THE INTRACELLULAR LOCATION OF THE GLYCOSYLATION OF HYDROXYLYSINE OF COLLAGEN*

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SUMMARY: The subcellular location at which hydroxylysine residues of collagen are glycosylated was studied in chick embryo fibroblasts. Ribosomes were isolated from ^{14}C-lysine pulse-labeled cells in tissue culture. Alkaline hydrolysis followed by amino acid analysis and scintillation counting of the effluent showed that glucosylgalactosyl hydroxylysine and galactosyl hydroxylysine as well as hydroxylysine and lysine were the major ^{14}C-labeled components. Acid hydrolysis destroyed the glycoconjugates and yielded only free ^{14}C-hydroxylysine and ^{14}C-lysine. These data indicate that glycosylation of peptide-bound hydroxylysine is initiated while the polypeptide chain is still in the stages of assembly on the ribosome.

Several of the hydroxylysine residues of collagen are known to be glycosidically linked to either a galactose or glucosylgalactose moiety. Both the relative amounts of mono- and disaccharide units and total extent of glycosylation vary depending on the origin of the tissues (1). The subcellular site at which the hydroxylysine of collagen is glycosylated is unknown. Early studies of other secreted glycoproteins suggest a multisite hypothesis (2). That is, glycosyl residues structurally close to the polypeptide chain are attached while the peptide chain is still in the process of synthesis on the ribosome. Residues farther from the peptide backbone are attached after the completed protein is released from the ribosome and as the protein traverses the lumen of the endoplasmic reticulum and the Golgi apparatus (1, 2, 3). Since collagen is a secreted protein whose glycoconjugates are very short and linked directly to the peptide backbone it seemed likely that, in this case too, the carbohydrate might be attached at the ribosomal stage of chain assembly. In this report we present evidence that ribosome bound nascent peptide chains of collagen are indeed glycosylated before release.

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EXPERIMENTAL METHODS

Tissue Culture: Nine-day-old chick embryos were eviserated and decapitated. The remaining tissues were treated, using standard enzymic digestion techniques, with 0.25% trypsin and 0.2% collagenase to release fibroblasts (4). These cells were plated at high density (2-4 X 10^6 cells/ml in a final volume of 15 ml) in Medium 199 with 10% fetal calf serum and 50 µg/ml Gentamicin. All operations were carried out using sterile technique in a laminar flow hood. The cells were incubated at 37°C in 5% CO_2 - 95% air and grew to confluency within one day.

After growing for one day, the spent medium was pipetted off and fresh medium containing in addition 50 µg/ml of sodium ascorbate was added.

Labeling of Cells and Polysome Preparation: TKM buffer (0.02 M Tris-HCl, pH 7.4; 0.24 M KCl; 0.0075 M $MgCl_2 \cdot 6H_2O$) was shaken with Macaloid to reduce ribonuclease activity. This buffer was used in preparing sucrose gradients; in preparing the washing buffer which also contained 0.12 M sucrose and 0.2% soybean trypsin inhibitor; and in preparing the lysing buffer which contained only the 0.12 M sucrose without the inhibitor. Detergents were prepared in a 10 X concentration in water the day before use. Labeling medium consisted of Hank's balanced salt solution to which had been added non-essential amino acids, vitamins, sodium ascorbate (50 µg/ml), and essential amino acids minus lysine. ^{14}C-lysine (125 µC) was added directly to this lysine-free medium. The cells were labeled in large Falcon flasks, 5 ml of medium/flask.

Labeling of cells was done after the cells had been in culture for two days. The spent tissue culture medium was decanted and the cells washed three times with Puck's saline at 37°C. Labeling medium containing no radioactivity was equilibrated with the cells at 37°C for 15 minutes. This was then removed and the radioactive labeling medium added. The cells were incubated at 37°C for 45 minutes. The medium was removed and modified phosphate buffered saline containing 10 µg/ml cycloheximide was added to

arrest protein synthesis. After a 10 minute incubation at 37°C, the solution was removed. The cells were dislodged by incubation at 37°C for five minutes with 0.25% trypsin and 10 μg/ml cycloheximide in modified phosphate buffered saline. Cell layers were freed by gently rocking the flasks. The cell layers were then pipetted into prechilled centrifuge tubes and spun at 1000 rpm in a Sorvall SS-34 rotor for 10 minutes at 4°C. Each supernatant was aspirated and the cell pellet resuspended in washing buffer. The cells were recentrifuged as before, the supernatant aspirated and the cells resuspended in lysing buffer. They were lysed by freezing the suspension in a dry ice/acetone bath and thawing in running tap water. This step was repeated once. The cell lysate was treated for five minutes at 4°C with detergents [final concentration sodium deoxycholate (1%) and Tween 40 (0.5%)]. The nuclei and mitochondria were pelleted by centrifugation at 11,000 rpm in the SS-34 for five minutes. An aliquot of the post-mitochondrial supernatant was treated with ribonuclease at 37°C for 10 minutes. The remainder was layered onto linear sucrose gradients (1.5 M to 0.5 M). The ribonuclease treated control was layered onto a separate gradient. The gradients were spun at 27,000 rpm in a SW 27 rotor for two hours in a Beckman L2-65B ultracentrifuge at 2-4°C. The gradient tubes were then punctured and the sucrose pumped from the bottom directly through a flow cell in a Gilford recording spectrophotometer. The absorbance at 260 nm was monitored and fractions of approximately 1 ml were collected.

Analysis: The combined ribosomal fractions were dialyzed against deionized water to eliminate sucrose and salts. They were then lyphilized in two equal aliquots. One aliquot was hydrolyzed in 2 N NaOH for 22 hours between 100-108°C. The other aliquot was hydrolyzed in 6 N HCl at the same conditions. After neutralization of the base hydrolysate, both were evaporated to dryness. Each aliquot was then charged to a JEOLCO amino acid analyzer. Using the stream splitting device, aliquots of approximately 1 ml were collected and 0.65 ml of each was counted in 10 ml Aquasol.

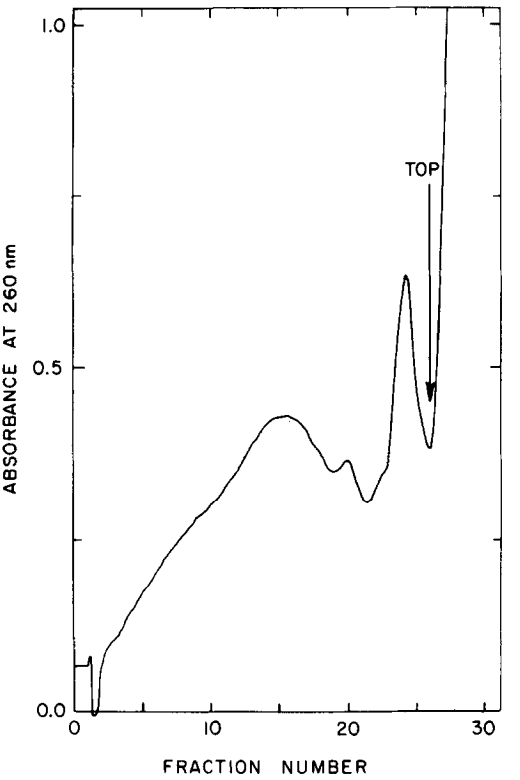

Figure 1 The polyribosomal profile obtained from chick embryo fibroblasts after 2 days in cell culture. The bottom of the gradient is 1.5 M sucrose while the top is 0.5 M sucrose prepared in TKM buffer. Fractions 1-25 were combined for analysis. Non-ribosome bound procollagen does not migrate into this gradient (unpublished results) and is found exclusively at the top.

RESULTS: Figure 1 shows the polyribosomal profile obtained from chick embryo fibroblasts after two days in tissue culture. Ribonuclease destroyed the absorbance in the polysome region and increased the absorbance at the monosome peak. Other experiments in our laboratory have shown that these polyribosomes contain nascent procollagen.

Alkaline hydrolysis of the ribosomal fractions yielded four major peaks of radioactivity when chromatographed, fractionated and counted as described above (Figure 2). The first peak eluted very early (~35 minutes) in the chromatogram and has been shown to be peptides which contain only ^{14}C-lysine and no ^{14}C-hydroxylysine (5). The second peak of radioactivity eluted be-

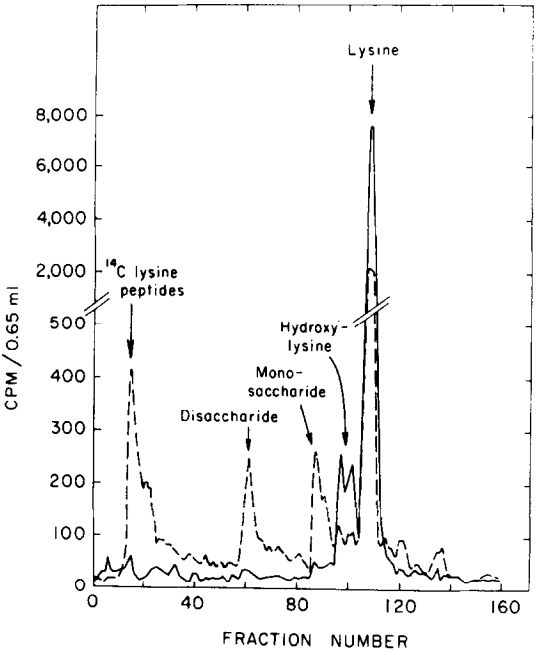

Figure 2 Recovery of ^{14}C-labeled components from the alkaline and acidic hydrolysates of the ribosomal fraction. Approximately the same number of cpm's were charged to the amino acid analyzer in both cases: alkaline hydrolysate, 104,000 cpm; acidic hydrolysate, 102,000 cpm. ----Alkaline hydrolysate; ——Acidic hydrolysate

tween 140-150 minutes and corresponded to the elution time of a standard of glucosylgalactosylhydroxylysine, while the third radioactive peak corresponded to the elution time of a standard of galactosylhydroxylsine (220-230 minutes). The final major peak of radioactivity was due to ^{14}C-lysine at 275-280 minutes.

Acid hydrolysis of these ribosomal fractions gave a somewhat different chromatogram. The initial peak of ^{14}C-lysine peptide radioactivity was diminished with a corresponding increase in radioactivity associated with ^{14}C-lysine. Significantly, the two peaks corresponding in elution time with the standard mono- and disaccharide-linked hydroxylysine were virtually abolished. A corresponding increase in the radioactivity associated with the elution of hydroxylysine (245-250 minutes) was seen (Table I).

TABLE I

RECOVERY OF ^{14}C-LYSINE, HYDROXYLYSINE AND HEXOSYLHYDROXYLYSINE FROM ACID AND ALAKALINE HYDROLYSIS OF ISOLATED POLYSOMES.

COMPONENT	ALKALINE HYDROLYSATE	ACID HYDROLYSATE
glucosylgalactosylhydroxylysine (t = ∿150 min)	DPM 1616	DPM 121
galactosylhydroxylysine (t = ∿230 min)	1743	212
hydroxylysine (t = ∿250 min)	1051	1730
lysine (t = ∿280 min)	10,000	27,000

*DPM integrated over peak.

The difference in dpm in lysine between the two hydrolysates is due to incomplete cleavage of lysine containing peptides in the alkaline hydrolysate. The peptides are totally degraded under acid hydrolysis conditions yielding more radioactivity in the lysine region. See text.

These data establish two important points. First, it is evident that the nascent collagen chains still bound to the ribosomes contain hexose bound hydroxylysine, demonstrating that some glycosylation does take place immediately upon hydroxylation of the lysine and before release of the nascent chains. Second, and surprisingly, the alkaline hydrolysis which produces the hexosyl bound hydroxylysine components yields less free hydroxylysine than hexosyl bound hydroxylysine. The chick embryo fibroblasts produce a collagen, in the final extracellular form, with less hexosyl bound hydroxylysine than free hydroxylysine. This observation suggests to us that those lysine residues destined to become glycosylated are preferentially hydroxylated while ribosome-bound. The remaining hydroxylysine residues are formed after release of the polypeptide. In a

more speculative vein we also suggest that separate enzymes may be involved in the ribosomal hydroxylation (i.e., that coupled with glycosylation) and in the hydroxylation taking place on the released chains in the cisternae of the endoplasmic reticulum.

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