera and by the demonstration of α1,2FT enzyme activity. Milk from transgenic animals also contained large quantities of 2′-fucosyllactose (Fucα1-2Galβ1-4Glc) and modified glycoproteins containing the H-antigen, whereas milk from control animals lacked these glycoconjugates. Expression levels of 2′-fucosyllactose were high in most animals and represented 1/2 to nearly 1/2 of the total milk oligosaccharides. These results demonstrate that heterologous transgenic expression of a glycosyltransferase can result in the expression of both the transgene and its secondary gene products and that the structures of milk oligosaccharides can be remodeled depending on expression of the appropriate enzyme. Furthermore, these results suggest that the lactating mammary gland may be a unique biosynthetic reactor for the production of biologically active oligosaccharides and glycoconjugates.

Despite the fact that animal oligosaccharides are important in many biological and pathological processes (Karlsson, 1989; Kornfeld, 1992; Drickamer and Taylor, 1993; McEver et al., 1995), little is known about the regulation of animal cell glycoconjugate biosynthesis. Biosynthesis is influenced by expression of appropriate glycosyltransferases, correct targeting of the enzymes to the Golgi apparatus, sugar nucleotide levels, competition between enzymes, residence time of glycoconjugates in intracellular compartments, acceptor specificity of the enzymes, and many other factors (Kornfeld and Kornfeld, 1985; Kobata and Takasaki, 1992; Cummings, 1992; Baenziger, 1994). Experimental modulation of these factors has been difficult. One approach that has proven somewhat successful in vitro is to attempt to alter oligosaccharide biosynthesis by genetic manipulation of glycosyltransferases (Lee et al., 1989; Smith et al., 1990; Lowe et al., 1990). This approach has so far been limited to cultured and immortalized cell lines and has been useful in producing new glycoconjugates for biological studies (Lowe et al., 1990).

One major site for glycoconjugate biosynthesis in animals, and one that has been of enormous historical importance in the field of glycobiology, is the lactating mammary gland. Milk contains, in addition to lactose, numerous glycoproteins (Patton et al., 1990; Fiat and Ollès, 1989) and a variety of free oligosaccharides (Kobata et al., 1972). The structure and composition of the free oligosaccharides in milk differ between mammals, but the human lactating mammary gland synthesizes the most complex mixture of free, reducing oligosaccharides, many of which contain fucose and other determinants for human blood groups, including the ABO and Lewis system. The structures of some of these oligosaccharides in humans are determined genetically by the expression of glycosyltransferases during lactation and the availability of sugar nucleotide substrates (Hill and Brew, 1975; Ginsburg, 1972; Smith et al., 1987), but the precise mechanisms regulating differential expression of individual milk oligosaccharides are not known.

Recent advances in transgenic technology have resulted in successful expression of transgene-encoded proteins in animal milk (Wilde et al., 1992; Paleyenda et al., 1991; Archer et al., 1994). Using this methodology, we have begun to explore factors regulating glycoconjugate biosynthesis. Our analyses of normal mouse milk demonstrated that it is deficient in fucose-containing oligosaccharides, and in particular it lacks oligosaccharides containing the H-blood group antigen. Based on this information, we chose to use a cDNA encoding a human α1,2-fucosyltransferase (α1,2FT)1 responsible for synthesis of the blood group H-antigen Fucα1-2Galβ1-3(4)-R (Rajan et al., 1989; Larsen et al., 1990). Our studies demonstrate that the human α1,2FT can be successfully introduced into transgenic animals causing the lactating mammary gland to synthesize relatively large quantities of oligosaccharides containing the H-antigen and at least one major glycoprotein containing this determinant. These studies provide new insights into the factors regulating biosynthesis of milk oligosaccharides within the

1 The abbreviations used are: α1,2FT, α1,2-fucosyltransferase; HPAC, high performance anion exchange chromatography; FACC, fluorophore-assisted carbohydrate electrophoresis; 3-FL, 3-fucosyllactose (Galβ1−1→4(Fucα1−3→Glc); DFL, difucosyllactose (Fucα1−2Galβ1−4(Fucα1−3→Glc); WAP, whey acidic protein; kb, kilobase(s); FBS, fetal bovine serum; bGH, bovine growth hormone; UEA I, U. europaes agglutinin I.

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lactating mammary gland and a new approach for studying factors controlling glycoconjugate biosynthesis.

EXPERIMENTAL PROCEDURES

Preparation of Vectors Containing the Human a1,2FT—The human a1,2FT was cloned by polymerase chain reaction from an epidermal carcinoma cell line (A431) cDNA library using primers derived from the previously published a1,2FT gene sequence (Larsen et al., 1990). Direct sequencing of the a1,2FT cDNA confirmed its identity with the known sequence. To demonstrate that the cDNA encoded a functional a1,2FT enzyme, the a1,2FT cDNA was inserted into an expression plasmid (pMet-FucT-bGH) and introduced into mouse L cells, which do not contain either a1,2FT enzyme activity or H-blood group antigens. This plasmid utilizes the mouse metallothionein-I promoter (Bayne et al., 1987) to direct expression and contains the bovine growth hormone polyadenylation signal (bGH poly(A)) for proper mRNA processing. Recombinant enzyme activity was demonstrated in the mouse L cells by indirect immunofluorescent staining of positive transfectants with Ulex europeaus agglutinin I (UEA I) (Sigma), which reacts with H-blood group antigen, and a monoclonal antibody against H-blood group antigen (Dako Corp.). To generate antibody to the a1,2FT, the cDNA encoding the a1,2FT was subcloned into an inducible expression vector (pQE11) (Qiagen, Inc.) in frame with a 6x histidine tag. This 6x histidine-tagged protein was purified by nickel affinity chromatography and injected in rabbits to generate polyclonal antibodies. For transgenic studies, the cDNA fragment encoding the a1,2FT was inserted into a second expression plasmid, pWAP-a1,2FT (Fig. 1). This plasmid contains bGH poly(A) and utilizes the murine whey acidic protein (WAP) promoter (Pittius et al., 1988) to direct gene expression primarily to lactating mammary gland tissue. An EcoRI-BamHI DNA fragment containing the WAP-a1,2FT-bGH poly(A) sequences was isolated from this plasmid and inserted into fortified mouse (B6/SJ L) eggs as described previously (McCranie et al., 1988). The WAP promoter was generously provided by Dr. Lothar Hennighausen (National Institutes of Health).

Preparation of Milk Samples—Individual milk samples were obtained from transgenic and non-transgenic mothers after nursing the pups for 5–12 days. Samples were collected using a mammary suction apparatus consisting of a 23-gauge needle and a vacuum line connected to a trap test tube. Samples were frozen on dry ice and stored at −70 °C. Oligosaccharide extracts were prepared using a method modified from Kobata et al. (1972). Typically, 50–100 μl milk aliquots were centrifuged for 15 min at 11,000 × g. The resulting lipid pads were removed from the top, and the liquid layer was transferred to a clean vial. A 2-fold volume of cold ethanol was added with mixing, and the samples were then centrifuged and the supernatants were removed to clean vials and dried for further analysis. Precipitates, containing the proteins, were dried and stored at −70 °C until used for further analysis. Isolation and Separation of Oligosaccharides—Oligosaccharides were separated, detected, and quantified by high performance anion exchange chromatography (HPAEC) and fluorophore-assisted carbohydrate electrophoresis (FACE). HPAEC was performed by a method adapted from Reddy and Bush (1991). Oligosaccharide standards for HPAEC and FACE were obtained from V-Labs Inc. (Covington, LA).

Individually dried oligosaccharide extracts from mouse milk, prepared as above, were resuspended in water to the original volume of their respective milk samples. Aliquots (20 μl) were injected onto a Dionex CarboPac PA1 analytical column. Analytes were detected using a pulsed electrochemical detector equipped with a gold working electrode and a pH reference electrode. Elution was started with a solution of 100 mM NaOH (RICCA Chemicals, Arlington, TX) held steady for 12 min and changed to 7% of 600 mM sodium acetate in 100 mM NaOH. After 8 min, this percentage was increased to 10% and held constant for 7 min. Flow rate was maintained at 1 ml/min. The column was washed in a mixture of 50% 100 mM NaOH, 50% 600 mM sodium acetate in 100 mM NaOH and equilibrated for 15 min in starting solution before reuse. After injection, the elution stream was diverted using a Dionex AMMSS-11 autosampler and membrane filter equipped with an AutoRegen apparatus equipped with an anion exchange cartridge and 0.15% H2SO4 regenerant solution. Fractions (0.5 ml) were collected and immediately frozen for lyophilization. Fractions obtained by HPAEC (or glycosidase digests from these fractions) were submitted to FACE analysis (J. Jackson, 1990; Stack and Sullivan, 1992). Electrophoresis equipment, imager, labeling, and reducing reagents for fluorophore labeling were from Glyko Inc. (Novato, CA). Manufacturer's procedures were followed.

Exoglycosidase Treatments—Fucosidases from Corynebacterium sp. (a1-2 specific) and Streptomyces (a1-3/4 specific) were from Takara Panvera (Madison, WI), and Escherichia coli galactosidase was from Boehringer Mannheim. All other chemicals used were from the highest purity available and, unless otherwise indicated, were from Sigma.

Immunoblotting of Milk Proteins—The milk protein pellets obtained by ethanol precipitation as described above were tested for the presence of the a1,2FT polypeptide by immunoblot using antisera raised against the recombinant enzyme. Pellets were resuspended in a volume of SDS equal to that of the original volume of the milk sample. Aliquots (5 μl) of resuspended pellets were electrophoresed on 12.5% polyacrylamide gels (Laemmli, 1970). Resolved proteins were transferred to nitrocellulose membranes for 1 h at 100 volts. The resulting membranes were blocked by incubation in 50 mM TBS, pH 7.5, containing 0.5 mM NaCl and 2% gelatin. Membranes were washed three times in TBS containing 0.05% Tween 20 and were incubated for 18 h in 1% gelatin/TBS containing 1:500 dilution of rabbit anti-a1,2FT. Membranes were then incubated with 1% gelatin/TBS containing goat anti-rabbit IgG previously conjugated to horseradish peroxidase and washed with TBS-Tween. Proteins were visualized by incubating in 50 ml of TBS containing 0.018% hydrogen peroxide and 10 ml of methanol containing 30 mg of 4-chloronapthol.

Western Blotting of Milk Proteins—Blotted proteins on nitrocellulose membranes were probed for the presence of a1,2-linked fucose by incubating for 18 h in a 2% gelatin/TBS containing a 1:500 dilution of the peroxidase labeled UEA-I (Sigma). The resulting membranes were then washed, and proteins were visualized by incubating in a mixture of 50 ml of TBS containing 0.018% hydrogen peroxide and 10 ml of methanol containing 30 mg of 4-chloronaphthol (Bio-Rad).

RESULTS

Analysis of Oligosaccharides in Milk from Control Mice—To determine a suitable glycosyltransferase for mammary gland-directed transgenic expression in mice, we analyzed mouse milk oligosaccharides using HPAEC. Mouse milk contains lactose, sialylactoses, and other oligosaccharides (Fig. 2, panel A). One of the minor oligosaccharides of murine milk coeluted with authentic 3-fucosylactosamine (3-FL, Galβ1-4(Fucα1-3)Gal) but was not detectable by low levels of 3-FL. 2’-Fucosylactose (Fucα1-2Galβ1-4Glc), which is an isomer of 3-FL, was not detected in the milk of control animals. In a separate experiment, a milk sample was exhaustively treated with a mixture of fucosidases, and the amount of released fucose was determined by HPAEC. Mouse milk was found to contain approximately 1 nmol of fucose per 100 μl. Although the amounts of total fucose and 3-FL in mouse milk are minuscule, their presence indicates that lactating mouse mammary glands most likely contain GDP-Fuc, which is the donor sugar nucleotide for fucosyltransferases.

Expression of the Human a1,2FT in Transgenic Mice—The
absence of 2'-FL in mouse milk suggests that the lactating mammary gland of the mouse lacks a 1,2-fucosyltransferase activity. A fusion gene containing a cDNA encoding the human a1,2FT was prepared and injected into mouse embryos as described under “Experimental Procedures.” A total of 85 pups were obtained from these injections, and 9 of these animals (8 male and 1 female) were found to contain at least one copy of the transgene by quantitative slot blots (McGrane et al., 1988). Six of the transgenic animals (all male) were mated to normal mice, resulting in a total of 98 F1 progeny. 38 of these animals (19 males/19 females) contained the a1,2FT gene. 15 of the females were bred with normal mice. Subsequent generations (F2 and F3) were obtained by interbreeding transgenic animals. Milk samples from transgenic and control non-transgenic animals were either directly tested for enzyme activity or, alternatively, treated with ethanol to obtain oligosaccharide extracts and protein pellets described under “Experimental Procedures.”

Milk samples from transgenic animals contained detectable a1,2FT activity as determined by a method modified from Rajan et al. (1989), while activity was absent in samples from control animals (data not shown). These results demonstrate that active a1,2FT enzyme was expressed in milk from transgenic animals.

The presence of a1,2FT polypeptide was determined by immunoblot analysis of milk protein using a polyclonal rabbit antibody against a1,2FT, as shown in Fig. 3. No immunoreactive protein was present in milk from control animals, whereas milk from transgenic animals contained four immunoreactive species of apparent molecular mass of 46, 32, 30, and 25 kDa (Fig. 3). The a1,2FT cDNA encodes the full-length type-2 transmembrane form of the enzyme that has a predicted size of approximately 46 kDa when fully glycosylated. Whether the 46-kDa form of the a1,2FT observed in transgenic milk represents a full-length form of the enzyme or a partly degraded form with altered glycosylation is not yet known. Nevertheless, the presence of discrete lower molecular weight forms of the enzyme in milk indicates discrete proteolytic cleavages of the polypeptide resulting in the species observed. These lower molecular weight forms were not found in cell extracts of a1,2FT transfected mouse L-cells. The potential proteolytic cleavage sites of the recombinant enzyme are under investigation.

Presence of Fucα1-2Galβ1-R Determinants in Milk Oligosaccharides from Transgenic Animals—Oligosaccharide extracts from milk samples from transgenic mice were analyzed by HPAEC and compared to those in milk from a control mouse (Fig. 2, panels B and C). A major neooligosaccharide found in the milk of the

FIG. 2. Chromatographic profiles of mouse milk oligosaccharides. Oligosaccharide extracts from a control non-transgenic mouse and two different transgenic mice, both of which were descendants of Founder 28, were subjected to Dionex high performance liquid chromatography. A, milk from control mice did not contain any DFL (α2 region) or 2'-FL (α3). A trace of 3-FL (α1) was detected and analyzed as described in the text. B, a second generation transgenic mouse (code 28-28) synthesized a large amount of 2'-FL (β3). Traces of 3-FL (β1) and DFL (β2) were detected. C, a larger amount of 2'-FL was present in milk from a homozygous third generation transgenic animal (code 28-28-37). The elution positions of monosaccharides and standard oligosaccharides are indicated.

FIG. 3. a1,2FT was present in milk of transgenic animals. Protein pellets from milk samples were separated by SDS electrophoresis, blotted, and assayed for binding to anti-a1,2FT antiserum as described under “Experimental Procedures.” Milk proteins from control animals were not specifically recognized by the antiserum (lane 1), while some proteins from transgenic animals (lanes 2 and 3) were recognized. One of the detected bands (identified by an arrow) was in the 46-kDa region, which corresponds to the expected molecular weight of the full-length a1,2FT. The migration positions of molecular weight markers are indicated.
Transgenic Expression of a Human Glycosyltransferase

The major neooligosaccharide found in milk of transgenic mice comigrated with authentic 2-FL in FACE analysis and was susceptible to a specific α1,2-fucosidase. Fractions corresponding to the major neooligosaccharide (region b3, Fig. 2, panel B) were pooled. An aliquot was directly labeled with 8-aminonaphthalene-2,3,6-trisulfonic acid and subjected to FACE analysis (lane 4). A second aliquot was treated with 20 milliunits of Corynebacterium sp. α1-2 fucosidase prior to labeling (lane 5). Authentic 2-FL was also labeled (lane 2) and treated with the enzyme (lane 3). Lane 1 contains labeled oligosaccharide standards.

To confirm that the neooligosaccharide in the milk of transgenic animals was 2-FL, the purified oligosaccharide collected after Dionex-HPAEC was subjected to FACE analysis and specific enzyme degradation. An electrophoretogram of the neooligosaccharide before and after treatment with α1,2-specific fucosidase from Corynebacterium sp. is shown in Fig. 4. The fluorophore-labeled neooligosaccharide (lane 4) comigrated with authentic 2-FL (lane 2) and, like the standard 2-FL, it was susceptible to the action of the α1,2-specific fucosidase (lanes 3 and 5). The neooligosaccharide was also exhaustively treated with a combination of the α1,2-specific fucosidase and E. coli β-galactosidase. This combined treatment resulted in the release of equimolar amounts of fucose, galactose, and glucose, as determined by FACE analysis (data not shown). These results demonstrate that the neooligosaccharide present in the milk of transgenic animals expressing the human α1,2FT is 2-FL.

A minor oligosaccharide that coelutes with the human milk tetrasaccharide difucosyllactose (DFL, Fucα1-2Galβ1-4(Fucα1-3)Glc) was also detected in the chromatographic profiles from transgenic animals (Fig. 2, panels B and C). Fractions corresponding to the elution time of this oligosaccharide were pooled and analyzed by FACE. The resulting band obtained from transgenic animal milk comigrated with authentic DFL (data not shown). Results were consistent with the presence of this oligosaccharide only in milk from transgenic animals. It is expected that this product is generated by the sequential action of the α1,2FT and the endogenous α1,3FT.

Presence of Glycoproteins Containing the H-blood Group Determinant in Milk of Transgenic Animals—The milk proteins of transgenic and control animals were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes by electroblotting. These blots were then assayed for the presence of Fucα1-2Galβ1-4(R determinants using the plant lectin UEA-I, which displays high affinity for H-blood group determinants (Allen et al., 1977; Hindsgaul et al., 1982). The milk of control animals lacked glycoproteins reactive with UEA-I, whereas milk of transgenic animals contained a major reactive glycoprotein of apparent molecular mass of 35–40 kDa in addition to a minor reactive band of 29 kDa (Fig. 5). This pattern is different from that observed for the recombinant α1,2FT (Fig. 3). This major fucosylated glycoprotein may be casein, but this is under investigation. These results demonstrate that at least one major glycoprotein is an acceptor for the transgenically expressed human α1,2FT and becomes highly modified by the enzyme, whereas other glycoproteins in the milk appear to be less efficiently modified.

Overall Expression of 2-FL in Milk of Transgenic Animals—Table I is a summary of results for control and transgenic animals generated by different founders and belonging to different generations. Transgenic mice obtained from founders 28 and 34 (Table I) produced typical amounts of 2-FL (average 2.2 μl) were resolved by electrophoresis and blotted in nitrocellulose membranes. The membranes were then incubated with a preparation of labeled UEA I, as described under “Experimental Procedures.” Samples obtained from transgenic animals (lanes 2–5) were specifically recognized by UEA I. Fucose/α1,2 linkages were not detected in samples from control animals (lanes 1 and 6). The migration positions of molecular weight markers are indicated.

DISCUSSION

Results presented in this report demonstrate that it is possible to dramatically alter the structure of the milk oligosaccharides and glycoproteins by the transgenic introduction of a heterologous glycosyltransferase under the control of a lacto-
Table I

| Analysis of oligosaccharides in milk of control and transgenic mice |
|---------------------------------------------------------------|
| Animal description | Generation | Number of transgene copies | 2'-Fucosyllactose total milk oligosaccharides% |
|---------------------|-------------|-----------------------------|-----------------------------------------------|
| Control             |             | 0                           | 0                                             |
| CD1                 | F0          | 0                           | 0                                             |
| Balb                | F0          | 0                           | 0                                             |
| B6SJ L              | F0          | 0                           | 0                                             |
| Transgenic          |             |                             |                                               |
| He 28-89-17F        | F2          | 1                           | 27                                            |
| He 28-89            | F1          | 1                           | 20                                            |
| He 28-89-17-5       | F3          | 1                           | 30                                            |
| Ho 28-89-129        | F2          | 2                           | 45                                            |
| He 34-34            | F1          | 3                           | 30                                            |
| He 34-23            | F1          | 3                           | 32                                            |
| He 34-35            |            | 3                           | 16                                            |
| Ho 34-23-102        | F2          | 6                           | 34                                            |
| He 6-14             | F1          | 4                           | <1                                            |
| Ho 6-14-120         | F2          | 8                           | 26                                            |

*Samples from control (CD1, Balb, B6SJ L) animals did not contain 2'-fucosyllactose, while samples from transgenic animals had varying numbers of the oligosaccharide.

**He and Ho represent animals hemizygous or homozygous, respectively, for the 1,2FT.**

***The first number in the identifying codes for transgenic animals indicates founder (F); second and third numbers identify specific transgenic mice in the progeny.***

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