EVIDENCE FOR THE TRANSFER OF MANNOSE TO THE EXTENSION PEPTIDES OF PROCOLLAGEN WITHIN THE CISTERNAE OF THE ROUGH ENDOPLASMIC RETICULUM

Henrik ANTTINEN, Aarne OIKARINEN, Lasse RYHÄNEN and Kari I. KIVIRIKKO
Department of Medical Biochemistry, University of Oulu, Kajaanintie 52 A, SF-90220 Oulu 22, Finland

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1. Introduction

Collagens from various sources contain galactose and glucosylgalactose in *O*-glycosidic linkage to hydroxylysyl residues (reviewed [1–3]). These are the only sugars in mammalian collagens, but the biosynthetic precursor of collagen, procollagen, contains additional carbohydrate units in its amino-terminal and carboxy-terminal peptide extensions [4–9]. These propeptides do not contain the hydroxylysyl-linked carbohydrate units [1–3]. The propeptides can be labelled by incubation with [2-^{3}H]mannose [5,7,8], most of the label being located in the carboxy-terminal peptide [8]. Direct carbohydrate analyses indicate the presence in the carboxy-terminal propeptide of chick embryo tendon procollagen of 2 residues of *N*-acetylglucosamine and 9–13 residues of mannose per peptide [9].

The hydroxylysyl-linked carbohydrate units are obviously synthesized within the cisternae of the rough endoplasmic reticulum [1,11–16]. Nothing is known, however, about the intracellular site of the glycosylation of the propeptides. This site was investigated in the present work by inhibiting the movement of procollagen from the cisternae of the rough endoplasmic reticulum into the Golgi vacuoles and by determining the effect of this inhibition on the extent of mannosylation of the intracellular procollagen. There should be no decrease in the extent of this reaction if the mannose transfer can occur within the cisternae, whereas a distinct decrease should be observed if the mannose transfer occurs within the Golgi vacuoles. The movement of procollagen was inhibited by preventing the triple helix formation, as it has been demonstrated that under such conditions the procollagen accumulates within the cisternae [17,18], and the fraction of the intracellular procollagen within the Golgi vacuoles decreases to about 1/10 [18]. No reduction was found in the extent of mannosylation of the pro-α chains, and our results thus strongly suggest that the glycosylation of the propeptides can occur within the rough endoplasmic reticulum.

Address correspondence to: Professor Kari I. Kivirikko

2. Materials and methods

Matrix-free cells were isolated from leg tendons of day 17 chick embryos by controlled digestion with trypsin and purified bacterial collagenase [19,20]. The cells were filtered through lens paper and washed 3 times with 25 ml a modified Krebs medium [19] containing 20% (w/v) foetal calf serum. They were washed once more and resuspended in a modified Krebs medium (termed the incubation medium) which contained 2% (w/v) foetal calf serum and in which the glucose had been replaced by 5 mM sodium pyruvate [7]. The cells were incubated at 37°C with moderate shaking, and L-[U-^{14}C]proline (270 Ci/mol), D-[1-^{14}C]mannose (59 Ci/mol) or D-[2-^{3}H]mannose (2 Ci/mol) (all from Radiochemical Centre, Amersham) were added as indicated in the legends to fig.1, 2 and table 1. The incubations were terminated by adding 0.3 vol. incubation medium to give a final concentration of 25 mM of ethylenediamine tetra-acetic acid, 10 mM *N*-ethylmaleimide, 1.5 g/l D-mannose and

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0.8 g/l L-proline. Propanol, 0.01 vol., containing phenylmethylsulphonylfluoride were then added to give final conc. 1 mM. The cells were separated from the medium by centrifugation at 1200 × *g* and the cell pellet washed with 1.5 ml incubation medium. After centrifugation, the cell pellets were again suspended in the incubation medium, heated to 100°C for 6 min and rapidly cooled to 0°C. The samples were dialyzed exhaustively against distilled water at 4°C and then dissolved in 0.5 ml or 1.0 ml a 'sample buffer' consisting of 0.125 M Tris–HCl, pH 6.8, at 22°C, 2% SDS, 10% glycerol and 0.001% bromophenol blue [21]. They were reduced with 5% β-mercaptoethanol for 3 min at 100°C, and analyzed by SDS–polyacrylamide slab-gel electrophoresis using 4% or 6% (w/v) acrylamide [21]. Radioactive spots were visualized by fluorography [22].

In some experiments the procollagen secreted into the medium was partially purified by ammonium sulphate precipitation [23] and a part of this preparation was subjected to limited pepsin digestion at 4°C for 15 h and at 15°C for 6 h [23], and another part was digested with purified bacterial collagenase [24]. The preparations were dialyzed against the sample buffer, reduced as above and analyzed by SDS–polyacrylamide slab-gel electrophoresis [21] using a concave acrylamide gradient from 4–20% (w/v).

3. Results

In order to ensure that the mannose label was actually in the peptide extensions of the procollagen, the tendon cells were incubated either with [^{14}C]proline or [2-^{3}H]mannose and the procollagen secreted into the medium was partially purified by ammonium sulphate precipitation. When these samples were analyzed by SDS–polyacrylamide slab-gel electrophoresis, only 2 major bands were seen in both preparations, their mobilities corresponding to those of the pro-α1 and pro-α2 chains (fig.1). Some of preparations were subjected to limited pepsin digestion to remove the propeptides, and in these samples 2 major bands were found with the [^{14}C]proline in the positions of the α1 and α2 chains. By contrast, only 1 band was seen in the dye front with [2-^{3}H]mannose, indicating that none of it was located in the helical portions of the α chains. After digestion with highly purified

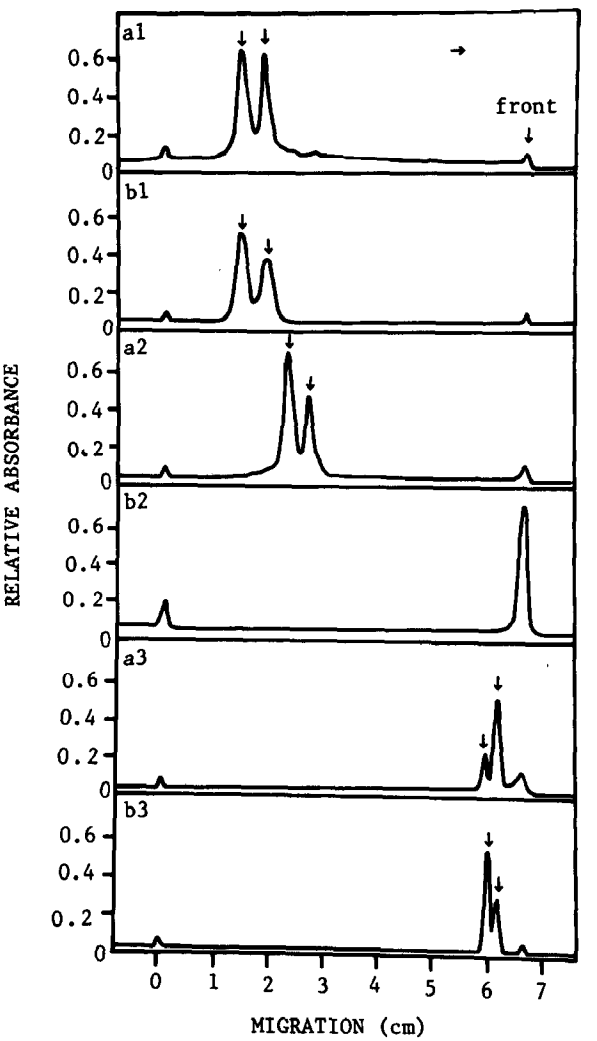

Fig.1. Densitometric tracings of fluorograms obtained after fractionation by SDS–polyacrylamide slab-gel electrophoresis of procollagen and its digestion products after treatment by pepsin or bacterial collagenase. Matrix-free chick embryo tendon cells were incubated with 30 μCi L-[U-^{14}C]proline (7×10^7 cells in 4 ml) or 1.4 mCi D-[2-^{3}H]mannose (6×10^8 cells in 20 ml) for 2 h, and the procollagen secreted into the medium partially purified by ammonium sulphate precipitation. Samples of approx. 15 000 dpm ^{14}C or approx. 100 000 dpm ^{3}H were applied to the gel and the electrophoresis was carried out on a concave gradient gel from 4–20% (w/v) acrylamide. (a) L-[U-^{14}C]Proline-labelled procollagen preparation; (b) D-[2-^{3}H]mannose-labelled procollagen preparation. (1) No treatment; (2) pepsin treatment; (3) collagenase treatment.

bacterial collagenase, 2 bands close to the dye front were present in both preparations (fig.1). Similar experiments were also performed with [^{14}C]mannose (not shown). Two bands were again seen in the positions of the pro-α1 and pro-α2 chains, but after limited pepsin digestion part of the label was found in the positions of the two α chains, suggesting that some of the [^{14}C]mannose had been converted to [^{14}C]galactose and/or [^{14}C]glucose, thus labelling the hydroxylysyl residues. After treatment of the [^{14}C]mannose-labelled samples with the highly purified bacterial collagenase, part of the label was again found in the propeptides.

Three different compounds were used to inhibit triple helix formation of the pro-α chains, namely α,α-bipyridyl [25,26], L-azetidine-2-carboxylic acid

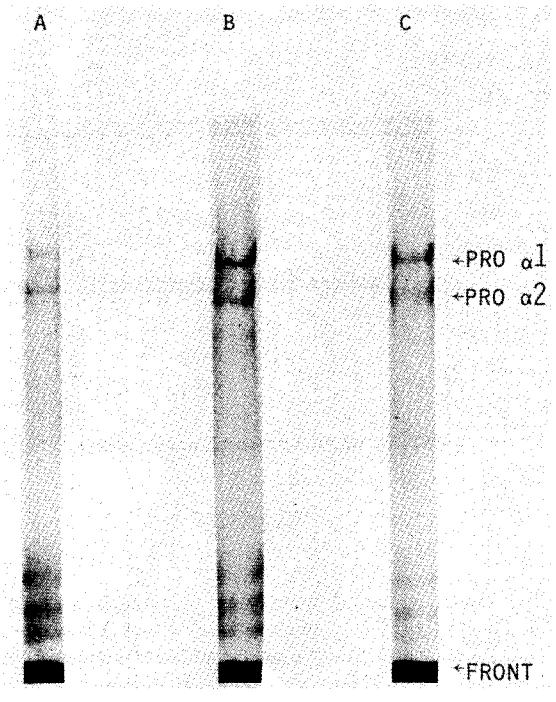

Fig.2. Fractionation by SDS–polyacrylamide (6% acrylamide) electrophoresis of intracellular D-[2-^{3}H]mannose-labelled protein synthesized by matrix-free chick embryo tendon cells. In each experiment 8.4 × 10^{7} cells were preincubated in 3 ml for 30 min with the compounds indicated below; 200 μCi D-[2-^{3}H]mannose was added, and the incubation was allowed to continue for 2 h. Samples of about 200 000 dpm were applied to the gel. The radioactivity was visualized by fluorography. (A) Control; (B) 0.3 mM α,α-bipyridyl; (C) 2.0 mM L-azetidine-2-carboxylic acid.

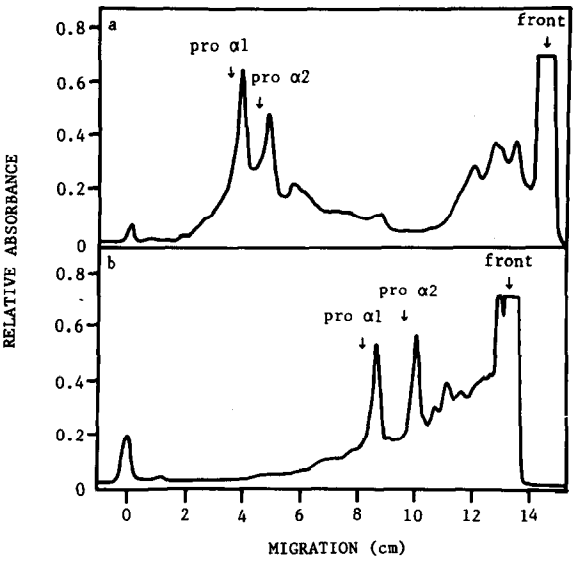

Fig.3. Densitometric tracings of the fluorograms in fig.2B (a) and of a corresponding fluorogram obtained after fractionation on a gel containing 4% acrylamide (b). The positions of the pro-α chains are shifted slightly towards the dye front due to inhibition in the prolyl and lysyl hydroxylations and hydroxylysyl glycosylations.

[27], and dithiothreitol [16,28]. The cells were incubated with [2-^{3}H]mannose for 2 h and the intracellular protein was analyzed by SDS–polyacrylamide slab-gel electrophoresis. Distinct bands were seen in the positions of the pro-α1 and pro-α2 chains after incubation with each of these three compounds (as shown in fig.2 for α,α-bipyridyl and L-azetidine-2-carboxylic acid) indicating that they did not interfere with intracellular mannosylation of the propeptides.

The amounts of label in the pro-α chains were quantified by densitometry (examples shown in fig.3). The cells incubated with α,α-bipyridyl or L-azetidine-2-carboxylic acid contained more [2-^{3}H]mannose label in the positions of the pro-α chains than did control cells (table 1). However, when cells were incubated with α,α-bipyridyl under identical conditions except that [^{14}C]proline was used, a similar increase was found in the radioactivity of these positions, suggesting that the increase in the [2-^{3}H]mannose label was due to an intracisternal accumulation of mannose-labelled pro-α chains rather than to excessive mannosylation of the chains. Incubation with dithio-

Table 1
Relative intracellular radioactivity in the presence or absence of compounds used to inhibit procollagen triple helix formation

Experiment	Relative intracellular radioactivity				
	Undialyzable		In pro-α chains		
	D-[2-^{3}H]Mannose	L-[^{14}C]Proline	D-[2-^{3}H]Mannose	L-[^{14}C]Proline	D-[2-^{3}H]Mannose / L-[^{14}C]Proline
Control	1.0	1.0	1.0	1.0	1.0
α,α-Bipyridyl, 0.3 mM	1.0	1.3	1.7	1.6	1.1
L-Azetidine-2-carboxylic acid, 2.0 mM	0.8	n.d.	1.3	n.d.	n.d.
Dithiothreitol, 0.6 mM	0.7	0.5	0.3	0.4	0.8

In each experiment 8.4 × 10^7 matrix-free chick embryo tendon cells were preincubated in 3 ml for 30 min with the compounds indicated; 200 μCi D-[2-^{3}H]mannose or 5 μCi L-[U-^{14}C]proline was added, and the incubation was allowed to continue for 2 h. The relative radioactivity in the positions of the pro-α chains was calculated from densitometric scanning of the fluorogram obtained after fractionation by SDS–polyacrylamide (4% acrylamide) electrophoresis of the intracellular radioactively-labelled protein. Each value is the mean of 2 experiments

threitol was accompanied by a decrease in the [2-^{3}H]-mannose label in the pro-α chain positions, but a similar decrease was also seen in the [^{14}C]proline label, suggesting, as reported [16], a decreased rate of synthesis of the pro-α chains with an unaltered extent of mannosylation.

4. Discussion

The [2-^{3}H]mannose label used here has the advantage that it would be lost during conversion to other neutral sugars or amino acids [7,8,29]. In agreement with this assumption, no label was found in the α chains obtained by limited pepsin digestion of the [2-^{3}H]mannose-labelled procollagen, whereas significant labelling of the α chains was found when [^{14}C]-mannose was used, indicating that this compound is not suitable for studying the mannosylation of the propeptides.

The experimental design should have shown a decrease in the amount of [2-^{3}H]mannose in the intracellular pro-α chains if mannosylation had taken place only within the Golgi vacuoles (see section 1). However, an increase in the mannose label of pro-α chains was seen, thus strongly suggesting that mannosylation of procollagen can occur within the cisternae of the rough endoplasmic reticulum. The increase in the label was evidently due to an accumulation of the pro-α chains within this compartment.

The propeptides also contain N-acetylglucosamine [7,9] and the oligosaccharide units are probably linked via this amino sugar to asparaginyl residues in the polypeptide chains [7–9]. Accordingly, the transfer of *N*-acetylglucosamine must also have occurred within the rough endoplasmic reticulum, since this amino sugar must be incorporated before the mannose in the oligosaccharide units.

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