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CALCITONIN IS A SUBSTRATE FOR OLIGOSACCHARYLTRANSFERASE IN VITRO

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Tumor-derived, large molecular weight forms of calcitonin, have been postulated to result from glycosylation of the hormone. To address this question we have examined the glycosylation of calcitonin in vitro and in cultured thyroidal C-cells. We show that native, undenatured calcitonin is an active substrate for oligosaccharyltransferase and that glycosylation of calcitonin by the transferase is inhibited by tunicamycin. In addition, calcitonin is an effective competitive inhibitor of the glycosylation of a known peptide substrate for oligosaccharyltransferase. Pulse-labelling of cultured medullary thyroid carcinoma cells with [^{3}H]-mannose indicate that detectable quantities of carbohydrate-containing forms of calcitonin are produced in these cells. These data indicate that glycosylation of calcitonin is one mechanism whereby tumor cells could produce higher molecular weight forms of the hormone.

Calcitonin is a 32-amino acid, nonglycosylated hormone which is synthesized in the C-cells of the thyroid and acts as a physiological regulator of calcium homeostasis in mammals (1,2). Synthesis of the hormone has also been detected in a number of tumor tissues where larger molecular weight forms of the hormone, postulated to result from glycosylation of calcitonin, are secreted (3,4). Earlier work had indicated that precursor forms of the hormone contain carbohydrate (5,6).

In the present report we have directly examined the glycosylation of calcitonin in vitro and in a cultured thyroidal C-cell line. From our present studies we conclude that calcitonin (MW=3,500) is an active substrate for oligosaccharyltransferase in vitro and can competitively

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inhibit the glycosylation of other substrates. In addition, detectable quantities of the hormone are glycosylated in cultured C-cells.

MATERIALS AND METHODS

Oviduct Microsome Preparation

The magnum portion of freshly killed laying hens was cleared of connective tissue, minced, homogenized, and centrifuged as described previously (7). Before use microsomes were stored at -70°C at approximately 30 mg/ml (8).

Oligosaccharyltransferase Assays

Reaction mixtures contained 100 uM UDP-GlcNac, 25 uM UDP-glucose, 1 uM GDP-[^{3}H] mannose (400,000 cpm/assay), 150 ug membrane protein, 50 mM Tris-HCl, pH 7.4, 10 mM $MnCl_2$, 140 mM sucrose, 25 mM NaCl, 1 mM EDTA, 0.1% NP-40 (v/v) and 150 uM acceptor peptide in total volume of 200 ul. After incubation for 30 min at 30°C assays were terminated by addition of 50 ul of 0.5 M EDTA. Membranes were pelleted by centrifugation in a Beckman Microfuge at setting 10 for 10 min and the supernatant fraction was applied to a G-50 Sephadex column (1.0 x 110 cm) equilibrated in 0.1 M NH_4HCO_3, pH 7.8. Fractions of 2.6 ml were collected at a flow rate of 30 ml/hour. The glycosylation of synthetic salmon and human calcitonin (Bachem, Torrance, CA) were compared with that of a known acceptor T_{17-58} of alpha-lactalbumin (9). Protein determinations were performed by the method of Lowry et al.(10).

1Endoglycosidase H and Tunicamycin Treatment of Glycosylation Assays

The supernatant of a standard reaction mixture was buffered to a final concentration of 50 mM Mes pH 5.5, incubated with 1 mUnit of Endo H at 30°C for 2 hours, and then another mUnit was added and the reaction was allowed to proceed overnight. The ph of the sample was then adjusted to 7.5 with NaOH and applied to G-50 Sephadex column as described above.

The effect of tunicamycin treatment upon transfer of ^{3}H-oligosaccharide to acceptor peptides was determined by performing standard transferase assays in the presence of 5 ug/ml HPLC purified tunicamycin. The supernatant was then applied to a Sephadex G-50 column as described above.

Competitive Inhibition of Acceptor Peptide Glycosylation by Calcitonin

Competition assays contained 90 uM nonradioactive synthetic human and salmon calcitonin (competing peptides), 30 uM N-alpha-[^{3}H] Ac-Asn-Leu-Thr-$NHCH_3$ radioactive acceptor (8), 50 mM Tris-HCl, pH 7.4, 140 mM sucrose, 25 mM NaCl, 1 mM EDTA, 10 mM $MnCl_2$, 0.1% NP-40 (v/v) and 200 ug membrane protein in a total volume of 50 ul. After incubation for 5 min at 30°C, the reactions were stopped with 50 ul of 20% TCA, spun in a Beckman Microfuge at setting of 10 for 10 min and 75% of the supernatant was removed for paper chromatography. The supernatants were backwashed 3 times with 1 ml of anhydrous ether, flushed with N_2 to remove residual ether and applied to a sheet of Whatman 3 MM paper for descending paper chromatography in Butanol:HoAc:H_2O (12:3:5). Glycosylation of N-alpha-[^{3}H] Ac-Asn-Leu-Thr-$NHCH_3$ was quantitated by measuring the radioactivity remaining at the origin (8).

Calcitonin Immunoprecipitations

Immunoprecipitations were performed utilizing a double antibody immunoprecipitation procedure as described previously for calcitonin-related products (5). Antisera to homogeneous, synthetic human calcitonin were employed which cross-react with rat calcitonin (5). Conditioned cell media was dissolved in 0.1 ml of a solution consisting of 10 mM NaH_2PO_4, pH 7.6, 1 mM Na_2 EDTA and 1% Triton X-100 and incubated overnight at 4°C with calcitonin antisera. Radioactivity in the washed immunoprecipitates was determined by liquid scintillation spectrometry (5).

Labeling of Calcitonin with [^{3}H]-Mannose and [^{35}S]-Methionine in Rat Medullary Thyroid Carcinoma Cells

Rat medullary thyroid carcinoma cells (rMTC 6-23) were kindly supplied by Robert Gagel, Baylor College of Medicine, Houston, Texas and were grown to confluency in 35-mm plastic dishes as described by Gagel et al. (11). To enhance production of calcitonin, cells were treated with 10^{-8}M dexamethasone for five days prior to labelling with radioactive sugars or amino acids (12). Cells (10^6) were incubated at 37°C under 95% air, 5% CO_2 for 48 h in the presence of 50 uCi [^{35}S]-methionine (980 Ci/mmol) or 150 uCi [^{3}H]-mannose, 27.2 Ci/mmol, (New England Nuclear). Media and fresh radiolabelled compounds were added after 24 h. At the end of the incubations the culture medium was collected and assayed for 1) total glycoprotein synthesis as measured by radioactivity in hot trichloroacetic acid (TCA) stable, alkali-stable, trichloroacetic acid precipitable fraction as reported by Carson et al (13); and 2) radioactivity in calcitonin immunoprecipitates (as described above).

RESULTS

Calcitonins Are Glycosylated In Vitro by Chicken Oligosaccharyltransferase

Two experiments were performed to determine whether the native, undenatured form of calcitonin is a substrate for glycosylation in vitro. In the first experiment salmon (sCT) or human (hCT) calcitonin was incubated with hen oviduct microsomes, containing endogenous oligosaccharyltransferase (7,9), and radioactive sugar nucleotide precursors for synthesis of labeled oligosaccharide (8). The glycopeptide product formed during the incubation was then analyzed by gel filtration chromatography on Sephadex G-50 as shown in Figure 1. When no peptide or a peptide without an acceptor site was used in this assay, no glycopeptide (marked by arrow) was formed (Fig. 1A). In contrast, the addition of a known acceptor peptide (9), to incubations led to formation of glycopeptide product (Fig. 1E). Addition of homogenous, synthetic sCT (Fig. 1B) to incubations also led to production of a glycosylated product. Formation of the sCT glycopeptide product was

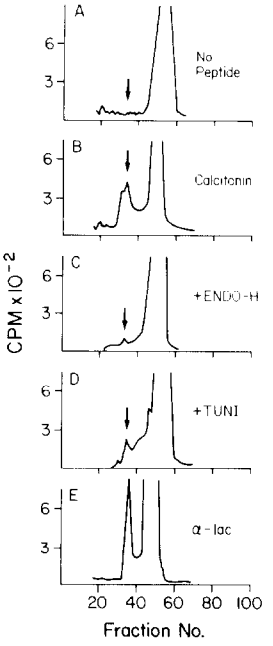

Figure 1: Sephadex G-50 gel filtration of reaction mixtures of oligosaccharyl transferase assays.
See "Materials and Methods" for details of the transferase assays. The following samples were added to the reaction mixtures: Panel A, no peptide; Panel B, synthetic salmon calcitonin; Panel C, synthetic salmon calcitonin plus endoglycosidase-H; Panel D, synthetic salmon calcitonin plus tunicamycin; Panel E, alpha-lac, the tryptic peptide comprising residues 17-58 of alpha-lactalbumin, a known acceptor peptide for N-linked glycosylatin (9). The arrow points to the mobility of glycosylated, synthetic salmon calcitonin on Sephadex G-50 chromatography as determined in Panel B.

inhibited by coincubation with tunicamycin (Fig. 1D). In addition, the glycosylated sCT which was produced in this assay was shown to be sensitive to endogylosidase H (Fig. 1C). Similar results were obtained for hCT as summarized in Table 1. It should be stressed that the above assays were performed in the absence of denaturing or reducing agents and, therefore, native calcitonins are enzyme substrates.

To demonstrate further that hCT and sCT are substrates <u>in vitro</u> for oligosaccharyltransferase, we examined whether calcitonins could competitively inhibit, by virtue of their substrate activity, the glycosylation of a known substrate for the enzyme, N-alpha-[^{3}H] Ac-Asn-Leu-Thr-$NHCH_3$ (8). In these studies either human or salmon calcitonin were present in a 3-fold molar excess over the tripeptide substrate. As shown in Table II both human and salmon calcitonins were

TABLE I

Calcitonins (CT) are Substrates for Oligosaccharyltransferase

Compound Added	Glycosylated Product Formed (cpm)
None	0
a-Lactalbumin T_{17-58} [a]	59,760
Salmon CT[b]	42,872
Salmon CT plus TM	6,386
Human CT	36,540

[a]a-lactalbumin T_{17-58} is the 41 residue tryptic peptide corresponding to residues 17-58 of bovine a-lactalbumin.
[b]Endoglycosidase H treatment released 67% of [^{3}H] oligosaccharide from glycosylated salmon CT as quantitated by gel filtration on a Sephadex G-50 column.

effective competitive inhibitors in this assay with human calcitonin being as potent an inhibitor of glycosylation as alpha-lactalbumin.

Examination of Calcitonin Glycosylation in Cultured C-Cells

As calcitonin is an active substrate for oligosaccharide transferase in vitro we next examined glycosylation of the hormone in its cellular site of synthesis. To perform these studies we utilized rat medullary thyroid carcinoma cells, a transformed C-cell line which synthesizes and secretes, as its predominant calcitonin product (11), a non-glycosylated peptide of MW=3500. Cells were treated with 10^{-8}M dexamethasone for five days to stimulate calcitonin synthesis (12) prior to pulse labelling with [^{35}S]-methionine or [^{3}H]-mannose. Pulse labellings of the cells were done with relatively high input of radioactive label in small

TABLE II

Calcitonins (CT) Competitively Inhibit Glycosylation of N-alpha-[^{3}H] Ac-Asn-Leu-Thr-$NHCH_3$

Peptide	Glycopeptide Formed (cpm)[b]	% Inhibition of Glycosylation of N-alpha-[^{3}H] Ac-Asn-Leu-Thr-$NHCH_3$
None	1,701	0
a-Lactalbumin T_{17-58} [c]	392	77
Salmon CT	955	44
Human CT	375	78

[a]Competing peptides were present at a three-fold molar excess over N-alpha-[^{3}H] Ac-Asn-Leu-Thr-$NHCH_3$.
[b]Numbers are average of duplicates.
[c]a-lactalbumin is the 41 residue tryptic peptide corresponding to amino acids 17 through 41 of bovine alpha-lactalbumin.

TABLE III

Calcitonin (CT) Glycosylation in Cultured Thyroidal C-cells

Treatment	Calcitonin Precipitated (CPM)[a]
A. Cells pulsed with ^{35}S -Methionine	
CT Antibody Alone	3800
CT Antibody +25 ug CT peptide[b]	350
B. Cells pulsed with ^{3}H -Mannose	
CT Antibody Alone	345
CT Antibody +25 ug CT peptide[b]	75
CT Antibody +Endoglycosidase H[c]	98

[a]See Materials and Methods for procedures on calcitonin immunoprecipitation.
[b]Twenty-five micrograms of synthetic calcitonin was added to competitively inhibit the immunoprecipitation reaction.
[c]See Materials and Methods for endoglycosidase H procedures.

volumes to maximize labelling of calcitonin-related proteins (See Materials and Methods). Conditioned media from pulse-labelled cells was immunoprecipitated with antisera raised to homogeneous, synthetic calcitonin. As shown in Table 3, we can specifically immunoprecipitate [^{35}S]-labelled calcitonin products from these cells as well as endoglycosidase H-sensitive, calcitonin-related products which have incorporated [^{3}H]-mannose.

DISCUSSION

In higher animals (rat and humans) calcitonin is synthesized in the C-cells of the thyroid gland as a higher molecular weight precursor and subsequently processed to its mature 32 amino acid form (14,15). The 32 amino acid peptide has a potential N-linked glycosylation site of -Asn-Leu-Ser- at residues 3-5 but glycosylated forms of the native peptide have not been reported. In the present study we have directly examined glycosylation of the hormone and show that synthetic calcitonins are potent acceptors for oligosaccharides *in vitro*. In addition, carbohydrate-containing forms of the hormone are detected in culture thyroidal C-cells. This is the first demonstration that calcitonin is an active substrate for N-linked glycosylation *in vitro* and indicates that glycosylation of the hormone may contribute to the heterogeneous, higher

molecular weight forms of the hormone seen in certain cancer cells (3,4,16,17).

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