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ADDITION OF MANNOSE TO BOTH THE AMINO- AND CARBOXY-TERMINAL PROPEPTIDES OF TYPE II PROCOLLAGEN OCCURS WITHOUT FORMATION OF A TRIPLE HELIX

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SUMMARY

Matrix-free cells obtained from chick embryo cartilage were incubated in the presence of α,α′-dipyridyl and radioactive mannose in order to examine the incorporation of mannose into the propeptide extensions of Type II procollagen. Cell proteins were digested with bacterial collagenase and the digests were examined by polyacrylamide gel electrophoresis. Radioactive mannose was found in fragments from both the N- and C-propeptides, and therefore the results provided the first indication that both these propeptides of Type II procollagen contain mannose. The results also supported previous indications that addition of carbohydrate to the propeptides of procollagen does not require folding of the collagen domain into a triple helix.

INTRODUCTION

Several studies have established that tendon and cartilagenous tissues contain genetically-distinct collagens, Type I and Type II, with the chain composition $[\alpha 1(I)]_2\alpha 2$ and $[\alpha(II)]_3$, respectively. Both of these collagens are first synthesized in a precursor form, called procollagen and they contain propeptide extensions at both the N- and C-terminals (1-7). The collagen domains of both Type I and Type II procollagens contain galactose and glucosylgalactose linked to hydroxylysine in O-glycosidic linkage (8,9), but much less is known about the sugar content of the propeptide extensions (10-15). Recent studies have indicated the presence of mannose, N-acetylglucosamine, and other sugars on the C-propeptide of Type I procollagen (12,14,15). In addition it has been suggested that the N-propeptide of Type I procollagen contains glucosamine but not mannose or glucose (15). The presence of sugar

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in the Type I N-propeptide, however, has not been thoroughly investigated. In the case of Type II procollagen, one report indicates that a collagenase-resistant peptide contains glucose, galactose and mannose, but it is not clear whether this peptide was N- or C-terminal (16).

We here report that mannose is present in both the N- and C-terminal propeptides of Type II procollagen and that addition of mannose to the propeptides occurs under conditions in which folding of the collagen domain into a triple helical conformation is prevented.

MATERIALS AND METHODS

Materials. L-[^{14}C(U)]Proline (262 mCi/mmole) was obtained from New England Nuclear, Boston Massachusetts. D-[1-^{3}H(N)]Mannose (13.4 mCi/μmole) and D-[2-^{3}H]mannose (2 mCi/μmole) were purchased from New England Nuclear, Boston, Massachusetts and Amersham/Searle Corp., Arlington Heights, Illinois, respectively. L-Ascorbic acid (sodium salt), pyruvic acid (sodium salt), Coomassie brilliant blue R, and α,α′-dipyridyl were obtained from Sigma Chemical Co., St. Louis, Missouri. The protease inhibitors, N-ethylmaleimide, phenylmethylsulfonylfluoride, p-aminobenzamidine-HCl, and ethylenediaminetetraacetic acid were also from Sigma. ABC Form III bacterial collagenase was purchased from Advance Biofacture Corp., Lynbrook, New York. SDS was obtained from Bio-Rad Laboratories, Richmond, California. Me_2SO was purchased from Mallinckrodt, Inc., St. Louis, Missouri and PPO was obtained from J.T. Baker Chemical Co., Phillipsburgh, New Jersey.

Preparation and Extraction of Cells Labeled with Mannose and Proline. Matrix-free cells were obtained from 17-day-old chick embryo sterna after proteolytic digestion as described elsewhere (17). Cells at a density of 10 x 10^6 cells/ml were incubated with either 600 μCi of [^{3}H]mannose or 25-50 μCi of [^{14}C]proline in 15 ml of modified Krebs glucose-free medium supplemented with 5 mM sodium pyruvate (12) and containing 10 μg/ml of sodium ascorbate. Incubations were carried out for 4 hr in the absence or presence of 0.3 mM α,α′-dipyridyl, which was dissolved in a small volume of absolute ethanol before being added to the medium. The media were separated from the cells by centrifugation (1,200 x g for 10 min) at room temperature. For comparison, identical experiments were carried out with matrix-free tendon cells obtained as described previously (18).

Cell pellets were suspended in 2 ml of boiling SDS[1/], if electrophoresis was carried out, or 2 ml of boiling deionized H_2O, if collagenase digestion was carried out. The suspensions were homogenized in a Teflon-glass homogenizer with 5 strokes up and down by hand. The homogenates were dialyzed against the following "sample buffer": 0.125 M Tris-HCl, pH 6.8, containing 2% SDS, 10% glycerol, and 0.001% bromophenol blue (19). The samples were then analyzed on 6% slab gel electrophoresis, or dialyzed against 0.4 M sodium chloride in 0.1 M Tris-HCl, pH 7.5 (NaCl-Tris) and digested with bacterial collagenase (see below). Aliquots of all samples were counted to measure total radioactivity.

[1/] Abbreviations employed here: SDS, sodium dodecyl sulfate; Tris, tris (hydroxymethyl)aminomethane; PPO, 2,5-diphenyloxazole; cpm, counts per minute.

Cell media were treated with protease inhibitors (19-21), adjusted to 40% ammonium sulfate and allowed to stand overnight at 4°C. The precipitates were suspended in 2 ml of boiling SDS, dialyzed against sample buffer and aliquots were analyzed on 6% slab gel electrophoresis (see below).

Treatment of Cell Extracts with Bacterial Collagenase. Collagenase digestion of the samples was carried out by a modification of previous methods (22,23) as described elsewhere (19). Fifty units per ml of ABC Form III bacterial collagenase and ^{3}H- or ^{14}C-labeled protein from the cell pellets were incubated in 4 mM $CaCl_2$, 2 mM N-ethylmaleimide and 0.4 M sodium chloride in 0.1 M Tris-HCl buffer, pH 7.5. Collagenase was omitted in the control samples. After the digestion for 3 hr at 37°C, the incubations were clarified by centrifugation for 10 min at 20,000 x g. The resulting supernatants were adjusted to 2% SDS, boiled for 5 min and dialyzed against sample buffer. Control and collagenase-treated samples were then analyzed on 15% slab gel electrophoresis to examine the labeling patterns of the collagenase-resistant peptides of procollagen.

Analysis of Cells and Media by Electrophoresis. The samples prepared as described above were analyzed by slab gel electrophoresis in SDS carried out according to a modification of the method of King and Laemmli (24). The running buffer consisted of 0.050 M Tris, 0.384 M glycine, and 0.1% SDS (pH 8.8). Before being applied to the gels, aliquots of about 100,000 cpm of ^{3}H-labeled proteins and about 20,000 cpm of ^{14}C-labeled proteins were then reduced by adding 2-mercaptoethanol to a final concentration of 5% and boiling for 3 min. The slab gels were 1.5 mm thick, 13 cm wide and 17 cm long. The separating gel was either 6% or 15% polyacrylamide and the stacking gel was 4.5% polyacrylamide. The electrophoresis was carried out with 40 mA until the dye reached the separating gel and then 70 mA until the dye migrated about 12 cm. The gels were rinsed for 10 min in deionized H_2O and stained by incubation for 1 hr in a solution containing 0.25% Coomassie brilliant blue R in 20% trichloroacetic acid. The gels were then rinsed in deionized H_2O for 20 min, destained in 7.5% acetic acid and 15% methanol, and photographed with a green filter (x 1, Nikon). After this procedure, the slab gels were permeated with Me_2SO and PPO for fluorescent autoradiography as described by Bonner and Laskey (25). The gels were then dried under vacuum, and exposed to RP Royal "X-OMAT" X-ray film which had been "preflashed" to make its fluorographic response linear (26). For quantitation the gels were scanned in a Joyce-Loebl 3CS microdensitometer. Molecular weight standards were run in both 6% and 15% gels.

RESULTS

Incorporation of Mannose into the Propeptides of Type I and Type II Procollagen. Freshly-isolated cartilage cells from chick embryos were incubated with either [^{3}H]mannose or [^{14}C]proline in the absence or the presence of 0.3 mM α,α′-dipyridyl. This iron chelator prevents hydroxylation of peptidyl proline and therefore prevents procollagen polypeptides from forming a stable triple helix. It has also been shown that the rate of procollagen secretion is significantly retarded when triple helix is not

formed, causing the retention of the procollagen chains within the cells (for review see ref. 2). This effect is apparent from the data in Table I, showing the intracellular incorporation of mannose and proline in cartilage and tendon cells. The data also shows that the ratio of mannose/proline incorporated is essentially the same in control and treated cells. As indicated in Table I, similar results were also obtained with tendon cells from chick embryos synthesizing Type I procollagen.

Analysis of the cell extracts and media on slab gel electrophoresis under conditions in which the intact procollagen chains were observed showed that [^{3}H]mannose was incorporated into the proα chains of both types of collagen whether or not α,α′-dipyridyl was present during the incubation of the cells (not shown).

To determine whether mannose was incorporated into the propeptides of the procollagen chains, cells were incubated in the presence of α,α′-dipyridyl as described above and cell homogenates were digested with bacterial collagenase prior to examining the samples by polyacrylamide gel electrophoresis. The migration positions of the collagenase-resistant fragments from the N- and C-propeptides of Type I and Type II procollagens were identified by molecular weight standards and on the basis of previous studies with these propeptides (see ref. 6,7,19,27). In confirmation of previous observations (12,14,15,28), [^{3}H]mannose was incorporated (Fig. 1A) into the two carboxyterminal extensions of Type I procollagen (col C1 and col C2). Little, if any, [^{3}H]mannose was incorporated in the N-propeptide of the proα1(I) chain. In contrast, [^{3}H]mannose was incorporated into both the C-propeptide [col C(II)red] and N-propeptide [col N(II)] of Type II procollagen (Fig. 1B). Similar results were obtained when the cells were incubated without α,α′-dipyridyl (not shown). The small amount of [^{3}H]-mannose migrating with col N(II) in the control sample not digested with collagenase probably reflects a small amount of cleavage of this propeptide during processing of the cell homogenates (see ref. 2).

Table I. Incorporation of [^{3}H]Mannose and [^{14}C]Proline into Freshly-Isolated Tendon and Cartilage Cells.

Cells	Radioactive Precursor	Non-dialyzable ^{3}H or ^{14}C	
		Minus α,α'-dipyridyl	Plus α,α'-dipyridyl
		Total cpm x 10^{-6}	
Cartilage	[^{3}H]Mannose	1.23	2.42
	[^{14}C]Proline	4.03	7.09
	Ratio $\frac{[^{3}H]Mannose}{[^{14}C]Proline}$	0.31	0.34
Tendon	[^{3}H]Mannose	2.18	3.63
	[^{14}C]Proline	9.76	21.30
	Ratio $\frac{[^{3}H]Mannose}{[^{14}C]Proline}$	0.22	0.17

DISCUSSION

The elucidation of the role(s) of the propeptides on the procollagen molecule depends, in part, upon chemical characterization of the entire precursor protein. The propeptides account for one third of the bulk of the procollagen molecule and several functions have been suggested for them (1-4). The suggested functions include prevention of premature fiber formation, initiation of folding of the proα chains into a triple helix, and feedback regulation of the amount of procollagen synthesized by cells. The possible role of the carbohydrate moities in the propeptide extensions remains entirely speculative at this time. Two studies with tunicamycin (13,29) indicated that the carbohydrates in the propeptides are not essen-

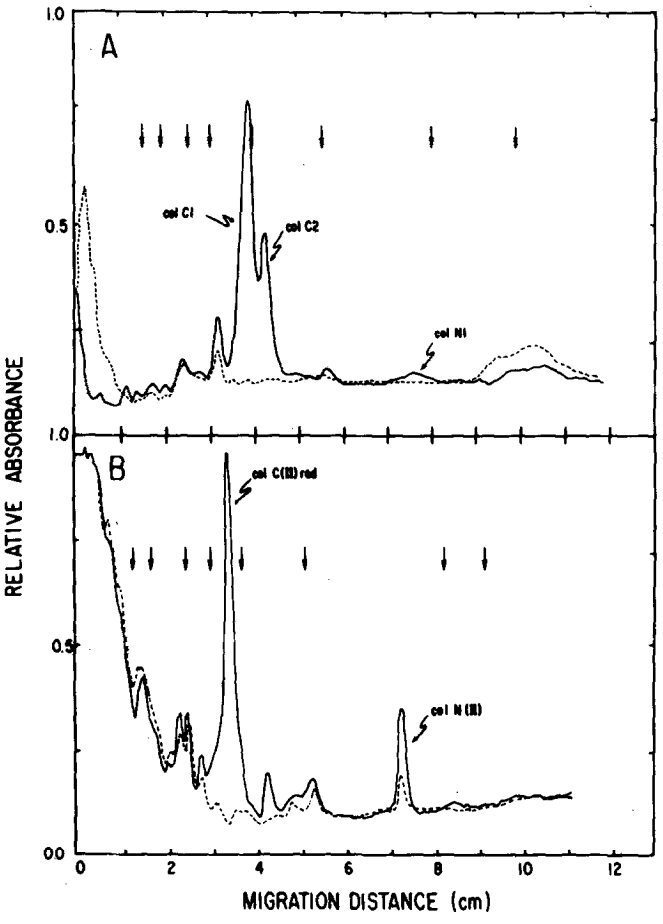

Figure 1. Analysis of [^{3}H]Mannose-labeled, Collagenase-Resistant Peptides on Polyacrylamide Gel Electrophoresis. Matrix-free tendon (Panel A) or cartilage cells (Panel B), at a density of 10 x 10^6 cells/ml, were incubated for 4 hr at 37°C in 15 ml of modified Krebs medium containing 600 µCi of [^{3}H]-mannose and 0.3 mM α,α′-dipyridyl. Cell extracts were analyzed on 15% slab gel electrophoresis as described in Materials and Methods. The arrows indicate molecular weight markers of 68000, 58000, 43000, 36000, 25700, 17200, and 11700 daltons. Solid lines represent control samples without collagenase.

tial for secretion of the procollagen to the extracellular milieu, even though current evidence indicates that glycosylation is required for the secretion of many, but not all, glycoproteins (29-32).

The results of the present investigation provide the first indication that radioactive mannose is incorporated into both the amino- and carboxy-terminal extensions of Type II procollagen. The addition of mannose to the procollagen molecule occurs under the conditions in which triple helix for-

mation is prevented. This strongly suggests that the addition of mannose to the collagenase-resistant peptides occurs in the cisternae of the rough endoplasmic reticulum, since previous studies have demonstrated that helix formation occurs either in this compartment or soon after the protein leaves this compartment (33). These studies are in agreement with recent reports obtained with Type I procollagen from chick embryo tendon cells (15,28).

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