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Biosynthesis of an Asparagine-Linked Oligosaccharide-Containing Calcitonin by a Rat Medullary Thyroid Carcinoma Cell Line†

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ABSTRACT: Calcitonin contains an amino acid sequence that provides a potential site for glycosylation of the peptide at the asparagine at position 3. Preliminary evidence has suggested that there are glycosylated forms of calcitonin and its precursor, procalcitonin. The CA-77 rat medullary thyroid carcinoma cell line, recently developed to study calcitonin biosynthesis, was used to demonstrate the synthesis of glycosylated forms of this hormone by intact cells. Cultures were incubated in medium containing either [^{3}H]mannose or [^{35}S]methionine. Two species incorporating both labels were specifically immunoprecipitated when cell extracts were treated with calcitonin antibodies. Gel filtration chromatography in 6 M guanidine hydrochloride indicated that one peptide had a molecular weight of 5500, approximately 2000 daltons larger than calcitonin, while the second peptide had a molecular weight of 14 400, the approximate size of procalcitonin. Treatment of the [^{3}H]mannose-labeled cell extract with endo-β-*N*-acetylglucosaminidase H before immunoprecipitation removed the labeled sugar from the calcitonin species. Microsequence analysis of the radiolabeled immunoreactive 5500-dalton calcitonin species showed methionine at cycle 8 and mannose at cycle 3, suggesting that this peptide is calcitonin containing an N-linked oligosaccharide at Asn-3. These results suggest that in this cell line a minor but significant biosynthetic pathway exists for the production of glycosylated calcitonin from glycosylated procalcitonin.

The 32 amino acid sequence of the peptide hormone calcitonin contains an N-linked glycosylation site (Raulais et al., 1976; Struck et al., 1978). This sequence, Asn-Leu-Ser, at residues 3–5 (Raulais et al., 1976), is a necessary but not compulsory condition for the transfer of an oligosaccharide consisting of glucose, mannose, and *N*-acetylglucosamine from a dolichol phosphate donor to the amide nitrogen of the asparagine (Waechter & Lennarz, 1976; Wagh & Bahl, 1981). In vivo such sites are often not recognized, or only a fraction of the molecules is modified (Eipper et al., 1976; Nisbet et al., 1981). The major form of calcitonin isolated from various mammalian and nonmammalian sources is not glycosylated (Guttman, 1981). Thus, it would appear that the signal was not recognized in vivo. Recently, several laboratories have reported that a fraction of the total immunoreactive calcitonin in extracts of calcitonin-producing tumors binds specifically to lectins, including concanavalin A (Dermody et al., 1981; O'Neil et al., 1981; Baylin et al., 1983). Furthermore, in the presence of microsomal membranes, cell-free translation of rat medullary thyroid carcinoma mRNA generates an N-linked glycosylated procalcitonin, the biosynthetic precursor of calcitonin (Jacobs et al., 1981), as expected from this type of experiment (Pless & Lennarz, 1977). In addition, synthetic calcitonin is a substrate for oligotransferases in the same in vitro system which will glycosylate procalcitonin (Jacobs et al., 1985).

Birnbaum et al. (1985) have described the major pathway for the biosynthesis of calcitonin in the dexamethasone-treated CA-77 cell line. Procalcitonin is proteolytically processed to a 6500-dalton biosynthetic intermediate, which is subsequently cleaved to the amidated, 32 amino acid calcitonin. We have observed (Birnbaum et al., 1984a) multiple forms of procalcitonin by reversed-phase high-performance liquid chromatography (HPLC)[1] fractionation of immunoprecipitates of radiolabeled CA-77 cell extracts or microsomal membrane

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[1] Abbreviations: HPLC, high-performance liquid chromatography; Endo H, endo-β-*N*-acetylglucosaminidase H; 3.4K etc., molecular weights ($\times 10^{-3}$) estimated by gel filtration chromatography; ACTH, adrenocorticotropin.

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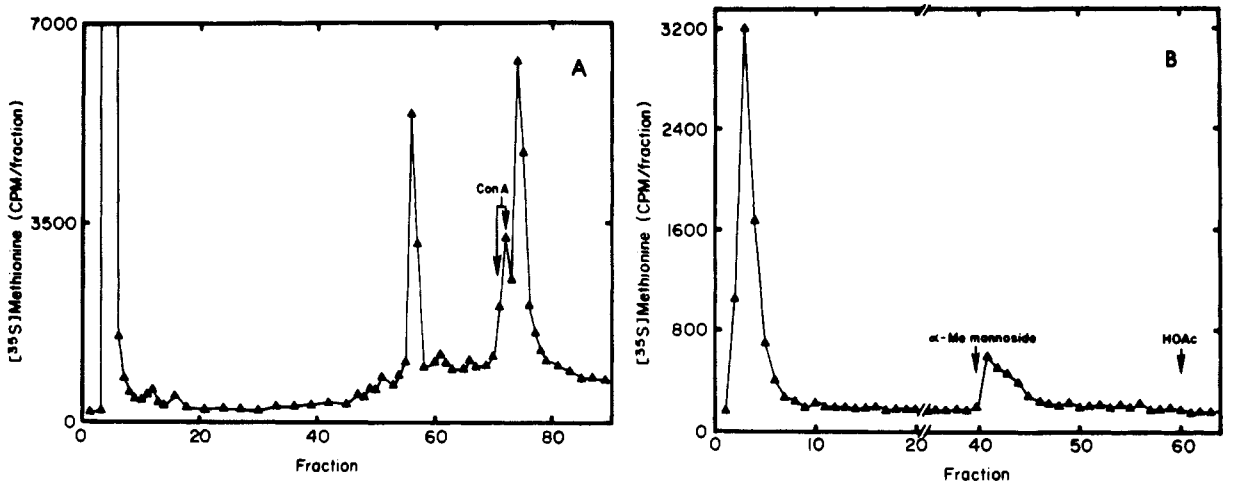

FIGURE 1: Concanavalin A affinity of procalcitonin. Cells were labeled with [^{35}S]methionine for 1 h, and the immunoprecipitated cell extract was fractionated by reversed-phase HPLC using a cyanopropyl column (A). The flow rate was 1 mL/min; 1-min fractions were collected. The fractions indicated by the arrows (Con A) were pooled, dried, and applied to a column of concanavalin A–agarose (B). After the column was washed extensively (note the break in fraction number), specifically bound glycosylated procalcitonin was eluted with 1 M methyl α-D-mannoside. No further radioactivity was recovered when the column was stripped with 1 M acetic acid.

containing in vitro translations using medullary thyroid carcinoma mRNA. These preliminary observations suggest that this cell line produces multiple forms of procalcitonin which may differ in a specific posttranslational modification, such as the addition of an oligosaccharide to the asparagine at position 3 of the calcitonin sequence. This study uses the CA-77 cell line to investigate the glycosylation of procalcitonin and its processing. CA-77 cultures were radiolabeled with [^{3}H]mannose, and extracts were fractionated by immunoprecipitation, gel filtration chromatography, and reversed-phase HPLC.

Experimental Procedures

Growth of CA-77 Cells. Cultures of CA-77 rat medullary thyroid carcinoma cells were grown in serum-free medium as previously described (Muszynski et al., 1983) for 5–7 days and then treated with 10^{-7} M dexamethasone for a 4-day period (Birnbaum et al., 1984a, 1986).

Radiolabeling of CA-77 Cells. Radiolabeling with L-[^{35}S]methionine was carried out as described previously (Birnbaum et al., 1984a). Labeling with [2-^{3}H]mannose (ICN, final specific activity of 1 mCi/mmol) was performed in modified culture medium: The glucose concentration was decreased to 3 mM, mannose was added to a concentration of 1 mM, and proline, aspartic acid, glutamic acid, and alanine were increased to concentrations of 20 mg/L (Guttman, 1981; Phillips et al., 1981). Incubations were 3–6 h. These changes had little effect on the incorporation of [^{35}S]methionine into immunoreactive calcitonin species. Cells were extracted as previously described (Birnbaum et al., 1984a).

Immunoprecipitations. The characterization and procedures for use of antiserum Marilyn to isolate immunoreactive calcitonin have been described previously (Birnbaum et al., 1984a, 1986).

HPLC. Chromatography was performed on the system previously described (Birnbaum et al., 1984a) using a Whatman Protesil 300 Octyl analytical column or a 300-Å pore-sized cyanopropyl column (ES Industries, 0.46 × 25 cm).

Gel Filtration Chromatography. Immunoprecipitates of radioactive material from HPLC fractions that had been dried in a Speed Vac concentrator (Savant Instruments, Inc.) were chromatographed on a column (0.9 × 55 cm) of Sephadex G-75 (Superfine, Pharmacia Fine Chemicals), equilibrated, and eluted with 6 M guanidine hydrochloride containing 0.02% bovine serum albumin as described previously (Birnbaum et al., 1984a).

Concanavalin A Chromatography. Samples were applied to and eluted from columns of concanavalin A–Sepharose (Pharmacia Fine Chemicals) as previously described (O'Neil et al., 1981), except that an ammonium acetate buffer was used instead of a sodium acetate buffer.

Microsequencing. HPLC fractions to be microsequenced were pooled and dried under a stream of purified nitrogen. The residue was subjected to automated Edman degradation as described previously (Birnbaum et al., 1984a).

Digestion with Endo-β-N-acetylglucosaminidase H. The dried residues of cell extracts labeled with [^{3}H]mannose were taken up in 60 μL of 50 mM sodium acetate (pH 5.5) and split evenly. Endo H (Miles Laboratories), 20 milliunits in 20 μL of sodium acetate, was added to one sample while the other received 20 μL of sodium acetate buffer. Following incubation at 37 °C for 20 h, the samples were immunoprecipitated as described above and fractionated by reversed-phase HPLC.

To establish the quantity of enzyme necessary to completely remove the high-mannose oligosaccharide from putative glycosylated forms of calcitonin, preliminary studies were performed with the glycoprotein ovalbumin. Affinity to concanavalin A was the criterion for removal of susceptible oligosaccharide. Since the amount of ovalbumin used was greater than the total amount of protein in the cell extracts, we concluded that the amount of Endo H used to deglycosylate our model protein would function similarly with the glycoproteins of the CA-77 cultures.

Results

Analyses of immunoprecipitates of cell-free translations in the presence of microsomal membranes of rat medullary thyroid carcinoma mRNA or of radiolabeled CA-77 cell extracts (Birnbaum et al., 1984a) suggested the existence of at least two forms of procalcitonin, probably differing in posttranslational modifications. A typical HPLC chromatogram, using a cyanopropyl column, of an immunoprecipitate of a cell extract labeled with [^{35}S]methionine is shown in Figure 1A. As demonstrated previously, fractions 70–80 contain partially resolved minor and major peaks of procalcitonin. When the indicated fractions of the minor peak were dried and applied to a concanavalin A–Sepharose column, 26% of the applied radioactivity was specifically eluted with methyl α-D-

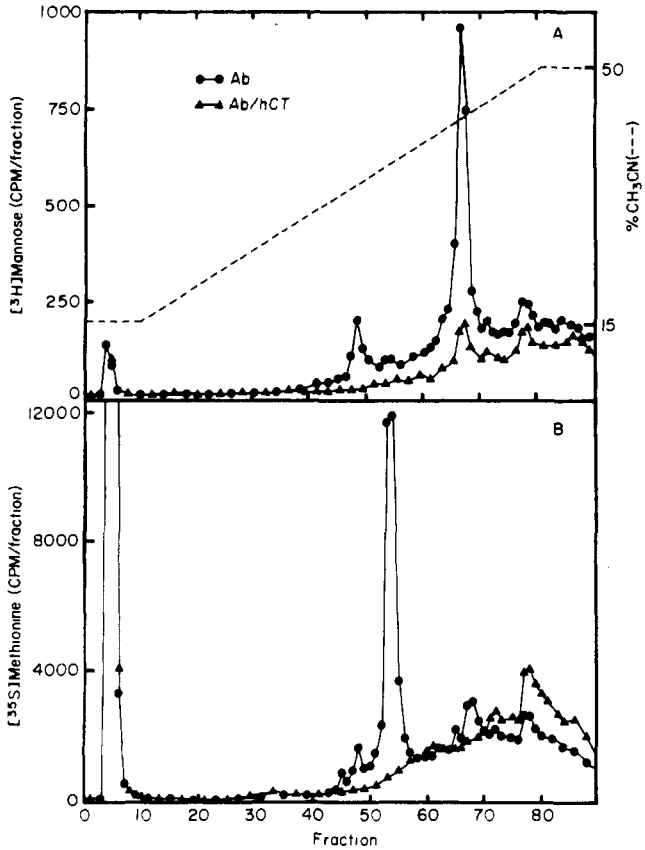

FIGURE 2: Reversed-phase HPLC profile of immunoprecipitated calcitonin from an extract of CA-77 cells. A C_8 column was used for this experiment. The flow rate was 1 mL/min; 1-min fractions were collected. (A) Cells were labeled for 6 h with [^{3}H]mannose. The peak eluting at 3–5 min is free mannose. (●) Extract incubated with antibody alone; (▲) extract incubated with antibody presaturated with calcitonin. (B) Cells were labeled for 3 h with [^{35}S]methionine. The peak eluting at 3–5 min is free methionine. Symbols are as given for panel A.

mannoside (Figure 1B). A much lower percentage of radioactive peptide was bound to the lectin if fractions from the major procalcitonin peak were tested. Complex oligosaccharide-containing species would not be detected by concanavalin A chromatography and, therefore, could be present in either peak of procalcitonin. However, these preliminary results suggested that an oligosaccharide was linked to the minor procalcitonin species and that posttranslational processing could yield a glycosylated calcitonin.

To test this hypothesis, an extract of CA-77 cells labeled for 6 h with [^{3}H]mannose was immunoprecipitated with calcitonin antibodies and fractionated by reversed-phase HPLC (Figure 2A). Radioactive peptides that eluted at fractions 48 and 66 were specifically immunoprecipitated since they were not detected if the antibodies were pretreated with synthetic human calcitonin. Chromatography of immunoprecipitates of [^{35}S]methionine-labeled cell extracts (Figure 2B) showed major calcitonin species eluting at fractions 54 and 68. Retention times are shorter than those observed in Figure 1A since a C_8 column, rather than the cyanopropyl column, was used. As noted above, the later-eluting peak has been identified as the form of procalcitonin that is the precursor for nonglycosylated calcitonin, the major product of the CA-77 cells (Birnbaum et al., 1984a,b, 1986). The peak that eluted at fraction 54 consists of the 6.5K biosynthetic intermediate and the 3.4K amidated calcitonin (Birnbaum et al., 1984b). The greater prominence of this peak, relative to the amount seen in the chromatogram in Figure 1A, is due to calcitonin

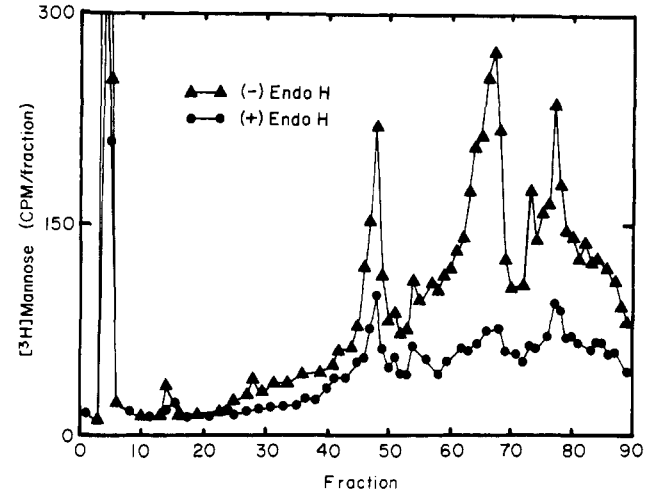

FIGURE 3: Reversed-phase HPLC profile of immunoprecipitated calcitonin from an extract of CA-77 cells labeled with [^{3}H]mannose for 3 h and treated with Endo H. (▲) Cell extract incubated in 50 mM sodium acetate, pH 5.5, at 37 °C for 20 h and then immunoprecipitated; (●) cell extract incubated with 20 milliunits of Endo H for 20 h and then immunoprecipitated.

accumulating during the longer (3-h) labeling time. Incubation for this period also revealed the presence of a minor calcitonin species at fraction 48, as well as a minor peak at fraction 65, which is presumably a form of procalcitonin. These results suggest that the immunoprecipitable species labeling with [^{3}H]mannose are glycosylated forms of calcitonin.

Aliquots of [^{3}H]mannose-labeled cell extracts were treated with Endo H, an enzyme that cleaves high-mannose asparagine-linked oligosaccharides between the two inner *N*-acetylglucosamine residues (Tai et al., 1977). The Endo H treated extract, along with the untreated extract, was then immunoprecipitated with calcitonin antibodies and fractionated by HPLC (Figure 3). The enzyme treatment removed most of the radioactivity from the peaks at fractions 48 and 66, as expected. The reason for incomplete removal of radioactivity from the peak at fraction 48 is under investigation, although sufficient Endo H was added to the cell extract to completely remove susceptible oligosaccharides (see Experimental Procedures). These results also demonstrated that the radioactivity was still present in sugar and not in amino acids. Even though the preparation of Endo H used for the digestion is reported to be protease free by the manufacturer, we also tested whether nonglycosylated forms of calcitonin were altered. Enzyme treatment of a [^{35}S]methionine-labeled cell extract followed by immunoprecipitation and HPLC revealed that Endo H did not substantially reduce the amount of radioactivity in nonglycosylated calcitonin and procalcitonin (data not shown). This result indicates that the loss of sugar residues resulted from specific cleavage of the oligosaccharide by Endo H rather than by digestion of the calcitonin peptide by proteases.

The molecular weights of the glycosylated forms of calcitonin were estimated by chromatography of peptides on a column of Sephadex G-75 (Superfine) in 6 M guanidine hydrochloride containing 0.02% bovine serum albumin. When the [^{3}H]mannose-labeled material that eluted from the HPLC at fraction 66 was applied to the gel filtration column, it eluted with a K_d of 0.18 (Figure 4), equivalent to M_r 14400. While this is slightly higher than the estimated molecular weight of 13400 reported for procalcitonin (Birnbaum et al., 1984a), it is not significantly different. Both the [^{35}S]methionine- and [^{3}H]mannose-labeled material which eluted at fraction 48

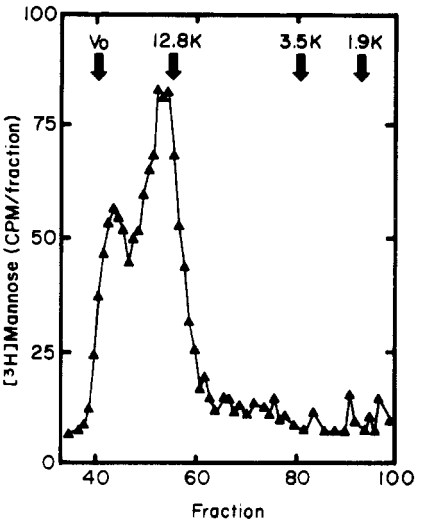

FIGURE 4: Gel filtration chromatography of a putative radiolabeled glycosylated procalcitonin. An extract of cells labeled for 3 h with [^{3}H]mannose was treated with calcitonin antibodies and fractionated by reversed-phase HPLC. Fractions 65–69 were pooled and dried. The residue was dissolved in 6 M guanidine hydrochloride containing 0.02% bovine serum albumin and chromatographed on a column of Sephadex G-75 (Superfine). 300-μL fractions were collected. 12.8K, for example, is a molecular weight of 12 800.

chromatographed with a K_d of 0.43 (Figure 5A,B), corresponding to M_r 5500. Alternatively, direct application of an immunoprecipitate of a [^{3}H]mannose-labeled cell extract to the gel filtration column resulted in peaks with K_d's of 0.43 and 0.18, as well as a large peak of nonspecifically precipitated protein eluting at the void volume (data not shown). The 5.5K species is approximately 2000 daltons larger than calcitonin, which would be consistent with the size of the hormone with an asparagine-linked oligosaccharide.

To provide additional evidence that this species was glycosylated calcitonin, the [^{35}S]methionine- and [^{3}H]mannose-labeled peptides that eluted from the HPLC at 48 min were further purified by gel filtration chromatography and then were microsequenced by automated Edman degradation. ^{35}S was found in cycle 8 (Figure 6A), while ^{3}H was found in cycle 3 (Figure 6B). As shown in Figure 7, cycle 8 corresponds to methionine at residue 8 of the calcitonin sequence, and cycle 3 corresponds to the oligosaccharide linked to the asparagine at residue 3.

Discussion

These studies have demonstrated the biosynthesis of glycosylated calcitonin and its precursor, glycosylated procalcitonin, in CA-77 cells. The oligosaccharide-containing calcitonin was identified from the incorporation of [^{3}H]-mannose into an immunoprecipitable peptide with an estimated molecular weight of 5500. The mannose was found at residue 3, an asparagine residue in the N-linked glycosylation signal Asn-Leu-Ser (Figure 7). Glycosylated procalcitonin was identified on the basis of concanavalin A affinity and incorporation of [^{3}H]mannose into an immunoprecipitable, 14 400-dalton calcitonin species. This peptide had previously been identified as a form of procalcitonin by in vitro translation of CA-77 mRNA in the presence of microsomal membranes (Birnbaum et al., 1984a). While we have not identified a glycosylated biosynthetic intermediate nor presented pulse–chase kinetic experiments that define the precursor–product relationships, these data together with the results presented by Birnbaum et al. (1986) strongly suggest that the biosynthesis of an oligosaccharide-containing calcitonin involves the same proteolytic cleavages as the major nonglycosylated pathway.

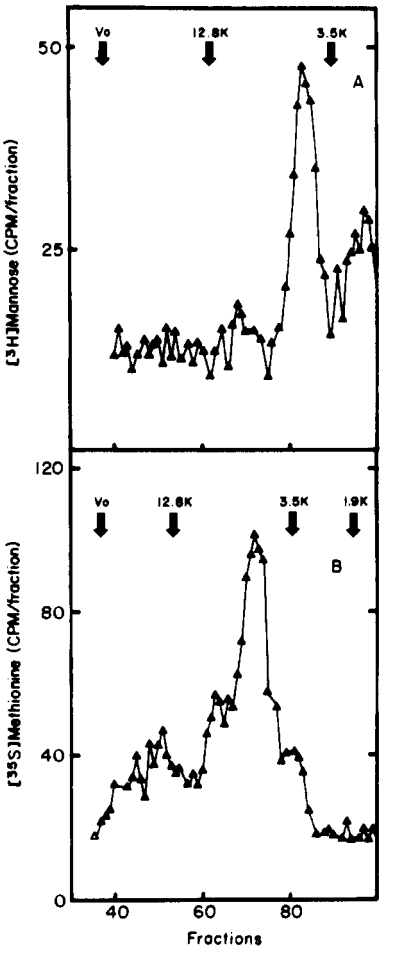

FIGURE 5: Gel filtration chromatography of putative radiolabeled glycosylated calcitonin. An extract of cells labeled for 3 h with either [^{3}H]mannose or [^{35}S]methionine was treated with calcitonin antibodies and fractionated by reversed-phase HPLC. Various fractions were pooled, dried, and chromatographed as described in Figure 4. (A) HPLC fractions 48 and 49 of a [^{35}S]methionine-labeled immunoprecipitate. (B) HPLC fractions 47–49 of a [^{3}H]mannose-labeled immunoprecipitate. 300-μL fractions were collected.

Endo H digestion of the [^{3}H]mannose-containing species completely removed the radioactivity from glycosylated procalcitonin, but only partially from glycosylated calcitonin. The enzyme requires the presence of at least four mannose residues linked to di-*N*-acetylchitobiose for full activity (Tai et al., 1977). The procalcitonin probably contains a core oligosaccharide (Waechter & Lennarz, 1976; Wagh & Bahl, 1981) that is further processed to a more complex unit, possibly less susceptible to Endo H cleavage. Further studies are necessary to confirm this hypothesis as well as to establish the time frame in which carbohydrate and protein processing occurs.

The unequivocal demonstration of the biosynthesis of glycosylated forms of calcitonin resolves conflicting reports in the literature. There have been both positive and negative reports of immunoreactive forms of calcitonin binding specifically to lectins (Goltzman & Tischler, 1978; Dermody et al., 1981; O'Neil et al., 1981; Baylin et al., 1983). Using the rMTC 6-23 cell line, Jacobs et al. (1985) have demonstrated incorporation of mannose into protein specifically immunoprecipitated with calcitonin antiserum. The immunoprecipitate was not fractionated, so it is unclear what forms of calcitonin were glycosylated. These authors have also shown, using an in vitro system, that synthetic calcitonin can act as a substrate for the dolichol phosphate oligotransferase present in microsomal

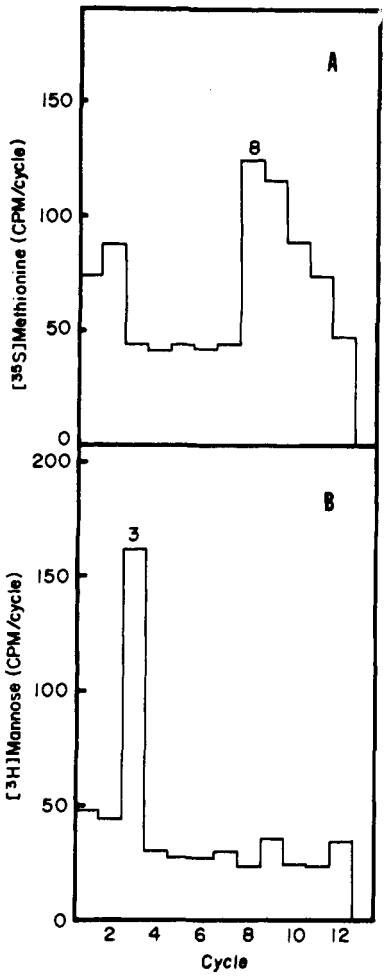

FIGURE 6: Microsequence analysis of putative glycosylated calcitonin radiolabeled with either (A) [^{35}S]methionine or (B) [^{3}H]mannose. The radiolabeled cell extracts were immunoprecipitated with calcitonin antibodies and fractionated by reversed-phase HPLC and gel filtration chromatography on Sephadex G-75 (Superfine). For the [^{35}S]-methionine-labeled sample, the peak with K_d = 0.43 was reapplied to a reversed-phase HPLC column, and the 48-min peak was microsequenced. The [^{3}H]mannose-labeled peptide was analyzed following gel filtration chromatography.

membranes. However, the 111 amino acid procalcitonin is the usual species glycosylated, since oligosaccharides are added shortly after translation (Hughes, 1983).

A structural feature common to all the calcitonins sequenced to date (Guttman, 1981) except the chicken I species (Homma et al., 1985) is the presence of the N-glycosylation signal Asn-Leu-Ser within the ring structure formed by the disulfide bond between Cys-1 and Cys-7 (Figure 7). An intact ring is essential for bioactivity (Guttman, 1981; Rittel et al., 1976). Like calcitonin, ACTH had been thought to be a non-glycosylated hormone, but it is now apparent that a significant proportion of murine ACTH has an oligosaccharide linked to an asparagine residue in the COOH-terminal region of the molecule (Eipper et al., 1976; Mains & Eipper, 1976), an area that is not essential for biological activity (Schwyzer et al., 1971; Seelig, 1971). Several years after this initial identification by pulse-labeling murine pituitary tumor cells (Eipper et al., 1976; Mains & Eipper, 1976), it was shown, not surprisingly, that glycosylated ACTH and nonglycosylated ACTH were equivalent in bioassays (Gasson, 1979, 1980). Therefore, calcitonin and its counterpart, glycosylated at a site within the biologically important ring, could provide a better model to investigate the role of oligosaccharide side-chain modifications.

The position of the oligosaccharide at Asn-3, close to a

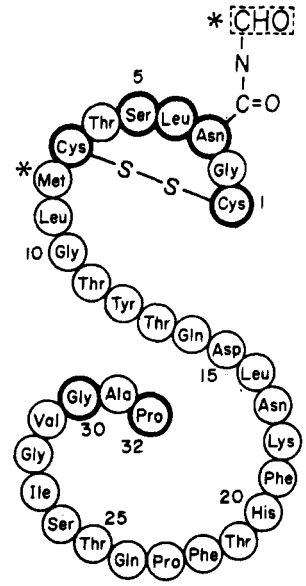

FIGURE 7: Sequence of glycosylated rat calcitonin. The asterisks indicate the sequencer cycles in which either [^{3}H]mannose or [^{35}S]methionine was found (Figure 6). *CHO*, oligosaccharide side chain. Note the N-linked glycosylation at residues 3–5, Asn-Leu-Ser.

proteolytic cleavage site, may also affect the processing of glycosylated procalcitonin. While the existence of glycosylated calcitonin suggests that the precursor can be processed, we have not presented any data concerning the relative rates for the glycosylated and nonglycosylated species. Our ability to resolve these species by reversed-phase HPLC should enable us to investigate the effect of glycosylation on processing without drugs such as tunicamycin, which has been extensively used for such studies (Waechter & Scher, 1979; Struck & Lennarz, 1980).

We have found that 5–10% of the total procalcitonin binds to concanavalin A. Processing of the glycosylated precursor constitutes a minor but significant biosynthetic pathway. We have detected glycosylated calcitonin in tissue culture medium, which indicates that the peptide is a secretory product of these cells (unpublished observations). While we believe that the CA-77 cell line mimics normal rat calcitonin producing cells, the percentage of oligosaccharide-containing forms of calcitonin could be different in various species, as for glycosylated ACTH which represents less than 1% of the total peptide in bovine pituitary but approximately 50% in murine pituitary (Eipper et al., 1976). The identification of glycosylated calcitonin in the CA-77 cell line should provide the impetus to identify these forms in normal and neoplastic tissues of other mammalian and nonmammalian species and evaluate their functions.

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Allosteric Sensitivity in Hemoglobin at the α-Subunit N-Terminus Studied by Hydrogen Exchange†

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ABSTRACT: Allosteric structure change in human hemoglobin was studied by hydrogen–tritium-exchange methods. The functional labeling method used takes advantage of the change in H-exchange rate at allosterically involved sites to selectively label, with tritium, H-exchange sites that are fast in one protein state and slow in another. The position of the labeled sites can then be located by the medium-resolution fragmentation–separation method. These methods reveal 5 allosterically sensitive, H-bonded, peptide NH's within the first 12 residues of the α chain. All five exchange with solvent protons at similar rates in deoxyhemoglobin (T form), and all shift to a new rate, about 30-fold faster, in the liganded protein (R) form. This indicates a decrease in structural stability at the α-chain N-terminus in going from the T to the R form, consistent with the loss of stabilizing interactions in that segment. The results indicate a loss of perhaps 2 kcal/mol in stabilization free energy and thus document a significant role for changes at the α-chain N-terminus in the allosteric transition.

Since the early work of Wyman and others on linked functions (Wyman, 1964, 1968; Monod et al., 1965; Koshland et al., 1966) and its widespread application to hemoglobin and other systems, the fundamental role of protein structure change in the regulation of protein function has become abundantly clear. Also fundamental in the linked function analysis is the issue of change in protein structural energy. In order to *understand* the regulation of function in any protein molecule, it will be necessary to detect the individual structure changes that contribute to the overall allosteric transition and to measure the contribution of each component change in terms of free energy.

At present, the molecular structures of about 200 protein molecules are known to atomic detail, and a large number of regulatory proteins that utilize the principles of structure change have been identified. Yet only in hemoglobin can it be said that the probable component changes are known in

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