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Biosynthesis of Prolyl Hydroxylase: Evidence for Two Separate Dolichol-Mediated Pathways of Glycosylation†

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ABSTRACT: Prolyl hydroxylase is a glycoprotein containing two nonidentical subunits, α and β. The α subunit of prolyl hydroxylase isolated from 13-day-old chick embryos contains a single high mannose oligosaccharide having seven mannosyl residues. Two forms of α subunit have been shown to exist in enzyme purified from tendon cells of 17-day-old chick embryos, one of which (α) appears to be identical in molecular weight and carbohydrate content with the single α of enzyme from 13-day-old chick embryos, as well as another form (α') that contains two oligosaccharides, each containing eight mannosyl units [see Kedersha, N. L., Tkacz, J. S., & Berg, R. A. (1985) *Biochemistry* (preceding paper in this issue)]. Biosynthetic labeling studies were performed with chick tendon cells using [2-^{3}H]mannose, [6-^{3}H]glucosamine, [^{14}C(U)]mannose, and [^{14}C(U)]glucose. Analysis of the labeled products using polyacrylamide gel electrophoresis in sodium dodecyl sulfate showed that only the oligosaccharides on α' incorporated measurable mannose or glucosamine isotopes; however, both α subunits incorporated ^{14}C amino acid mix and [^{14}C(U)]glucose [metabolically converted to [^{14}C(U)]mannose] under similar conditions. Pulse–chase labeling studies using ^{14}C amino acid mix demonstrated that both glycosylated polypeptide chains α and α' were synthesized simultaneously and that no precursor product relationship between α and α' was apparent. In the presence of tunicamycin, neither α nor α' was detected; a single polypeptide of greater mobility appeared instead. Incubation of the cells with inhibitory concentrations of glucosamine partially depressed the glycosylation of α' but allowed the glycosylation of α. Therefore, although both α and α' are synthesized and glycosylated simultaneously, the former appears to be glycosylated exclusively with unlabeled mannose or labeled mannose derived from [^{14}C(U)]glucose but not [2-^{3}H]mannose whereas the latter readily utilizes [2-^{3}H]mannose, [6-^{3}H]glucosamine, or labeled mannose derived from [^{14}C(U)]glucose. These results suggest the existence of two separate pathways of oligosaccharide biosynthesis, both mediated by dolichol but differing in their ability to be labeled by exogenous sugars, in the size of the high mannose oligosaccharides ultimately present on prolyl hydroxylase, and in their susceptibility to inhibition by high levels of glucosamine.

The dolichol-mediated biosynthesis of N-linked oligosaccharides has in the past 10 years been the subject of intensive study [for reviews, see Parodi & Leloir (1979), Kornfeld & Kornfeld (1980), Struck & Lennarz (1980), Hubbard & Ivatt (1981), Snider & Robbins (1981), and Bergman & Kuehl (1982)]. It is generally accepted that these oligosaccharides are synthesized by the ordered stepwise transfer first of *N*-acetylglucosamine 1-phosphate and then *N*-acetylglucosamine, mannose, and glucose from their nucleotide or lipid donors to the lipid carrier–acceptor dolichol phosphate (Chapman et al., 1978; Vijay et al., 1980; Rearick et al., 1981; Hubbard & Robbins, 1980). In mammalian systems, the ultimate lipid-linked product is apparently $Glc_3Man_9GlnNAc_2$ (Chapman et al., 1979; Henner et al., 1981), which is transferred "en bloc" to recipient polypeptides. In chick embryo fibroblasts the pathway has been especially well characterized (Hubbard & Robbins, 1979; 1980).

The majority of oligosaccharide biosynthetic studies in vivo have relied heavily on the use of radioisotopes, usually [2-^{3}H]mannose. Such studies have led to the accepted view of

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dolichol-mediated biosynthesis of N-linked oligosaccharides as described above. Several alternative pathways involving dolichol have been proposed. One of these involves the transfer of the truncated species $Glc_3Man_5GlcNAc_2$ (Bailey et al., 1981; Eggers & Dallner, 1980), occurring as a minor pathway under normal conditions or as the major pathway under conditions of glucose deprivation (Gershman & Robbins, 1981; Rearick et al., 1981; Turco, 1980) or energy depletion (Datema & Schwartz, 1981). An additional dolichol-mediated pathway has been demonstrated in cell-free systems, in which the unmannosylated diacetylchitobiosyl unit is transferred to protein (Chen & Lennarz, 1977; Lehle & Tanner, 1978). This latter pathway may result in posttransfer mannosylation of the diacetylchitobiosyl to form high mannose oligosaccharides in vivo (Hoflack et al., 1982).

A common approach in studies of the glycosylation of proteins has been to use specifically radiolabeled sugars and to monitor their transfer either to selected proteins or to "endogenous acceptors". Our attempts to utilize [2-^{3}H]-mannose to radiolabel the partially characterized high mannose oligosaccharides of prolyl hydroxylase resulted in observations consistent with the following contention: two dolichol-mediated, tunicamycin-sensitive pathways are operative in chick embryo fibroblasts (CEF).[1] One of these readily utilizes exogenously supplied [2-^{3}H]mannose, [^{14}C(U)]glucose, and [6-^{3}H]glucosamine, produces a $Man_8GlcNAc_2$ oligosaccharide on the native α' subunit of prolyl hydroxylase, is inhibited or partially inhibited by high concentrations of glucosamine, and generally behaves as predicted by the accepted view of dolichol-mediated N-linked oligosaccharide biosynthesis. The other pathway utilizes [^{14}C(U)]glucose which is converted to [^{14}C]mannose, excludes [2-^{3}H]mannose and [6-^{3}H]glucosamine, and produces a $Man_7GlcNAc_2$ oligosaccharide on the native α subunit of prolyl hydroxylase which appears resistant to inhibitory concentrations of glucosamine. This may represent the pathway suggested by the results of Gottleib & Wallace (1982) and Parodi et al. (1981). The existence of such an alternate pathway may help explain some of the conflicting reports about the topology and timing of the initial N-linked glycosylation of newly synthesized proteins (Snider et al., 1982; Bailey et al., 1981; Bollini et al., 1983; Nilsson et al., 1978; Eggens & Dallner, 1980). Furthermore, in light of its specific exclusion of [2-^{3}H]mannose, this pathway may be of more widespread occurrence than is currently realized.

Experimental Procedures

Materials. The labeled sugar D-[2-^{3}H]mannose was purchased from Amersham Corp.; ^{14}C amino acid mix, D-[6-^{3}H]glucosamine, D-[^{14}C(U)]mannose, and D-[^{14}C(U)]glucose were purchased from New England Nuclear. Hemocyanin, cyanogen bromide, and deoxycholate were obtained from Sigma Chemical Co., protein A–Sepharose was from Pharmacia, Inc., and collagenase (form III) was from Advanced Biofactors.

Freshly Isolated Chick Tendon Cells and Organ Cultures of Embryonic Tendons. Freshly isolated tendon cells were prepared from 17-day-old chick embryos. The cells were incubated (20×10^6/mL) in modified Krebs medium II (Dehm & Prockop, 1971), containing either ^{14}C amino acids, D-[2-^{3}H]mannose, D-[6-^{3}H]glucosamine, or D-[^{14}C(U)]glucose. When cells were incubated with radiolabeled sugars or in the presence of inhibitors glucosamine or tunicamycin, Krebs medium II was prepared without glucose but supplemented instead with 5 mM sodium pyruvate and 10 μg/mL sodium ascorbate. Cells were harvested by centrifugation, frozen, thawed, and resuspended in enzyme buffer (0.01 M Tris, pH 7.8 at 4 °C, containing 0.1 M glycine, 0.2 M NaCl, and 1.5 μM dithiothreitol) containing 0.5% Nonidet P-40 to obtain a concentration of 10^8 cells/mL. The mixture was homogenized by 10 strokes in a Teflon–glass homogenizer, allowed to stand on ice for 30 min, and centrifuged at 20000g for 20 min to remove cell debris. Prolyl hydroxylase in the supernatant was then either immunoprecipitated or affinity precipitated.

To provide an organ culture system, freshly isolated tendons from 17-day-old chick embryos were washed with Krebs II containing 100 units/mL penicillin and 100 μg/mL streptomycin to inhibit bacterial growth and subsequently incubated in Dulbecco's modified Eagle medium (Gibco) containing 1000 mg/L glucose as described (Olsen et al., 1977). After incubation tendons were either digested with collagenase or disrupted by using a Polytron followed by homogenization in enzyme buffer containing 0.5 Nonidet P-40 detergent (Bethesda Research Laboratories).

Antibody Preparation. Subunits of chick prolyl hydroxylase were prepared as described previously (Berg et al., 1979). Approximately 50–100 μg of antigen was conjugated to 1 mg of hemocyanin with glutaraldehyde (Avrameas & Ternynck, 1969) and emulsified in Freund's adjuvant prior to intramuscular injection into rabbits. Freund's complete adjuvant was used for the initial immunization; subsequent boosters of 100 μg were made biweekly with Freund's incomplete adjuvant (Gibco). Antibodies were purified from the sera by immunoadsorption on an agarose column (Bio-Gel A-5M, 50–100 mesh; Bio-Rad Laboratories) to which native prolyl hydroxylase had been conjugated by cyanogen bromide. The bound antibodies were eluted with 3 M sodium thiocyanate (Fisher Chemicals), dialyzed against phosphate-buffered saline, and concentrated by precipitation with 50% ammonium sulfate. Following dialysis against phosphate-buffered saline, antibodies were stored frozen until used. Ouchterlony double immunodiffusion was performed as described previously (Berg et al., 1980).

Immunoprecipitation of Intracellular Prolyl Hydroxylase. Nonidet P-40 and deoxycholate were added to the cell extracts to obtain final concentrations of 0.5% each. Approximately 50–100 μg of purified anti-α or anti-β antibodies were added to the samples, each containing 0.5 mL of cell homogenate (1×10^6 cells/mL). Samples were incubated at 4 °C for at least 4 h with constant shaking. To each sample was added 10 mg of washed protein-A Sepharose suspended in 100 μL of buffer (0.5% Nonidet P-40, 0.5% sodium deoxycholate, 0.135 M NaCl, and 0.01 M sodium phosphate, pH 7.5 at 4 °C). The incubations were continued at 4 °C overnight with constant gentle shaking. The samples were centrifuged at 8000g for 5 min to obtain the protein A–Sepharose pellets which were then washed twice in the same buffer containing 0.1% SDS and resuspended in 0.1 mL of collagenase buffer (5 mM $CaCl_2$ and 10 mM Tris/HCl buffer, pH 7.5 at 22 °C, containing 2.5 mM *N*-ethylmaleimide). To each sample was added 10 units of collagenase, and the samples were incubated at 4 °C for 16 h. Following the addition of 20 μL of 20% sodium dodecyl sulfate and 10 μL of β-mercaptoethanol, each sample was boiled for 5 min and centrifuged. The supernatants were saved, and the pellets were washed with SDS buffer (Kao

[1] Abbreviations: Endo H, endo-β-*N*-acetylglucosaminidase H; Con A, concanavalin A; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CEF, chick embryo fibroblast; WCE, whole chick embryo; DTT, dithiothreitol; PPO, 2,5-diphenyloxazole.

et al., 1977). Washes were pooled with the corresponding supernatants, and the mixtures were dialyzed against slab gel sample buffer as previously described (Berg et al., 1979).

Affinity Purification. Poly-L-proline was linked to agarose by using a method described previously (Tuderman et al., 1975; Kedersha & Berg, 1981), and the conjugate was suspended in 2 volumes of 0.05 M Tris, 0.05 M glycine, and 0.05 M NaCl buffer, pH 7.8 at 4 °C. One hundred microliters of this suspension was added to each cell extract derived from approximately 10^6 cells, and the mixtures were incubated at 4 °C for 4 h with gentle agitation. Samples were subsequently washed as described under immunoprecipitation. After the samples were washed, no collagenase digestion was necessary as the prolyl hydroxylase was bound to the inhibitor through its active site, so that there was no coprecipitation of procollagen as observed with the antibody precipitation (Berg et al., 1980; Kedersha & Berg, 1981). Washed pellets were boiled in 100 μL of sample bufer containing 10 μL of β-mercaptoethanol and then centrifuged; the resulting supernatants were examined by PAGE in SDS.

Paper Chromatography. In order to confirm that the radioactivity released by prolonged incubation with α-mannosidase was indeed mannose, prolyl hydroxylase was affinity purified from CEF cells that were labeled for 5 h with 100 μCi of [^{14}C(U)]glucose by using the conditions described in the text. The affinity-purified prolyl hydroxylase, which contained 2200 cpm, was digested with α-mannosidase for 12 h at 37 °C (Kedersha et al., 1985). The cleaved sugars were extracted from the protein with 90% ethanol. The extracted material was analyzed by descending paper chromatography in ethyl acetate/pyridine/water (8:3:1) as described by Hough & Jones (1962).

Other Procedures. Tunicamycin was dissolved in 50 mM NaOH at a concentration of 100 μg/mL and diluted to 1 μg/mL for use. Cells were incubated with the antibiotic for 1 h at 37 °C prior to the addition of radioisotope. Endoglucosaminidase H and α-mannosidase were purified and used as described elsewhere [see Kedersha et al., 1985)]. The Endo H cleaved oligosaccharides were subjected to chromatography on Bio-Gel P4 (200–400 mesh, 1 × 114 cm; Bio-Rad Laboratories) as described in the preceding paper (Kedersha et al., 1985). Polyacrylamide slab gel electrophoresis was performed as described previously (Berg et al., 1979), and fluorography was performed as described in the preceding paper (Kedersha et al., 1985). Fluorograms were scanned on an LKB Ultra Scan laser densitometer and integrated on an HP 3390A integrating printer plotter.

Results

Prolyl hydroxylase biosynthesis was first examined by labeling freshly isolated tendon cells with either [2-^{3}H]mannose or ^{14}C amino acid mix. The labeled enzyme was purified by immunoprecipitation with antibodies specific for each subunit (Figure 1). Since all of the intracellular α subunits are present in the tetrameric form of prolyl hydroxylase (Kao et al., 1980), antibodies specific for this subunit precipitate only newly synthesized, fully assembled enzyme, whereas antibodies to the β subunit precipitate both $\alpha_2\beta_2$ tetramers and cross-reacting protein, a structurally related precursor form of the β subunit (Chen-Kaing et al., 1977; Berg et al., 1980). The data shown in Figure 2 (lanes 1 and 2) demonstrate that either anti-α or anti-β antibodies precipitate only a single, [2-^{3}H]mannose-labeled species as resolved on PAGE in SDS. In cells labeled for 5 h with [6-^{3}H]glucosamine or [^{14}C(U)]mannose, the same pattern was observed (data not shown). Overexposure of gels revealed a trace of sugar-labeled α-like material (data not shown). In the immunoprecipitates of ^{14}C amino acid labeled material, the larger amount of β-like protein appearing relative to the α and α′ (Figure 2, lane 4) is due to cross-reacting protein (Berg et al., 1980).

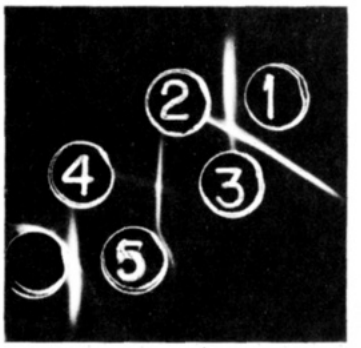

FIGURE 1: Antibodies specific for each of the two subunits of prolyl hydroxylase, demonstrated by Ouchterlony double immunodiffusion. Well 1, 11 μg of α subunit mixed with 19 μg of the β subunit; well 2, 250 μg of purified anti-α antibody; well 3, 400 μg of purified anti-β antibody; well 4, 20 μg of purified β subunit; well 5, 15 μg of purified α subunit. In order to enhance the solubility of the isolated subunits, both the gel and the reagents contained 0.5% Nonidet P-40 (v/v) and 0.5% deoxycholate (w/v).

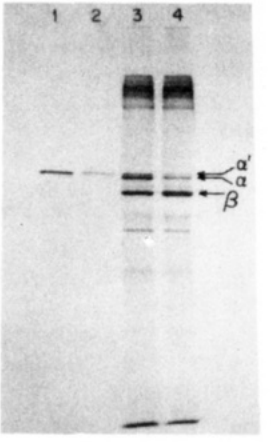

FIGURE 2: Labeling of newly synthesized prolyl hydroxylase with [2-^{3}H]mannose and ^{14}C amino acids. Freshly isolated fibroblasts were continuously labeled for 4 h with either 2 μCi/mL [2-^{3}H]mannose or ^{14}C amino acids, homogenized, immunoprecipitated, and subjected to PAGE in SDS and fluorography as described in the text. Lane 1, [2-^{3}H]mannose-labeled cells precipitated with anti-α antibodies; lane 2, [2-^{3}H]mannose-labeled cells precipitated with anti-β antibodies; lane 3, ^{14}C amino acid labeled cells precipitated with anti-α antibodies; lane 4, ^{14}C amino acid labeled cells precipitated with anti-β antibodies.

Amino acid labeling of cells in the presence of tunicamycin gave neither α nor α′ but a new band of higher mobility (see below; Figure 6, lane 5) that migrated similarly to the single α_0 band resulting from Endo H treatment of enzyme purified from control cells (Figure 3, lane 2). The alteration in mobility of the α band by tunicamycin is indicative that native α is glycosylated although it was not labeled by any of the radioactive sugars used (Figure 2, lanes 1 and 2). That α is glycosylated is further suggested by the fact that its mobility is increased by treatment with α-mannosidase (Figure 3, Lane 2) and Endo H (Figure 3, lane 3).

Because of the difficulty in labeling the α subunit with [2-^{3}H]mannose, additional labeling experiments were performed. A 2-h labeling with [2-^{3}H]mannose followed by a 6-h chase did not alter the distribution of label (data not shown); the α′ subunit was labeled while the α subunit remained unlabeled. This suggests that the α subunit does not arise from the α′ subunit through the removal of the mannose

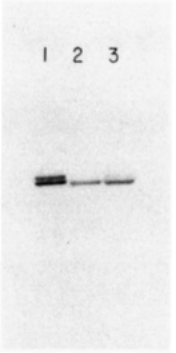

FIGURE 3: Effect of glycosidases on the mobility of the two α subunits in SDS–PAGE. Prolyl hydroxylase was purified from cell extracts labeled with amino acid mix and then treated with glycosidases as indicated in the text. Lane 1, control enzyme labeled for 2 h with ^{14}C amino acid mix; lane 2, α-mannosidase-treated enzyme; lane 3, enzyme treated with Endo H. The β subunit is not labeled under these conditions, and hence does not appear.

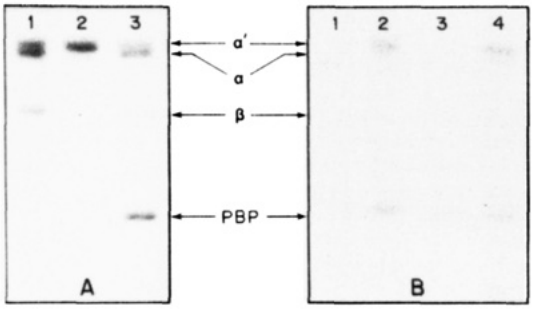

FIGURE 4: (A) Fluorogram of PAGE in SDS showing the labeling of prolyl hydroxylase with various radioisotopes. Whole tendon suspensions were incubated for 22 h with different radiolabeled substrates, the cells were isolated, and the prolyl hydroxylase was purified by affinity chromatography. Lane 1, [$^{14}C(U)$]glucose; lane 2, [6-^{3}H]glucosamine; lane 3, ^{14}C amino acid mix. (B) Freshly isolated cells (2.4×10^9) were labeled in suspension culture for 4 h in 100 mL of modified Krebs II media containing 250 μCi of [$^{14}C(U)$]glucose. At the end of the labeling period the cells were frozen, thawed, and affinity precipitated as described in the text. The affinity precipitates were digested with either Endo H or α-mannosidase for 12 h at 37 °C over layered with toluene. The stained gel (not shown) revealed only the mobility changes shown in Figure 3. Lane 2, control; lane 1, Endo H digest; lane 4, control; lane 3, α-mannosidase digest.

residues. The labeling period was extended to 22 h by using an organ culture system in which whole tendons were incubated in Dulbecco's medium containing 1000 mg/L glucose. These conditions were used in order to ensure that the normal cellular processes of oligosaccharide biosynthesis were not altered by glucose deprivation, as has been observed in some studies. The results confirmed that asymmetric incorporation of both [2-^{3}H]mannose (not shown) and [6-^{14}C]glucosamine (see Figure 4A, lane 2) occurred in long-term glucose-containing organ culture as well as in short-term, glucose-free cell suspensions (see Figure 2, lanes 1 and 2). A radiolabeled saccharide could be incorporated into α' but not into α, while ^{14}C amino acids were found in both α and α' (Figure 4A, lanes 2 and 3). However, [$^{14}C(U)$]glucose was found to label α, α', and, to a much lesser extent, the β subunit (Figure 4A, lane 1). The protein band labeled PBP is a polypeptide present in chick embryos that copurifies with prolyl hydroxylase using the affinity technique (Kedersha & Berg, 1981).

In order to confirm that the label was still present as carbohydrate, the glucose-labeled material was treated with Endo H and α-mannosidase. The results (Figure 4B, lanes 2 and 4) show that the radiolabel originally supplied by [$^{14}C(U)$]-glucose was removed by both Endo H and α-mannosidase,

Table I: Incorporation of Radioactivity from Labeled Precursors into the α and α' Subunits of Prolyl Hydroxylase

isotope	radioactivity in subunits (dpm) α'	α	% in α'	% in α
^{14}C amino acid mix	10860	15245	41.6	58.4
[$^{14}C(U)$]glucose	48635	54832	48.2	51.8
[6-^{3}H]glucosamine	32000	0	100	0
[2-^{3}H]mannose	71000	0	100	0

indicating that the label had not been metabolically converted to amino acids and then incorporated into the polypeptide portions of α and α'. The digested material was dried under nitrogen, and the cleaved sugars were extracted twice with 90% ethanol. The extracts were pooled and dried under nitrogen. The remaining sample was applied in duplicate to a sheet of Whatman chromatography paper, unlabeled mannose and glucose were applied over the sample spots, and the sugars were separated by descending chromatography using a pyridine/ethyl acetate/water (2:8:1) solvent system described by Hough & Jones (1962). After 16 h, the chromatography was stopped, and one of the sample lanes was developed with silver nitrate–sodium hydroxide. The elution positions of glucose and mannose in the presence of half of the sample extract were found to be unchanged relative to the other standard lanes run on the same chromatogram. The remaining sample lane was cut into 1-cm strips; each strip was placed in a liquid scintillation counting vial and incubated with 1 ml of water for 30 min. Ten milliliters of Aquamix was added to each vial, and the vials were counted. In the solvent system chosen, mannose migrates further than does glucose. All detectable counts coeluted with the mannose standard, indicating that a metabolic conversion of glucose to mannose had occurred (data not shown). Its removal by highly purified α-mannosidase and its comigration with mannose on paper chromatography suggest that the [$^{14}C(U)$]glucose had been converted into [$^{14}C(U)$]mannose and incorporated into the dolichol oligosaccharide prior to its transfer to the α and α' subunits. Should such an isomerization be required in the glycosylation of α, this would explain its long-term ability to exclude [2-^{3}H]mannose, since during the isomerization of mannose to glucose the 2-H is lost. Further support for this conclusion are previous studies indicating that no glucose residues are present on any of the Endo H sensitive oligosaccharides found on α or α' (Kedersha et al., 1985).

To summarize the distribution of label on α and α' in various labeling experiments, the fluorograms in Figures 2 and 4 were scanned for radioactivity in α and α', and the relative distribution of radioactivity was determined. The dpm applied to each lane was used to determine the dpm found in α and α'. The results are shown in Table I. When chick tendons in organ culture are labeled for 22 h with ^{14}C amino acid mix, approximately 58% of the label is found in α and 42% in α'. When [$^{14}C(U)$]glucose is incubated with whole tendons for 22 h, the relative distribution of label into α and α' is approximately equal (52% α; 48% α'). Since the label on the enzyme is removed by either Endo H or α-mannosidase, we conclude that the labeled glucose was isomerized to mannose which then became utilized for the synthesis of the dolichol-mediated high mannose oligosaccharide. In contrast, when [2-^{3}H]mannose was used to label the embryonic tendons, all of the labeled mannose was present on the α' subunit and none on the α subunit.

As for the radiolabeled glucose apparently found in the β subunit, it has previously been shown to contain a trace of glucose (Berg et al., 1979), and although it does not bind to

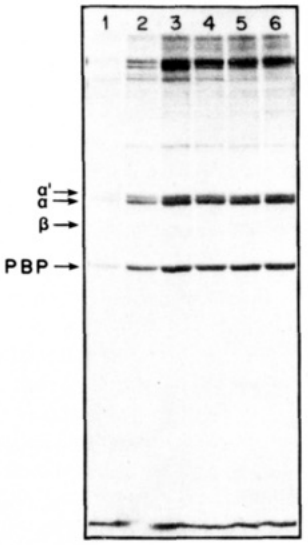

FIGURE 5: Pulse–chase labeling of prolyl hydroxylase, showing no precursor–product relationship between the two forms of α subunit. Freshly isolated cells were incubated at 50×10^6 cells/mL in suspension culture, in medium containing 2 μCi/mL ^{14}C amino acids. At the end of the 30-min chase, the cells were centrifuged, washed once in media containing a 20-fold excess of amino acids, and then resuspended in the same media. At the end of each time point, cycloheximide was added to a final concentration of 100 μg/mL, the cells were separated from the medium by centrifugation, and the cell pellet was frozed in liquid nitrogen. Prolyl hydroxylase was subsequently affinity purified and subjected to electrophoresis as described in the text. Lane 1, 15-min pulse; lane 2, 30-min pulse; lane 3, 10-min chase; lane 4, 20-min chase; lane 5, 30-min chase; lane 6, 90-min chase.

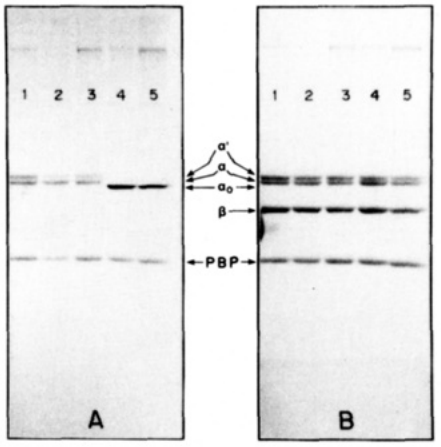

FIGURE 6: (A) Effect of various inhibitors of glycosylation on the biosynthesis of the α subunits. Cells were preincubated for 1 h at a concentration of 50×10^6/mL in the presence of either 20 μM glucosamine or 1 μg/mL tunicamycin. Cells were then labeled for 2 h with ^{14}C amino acids, washed, resuspended in inhibitor-free media, and incubated for an additional 2 h. Cells were harvested at the indicated time points, and prolyl hydroxylase was affinity purified and subjected to SDS–PAGE as described in the text. Lane 1, control (no inhibitors, incubated in glucose-free media); lane 2, 2-h pulse labeling in the presence of glucosamine; lane 3, 2-h pulse in the presence of glucosamine and 2-h chase in the absence of inhibitors; lane 4, 2-h pulse in the presence of tunicamycin; lane 5, 2-h pulse in the presence of tunicamycin followed by a 2-h chase in the absence of tunicamycin. (B) Stained gel from (A), showing the resolution of preexisting prolyl hydroxylase.

Con A (Guzman et al., 1977), it is retained by columns of immobilized boronic acid (not shown), suggesting it may contain carbohydrate.

In contrast to the labeling with [6-^{3}H]glucosamine and [2-^{3}H]mannose, ^{14}C amino acids were incorporated into the α and α' subunits simultaneously. This was shown by experiments in which cells were incubated for short periods with labeled amino acids, and the prolyl hydroxylase formed was examined by PAGE in SDS (Figures 2 and 5). Radioactive α and α' appear in 15 min (Figure 5, lane 1). The appearance of labeled β subunit in the enzyme is delayed due to its dilution in a preformed pool of free subunits (i.e., the nontetrameric β-like protein that reacts with anti-β antibodies; Berg et al., 1980). During the chase period, no apparent conversion of α' to α is observed (Figure 5, lanes 2–5), as would be expected if α were a trimmed or processed form of α'. No apparent precursor–product relationship between the two forms of α subunits is observed here because both subunits incorporated ^{14}C amino acid at the same rate.

The effect of various inhibitors on the labeling of α and α' are shown in Figure 6. Lane 1 demonstrates that, even in the absence of glucose, α and α' are synthesized in nearly equal amounts, as in Figure 2 (lanes 3 and 4). Lanes 2 and 3 show the effect of high concentration (20 mM) of glucosamine, which has been shown to inhibit N-linked glycosylation (Schwartz & Datema, 1982). When incubated in the presence of glucosamine, the appearance of α' is partially blocked (Figure 6, lane 2) while the appearance of α or of a partially glycosylated form of α' seems unaffected. Further protein synthesis was blocked by the addition of cyclohexamide, and the cells were washed in several changes of glucosamine free media. The glucosamine was chased by a 90-min incubation in the presence of excess glucose; some apparent conversion of α to α' was observed (Figure 6, lane 3), indicative of a small amount of posttranslational glycosylation.

In contrast, the effect of tunicamycin extends to both α and α'. Neither α subunit appears when cells are incubated in the presence of tunicamycin (Figure 6, lane 4), nor can this effect be reversed after removal of tunicamycin from the media (Figure 6, lane 5). This is not unexpected despite some reports indicating it can be "chased" in some systems (Gottleib & Wallace, 1982), as in most systems tunicamycin behaves as a "tight-binding" inhibitor (Tkacz, 1983) that cannot be chased from cells.

Discussion

Prolyl hydroxylase effects an important posttranslational modification of procollagen, the major secretory protein of chick embryo fibroblasts (Davidson & Berg, 1981). Both electron microscopical localization (Olsen et al., 1973) and subcellular fractionation studies (Peterkofsky & Assad, 1976) indicate that prolyl hydroxylase is largely confined to the rough endoplasmic reticulum of fibroblasts. Therefore, it resides at a point that represents the beginning of the secretory pathway, and its high mannose oligosaccharide may reflect this intracellular location in that it possesses no peripheral sugars of complex type oligosaccharides acquired by some glycoproteins in the Golgi (Schachter, 1981).

Structural studies of the glycosylated subunits indicated that the larger α' subunit contains two oligosaccharides of eight mannosyl residues each, while the smaller α subunit contains a single seven-mannosyl oligosaccharide [see Kedersha et al. (1985)]. Since it is now clear that in many systems the $Glc_3Man_9GlcNAc_2$ oligosaccharide initially transferred to an Asn residue in a protein is subject to "processing" glycosidases that remove sugar residues (Hubbard & Ivatt, 1981; Schacter, 1981; Kornfeld & Kornfeld, 1980), it appeared possible that the smaller α was a more trimmed product of α', in the context of the commonly accepted view of dolichol-mediated oligosaccharide biosynthesis; therefore, labeling studies were undertaken partly to ascertain if this were the case. Although the [2-^{3}H]mannose labeling results showing that α' is easily

Table II: Comparison of the α and α' Subunits of Chick Embryo Fibroblast (CEF) Prolyl Hydroxylase

α subunit	α' subunit	α and α' subunits
contains a single Endo H sensitive Man_7 oligosaccharide	contains 2 Endo H sensitive Man_8 oligosaccharides	differ in charge, as indicated by their separation by isoelectric focusing
glycosylation blocked by tunicamycin	glycosylation blocked by tunicamycin	yield similar but not identical peptide maps when each is treated with cyanogen bromide
glycosylation apparently unaffected by 20 mM glucosamine	glycosylation partially blocked by 20 mM glucosamine	
does not utilize [2-^{3}H]mannose	readily utilizes [2-^{3}H]mannose	
does not utilize [6-^{3}H]glucosamine	readily utilizes [6-^{3}H]glucosamine	
readily labels with ^{14}C amino acids	readily labels with ^{14}C amino acids	
readily labels with [^{14}C(U)]glucose	readily labels with [^{14}C(U)]glucose	

labeled even after 22 h might be consistent for a precursor–product relationship requiring a very long turnover period (e.g., greater than 22 h), the data obtained from short pulse labeling of the protein with ^{14}C amino acids (Figure 5) indicate this is not the case, as do previously published results (Berg et al., 1980). Both forms, α and α', appear within 15 min in equal amounts (Figure 5, lane 1), and pulse–chase experiments indicate that ^{14}C amino acid labeled α' is not converted to α during a 90-min chase period. Tunicamycin prevents the glycosylation and appearance of both α and α', whereas glucosamine has no obvious effect on the glycosylation of α but does partially inhibit the glycosylation of α' (see Figure 6). It therefore appears that there are two separate glycosylation mechanisms operative in this system, distinguishable both in terms of their ability to incorporate exogenously supplied radioisotopes and in terms of their sensitivity to inhibitors (see Table II). Both mechanisms are completely blocked by tunicamycin, but glucosamine partially inhibits one mechanism while having no visible effect upon the other.

It is reasonable to assume one of the two α species detected here is glycosylated by the same pathway studied by Hubbard and Robbins in chick fibroblasts (Hubbard & Robbins, 1980). In view of the ease with which it incorporated [2-^{3}H]mannose, it would seem likely that α' is probably the recipient of the $Glc_3Man_9GlcNAc_2$ transfer species. Since α' contains eight mannosyl units [see Kedersha et al., (1985)], it is probable either that an α-mannosidase exists in the endoplasmic reticulum which is capable of cleaving at least one mannosyl unit from a Man_9-containing species as has been suggested for the processing of thyroglobulin (Godelaine et al., 1981) or else that prolyl hydroxylase is transiently present in the Golgi. We cannot distinguish between these possibilities here; however, no studies to date have detected prolyl hydroxylase in the Golgi (Olsen et al., 1973; Peterkofsky & Assad, 1976).

The majority of published reports regarding the glycosylation of Asn residues in proteins favor the view that the species $Glc_3Man_9GlcNAc_2$ is the major oligosaccharide species transferred in a large number of cells and specifically in chick embryo fibroblasts (Hubbard & Robbins, 1979, 1980). Most, if not all, of these studies have relied heavily on the use of radioisotopes, particularly [2-^{3}H]mannose. The results presented here indicate that at least in vivo a different dolichol-mediated pathway may exist which apparently draws upon a pool of sugars that is not readily saturated by exogenously added [2-^{3}H]mannose or [6-^{14}C]glucosamine but does utilize [^{14}C(U)]glucose and which may produce a smaller oligosaccharide than does the generally studied pathway.

There are currently a number of reports in the literature indicating that more than one route of N-glycosylation exists (Bollini et al., 1983; Chen & Lennarz, 1977; Eggens & Dallner, 1980; Gottlieb & Wallace, 1982; Hoflack et al., 1982; Lehle & Tanner, 1978; Parodi et al., 1981). Several authors (Chen & Lennarz, 1977; Hoflack et al., 1982; Lehle & Tanner, 1978) have reported the direct transfer of *N,N'*-diacetylchitobiose from dolichol pyrophosphate to protein in vitro. Lehle & Tanner (1978) also reported the transfer of $Man_1GlcNAc_2$ to protein in yeast membrane preparations. Montreiul's group (Hoflack et al., 1982) used a lymphocyte system in which they observed GDP-mediated mannosylation of the chitobiosyl group after its transfer to protein. Their study differed from the others in that they used whole lymphocytes (which possess cell surface glycosyltransferases); so they were able to use a membrane system without the disrupting procedures employed in earlier studies (Chen & Lennarz, 1977) in which mannosylation of protein-bound $GlcNAc_2$ was not observed.

Results similar to those reported here were recently reported by Gottleib & Wallace (1982), who observed that the glycoprotein vitellogen in *Xenopus* liver was resistant to labeling with [2-^{3}H]mannose despite the demonstrable presence of mannose as determined by gas–liquid chromatography. They further demonstrated that the glycosylation of vitellogen occurred in a smooth membrane compartment of the cells, not the rough endoplasmic reticulum as is generally believed. While unable to explain the inability of *Xenopus* liver to utilize exogenously supplied [2-^{3}H]mannose in the synthesis of vitellogen, the authors suggest that glycogen might supply the mannose used. Our studies presented here used freshly isolated embryonic chick fibroblasts, which would not be expected to contain large glycogen reserves. In any case, it appears that the inability of cells to utilize labeled sugars in the synthesis of certain proteins is not an isolated event. Furthermore, since the relative contributions of mannose "labelable" vs. "unlabelable" pathways have not been measured in any system to date, it is possible that the unlabelable (and, hence, largely unstudied) mechanism is widely used by cells but undetected in the studies relying on the use of [2-^{3}H]mannose.

Parodi et al. (1981) has recently reported that a $Man_7GlcNAc_2$ lipid-linked species is the largest lipid-linked species detectable in an insect parasite; no glucose-containing species were detected. The authors suggest that in this system a nonglucosylated $Man_7GlcNAc_2$ is transferred directly to protein. This report is of significance here when one notes that their studies employed [^{14}C(U)]glucose in the detection of all lipid-linked species, and the authors confirmed its chemical conversion to mannose by chemical analysis. As the oligosaccharide of α can *only* be labeled with [^{14}C(U)]glucose, and as it contains seven mannose residues and no glucose (Kedersha et al., 1985). It may be that the pathway observed by Parodi et al. is operating in the chick embryo fibroblasts studied here and constitutes the pathway that mediates the glycosylation of α but not α'.

The existence of two separate dolichol pyrophosphate mediated pathways may explain much of the controversy in the

literature concerning the nature of glycosylation. For example, there is evidence to support both cotranslational and posttranslational glycosylation (Hubbard & Ivatt, 1981). It would appear likely that only the glycosylation occurring in the rough endoplasmic reticulum could occur cotranslationally, whereas N-glycosylation occurring in smooth membranes as observed by Gottleib & Wallace (1982) could only occur posttranslationally. Should the posttranslation pathway be generally resistant to labeling, its possible presence in many cell types would be undetected by isotopic studies and therefore its importance in glycosylation unknown.

The topological arrangement of the sugar transferases in the membrane of the endoplasmic reticulum has not been firmly established. Evidence has accumulted for physically distinct dolichol pathways segregated by the rough endoplasmic reticulum membrane itself, i.e., one intraluminal (Hanover & Lennarz, 1982) and one cytoplasmic (Eggens & Dallner, 1980), but it is not yet clear whether these physically separate pathways exist (Snider & Robbins, 1981; Snider et al., 1982). The existence of two physically segregated, metabolically distinct pathways could explain our observed differences in labeling with glucosamine as well as mannose.

In the CEF system studied here it appears that two pathways are involved in the glycosylation of α and α' (Table II). As each pathway appears to glycosylate only one of the two clearly related polypeptides, it seems likely that some differences in primary sequence must ultimately be responsible for this separation (Kedersha et al., 1985). The results presented here indicate that the α subunit of prolyl hydroxylase is glycosylated by (1) a tunicamycin-sensitive process that (2) does not readily utilize mannose or glucosamine but does use glucose, (3) results in an Endo H and α-mannosidase sensitive structure, and (4) may be of more widespread occurrence than studies employing [2-^{3}H]mannose are likely to detect.

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Generation of pH-Sensitive Liposomes: Use of Large Unilamellar Vesicles Containing *N*-Succinyldioleoylphosphatidylethanolamine†

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ABSTRACT: By use of a carboxylated derivative of phosphatidylethanolamine, *N*-succinyldioleoylphosphatidylethanolamine (COPE), pH-sensitive liposomes have been designed that have a wide range of leakage properties. The leakage rate of the vesicle contents, as determined by the release kinetics of the water-soluble fluorophore 8-aminonaphthalene-1,3,6-trisulfonic acid complexed with the quencher *p*-xylenebis(pyridinium) dibromide [Ellens, H., Bentz, J., & Szoka, F. C. (1984) *Biochemistry 23*, 1532], was found to be dependent on the lipid composition and the pH of the incubation medium. Pure COPE vesicles released their encapsulated contents at pH 7.4 but not at pH 4.0. Leakage of these vesicles appears to be due to the electrostatic interactions between the COPE molecules. A dramatic reversal of the leakage properties was observed in mixed-lipid vesicles composed of COPE containing increasing amounts of dioleoylphosphatidylethanolamine (DOPE). Unlike pure COPE vesicles, COPE/DOPE (3:7) vesicles were more leaky under acidic conditions (pH 4.0) than they were at neutral pH. Studies employing a fluorescent COPE analogue, *N*-succinyl-1-acyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]phosphatidylethanolamine, suggested that the mechanism of leakage might be a result of lipid-packing defects due to the nonbilayer properties of DOPE and protonation of the COPE molecules. Hence, the mechanism of release is different from that of other recently described pH-sensitive liposomes where either fusion or aggregation of the vesicles results in the release of vesicle contents.

Liposomes have recently attracted considerable interest as a potential modality for selective drug delivery in vivo (Gregoriadis & Allison, 1980). Many concerns have been raised, however, about their effectiveness in delivering drugs to extravascular sites because of anatomical barriers imposed by the peripheral circulation (Poste, 1983). In an effort to circumvent these problems, strategies have been suggested that take advantage of the reduced local pH environments of pathologic tissues (Yatvin et al., 1980) by employing pH-sensitive liposomes. Indeed, it has been shown that sites of primary tumors, metastasis, inflammation, and infection have an ambient pH considerably lower than that of normal tissues (Kahler & Robertson, 1943; Gullino et al., 1965; Meyer et al., 1948; Naeslund & Swenson, 1953). Conversely, the passive localization of liposomes to cells of the reticuloendothelial system has been exploited to deliver immunomodulators to these cells (Alving, 1983). Since the primary mechanism of liposome uptake by these cells is via endocytosis followed by sequestration of the vesicles into acidic intracellular vacuoles (Straubinger et al., 1983), utilization of this "pH-sensitive pathway" has been suggested as a means of improving the delivery of liposome-encapsulated compounds to macrophages (Poste & Kirsh, 1983; Straubinger et al., 1983; Straubinger et al., 1985).

The general strategy employed in the construction of pH-sensitive liposomes has been to include lipids containing pH-sensitive groups such as *N*-palmitoyl-L-homocysteine (Yatvin et al., 1980) and cholesteryl hemisuccinate (Ellens et al., 1984). These lipids have the ability to destabilize the lipid bilayer when exposed to acidic environments, which results in liposome–liposome fusion (Conner et al., 1984) and liposome–liposome aggregation (Ellens et al., 1984), respectively, followed by concomitant release of the entrapped compounds. The acid-induced leakage from liposomes that contain *N*-palmitoyl-L-homocysteine required the presence of lipids near their phase transition (Yatvin et al., 1980), and the release of contents from cholesteryl hemisuccinate containing liposomes has been shown to depend on the presence of auxiliary lipids, in particular, on the inclusion of phosphatidylethanolamine (Ellens et al., 1984).

In this paper, we describe the preparation and properties of COPE,[1] which, when incorporated into liposomes, conveys

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