

Polypeptide GalNAc-transferase T3 and Familial Tumoral Calcinosis

SECRETION OF FIBROBLAST GROWTH FACTOR 23 REQUIRES O-GLYCOSYLATION*

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Mutations in the gene encoding the glycosyltransferase polypeptide GalNAc-T3, which is involved in initiation of O-glycosylation, were recently identified as a cause of the rare autosomal recessive metabolic disorder familial tumoral calcinosis (OMIM 211900). Familial tumoral calcinosis is associated with hyperphosphatemia and massive ectopic calcifications. Here, we demonstrate that the secretion of the phosphaturic factor fibroblast growth factor 23 (FGF23) requires O-glycosylation, and that GalNAc-T3 selectively directs O-glycosylation in a subtilisin-like proprotein convertase recognition sequence motif, which blocks processing of FGF23. The study suggests a novel posttranslational regulatory model of FGF23 involving competing O-glycosylation and protease processing to produce intact FGF23.

The UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase (GalNAc-transferase)² isoform GalNAc-T3 was recently implicated in familial tumoral calcinosis (FTC) (1, 2). GalNAc-T3 is a member of the large polypeptide GalNAc transferase gene family containing 20 genes of which 15 have been shown to encode functional enzymes. The GalNAc-transferase gene family is the largest mammalian family of glycosyltransferases, which are involved in catalysis of a single glycosidic linkage, GalNAc α 1-O-Ser/Thr, and collectively the GalNAc-transferase isoforms control the initiation of mucin-type O-glycosylation in the Golgi complex (3, 4). Mucin-type O-glycosylation is one of the most abundant forms of glycosylation of proteins, and is found on a large variety of cell membrane and secreted glycopeptides and glycoproteins. O-Glycosylation imparts unique physicochemical features to glycoproteins and O-glycans have been shown to play important functions in almost all known biological processes including intracellular sorting, cell-cell adhesion, and microbial adhesion events (5).

Inactivating mutations in *GALNT3* were originally discovered in FTC (1, 2, 6, 7), but more recently mutations in the gene encoding the phosphaturic factor FGF23 have also been identified in FTC (8–10). Thus, FGF23 mutations affecting folding and secretion were identified in FTC patients without mutations in GalNAc-T3. Furthermore, ablation of FGF23 in mice leads to hyperphosphatemia resembling FTC (11). FTC patients with mutations in GalNAc-T3 or FGF23 exhibit hyperphosphatemia and have reduced serum intact FGF23 levels, which may suggest that GalNAc-T3 and FGF23 act in a common pathway. GalNAc-T3 and FGF23 were found to be co-expressed in a number of tissues (2).

FGF23 is a key regulator of phosphate homeostasis (12, 13), and is an O-glycosylated glycoprotein of ~32 kDa (14). FGF23 is partially processed intracellularly by subtilisin-like proprotein convertases (SPC) at the consensus sequence RXXR↓ (RHTR¹⁷⁹) between Arg¹⁷⁹ and Ser¹⁸⁰ (14–16). This processing step appears to be essential in the regulation of phosphate homeostasis, because mutations in the SPC cleavage sequence prevent processing and result in autosomal dominant hypophosphatemic rickets (12). Many secreted proteins and peptides essential for regulation of biological activities are synthesized as inactive proproteins, which are subsequently activated by site-specific proteolysis mainly by SPCs to form the intact active forms (17). Examples of inactivation of proteins by SPCs have emerged more recently including FGF23 (14–16), endothelial lipase (18), and matrix metalloproteinase-2 (19). Interestingly, the SPC site in FGF23 contains the sequence RHTR¹⁷⁹↓ with a potential O-glycosylation site at Thr¹⁷⁸.

We hypothesized that O-glycosylation and in particular O-glycosylation directed by GalNAc-T3 could play a role for processing and secretion of FGF23. The results presented in this study demonstrate that secretion of FGF23 is dependent on O-glycosylation in CHO Id1D cells and that co-expression of GalNAc-T3 in these cells results in a marked increase of secretion. Furthermore, *in vitro* studies demonstrate that GalNAc-T3 can glycosylate the SPC signal sequence of FGF23 at Thr¹⁷⁸, which suggests that the underlying mechanism for GalNAc-T3 involvement in FTC is at least in part a role in O-glycosylation of the furin protease consensus sequence in FGF23.

EXPERIMENTAL PROCEDURES

Expression of FGF23 and Glycosyltransferases in CHO Id1D Cells—An expression construct of the entire coding sequence of human FGF23 cloned into pcDNA3.1/myc-His (C-terminal tags) was prepared as previously reported (15). Full coding expression constructs of human GalNAc-T3 (20) and human core 3 β 3GlcNAc-transferase T6 (21) were prepared as follows. The 5'-end of GalNAc-T3 (nucleotide 1–1032) was amplified by PCR using primer pair EHBC238 (5'-GCGGGATCCGT-

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² The abbreviations used are: GalNAc-transferase, UDP-N-acetyl-o-galactosamine: polypeptide N-acetylgalactosaminyltransferase; FTC, familial tumoral calcinosis; FGF23, fibroblast growth factor 23; SPC, subtilisin-like proprotein convertase; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; HPLC, high pressure liquid chromatography; MES, 4-morpholineethanesulfonic acid; CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

CAGAATGGCTCACCTAAAGCGA-3')/EBHC222 (5'-ATCAGGA-AGCGACTCCCAGCC-3') with salivary gland mRNA (Clontech). An internal EcoRI site was used together with the BamHI site contained in EBHC238 to directionally clone the entire 5'-end of GalNAc-T3 into an existing secreted GalNAc-T3 construct (20). The full coding core 3 β 3GnT6 construct was amplified using primer pair MTHC2307 (5'-GCGGATCCACCATGGCTTTTCCCTGCCGACAGG-3')/MTHC2302 (5'-GCGAATTCTCAGGAGACCCGGTGTCCCCG-3') with human genomic DNA, and the product was cloned into the BamHI/EcoRI site of pBluescript (Stratagene, La Jolla, CA). Both constructs were cloned into pcDNA3.1. Chinese hamster ovary mutant cells, CHO ldlD (22), with defective UDP-Gal/UDP-GalNAc 4-epimerase were grown in Ham's F-12/Dulbecco's modified Eagle's medium (1:1, v/v) supplemented with 3% fetal bovine serum and 0.03% glutamine. Cells were transfected with pcDNA3.1 constructs and stable transfectants were selected in 0.4 mg/ml G418 and/or 0.4 mg/ml Zeocin (Invitrogen) using immunostaining for expression of FGF23 (monoclonal anti-myc antibody (Invitrogen)) and GalNAc-T3 (monoclonal antibody UH5) (23). In the case of core 3 β 3GnT6, stable transfectants were selected by loss of reactivity with an anti-Tn (GalNAc α 1-O-Ser/Thr) monoclonal antibody (5F4) when cells were grown in medium with 1 mM GalNAc (24). Double transfectants were prepared sequentially with FGF23 followed by glycosyltransferases. To evaluate the effects of O-glycosylation CHO ldlD transfectant cells were grown in medium supplemented with 0.1 mM Gal, 1 mM GalNAc, or a combination.

Immunocytology—Immunostaining of CHO ldlD cells was performed with washed air-dried cells on multiwell slides using fixation with 3% paraformaldehyde and permeabilization with 0.5% Triton X-100. Slides were incubated with monoclonal antibodies overnight at 4 °C, followed by fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulins (F261, Dako) and examination on a Zeiss fluorescence microscope using epi-illumination.

ELISA for Quantification of FGF23—A commercial ELISA kit for quantification of intact FGF23 was used with culture medium from CHO ldlD transfectant cells according to the manufacturer's protocol (Kainos Laboratories International). Culture medium from cells was diluted to 1:500 and the assay run with an internal standard.

Glycosyltransferase Assays and Chemoenzymatic Synthesis of Glycopeptides—All glycosyltransferases were expressed as soluble secreted truncated proteins in insect cells as previously described (24, 25). Screening assays for GalNAc-transferases with peptides were performed with UDP-[¹⁴C]GalNAc as previously described (25) or as product development assays in 25 μ l of 25 mM cacodylic acid sodium, pH 7.4, 10 mM MnCl₂, 0.25% Triton X-100, 1.5 mM UDP-GalNAc (Sigma), 8.5 μ g of acceptor peptides, and 0.4 μ g of purified recombinant GalNAc-transferases. Peptides were custom synthesized by Schafer-N (Copenhagen, Denmark). Microsomal fractions derived from 200 μ l of packed cell pellets were resuspended in 200 μ l of 10 mM Hepes, 150 mM NaCl, 1% Triton X-100 and incubated on ice for 1 h, followed by centrifugation at 100,000 $\times$ g for 1 h, and the supernatant used for assays with or without immuno-clearance of human GalNAc-T3. Immuno-clearance was performed by incubating the supernatant overnight with anti-GalNAc-T3 monoclonal antibody UH5 (23), followed by incubation with protein G-Sepharose (Amersham Biosciences) for 1 h and centrifugation. The resulting supernatants were used for GalNAc-transferase assays and incorporation of GalNAc was quantified by scintillation counting after Dowex-1 chromatography. Synthesis of glycopeptides was performed sequentially with HPLC purification between each step. GalNAc-O-glycosylation was performed in 25 mM cacodylic acid sodium, pH 7.4, 10 mM MnCl₂, 0.25% Triton

X-100, 1.5 mM UDP-GalNAc (Sigma), 0.4 mg/ml of acceptor peptides, and 1.6 μ g/100 μ l of GalNAc-T3 (24). Core 1 galactosylation was performed in 100 mM MES, pH 6.0, 20 mM MnCl₂, 0.1% Triton X-100, 2.6 mM UDP-Gal (Sigma), 0.4 mg/ml of GalNAc-glycosylated FGF23b peptide, and dC1Gal-T1 (24, 26). Sialylation with ST3Gal-I was performed in 25 mM Tris-HCl, pH 6.5, 0.1% Triton X-100, 2 mM CMP-NANA (Sigma), 0.4 mg/ml of Gal β 1-3GalNAc α 1-O-glycosylated FGF23b peptide, and human ST3Gal-I (27, 28). Sialylation with ST6GalNAc-I was performed in 50 mM MES, pH 6.0, 20 mM EDTA, 2 mM dithiothreitol, 2 mM CMP-NANA, 0.4 mg/ml of GalNAc-glycosylated or Sial α 2-3Gal β 1-3GalNAc α -O-glycosylated FGF23b, and human ST6GalNAc-I (29). All glycopeptides were purified by HPLC on a C18 column (ZORBAX 300 SB C-18, 9.4 $\times$ 250 mm) and analyzed by MALDI-TOF.

MALDI-TOF MS Analysis—Evaluation of the number of GalNAc residues and subsequently Gal and NeuAc residues incorporated into peptides was done by MALDI-TOF MS as previously described (24). Briefly, 0.5 μ l of enzyme reaction mixtures were diluted with 3.5 μ l of 0.1% trifluoroacetic acid, H₂O, and 1 μ l was applied and mixed with 1 μ l of 25 mg/ml 2,5-dihydroxybenzoic acid dissolved in H₂O/CH₃CN (2:1) solution. Mass spectra were obtained on a Voyager-DETM instrument (Applied Biosystems) operating at an accelerating voltage of 20 kV (grid voltage 96.5%, guide wire voltage 0.05%) in the linear mode with the delayed extraction setting. Recorded data were processed using GRAMS software.

In Vitro Furin Cleavage Assay—An *in vitro* SPC cleavage assay with a human furin protease (Sigma) was developed with FGF23-related peptides covering the RHTR cleavage site. Assays were performed in 100 mM Hepes, pH 7.5, 1 mM CaCl₂, 0.5% Triton X-100, 1 mM 2-mercaptoethanol using 10 μ g of (glyco)peptides substrate and 1 unit of enzyme in a total volume of 50 μ l with incubations at 37 °C for 1, 4, and 24 h. Product development was evaluated by MALDI-TOF.

RESULTS

The effects of O-glycosylation of FGF23 were studied using the CHO ldlD mutant cell line. This cell line was originally developed and used to demonstrate that O-glycosylation of the low density lipoprotein receptor was required for stable cell-surface expression by preventing proteolytic cleavage of the extracellular domain (30). CHO ldlD cells are deficient in the UDP-Gal/UDP-GalNAc 4-epimerase, which catalyzes the reaction from UDP-glucose to UDP-galactose. The defect can be selectively restored by addition of GalNAc and/or Gal. Interestingly, addition of GalNAc alone results in O-glycosylation limited to GalNAc with no apparent elongation or sialylation (24), whereas the substitution of GalNAc and Gal leads to a complete restoration of O-glycosylation.

Secretion of FGF23 Is Dependent on GalNAc O-Glycosylation—FGF23 has previously been expressed in CHO cells and both intact 32 kDa and processed fragments are secreted suggesting that CHO cells process FGF23 partially (31). As shown in Fig. 1 secretion of FGF23 in CHO ldlD cells requires O-glycosylation with at least GalNAc, and the level of secretion is dramatically increased when GalNAc-T3 is co-expressed. All cells were characterized for expression of respective genes by immunocytology (Fig. 1A) and SDS-PAGE Western blot analysis (Fig. 1B, top panel). The stable transfectants of CHO ldlD cells with FGF23 alone or subsequent introduced GalNAc-T3 or control β 3GnT6 expressed similar levels of FGF23 evaluated by anti-Myc reactivity. The β 3GnT6 construct encodes the human β 3GlcNAc-transferase that forms the core 3 mucin-type O-linked structure (GlcNAc β 1-3GalNAc α 1-O-Ser/Thr), and is expected to elongate and mask the Tn structures expressed in CHO ldlD cells grown in only GalNAc (21). The GalNAc-T3 transfectant cells exhibited the expected supranuclear Golgi-like staining (23).

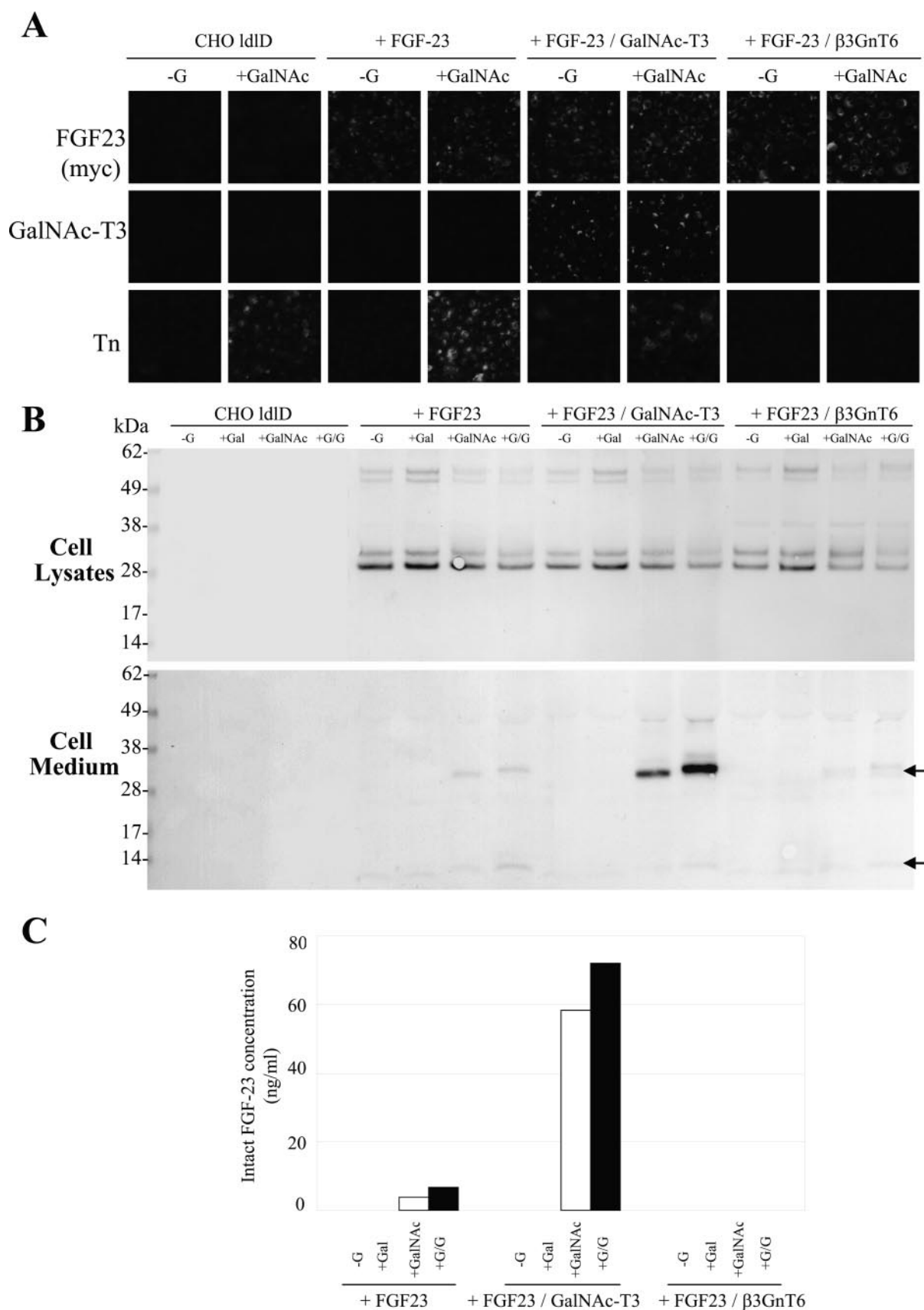


FIGURE 1. Secretion of FGF23 in CHO IdID cells requires O-glycosylation and is strongly up-regulated by co-expression of GalNAc-T3. A, immunofluorescence staining of wild type and stably transfected CHO IdID cells for FGF23 with anti-myc, GalNAc-T3 with monoclonal antibody UH5, and Tn with monoclonal antibody 5F4 (magnification $\times 200$). Designations: +FGF23, CHO IdID clone stably transfected with myc-tagged FGF23; +FGF23/GalNAc-T3, FGF23 and GalNAc-T3; +FGF23/ β 3GnT6, CHO IdID clone stably transfected with FGF23 and β 3GnT6. B, anti-myc SDS-PAGE Western blot analysis of cell lysates (upper panel) and culture medium (lower panel) of CHO IdID cells harvested after 72 h growth in

TABLE 1

Substrate specificities of cell lysates with or without immunoprecipitation with acceptors

Acceptor substrate	MUC1a, AHGV <u>T</u> SAPD <u>T</u> R		FGF23b, PIPRRH <u>T</u> RS <u>A</u> EDD <u>S</u> ERDP	
	<i>cpm (pmol/min)^a</i>			
Detergent-soluble cell lysates				
CHO ldlD FGF23 clone	134	(3.4)	7	(0.2)
CHO ldlD FGF23/T3 clone	270	(6.9)	110	(2.8)
Anti-GalNAc-T3 cleared lysates				
CHO ldlD FGF23 clone	160	(4.1)	6	(0.2)
CHO ldlD FGF23/T3 clone	160	(4.1)	2	(0.1)

^a Activities expressed as measured counts in reactions. Eight μ l of 200 μ l of microsome detergent lysates derived from 200 μ l of packed cell pellets were assayed as described under "Experimental Procedures" in a 25- μ l reaction volume. Background of 70 cpm was subtracted.

CHO ldlD cells grown in GalNAc-substituted medium express very little Tn glycoproteins (Fig. 1A), but cells transfected with FGF23 as well as FGF23 and GalNAc-T3 produced the expected diffuse cytoplasmic staining with anti-Tn (GalNAc α 1-O-Ser/Thr). This suggests that indeed FGF23 is GalNAc-glycosylated in cells grown in GalNAc (Fig. 1A). The control β 3GnT6-transfected cells when grown in GalNAc exhibited marked reduction in reactivity with anti-Tn compared with the parent FGF23 cells, in agreement with the expected function of this enzyme in elongation of Tn to core 3 structures (GlcNAc β 1-3GalNAc α 1-O-Ser/Thr). Analysis of lysates of all transfectant cells grown with or without addition of sugars by anti-myc Western blotting revealed comparable levels of intracellular FGF23, although there was a clear tendency that cells grown in GalNAc as well as GalNAc and Gal exhibited slightly reduced levels compared with cells grown without GalNAc (Fig. 1B, top panel). Cell lysates contained a major anti-myc reactive protein with a molecular mass of $\sim$ 30 kDa with a trace of a higher molecular mass band of 32 kDa. The upper band does not appear to represent an O-glycosylated glycoform because it does not vary with growth conditions of cells, *i.e.* presence or absence of added Gal or GalNAc. Analysis of FGF23 levels in medium of cells grown for 72 h by Western blot analysis as well as ELISA showed complete lack of FGF23 when cells were grown without GalNAc, whereas two distinct molecular species of $\sim$ 31 and 32 kDa were found in the medium when cells were grown in GalNAc or GalNAc and Gal, respectively (Fig. 1, B and C). CHO ldlD cells grown in both GalNAc and Gal produce sialylated core 1 structures, which should account for the apparent higher molecular weight. A faint band of $\sim$ 14 kDa was also observed in medium from all cells grown in GalNAc, which represents the C-terminal myc-tagged fragment (FGF23-(180–251)) (14). ELISA analysis of levels of secreted FGF23 from CHO ldlD FGF23 and the double FGF23 and β 3GnT6 transfectants showed low or undetectable levels, whereas the GalNAc-T3 transfectant cells showed a marked increase. This increase was especially significant with cells grown in both GalNAc and Gal, which suggests that GalNAc-glycosylation alone promotes secretion to a lesser degree than the more complex glycosylation achieved with addition of Gal and GalNAc. Interestingly, lack of GalNAc-T3 does not appear to result in significant intracellular accumulation of FGF23 or degradation products (Fig. 1B). The major anti-myc reactive species of $\sim$ 30 kDa in cell lysates did appear to exhibit a weak inverse correlation with levels of secreted FGF23. It may therefore be assumed that rapid degradation controls the intracellular pool similar to what was observed with a FGF23 mutant in HEK293 cells (8).

O-Glycosylation Capacity in CHO ldlD Cells—The remarkable induction of secretion of FGF23 by co-expression of GalNAc-T3 suggests that CHO ldlD cells do not express an endogenous hamster GalNAc-T3 ortholog or other GalNAc-transferase isoforms with the same specificity; however, the repertoire of GalNAc-transferases in CHO ldlD cells is unknown. To evaluate the presence of such activity, we tested detergent lysates of CHO ldlD cells for GalNAc-transferase activity with a peptide sequence derived from FGF23 (FGF23b) and a MUC1-derived peptide sequence as control (Table 1). CHO ldlD cell lysates contained significant activity with Muc1a peptide and essentially no detectable activity with the FGF23b peptide. The GalNAc-T3 CHO ldlD transfectant cell lysates contained almost 2-fold higher activity with Muc1a and significant activity with the FGF23 peptide. Clearing the lysates with anti-GalNAc-T3 immobilized antibodies resulted in selective removal of the activity with FGF23. We did test for activity in the immunoprecipitates, because our previous studies of immunoprecipitation of soluble recombinant GalNAc-T3 by UH5 was found to be active (32), however, no activity above background could be demonstrated in precipitates of CHO ldlD GalNAc-T3 cells. This is likely due to the fact that the enzyme is the membrane-bound form, which is known to be very unstable. These experiments indicate that CHO ldlD cells do not express GalNAc-T3 or isoforms with FGF23 substrate specificities in a significant degree. We have previously identified unique *in vitro* substrate specificities for the GalNAc-T3 isoform and its close subfamily member GalNAc-T6 (23). One peptide substrate identified from the V3-loop of HIV gp120 has been confirmed as an exclusive *in vivo* substrate for GalNAc-T3 in COS7 cells, which is the only available correlation of *in vitro* and *in vivo* functions of GalNAc-transferases to date (33). GalNAc-T3 and -T6 have similar substrate specificities as revealed by *in vitro* analysis, however, the two isoforms have markedly different cell and tissue expression patterns (23).

Unique GalNAc-T3-mediated O-Glycosylation of Peptide Substrates from FGF23—To further explore the role of GalNAc-T3 we tested a panel of recombinant human GalNAc-transferases with peptide substrates designed to cover potential O-glycosylation sites in FGF23. Shimada *et al.* (14) found evidence of variable O-glycosylation in two peptide fragments (162–175 (1 site) and 176–187 (2 sites)) around the furin protease processing site and also O-glycosylation in a more C-terminal fragment (199–228) of mutant FGF23 (double R176Q and R179Q) expressed in PEAKrapid cells. The actual sites of attachments have not been defined. We focused on the potential Ser/Thr sites around the RHTR furin protease cleavage signal (Fig. 2). GalNAc-T3

medium supplemented as indicated. Arrows indicate intact FGF23 and cleaved C-terminal fragment. C, ELISA for quantification of intact FGF23 in culture medium. A 1:500 dilution of culture medium was used and titrated together with a standard (50–500 pg/ml) provided with the ELISA kit (Kainos Laboratories International). Designations: –G, no sugars added; +G/G, both Gal and GalNAc added. Cells were seeded with 1×10^5 cells/well in 24-well plates (NUNC) and cultured at 37 °C in 1 ml of medium without sugar for 24 h. After 24 h the medium was exchanged with 1 ml of medium without sugar or with 0.1 mM Gal, 1 mM GalNAc, or 0.1 mM Gal and 1 mM GalNAc, and cells were further cultured for 72 h. Culture medium and cell pellets were collected and used for assays. Cell pellets were solubilized in 125 μ l of NuPAGE LDS Sample Buffer (4 times) (Invitrogen), 25 μ l of 10% SDS, and 350 μ l of dH₂O by vortexing. 20 μ l of the lysates and conditioned media were analyzed by 4–12% BisTris gradient NuPAGE (Invitrogen) and blotted on nitrocellulose membrane (Bio-Rad) and stained with anti-myc (1:5,000, Invitrogen) followed by visualizing with the Western Breeze kit (Invitrogen).

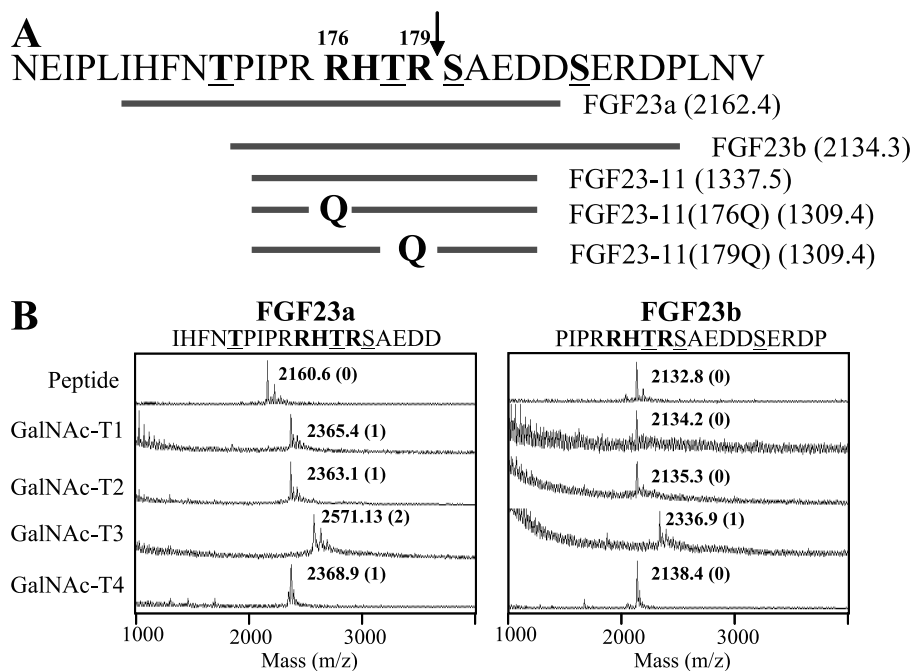


FIGURE 2. Identification of a unique acceptor substrate site for GalNAc-T3 at Thr¹⁷⁸ in FGF23. A, schematic representation of peptides derived from FGF23 used in the study. Arrow indicates the furin protease cleavage site in FGF23 and the **bold** sequence indicates the furin protease recognition motif. Potential O-glycosylation sites are **bold** and underlined. B, MALDI-TOF characterization of products formed in exhaustive glycosylation reactions with FGF23a and FGF23b peptides using four different recombinant human GalNAc-transferase isoforms. Masses of peptides and glycopeptide products are indicated with the predicted number of GalNAc residues in parentheses.

was tested together with three other broadly expressed isoforms, GalNAc-T1, -T2, and -T4, with different acceptor substrate specificities (34, 35). Whereas all isoforms utilized Thr¹⁷¹, only GalNAc-T3 incorporated GalNAc into Thr¹⁷⁸. The site of incorporation was determined by MALDI-TOF analysis of the furin-cleaved product of the FGF23b GalNAc glycopeptide, where the N-terminal peptide fragment was found as the expected mass (calculated mass 1032.2) plus the saccharide component (calculated mass 1235) (Fig. 3). The close subfamily homolog GalNAc-T6 exhibited the same substrate specificity as T3 (not shown) (23). The studies of O-glycosylation of mutant FGF23 by Shimada *et al.* (14) are in agreement with the *in vitro* O-glycosylation of Thr¹⁷⁸ as they found two O-glycans in the peptide fragment ¹⁷⁶QHTQSAEDDSER¹⁸⁷ with three potential acceptor sites of which Thr¹⁷⁸ and Ser¹⁸⁰ are most likely sites.

Specific O-Glycosylation of FGF23 Block Furin Protease Cleavage of FGF23 Peptides—The peptides FGF23b and FGF23-11, which contain the GalNAc-T3 Thr¹⁷⁸ glycosylation site, were then used for further studies of the effect of O-glycosylation for *in vitro* furin protease cleavage. To demonstrate the validity of this assay, we tested two mutant peptides based on identified mutations in the furin cleavage signal in patients with autosomal dominant hypophosphatemic rickets (12, 16). As shown in Fig. 3A the wild type sequences were efficiently cleaved to the expected fragments, whereas the R179Q mutant peptide was completely resistant and the R176Q peptide partially resistant to cleavage. The mutant peptides were both equally efficient selective substrates for GalNAc-T3 and none of the other isoforms (GalNAc-T1, -T2, and -T4) utilized these substrates (not shown). A series of glycopeptides with all the expected O-glycan glycoforms, which can be produced in CHO cells, were tested in the furin cleavage assay (Fig. 3B). Interestingly, GalNAc alone or Galβ1–3GalNAc did not seem to have a significant inhibitory effect, whereas α2,6-sialylation on either structure of the GalNAc residue blocked cleavage. The α2,3-sialylation to the Gal residue of Galβ1–3GalNAc did not significantly block, suggesting that the major inhibitory effect is exerted by α2,6-sialylation of GalNAc (NeuAcα2–6GalNAcα1-O-Thr).

DISCUSSION

This study provides a novel mechanism for the regulation of processing and secretion of the phosphaturic factor, FGF23, which plays a pivotal role in phosphate homeostasis. Inactivating mutations in FGF23 or GALNT3 both result in FTC with reduced intact FGF23 serum levels and hyperphosphatemia thereby suggesting an interaction of these proteins (1, 2, 6, 8, 9). Our results show that loss of function of GalNAc-T3 selectively affects O-glycosylation and secretion of FGF23. A single unique O-glycan acceptor site in a SPC cleavage site of FGF23 likely directs the requirement for the GalNAc-T3 isoform.

Regulation of secretion of FGF23 appears to include an intracellular processing event in which intact FGF23 is partially cleaved and inactivated by subtilisin-like proprotein convertases at the RHTR¹⁷⁹ ↓ cleavage site (15). The cleavage signal sequence with a potential O-glycosylation site is conserved to rodents. Mutations in autosomal dominant hypophosphatemic rickets (14, 16) leads to a complete block of proteolytic cleavage and increased levels of circulating FGF23 causing hypophosphatemia (37). Furthermore, in cell lines this block of proteolysis leads to increased secretion into the medium (14). Here, we found that GalNAc-T3-mediated O-glycosylation in the protease recognition sequence (Thr¹⁷⁸) blocked proteolysis in an *in vitro* assay, however, only when the O-glycan was α2,6-sialylated (Fig. 3). This was supported by *in vivo* studies as GalNAc-T3 directed GalNAc glycosylation in CHO IdID cells substituted with only GalNAc showed a lower ratio of intact FGF23 to the C-terminal fragment compared with cells with further galactosylation and sialylation (Fig. 1B). O-Glycosylation in CHO cells is predicted to produce a mixture of disialyl and monosialyl core 1 (T) structures (NeuAcα2–3Galβ1–3[NeuAcα2–6]GalNAcα1-O-Ser/Thr) with variable α2,6-sialylation (38). The α2,6-sialylation is controlled by the ST6GalNAc-I, -II, or -IV sialyltransferases (39–41), which are differentially expressed and have different substrate specificities (42), however, α2,6-sialylation of O-glycans is normally not complete. It therefore emerges that regulation of FGF23 secretion may include a posttranslational processing step in which competition between proprotein convertase cleavage and block of cleavage by O-glycosylation determines the level of secretion of active intact FGF23. This would be consistent

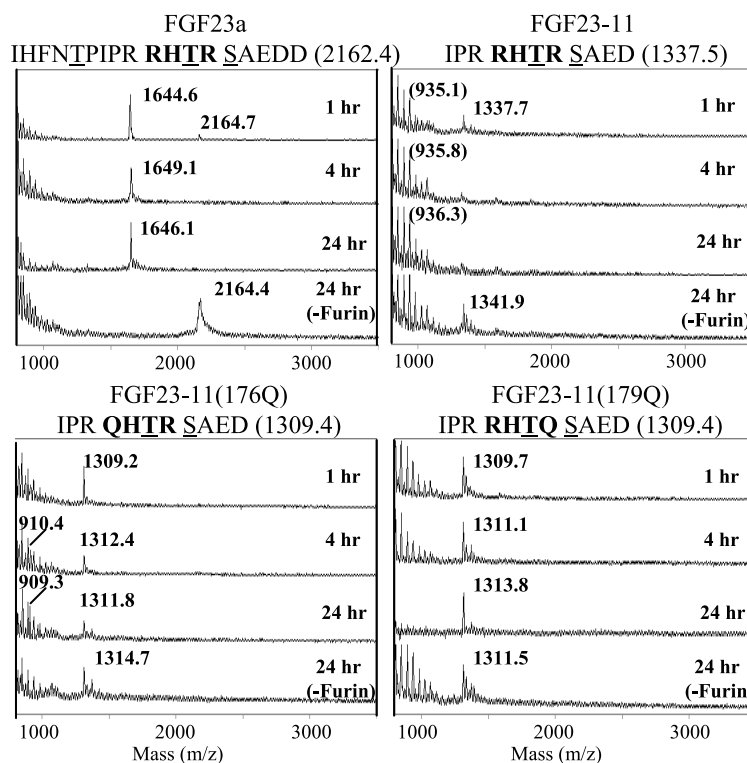
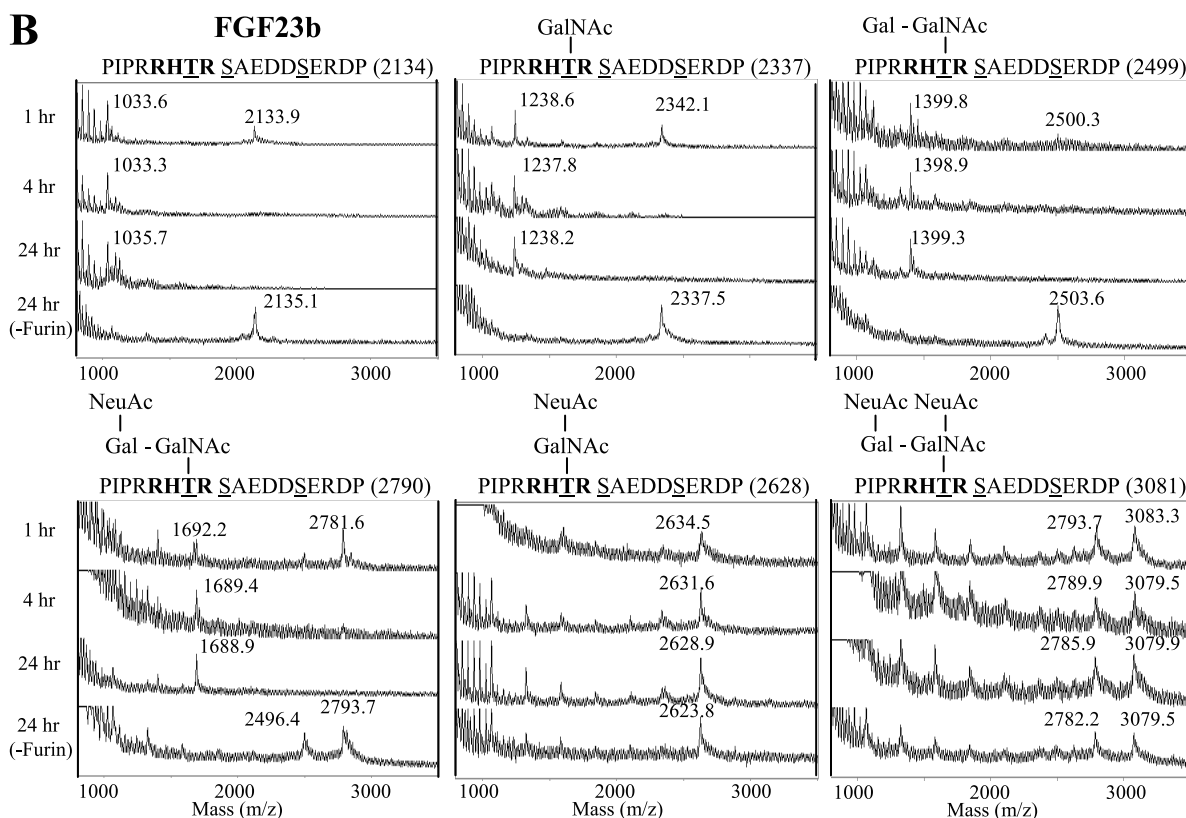
A

B


FIGURE 3. Time course MALDI-TOF analysis of *in vitro* furin cleavage of FGF-23 (glyco)peptides. A, analysis of the influence of Gln mutations in the RXRR recognition motif. The FGF23a and FGF23-11 wild type peptides were efficiently cleaved at the RXRR ↓ (RHTR¹⁷⁹) motif yielding N-terminal fragments of predicted sizes (1644.9 and 935.1, respectively). The mutant peptides with QHTR¹⁷⁹ and RHTQ¹⁷⁹ sequences were largely not cleaved, although the QHTR¹⁷⁹ peptide did partly produce an N-terminal fragment of the predicted size (907.0). B, analysis of the influence of glycosylation using FGF23b glycopeptides. The unglycosylated FGF23b peptide was rapidly cleaved with emergence of the predicted N-terminal fragment (1032.2). The expected C-terminal fragment (1120.1) was invariably observed as a smaller peak. The glycopeptides with GalNAc, Galβ1-3GalNAc, and NeuAcα2-3Galβ1-3GalNAc were equally efficiently cleaved to form the predicted N-terminal fragments (1235, 1397, and 1688, respectively). In contrast, the glycopeptides with NeuAcα2-6GalNAc and NeuAcα2-3Galβ1-3(NeuAcα2-6)GalNAc were largely resistant to cleavage, and no clear masses for the predicted N-terminal fragments (1526 and 1799, respectively) were observed. The disialylglycopeptide produced two masses corresponding to the predicted masses of both mono- (2790) and disialylglycopeptides (3081), which may indicate that the sialylation was only partial or loss of one sialic acid residue in the analysis. Analysis of the sialylated glycopeptides were complicated by the need to use high laser intensity with resulting higher noise levels. The furin cleavage assay was performed at 37 °C with 0.2 mg/ml (glyco)peptides and reactions were monitored at 1, 4, and 24 h.

with the knowledge that SPC processing takes place in the trans-Golgi complex where final processing including sialylation of O-glycans occur.

It may be interesting to note that the tumor-induced osteomalacia condition is a result of hypersecretion of FGF23 usually by benign mesenchymal tumors (43). The expression of GalNAc-T3 as well as its close subfamily member GalNAc-T6 is dramatically altered in epithelial carcinomas (44, 45), however, to our knowledge expression of these enzymes has not been studied in tumor-induced osteomalacia cases.

O-Glycosylation has long been known to confer resistance to proteolysis (5). The low-density lipoprotein receptor represents an excellent example where lack of O-glycosylation in the extracellular stalk region results in rapid proteolytic release of the receptor domain (22, 46). O-Glycosylation of the β -chain of the zinc metalloendoproteinase meprin has been shown to affect intracellular processing and inhibition of O-glycosylation leads to increased secretion (47). Studies may also suggest a role for O-glycosylation in processing of hormones including pro-ACTH/endorphin (48) and insulin-like growth factor II (49, 50), but conclusive evidence is lacking. O-Glycosylation has been implicated in intracellular sorting of membrane proteins (30, 51–53), however, the molecular mechanisms behind these remain unclear. Other studies have shown that O-glycosylation may affect endocytosis (54, 55). In none of these studies have a single GalNAc-transferase isoform been implicated, which may be related to redundancy in GalNAc-transferases in cells. In a recent study in yeast, however, a mannose O-glycosylation-mediated intracellular sorting event was shown to be directed by one specific O-mannosyltransferase, PMT4, out of the family of seven O-mannosyltransferase isoforms in yeast (56). In this study the O-glycosylated sequence required was narrowed down to a 33-amino acid stretch immediately after a transmembrane sequence with 15 potential O-glycosylation sites. It is unlikely that all 15 potential sites are exclusive substrates for PMT4, which suggests that a subset of O-glycosylation sites directs the sorting event.

The large family of 20 polypeptide GalNAc-transferases control the initiation step of mucin-type O-glycosylation, which defines the sites and patterns of O-glycan decoration of glycoproteins. The polypeptide GalNAc-transferase isoforms have been demonstrated in *in vitro* studies to have different peptide substrate specificities, however, a significant degree of overlap in specificities exists especially with mucin-like substrates with high-density clustered acceptor sites (3, 57). The cell and tissue expression patterns of individual GalNAc-transferase isoforms are also distinctly different but with significant overlap, and it is expected that all cells express multiple isoforms (32, 58). Therefore, the large number of GalNAc-transferase isoforms available has been interpreted to provide a high degree of redundancy, and in fact ablation studies in mice have not yet provided unambiguous information of unique roles for individual GalNAc-transferase isoforms (59–62), although a recent reinvestigation of GalNAc-T1-ablated mice appear to have defects in lymph node B-cell retention (63). A case for unique *in vivo* functions of GalNAc-transferase isoforms was, however, provided by studies in *Drosophila melanogaster*, where it was found that a single GalNAc-transferase isoform out of 15 putative GalNAc-transferase genes in *Drosophila* was essential for pupal development (25, 36). Although the molecular basis for this is unknown, the result clearly points to unique non-redundant functions of GalNAc-transferase isoforms. The present study provides the first example of a defined molecular mechanism underlying deficiency in a single GalNAc-transferase isoform, GalNAc-T3, and it is clear that GalNAc-transferase isoforms may serve unique functions

in cells where the repertoire of isoforms does not represent appropriate redundancy.

The initiation step of O-glycosylation is clearly the single glycosidic linkage covered by the highest degree of potential biosynthetic redundancy. Yet, deficiency of GalNAc-T3 apparently results in a limited and defined molecular defect in FGF23 secretion that leads to hyperphosphatemia in FTC. This is the first disease associated with mutations in a specific GalNAc-transferase isoform, but given the subtle and indirect model proposed for the effect of deficiency of GalNAc-T3, it is not unreasonable to predict that further disease-causing GalNAc-transferase genes will emerge in the future. Although studies have suggested a role of O-glycosylation in processing of other glycoproteins (48, 49), it is presently unclear if the proposed posttranslational regulatory model for FGF23 involving competing O-glycosylation and protease processing may have more widespread applicability.

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**Polypeptide GalNAc-transferase T3 and Familial Tumoral Calcinosis:
SECRETION OF FIBROBLAST GROWTH FACTOR 23 REQUIRES
O-GLYCOSYLATION**

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