Incorporation of copper into lysyl oxidase

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Lysyl oxidase is a copper-dependent enzyme involved in extracellular processing of collagens and elastin. Although it is known that copper is essential for the functional activity of the enzyme, there is little information on the incorporation of copper. In the present study we examined the insertion of copper into lysyl oxidase using ^{67}Cu in cell-free transcription/translation assays and in normal skin fibroblast culture systems. When a full-length lysyl oxidase cDNA was used as a template for transcription/translation reactions *in vitro*, unprocessed prolysyl oxidase appeared to bind copper. To examine further the post-translational incorporation of copper into lysyl oxidase, confluent skin fibroblasts were incubated with inhibitors of protein synthesis (cycloheximide, 10 μg/ml), glycosylation (tunicamycin, 10 μg/ml), protein secretion (brefeldin A, 10 μg/ml) and prolysyl oxidase processing (procollagen C-peptidase inhibitor, 2.5 μg/ml) together with 300 μCi of carrier-free ^{67}Cu. It was observed that protein synthesis was a prerequisite for copper incorporation, but inhibition of glycosylation by tunicamycin did not affect the secretion of ^{67}Cu as lysyl oxidase. Brefeldin A inhibited the secretion of ^{67}Cu-labelled lysyl oxidase by 46%, but the intracellular incorporation of copper into lysyl oxidase was not affected. In addition, the inhibition of the extracellular proteolytic processing of prolysyl oxidase to lysyl oxidase had minimal effects on the secretion of protein-bound ^{67}Cu. Our results indicate that, similar to caeruloplasmin processing [Sato and Gitlin (1991) J. Biol. Chem. **266**, 5128–5134], copper is inserted into prolysyl oxidase independently of glycosylation.

INTRODUCTION

Lysyl oxidase (EC 1.4.3.13) is a cuproprotein that is present in high concentrations in dense connective tissue [1,2]. It catalyses the oxidative deamination of lysine residues in elastin and collagens as an initial step in their extracellular assembly into insoluble fibres [2,3]. Although levels of dietary copper intake do not influence lysyl oxidase expression, i.e. lysyl oxidase steady-state mRNA concentrations are similar in copper-depleted and copper-adequate animals, the amount of dietary or cellular copper can markedly influence its functional activity [1,4,5]. Lysyl oxidase activity is decreased on nutritional copper deprivation, which can result in connective tissue defects, including obstructive pulmonary disease, aortic aneurysms and various skin and bone defects. Animals are more susceptible to the effects of copper deficiency during periods of rapid growth and development [6].

Despite the importance of copper to lysyl oxidase activity, and its functions in electron transport and oxidant defence [7], the metabolism and intracellular sites at which copper is incorporated into proteins such as lysyl oxidase are not well characterized. A detailed elucidation of cellular copper metabolism is also vital for understanding the formation of quinone cofactors in lysyl oxidase and other copper-dependent amine oxidases [8,9]. It is presumed that the formation of the lysine tyrosylquinone in lysyl oxidase is the result of direct catalysis mediated by copper at the active site of lysyl oxidase [10].

In the present study we investigated the incorporation of copper into unprocessed prolysyl oxidase using a cell-free transcription/translation system with a full-length lysyl oxidase cDNA as template for the nascent protein. The post-translational processing of lysyl oxidase and the copper incorporation into lysyl oxidase were evaluated in cultured skin fibroblasts incubated with inhibitors of protein synthesis (cycloheximide), glycosylation (tunicamycin), protein secretion (brefeldin A) or prolysyl oxidase processing (procollagen C-peptidase inhibitor). Results indicate that the majority of protein-bound copper secreted from skin fibroblasts is associated with lysyl oxidase, and that protein synthesis is a prerequisite for this process. In addition, we demonstrate that glycosylation of lysyl oxidase is not required for copper incorporation into, or secretion of, lysyl oxidase.

EXPERIMENTAL

Materials

Chemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and Fisher Scientific (Pittsburgh, PA, U.S.A.). Tissue culture supplies were products of Gibco–BRL Life Technologies (Grand Island, NY, U.S.A.). Reagents for PCR were purchased from Invitrogen (San Diego, CA, U.S.A.) and United States Biochemical Corporation (Cleveland, OH, U.S.A.). Translation-grade [^{35}S]methionine (1000 mCi/mmol) was from DuPont–NEN (Boston, MA, U.S.A.). Cell-free transcription/translation reagents were products of Promega (Madison, WI, U.S.A.), and electrophoresis supplies and materials were obtained from Bio-Rad (Richmond, CA, U.S.A.). Chromatographic supplies were from Pharmacia Biotech (Uppsala, Sweden). Carrier-free copper as ^{67}Cu was prepared at the National Laboratories of Brookhaven (Upton, NY, U.S.A.) or Los Alamos (Los Alamos, NM, U.S.A.) (specific radioactivity 3.8–4.5 mCi/μg of Cu or 9–10 mCi/mg of Cu respectively). Goat anti-chicken serum conjugated to horseradish peroxidase used for detection in ELISA was purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD, U.S.A.). Antibodies to rat lysyl oxidase for ELISA were prepared in chickens as described by Romero-Chapman et al. [5]. Finely ground insoluble elastin isolated from copper-deficient chick aortae was used as a support for affinity chromatography columns [1,12].

Plasmid construction

A full-length lysyl oxidase cDNA was prepared by reverse transcription PCR from rat aortic smooth-muscle cell RNA [13]. The 20 μl reverse transcription reaction mixture consisted of 1 μg of total RNA, 3.5 mM $MgCl_2$, 1 mM each dATP, dCTP, dGTP and dTTP, 20 units of RNase inhibitor, 1× reaction buffer,

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50 units of Moloney murine leukaemia virus reverse transcriptase and a 0.75 μM concentration of the 3′ untranslated region (downstream) primer, 5′-GGACCTATGAAAACCGTAG-3′. This reaction mixture was incubated at 37 °C for 30 min, and stopped by heating (99 °C for 5 min). For the subsequent PCR amplification, reaction volumes were adjusted to 100 μl, and the final $MgCl_2$ concentration was adjusted to 1.9 mM. This concentration of $MgCl_2$ was determined to be optimal for amplification of lysyl oxidase. The 5′ untranslated region (upstream) primer, 5′-GATC7GAGTCCC7GTCTTC-3′ (final concentration of 0.15 μM) and 2.5 units of *Taq* polymerase were added to the reaction mixture. The PCR amplification consisted of 35 cycles: dissociation at 96 °C (1 min), annealing at 60 °C (1 min) and amplification at 72 °C (5 min). PCR products were separated by electrophoresis in 1 % agarose and purified using Sepharose CL-4B spin-columns (Pharmacia Biotech, Piscataway, NJ, U.S.A.). The purified lysyl oxidase cDNA was subcloned into pcr™-II plasmid (Invitrogen) and sequenced using the chain-termination DNA-sequencing method [14]. The sequence consisted of the entire coding sequence (bases 287–1522) of rat lysyl oxidase and short regions of 5′ and 3′ untranslated sequences extending from base 217 to 1585 as reported for rat aortic lysyl oxidase (GenBank accession no. U11038) [15,16], except for a substitution of C for T at position 1236. The lysyl oxidase cDNA was next subcloned into pSP64(polyA) plasmid vector (Promega) for use in cell-free coupled transcription/translation assays. The plasmid construct is called pSPLOX.

In vitro cell-free coupled transcription/translation

An *in vitro* cell-free transcription/translation system provided by Promega was used to produce nascent chains of prolysyl oxidase. Each 50 μl reaction contained 25 μl of rabbit reticulocyte lysate, 1.0 μg of pSPLOX plasmid and other reagents as described by Promega. In control reactions, 40 μCi of translation-grade [^{35}S]methionine (1000 Ci/mmol) was used to label proteins. When ^{67}Cu was used, 300–400 μCi of carrier-free ^{67}Cu (3.8–4.5 mCi/μg of Cu) was added per reaction. The ^{67}Cu was supplied in 0.1 M HCl, which was neutralized by adding an equal volume of 0.1 M NH_4OH, and taken to dryness in a vacuum evaporator (Labconco, Kansas City, MO, U.S.A.). The dried product was redissolved in type-I water ($>$ 16.3 MΩ), and the process was repeated twice. It is important to note that neutralization of 0.1 M HCl with NaOH or sodium acetate interfered with the transcription/translation of both luciferase and prolysyl oxidase, whereas no inhibition was observed with the NH_4OH procedure. Reactions without added cDNA construct were used as controls for background incorporation of [^{35}S]methionine or ^{67}Cu. Ribonuclease inhibitor (40 units/μl of RNasin; Promega) was included in all reactions to inhibit degradation of mRNA, and all reactions were incubated for 2 h at 30 °C. A plasmid encoding luciferase was used as a control for the coupled transcription/translation reaction. β-Lactamase and α-mating factor mRNAs were used as controls to confirm the ability of canine pancreatic microsomes to carry out signal peptide cleavage and glycosylations respectively. For each of these assays, manufacturer's specifications (Promega) were followed.

Lysyl oxidase purification and isolation

Lysyl oxidase was isolated and purified by the methods of Romero-Chapman et al. [5] and Shackleton and Hulmes [17] with slight modification. Lysyl oxidase was extracted from gravid rat uteri (gestation day 18) into 0.05 M Hepes buffer, pH 7.5, containing 6 M urea (1:5, w/v; 12 h at 4 °C with constant stirring). After centrifugation (12000 ***g***; 60 min; 4 °C), DEAE-cellulose was added to the extract (1 g of DEAE-cellulose per 20–40 mg of protein), stirred for 60 min and washed thoroughly with buffered 4 M urea. Lysyl oxidase activity was eluted with five column volumes of buffered 4 M urea containing 2 M NaCl. The eluates collected from DEAE-cellulose chromatography were diluted to reduce urea concentration to 0.5–1.0 M. Eluates were next loaded into an affinity column 2 cm × 5 cm made of hydrated insoluble elastin and Sephacryl S-200 (mixed at a ratio of 3:1). When applied in 0.5–1.0 M urea, lysyl oxidase binds with high avidity to insoluble elastin and Sephacryl S-200 resin [1,17]. The column was washed with four column volumes of 0.05 M Hepes buffer containing 0.5 M urea. Lysyl oxidase was eluted with the addition of two column volumes of 0.05 M Hepes buffer containing 6 M urea. The final step in lysyl oxidase purification was gel-exclusion chromatography on a Sephadex G-100 column (1 cm × 30 cm; 4 M urea in 0.1 M borate buffer, pH 8.2) as described previously [1,5]. The functionally active form of lysyl oxidase was eluted in an approx. 30 kDa protein fraction, and was subsequently used as the standard for characterization of ^{67}Cu-labelled proteins. In some transcription/translation experiments, the proforms of lysyl oxidase were also partially purified using the insoluble elastin–Sephacryl S-200 affinity columns.

In some experiments, lysyl oxidase was separated on Superose 12 or Superdex 75 HR 10/30 FPLC columns (Pharmacia). The Superose 12 column was used for the separation of ^{67}Cu-associated proteins, peptides and unbound ^{67}Cu resulting from the addition of ^{67}Cu to cell-free transcription/translation reactions. The elution buffer contained 25 mM Hepes, pH 7.4, and 0.15 M NaCl. Fractions of 0.5 ml were collected at a flow rate of 0.5 ml/min. Cell culture experiments employed a Superdex 75 FPLC column which enhanced resolution of proteins in the 30–50 kDa range, i.e. the size range for different forms of lysyl oxidase. The inclusion of 4 M urea was necessary to maintain lysyl oxidase in an unaggregated state and dissociated from the structural proteins in the cell culture matrix. The presence of urea in the buffers acts also to decrease non-specific interactions between ^{67}Cu and proteins. All buffers were filtered twice through 0.2 μm nitrocellulose (Corning, Corning, NY, U.S.A.) and degassed before use. Proteins were eluted at a flow rate of 0.5 ml/min, and 0.16 ml fractions were collected. The fractions were stored at 4 °C. Absorbance was measured continuously at 280 nm in a flow cell spectrophotometer (Bio-Rad). ^{67}Cu was estimated by Minaxi γ-counter (Packard Instrument Company, Downers Grove, IL, U.S.A.). Radioactivity was corrected for isotope decay, and adjusted for total radioactivity added to the culture plates. BSA (57 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), cytochrome *c* (12.5 kDa) and aprotinin (6.5 kDa) were used as molecular-mass standards to calibrate the FPLC chromatograms.

Radioactivity eluted as lysyl oxidase was defined relative to: (1) elution of the lysyl oxidase standard, (2) estimation of lysyl oxidase activity using a fluorescence assay and (3) detection of lysyl oxidase by ELISA [5] in selected FPLC fractions. Data are expressed as the total amount of ^{67}Cu in lysyl oxidase-containing fractions divided by the amount of DNA in a given culture, and adjusted for radiochemical decay and total radioactivity added to a given culture.

Cell culture

Normal human skin fibroblasts (GM 05565) were purchased from the National General Medical Sciences, Human Genetic Mutant Cell Repository (Camden, NY, U.S.A.). Cell cultures

were maintained as described previously [18] in humidified atmosphere with 5 % CO_2 at 37 °C. Fibroblasts were propagated in plastic culture flasks in complete Dulbecco's modified Eagle's medium containing 10 % (v/v) fetal bovine serum, and penicillin and streptomycin at final concentrations of 100 units/ml and 100 μg/ml respectively. For the ^{67}Cu-incorporation experiments, cells at passages 6–8 were plated at 1×10^6 cells per 100 mm culture dish. When fibroblasts reached confluency (in 2–3 days), complete medium was replaced with serum-free medium (7 ml/plate).

To perturb steps in post-translational processing, fibroblast cultures were preincubated (3 h) with either tunicamycin (10 μg/ml), to inhibit N-linked glycosylation, or cycloheximide (10 μg/ml), to inhibit protein synthesis. Brefeldin A (10 μg/ml) was used to inhibit protein secretion. The peptidyl inhibitor, HS-CH_2-R-CH(CH_2CH[CH_3]$_2$)-CO-Phe-Ala-N_2, known to inhibit conversion of prolysyl oxidase into lysyl oxidase [19], and inactive control peptide, Ac-S-CH_2-CH(CH_2CH[CH_3]$_2$)-CO-Phe-Ala-NH_2 (Peptides International, Louisville, KY, U.S.A.), were added to fibroblast cultures at final concentrations of 2.5 μg/ml. Inhibitor, HS-CH_2-R-CH(CH_2CH[CH_3]$_2$)-CO-Phe-Ala-NH_2, was used to assess whether copper retention in lysyl oxidase was influenced by inhibiting the extracellular cleavage of prolysyl oxidase. For each treatment, the fibroblasts were incubated with 300 μCi of carrier-free ^{67}Cu (specific radioactivity 9–10 mCi/mg of Cu) per plate in serum-free medium for 5 h. Tunicamycin and cycloheximide were replenished at the beginning of the 5 h ^{67}Cu incubation, and the active and inactive inhibitor were replenished at 2.5 h. All treatments and controls were performed in triplicate.

At the end of the ^{67}Cu incubation, culture media were collected and solid urea was added to a 4 M final concentration. Cell layers were rinsed once with PBS and then scraped into 0.9 ml of 0.05 M Hepes-buffered 4 M urea. To prevent proteolysis, PMSF was added to a final concentration of 1 mM. Cells were disrupted by sonication, centrifuged at 10000 ***g***, at 4 °C for 30 min, and supernatants were collected and stored at 4 °C for analysis.

Lysyl oxidase functional activity

Lysyl oxidase functional activity was measured by a fluorescence assay based on H_2O_2 release as described by Trackman et al. [20]. Tropoelastin purified from copper-deficient chick aortae (50 μg per assay) or cadaverine (1 mM) was used as a substrate. The total assay volume was 2 ml.

Immunological detection of lysyl oxidase

Lysyl oxidase protein was detected in selected FPLC fractions by ELISA [5]. Selected FPLC fractions were dialysed overnight against PBS to remove urea and concentrated in a vacuum evaporator. The presence of lysyl oxidase was determined by ELISA using anti-(lysyl oxidase) antibodies produced in chickens [5]. Anti-(lysyl oxidase) antibodies were diluted 1:500 before addition to the antigen-coated microtitre plates (Dynatech Labs, Alexandria, VA, U.S.A.). Goat anti-chicken serum conjugated to horseradish peroxidase (diluted 1:1000) was used to detect lysyl oxidase–antibody complexes using 3,3,5,5-tetramethylbenzidine and H_2O_2 as substrates in 0.1 M sodium acetate/citric acid buffer, pH 6 [5].

Electrophoresis

In cell-free transcription/translation experiments, translation products were separated by discontinuous SDS/PAGE (6 % stacking gel, 10 % resolving gel) under reducing conditions [21]. In cell culture experiments, selected FPLC fractions were first concentrated in micro-concentrators with a molecular-mass cut-off of 3000 Da (Amicon, Beverly, MA, U.S.A.) and subjected to electrophoresis using either reducing SDS/PAGE conditions in the presence or absence of 4 M urea incorporated into the gels (6 % stacking gel, 10 % resolving gel) or native conditions in precast 4–20 % gradient gels (Bio-Rad). A commercial autoradiographic fluor was used according to the manufacturer's instructions to enhance the ^{35}S signal (Amplify, Amersham Life Sciences, Arlington Heights, IL, U.S.A.). In all experiments, gels were air-dried overnight between two cellulose sheets in a gel-drying frame (Promega). Dried gels were exposed to X-ray film (Hyperfilm-MP; Amersham Life Sciences) for autoradiographic or fluorographic detection of ^{67}Cu or ^{35}S respectively.

Protein and DNA assays

Protein concentration in cell extract supernants was measured by the method of Bradford using BSA as a standard [22]. The DNA concentration in cell layer extracts was determined fluorimetrically using fluorochrome H33258 [23].

RESULTS

In vitro cell-free transcription/translation experiments

The cell-free transcription/translation of lysyl oxidase from the pSPLOX plasmid in the presence of [^{35}S]methionine resulted in a non-glycosylated prolysyl oxidase migrating at 46 kDa in SDS/PAGE (Figure 1A). The expected product was observed only when the pSPLOX plasmid was included in the lysate as a

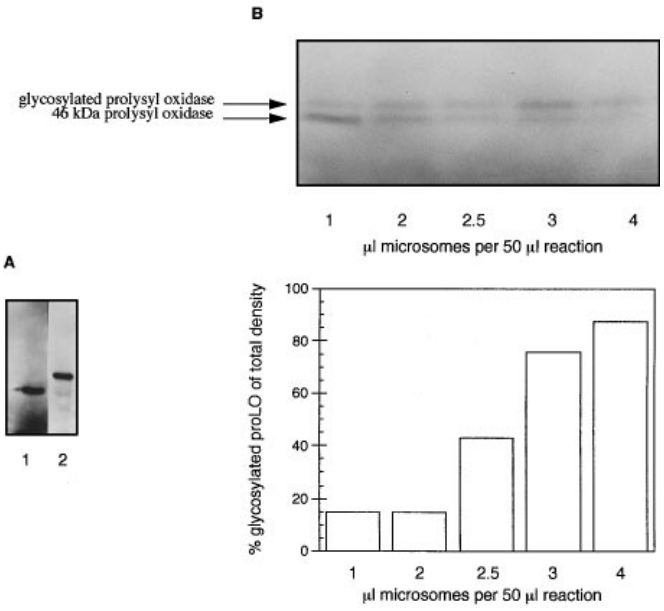

Figure 1 ***In vitro*** **cell-free transcription and translation of prolysyl oxidase in the presence of [^{35}S]methionine**

(**A**) *In vitro* transcription/translation of the pSPLOX plasmid using a cell-free rabbit reticulocyte lysate system as described in the Experimental section resulted in the expected 46 kDa product (lane 1). Luciferase cDNA was used as a control for *in vitro* transcription/translation (lane 2). (**B**) The addition of canine pancreatic microsomal vesicles resulted in a shift in the molecular mass of the product to 50 kDa, which corresponds to the size of the glycosylated prolysyl oxidase. The glycosylation of prolysyl oxidase was directly related to the amount of microsomal vesicles (added up to 4 μl per 50 μl of reaction mixture). The concentration of microsomal vesicles at 3 μl/50 μl of reaction mixture was used in subsequent ^{67}Cu-labelling experiments. LO, lysyl oxidase.

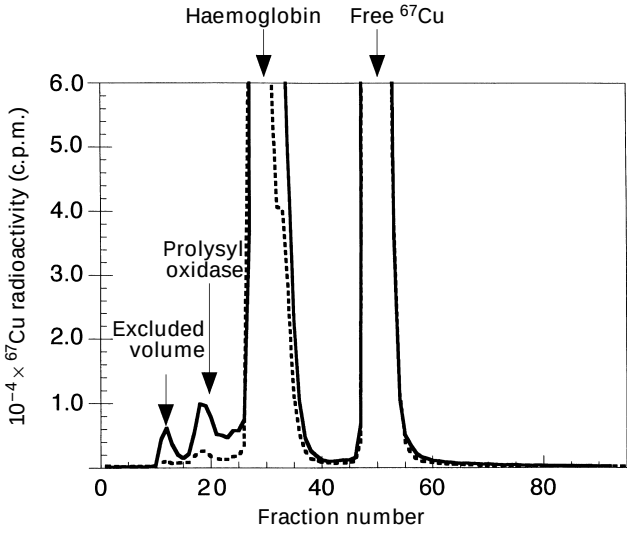

Figure 2 Fractionation of *in vitro* transcription/translation products on Superose 12

Aliquots of a cell-free transcription/translation reaction mixture containing pSPLOX plasmid (——) or control lysate without pSPLOX (– – – –) were fractionated on a Superose 12 column using 0.025 M Hepes buffer (pH 7.4)/0.15 M NaCl as eluent (flow rate, 0.5 ml/min). Radioactivity in 0.5 ml fractions was analysed by γ-counting and corrected for isotope decay. ^{67}Cu eluted between the void volume and haemoglobin was taken to represent prolysyl oxidase in an aggregated state. ^{67}Cu was also associated with the haemoglobin α- and β-chains, which are endogenously present in the reticulocyte lysate. Unbound ^{67}Cu was eluted in fractions 45–60.

template for transcription and translation. In the presence of microsomal vesicles, the 46 kDa product was glycosylated to a 50 kDa product. The processing of nascent prolysyl oxidase chains to the 50 kDa product was directly dependent on the canine pancreatic microsomal vesicle concentration (Figure 1B).

In attempts to separate ^{67}Cu-labelled products arising from transcription/translation in cell-free lysates, prolysyl oxidase was first partially purified using insoluble elastin–Sephacryl S-200 affinity columns. Recovery of lysyl oxidase as protein from each transcription/translation reaction was estimated to be 10–50 ng, based on the quantity of ^{67}Cu-associated protein retained on the elastin affinity or Superose 12 columns (Figure 2). Regrettably, insufficient amounts were generated for functional assay. The lysyl oxidase functional assay described by Trackman et al. [20] is sensitive to 100–200 ng of enzyme. Although ^{35}S-labelled glycosylated and non-glycosylated forms of prolysyl oxidase were easily detected, no protein-bound ^{67}Cu was observed when SDS was included in the PAGE system. Separation of ^{67}Cu-proteins by gradient PAGE using gels without SDS also resulted in artifacts. Electrophoresis of ^{67}Cu as copper chloride in the absence of protein resulted in broad bands of radioactivity in the region corresponding to the migration of 40–60 kDa proteins (results not shown).

Given the above, an FPLC system using a Superose 12 column was used to determine ^{67}Cu-labelled protein products in the lysate. As shown in Figure 2, the elution characteristics of ^{67}Cu from the Superose 12 column suggest that protein products arising from pSPLOX plasmid were capable of binding ^{67}Cu. The binding of ^{67}Cu to haemoglobin α- and β-chains was used as an internal marker. Although qualitative, these observations indicate that protein capable of binding copper was produced from cell-free transcription/translation of pSPLOX.

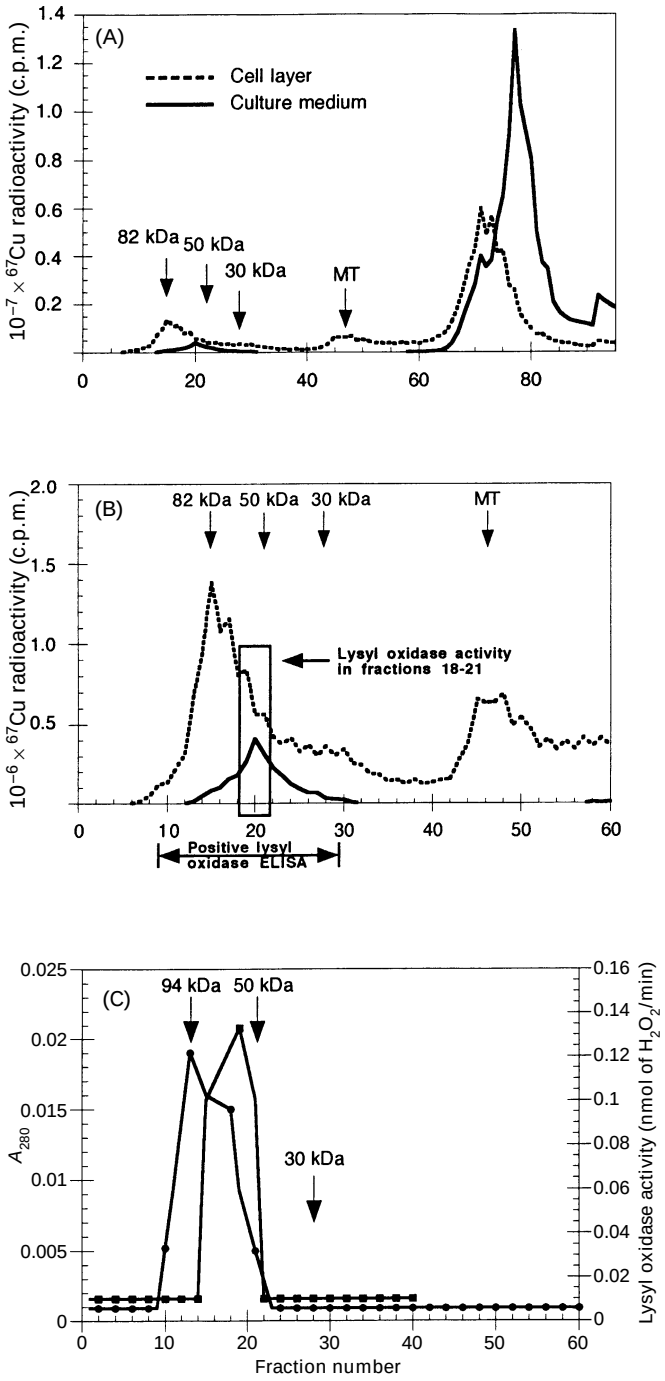

Figure 3 Fractionation of ^{67}Cu associated with the urea extracts of fibroblast cell layer or culture medium, and the elution of lysyl oxidase standard on Superdex 75

Aliquots (200 μl) of cell layer extract, culture medium and rat uterine lysyl oxidase standard were fractionated on a Superdex 75 column using 0.05 M Hepes buffer, pH 7.4, containing 4 M urea, 0.3 M NaCl and 0.02% NaN_3 as eluent (flow rate 0.5 ml/min; fraction volume 0.17 ml). Elution positions of 94, 82, 50 and 30 kDa proteins and metallothionein (MT) are indicated. (**A**) Representative elution profiles for a control cell layer (– – – –) and culture medium (——). (**B**) The region of the cell layer extract (——) and culture medium corresponding to 3–100 kDa includes lysyl oxidase and metallothionein. Lysyl oxidase as assessed by ELISA was observed in fractions 10–30. Most of the lysyl oxidase functional activity (> 80%) was eluted in fractions 18–21; for culture medium, the total lysyl oxidase activity corresponded to 0.8 nmol of H_2O_2/min or about 1 μg of lysyl oxidase. (**C**) Elution of the lysyl oxidase standard on Superdex 75 measured by A_{280} (●), and detection of lysyl oxidase activity as nmol of H_2O_2/min per fraction (■).

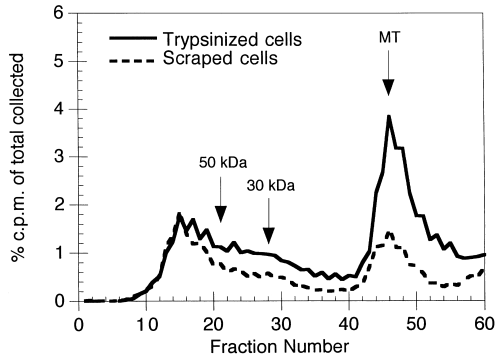

Figure 4 Fractionation of ^{67}Cu associated with the urea extracts of fibroblast cell layer (cells and matrix) or cells

To distinguish between the association of ^{67}Cu in cells and matrix, the fibroblasts were released from culture dishes either by treating them with trypsin or scraping them and associated matrix with a plastic cell lifter. Aliquots (200 μl) of urea extracts were fractionated on Superdex 75 column as described in Figure 3 and in the Experimental section. On average, cells collected by trypsin treatment (——) had 1.7 fold more ^{67}Cu eluted in the 30–50 kDa range, and twice as much ^{67}Cu radioactivity eluted as proteins and peptides in the 4–14 kDa range than the cells and matrix collected by scraping (– – – –).

Cell culture experiments

The elution of gravid rat uterus lysyl oxidase standard from Superdex 75 is shown in Figure 3(C). Although the starting material had an apparent molecular mass of approx. 30 kDa, upon storage and concentration, aggregation to dimeric and higher-molecular-mass forms usually occurred. This behaviour is similar to that reported for lysyl oxidase isolated from rat skin [1] and similar to observations by others [24,25]. Consequently, an unambiguous assessment of lysyl oxidase (based on size) elution from Superdex 75 was complicated by apparent aggregation. Therefore considerable emphasis was given to measurements of lysyl oxidase functional activity and response in ELISA in defining Superdex 75 fractions as lysyl oxidase. Lysyl oxidase activity was detected in fractions corresponding to a molecular mass of 50–60 kDa, whereas lysyl oxidase protein was immunologically detected in fractions corresponding to a molecular mass of 25–88 kDa (Figures 3A and 3B). With regard to protein secretion, all of the ^{67}Cu incorporated into protein in cultured fibroblast and released into the medium was eluted in fractions corresponding to the elution of lysyl oxidase standard (Figure 3C).

In attempts to separate matrix-associated radioactivity from that associated with cells, fibroblasts were released from the matrix by trypsin digestion using 2 ml of 0.25 % trypsin/1 mM EDTA. After 4 min, the reaction was stopped by the addition of an equal amount of soya-bean trypsin inhibitor. The elution profiles for ^{67}Cu-associated proteins from cells collected using trypsin treatment were similar to those for cells collected by scraping with a plastic cell lifter (Figure 4). However, there was a 1.7-fold increase in the percentage of total radioactivity eluted as proteins in the 30–50 kDa range and a 2-fold increase in the percentage of total radioactivity eluted as proteins and peptides, including metallothionein, in the 4–14 kDa range, in the trypsinized compared with scraped cells.

Excellent recoveries (> 80 %) of ^{67}Cu were obtained for all chromatographic runs. The observed distribution of ^{67}Cu represented proteins that have relatively high avidity for copper, because urea is effective in removing or partitioning copper from weak to moderate binding sites ($K_a < 10^7$). Of the total ^{67}Cu-labelled proteins (medium plus cells and matrix), 10–43 % was bound to proteins in the culture medium.

Inhibitors of protein processing and secretion

The responses of fibroblasts to cycloheximide, tunicamycin, brefeldin A or a procollagen C-peptidase inhibitor are given in Table 1. Since protein-bound ^{67}Cu in the medium was found to be principally lysyl oxidase (Figures 3A–3C), radioactivity in medium fraction relative to the ^{67}Cu-labelled protein in the matrix was taken to reflect the effects of inhibitors or incorporation of ^{67}Cu into lysyl oxidase, particularly the relative secretion of ^{67}Cu lysyl oxidase. Protein synthesis was clearly a prerequisite for copper incorporation and secretion. Concentrations of cycloheximide known to inhibit protein synthesis [27,28] blocked the appearance of ^{67}Cu-labelled proteins in medium FPLC fractions. However, glycosylation of lysyl oxidase did not appear to be essential for copper insertion and secretion. At concentrations of tunicamycin known to completely inhibit protein glycosylation in fibroblasts [29], there was a 39 % reduction in the secretion of ^{67}Cu-labelled protein into the medium relative to controls. However, no reduction was observed

Table 1 Inhibition of protein processing in cultured fibroblasts: ^{67}Cu incorporation into and secretion of ^{67}Cu-containing protein

Urea extracts of the cell layer or culture medium were fractionated on a Superdex 75 column, and collected fractions (0.17 ml) were analysed for ^{67}Cu. Incorporation of ^{67}Cu into protein was defined as ^{67}Cu eluted in fractions 8–60 (urea extract of cell layers) or fractions 8–40 (medium) as described in Figure 3. Values are means ± S.E.M. for three cultures and expressed as a percentage of the control cultures with no additions. Arsine transformation was applied to the percentage and ratio data before statistical analysis [26]. Values with different superscripts are significantly different at $P < 0.075$. The values designated medium are the percentage of total ^{67}Cu as protein relative to the total radioactivity as protein per culture. Radioactivity in the medium relative to that in the cell matrix is presented as a ratio. The inactive form of the peptidase inhibitor is acetylated at its thiol moiety.

	Control	Cycloheximide (10 μg/ml)	Tunicamycin (10 μg/ml)	Brefeldin A (10 μg/ml)	Peptidase inhibitor (2.5 μg/ml)	Inactive peptidase inhibitor (2.5 μg/ml)
Medium (% of total radioactivity)	7.8 ± 3.0^{a}	0.47 ± 0.01^{b}	$4.76 \pm 0.03^{a,c}$	4.2 ± 3.0^{c}	8.5 ± 3.0^{a}	10.0 ± 0.1^{a}
Medium/cell layer ratio	0.086^{a}	0.0046^{c}	0.088^{a}	0.058^{b}	0.115^{a}	0.193^{d}
$10^{-6} \times$ Radioactivity per culture (d.p.m.)	$1.69 \pm 0.51^{a,b}$	0.63 ± 0.02^{c}	$0.82 \pm 0.25^{b,c}$	2.21 ± 0.31^{a}	1.13 ± 0.14^{b}	$0.83 \pm 0.29^{b,c}$

in the amount of ^{67}Cu-labelled protein in medium when a correction was made for the decrease in cellular uptake/retention of ^{67}Cu caused by the addition of tunicamycin. In contrast, concentrations of brefeldin A known to transiently inhibit secretory vesicle assembly at the Golgi [30] caused a 46% reduction in ^{67}Cu secretion as lysyl oxidase after the 5 h exposure. Note that for many types of cell, protein secretion gradually returns to pretreatment level after prolonged exposure (> 4 h) to brefeldin A [30]. In a separate experiment, we confirmed that secretion of ^{35}S-labelled proteins was still significantly reduced (27% of the controls) after 5 h incubation with brefeldin A at concentration of 10 μg/ml. The presence of prolysyl oxidase peptidase inhibitor at a concentration known to inhibit the conversion of prolysyl oxidase into lysyl oxidase [19] had no inhibitory effects on the amount of protein-bound ^{67}Cu secreted into the medium, although ^{67}Cu uptake may be influenced, which is perhaps related to copper chelation.

DISCUSSION

Rayton and Harris [27] reported that chick aortae labelled with ^{64}Cu in culture produced a ^{64}Cu-labelled protein with the characteristics of prolysyl oxidase and lysyl oxidase dimers. They also reported that active protein synthesis was required before ^{64}Cu-labelled protein was detected in extracellular compartments. To our knowledge, no further studies have been published that define the specific details of copper incorporation into lysyl oxidase. This study was therefore designed to examine the incorporation of copper into lysyl oxidase using human skin fibroblasts in culture. Skin fibroblasts were chosen because 0.1–0.3% of the protein secreted from fibroblasts is lysyl oxidase. In addition, the majority of the protein-bound copper secreted from fibroblasts also appears to be associated with lysyl oxidase [1].

Lysyl oxidase activity detected in the FPLC fractions from the medium and cell layer corresponded to a 50–60 kDa protein. However, in defining the fraction containing lysyl oxidase, more emphasis was given to the ELISA detection because lysyl oxidase interacts with a number of matrix proteins [31,32]. For example, lysyl oxidase extracted from skin often co-purifies with a 22 kDa tyrosine-rich acidic matrix protein [33,34]. In addition, lysyl oxidase interacts to varying degrees with the solid-phase materials used for gel-exclusion chromatography [17] producing distortions in its elution profiles. Therefore it can be reasonably argued that most of the ^{67}Cu-labelled protein found in culture medium represented lysyl oxidase in various forms including free and aggregated (pro)lysyl oxidase and possibly aggregates of (pro)-lysyl oxidase with extracellular proteins.

Our studies, which indicate that active protein synthesis is required for copper incorporation, are consistent with findings of Harris and co-workers [27,35]. Cycloheximide blocked copper incorporation into secreted protein; moreover, pre-existing apoforms of lysyl oxidase did not appear to bind copper. In contrast, the relative incorporation of ^{67}Cu into lysyl oxidase was not inhibited by exposure of cells to high levels of tunicamycin. Similar responses have been observed for the binding of copper by unglycosylated caeruloplasmin [11] and for the binding of iron by unglycosylated lactoferrin [36]. Tunicamycin did not significantly affect the relative secretion of lysyl oxidase-associated ^{67}Cu. Thus, for many metalloproteins, glycosylation does not appear to be essential for the insertion of metal cofactors nor is it required for the secretion of these proteins.

Brefeldin A, which causes the disassembly of the Golgi leading to inhibition of protein secretion [37], inhibited the secretion of protein-bound ^{67}Cu into the culture medium by 46% as compared with controls. Inhibition of protein secretion by brefeldin A often results in accumulation of secretory proteins in the endoplasmic reticulum. However, our experiments indicate no accumulation of ^{67}Cu in cellular proteins, which suggests that either disassembly of the Golgi inhibited the incorporation of copper into lysyl oxidase or, if ^{67}Cu-labelled lysyl oxidase accumulated in the endoplasmic reticulum, it was rapidly degraded by intracellular proteinase(s). Similar observations to the latter alternative have been reported for another extracellular matrix protein, tropoelastin [38].

The proteolytic processing of prolysyl oxidase to lysyl oxidase is extracellular [39]. Recently, it has been suggested that procollagen C-peptidase is the enzyme that cleaves the prolysyl oxidase ($\sim$ 50 kDa) to lysyl oxidase ($\sim$ 30 kDa) [19,40]. Procollagen C-peptidase is a glycoprotein that cleaves the C-terminal propeptides in type-I, -II and -III procollagens extracellularly. This enzyme is secreted from mouse and human fibroblasts [41–43]. Importantly, a potent inhibitor of procollagen C-peptidase did not interfere with the relative secretion of ^{67}Cu-labelled lysyl oxidase. Thus our results indicate that neither inhibiting glycosylation of prolysyl oxidase (the propeptide contains the carbohydrate) nor cleavage of the glycosylated lysyl oxidase propeptide is qualitatively important to copper incorporation or binding to lysyl oxidase. Moreover, unglycosylated nascent chains of prolysyl oxidase generated by *in vitro* transcription/translation in cell-free reticulocyte lysate appeared to be capable of binding copper. The copper-binding domains of lysyl oxidase extend from amino acid 278 to 295 [44] or from 326 to 399 [16,45]. The lysyl oxidase clone generated in our laboratory had a single base substitution at position 1236 as compared with the sequence reported by Trackman et al. [15,16], resulting in a change of phenylalanine to serine in position 317. This residue is not thought to participate in the direct binding of copper to lysyl oxidase.

Copper incorporation into lysyl oxidase takes place either in the endoplasmic reticulum or during protein trafficking through the Golgi elements. In order to insert copper into secretory proteins, there needs to be a mechanism to shuttle cytosolic copper into the subcellular organelles. New data emerging for the eukaryotic copper transporter, a P-type Cu-ATPase [46–50], suggest such a mechanism. The cellular location of this protein is thought to reside in membranes of *trans*-Golgi vesicles [47a]. Secretory proteins are transported through the Golgi and to the cell membrane in vesicles formed by budding of, and fusion with, the subcellular membranes. It may be that the same vesicular events are required for copper secretion as for lysyl oxidase secretion. In Menkes disease, a genetic disorder characterized by abnormal copper metabolism, the mRNA of the copper transporter is either absent or very low [47a,48]. Lysyl oxidase activity is also greatly reduced in Menkes fibroblasts [18,47a–50]. A common pathway for the concentration and secretion of copper and lysyl oxidase could provide a basis for low lysyl oxidase activity in such cells, even though they accumulate high concentrations of copper.

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