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Role of Lysyl Oxidase Propeptide in Secretion and Enzyme Activity

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ABSTRACT

Lysyl oxidase (LOX) is secreted as a proenzyme (proLOX) that is proteolytically processed in the extracellular milieu to release the propeptide and mature, active LOX. LOX oxidizes lysyl residues of a number of protein substrates in the extracellular matrix and on the cell surface, which impacts several physiological and disease states. Although the LOX propeptide (LOX-PP) is glycosylated, little is known about the role of this modification in LOX secretion and activity. To gain insight into this issue, cells were transfected with native, full-length LOX cDNA (pre-pro-LOX), the *N*-glycosylation null pre-[N/Q]pro-LOX cDNA and the deletion mutant pre-LOX cDNA, referred to as secretory LOX, in which mature LOX is targeted to the secretory pathway without its N-terminal propeptide sequence. The results show that glycosylation of the LOX-PP is not required for secretion and extracellular processing of pro-LOX but it is required for optimal enzyme activity of the resulting mature LOX. Complete deletion of the propeptide sequence prevents mature LOX from exiting the endoplasmic reticulum (ER). Taken together, our study points out the requirement of the LOX-PP for pro-LOX exit from the ER and is the first to highlight the influence of LOX-PP glycosylation on LOX enzyme activity. J. Cell. Biochem. 111: 1231–1243, 2010.

KEY WORDS: ENDOPLASMIC RETICULUM; ER-ASSOCIATED PROTEIN DEGRADATION; LYSYL OXIDASE; PROPEPTIDE; GLYCOSYLATION; SUBCELLULAR LOCALIZATION

Lysyl oxidase (LOX) is a secretory amine oxidase which catalyzes the oxidative deamination of peptidyl lysine in elastin and peptidyl lysine and hydroxylysine in collagen, leading to inter- or intra-molecular crosslinks required for the insolubilization and stabilization of these proteins in the extracellular milieu [Kagan, 1986]. LOX activity is thus required for the biosynthesis of a functional extracellular matrix and in the morphogenesis and repair of connective tissue [Vater et al., 1979]. In addition to LOX, four LOX-like proteins (LOXL1, LOXL2, LOXL3, and LOXL4) of the same gene family have also been identified [Csiszar, 2001]. LOX activity has more recently been implicated in a variety of diverse biological processes including cell motility and migration, cell signaling and transcriptional regulation, altered chromatin condensation, tumor suppression and metastasis promotion [Payne et al., 2007], the modulation of PDGFR-β [Lucero et al., 2008] and TGF-β [Atsawasuwan et al., 2008] signaling.

The primary sequence and, presumably, the secondary and tertiary structural features of the LOX protein are highly conserved in mammals [Lucero and Kagan, 2006]. Human LOX is synthesized as a pre-proprotein (pre-pro-LOX) of 417 amino acids which undergoes a number of post-translational modifications within the endoplasmic reticulum (ER). After cleavage of the 21 amino acid signal sequence, the N-terminal propeptide, comprising 147 amino acid residues, is *N*-glycosylated [Trackman et al., 1992] and the C-terminal sequence containing the 249 amino acid residue mature protein is distinctively folded to acquire at least three disulfide bonds [Williams and Kagan, 1985]. Copper is a cofactor of the functional catalyst [Gacheru et al., 1990], presumably incorporated into the nascent enzyme within the ER [Kosonen et al., 1997]. The enzyme also contains a peptidyl organic cofactor, lysyltyrosine quinone (LTQ) generated by an intramolecular crosslink between lysine 320 and the copper-dependent oxidation

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product of tyrosine 355 [Wang et al., 1996]. Following secretion of proLOX to the extracellular space, the glycosylated N-terminal propeptide of the proenzyme is proteolytically separated to release the catalytically active mature LOX. This cleavage is catalyzed by procollagen-C-proteinase (BMP-1) or BMP-1-related metalloproteinases, generating the free propeptide and the fully active LOX [Cronshaw et al., 1995; Panchenko et al., 1996; Uzel et al., 2001].

Propeptides of secretory proproteins are released by proteolytic cleavage within the secretory pathway or in the extracellular compartment. This event generates mature proteins eliciting their biological activities at their destination. A key intracellular function of LOX-PP is most likely the maintenance of LOX in an inactive state within the secretory pathway [Kagan and Li, 2003]. Propeptides may also function as intramolecular chaperones to facilitate correct folding and the eventual targeting of these proteins to their destinations [Yasuda et al., 2005; Romero et al., 2008]. While the extracellular processing and activation of secretory pro-LOX upon release of the propeptide moiety have been elucidated [Cronshaw et al., 1995; Panchenko et al., 1996; Uzel et al., 2001] the intracellular role of LOX-PP within the secretory pathway has not been definitively described.

The unique architecture of proLOX, comprising a glycosylated pro-sequence upstream of the catalytic domain, with the latter structurally reinforced by six covalent bonds, suggested the possibility that glycosylation could be involved in targeting this molecule to the calnexin/calreticulum/Erp57/glucosyl transferase quality control complex in the ER [Parodi, 2000; Ellgaard and Helenius, 2001; Trombetta and Parodi, 2003]. Thus, we have hypothesized that the glycosylation of LOX-PP may act as an intramolecular chaperone for the folding of its catalytic domain. In the present investigation, we have tested this hypothesis and have revisited the intracellular role of the propeptide and its glycosylation in the secretion of pro-LOX and the subsequent generation of extracellular, active, mature LOX. By expressing different forms of LOX proteins in Chinese hamster ovary (CHO) cells, we have found that glycosylation of the LOX-PP is not essential for secretion and processing of pro-LOX, but is required for optimal enzymatic activity of secreted, mature LOX.

MATERIALS AND METHODS

CELL CULTURE AND STABLE TRANSFECTION

CHO-K1 cells were selected for expression of human LOX reconstructed genes since these cells do not express a detectable level of endogenous LOX [Kagan et al., 1995]. In addition, the expressed LOX constructs were tagged with V5/His epitope to allow for the antigenic detection of only recombinant proteins. Furthermore, the glycosylation and glycan processing systems in CHO and human cells are very similar and have been used in seminal contributions unraveling canonical molecular mechanisms of the early secretory pathway, including ER protein folding, ER quality control, and ER-associated protein degradation [Hammond and Helenius, 1994]. CHO cells (150,000 cells/T-75 tissue culture flask) were plated and maintained at 37°C in a 5% CO_2 atmosphere in DMEM/F-12 medium (GIBCO) containing 3.7 g/L sodium bicarbonate, 100 U/ml penicillin, 100 μg/ml streptomycin, non-essential amino acids (GIBCO), 1 mM sodium pyruvate (GIBCO), and 10% fetal bovine serum. At 70% confluence, cells were transfected for 24 h using Lipofectamine 2000 reagents (Invitrogen) at a ratio of 1 μg DNA/5 μl lipofectamine. Cells were passed into fresh growth medium and allowed to recover for an additional 24 h. Selective growth medium, containing 500 μg/ml of the antibiotic Geneticin (G418, Gibco-BRL), was added the following day. Cells were grown for 14 days or until all control (non-transfected cells) died. All surviving clones were collected as a heterogeneous pool of stable clones. Expression of the various recombinant forms of LOX was observed in all the cells analyzed by immunofluorescence (see below). In addition, integration of the reconstructed LOX genes into the chromosomal DNA of the stable cell clones was confirmed by PCR amplification of DNA fragments using the forward primer 5′-tgactatggctaccacaggcgatt-3′ within the coding region of mat-LOX and the reverse primer 5′-tctgcagaattccaccacactgga-3′ within the coding region of the C-terminal V5 peptide, to prevent the amplification of any endogenous form of LOX sequence potentially present in CHO cells.

PLASMID CONSTRUCTS DESIGN

The cDNA containing the complete open reading frame of human LOX (GenBank accession number NM_002317) flanked by *Eco*RI/*Bam*HI restriction sites within a pEGFP-N1 vector, kindly provided by Dr. Katalin Csiszar, was subcloned into a pcDNA 3.1/V5-His C vector (Invitrogen) using *Hin*dIII/*Bam*HI sites to produce pre-pro-LOX, C-terminally tagged with the V5/His epitope. The asparagine residues within the three consensus *N*-glycosylation sites at residues 81, 97, and 144 of the LOX protein sequence were mutated to glutamines by site-directed mutagenesis (Finnzymes, NEB) and additional PCR methods (TopGene Technologies, Montreal, Canada) to produce the glycosylation-null pre-[N/Q]-pro-LOX. The sequences of the signal peptide and the catalytic domain of pre-pro-LOX were independently amplified by PCR from the pEGFP-N1 pre-pro-LOX construct described above using the corresponding primers 5′-aatcaattacggggtcattagttcatagcccatatat ggagttccgcg-3′/5′-aggggttgtaagggtcgtcacagtgcactagcgcgcagagctgcaaaggc-3′ and 5′-gtgcactgcgacgaccttacaacccctacaagtactctgacg-3′/5′-cgcggatccc-gatacggtgaaattgtgcagcctgaggcatacgc-3′. These PCR products were then fused in frame by overlapping-extension using the primers 5′-aatcaattacggggtcattagttcatagcccatatatggagttccgcg-3′/5′-cgcgg-atcccgatacggtgaaattgtgcagcctgaggcatacgc-3′. The resultant PCR fragment was amplified by subcloning into a pCRII-TOPO vector (Invitrogen), then subcloned into a pEGFP-N1 plasmid using *Eco*RI/*Bam*HI sites and finally into a pcDNA3.1/V5-His C vector (Invitrogen) using *Hin*dIII/*Bam*HI sites, to generate pre-LOX, wherein the entire propeptide is deleted and the signal sequence and mature protein are fused in frame. Sequence fidelity of all the constructs utilized in this study was confirmed by sequencing the final cDNA constructs (Tufts Core Sequencing Facility; Physiology Department, Boston, MA). A schematic representation of the reconstructed LOX genes is shown in Figure 1A. Pre-pro-LOX is the native construct encoding the signal sequence (pre), the propeptide (pro), and the mature enzyme (LOX). Based on the premise that *N*-glycosylation of secretory proteins is essential for their interaction with the ER conformational quality control systems

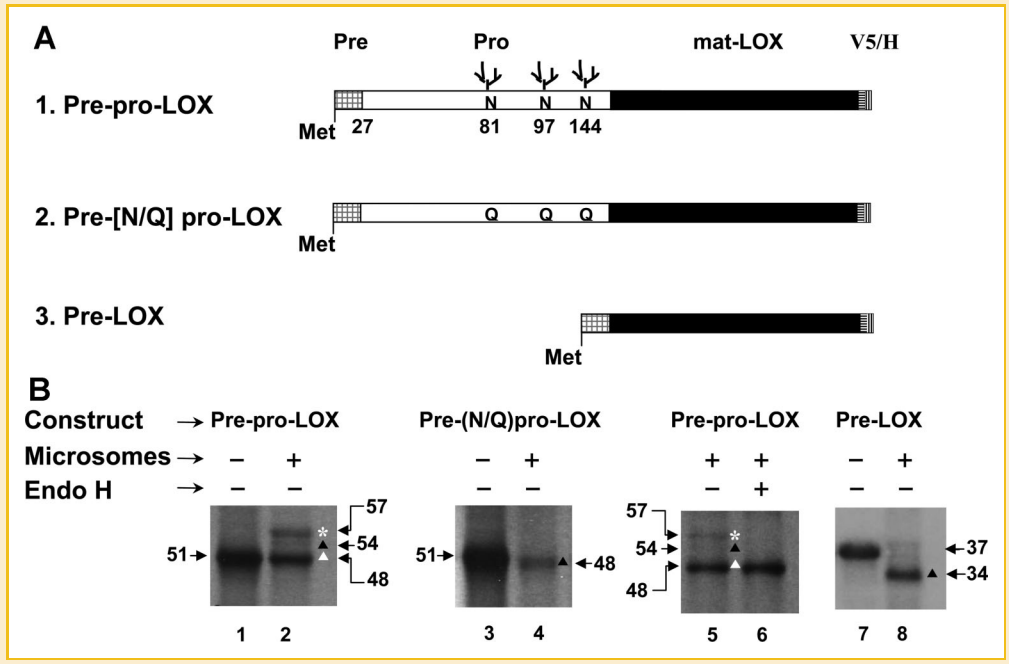

Fig. 1. A: The configuration of the constructs is represented by bar diagrams. (1) Pre-pro-LOX includes the complete open reading frame of human lysyl oxidase and encodes the signal sequence (pre, grid-pattern bars), the propeptide (pro, white bars), and the mature enzyme (mat-LOX, black bars). (2) Pre-[N/Q] pro-LOX encodes the glycosylation null mutant. (3) Pre-LOX encodes mature LOX targeted to the ER. Numbers under the pre-pro-LOX and pre-[N/Q] pro-LOX bar diagrams indicate the position in the encoded primary protein sequence starting from the initiation methionine (Met). B: In vitro transcription, translation, and translocation of LOX cDNA constructs. SDS–PAGE of in vitro transcription–translation products. Additions or omissions to the reaction mix are indicated by the + and − symbols, respectively. The molecular mass (kDa) of the translation products are indicated on the side of the lanes. The 57 and 54 kDa bands are the triple-glycosylated pre-pro-LOX and pro-LOX, respectively. The 51 and 48 kDa bands are the non-glycosylated pre-pro-LOX and pro-LOX, respectively. The 37 and 34 kDa bands are pre-LOX and mat-LOX, respectively.

[Ellgaard and Helenius, 2001], the asparagine residues of the three predicted *N*-glycosylation sites within the propeptide domain of pre-pro-LOX were mutated to glutamine residues to generate the *N*-glycosylation-null mutant construct, pre-[N/Q]pro-LOX. The role of the propeptide in the secretion of the proenzyme (pro-LOX) was assessed by using a construct (pre-LOX) generated by the deletion of the entire propeptide sequence from pre-pro-LOX. This yielded the LOX signal peptide fused in frame with the catalytic domain and endowed the catalytic domain with the potential to be secreted. This form of LOX, artificially engineered to be a secretory molecule by itself, will be henceforth referred to as secretory LOX (sec-LOX), to distinguish it from the mature LOX (mat-LOX) which results from the processing of pro-LOX or [N/Q]pro-LOX in the extracellular milieu. The constructs were tagged with the V5 epitope at their C-termini to specifically monitor the expressed proteins. Recombinant LOX bearing a V5 epitope tagged at its C-terminal end has been shown to be normally secreted [Thomassin et al., 2005].

IN VITRO TRANSCRIPTION, TRANSLATION, AND TRANSLOCATION ASSAYS

In vitro transcription of wild-type pre-pro-LOX, the glycosylation-null mutant (pre-[N/Q]pro-LOX) and pre-LOX cDNA constructs was performed with T7 RNA polymerase in a reaction mixture (50 μl) containing 1 μg cDNA. Transcripts were translated with rabbit reticulocyte lysate in the presence or absence of canine pancreatic microsomal membranes, highly enriched in ER membranes, at 30°C for 90 min using the TNT Quick Coupled Transcription/Translation kit (Promega). Aliquots (5 μl) of each sample were dispersed in 20 μl SDS–polyacrylamide gel electrophoresis (PAGE) sample buffer containing 50 mM DTT and 10 μl aliquots of these mixtures were analyzed by 12% SDS–PAGE (see below) and autoradiography using BioMax MS film (Kodak) and a BioMax TranScreen LE screen (Kodak). Aliquots (5 μl) of the pre-[N/Q]pro-LOX translation–translocation products were incubated for 1 h at 37°C in a medium (10 μl) containing 750 U of EndoH (New England Biolabs) and 1% SDS. The reactions were terminated by addition of 40 μl SDS–PAGE sample buffer containing 50 mM DTT and samples (20 μl) were analyzed by SDS–PAGE (see below) and autoradiography, as described above.

ANALYSIS OF INTRACELLULAR AND SECRETED RECOMBINANT LOX

CHO cells, expressing different LOX constructs, were plated (900,000/60 mm plate) and grown to post-confluence. The medium was changed to serum-free and phenol red-free medium (4 ml per 60 mm plate) and cells were further incubated for 24 h. Conditioned medium, containing the secreted LOX proteins, was collected and saved, and cell monolayers were rinsed twice with Hank's balanced salt solution (HBSS). To recover the intracellular recombinant LOX proteins, the cell monolayers were scraped in 1 ml of lysis buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris, pH 8.0, 0.2 mM PMSF, and 1 μl/ml Sigma protease inhibitor cocktail) and the resulting cell lysate was collected by centrifugation at 16,000*g* for 20 min at 4°C.

Supernatants were stored at −80°C prior to analysis by SDS–PAGE/Western blotting (see below). The conditioned medium saved as described above was supplemented with 0.2 mM PMSF, 1 μl/ml Sigma protease inhibitor cocktail, centrifuged at 200*g* for 5 min to clear it of cells and the supernatant was stored at −80°C until immunoprecipitation and SDS–PAGE/Western blotting analysis were performed (see below).

IMMUNOPRECIPITATION FOR DETECTION OF SECRETED RECOMBINANT LOX

The saved aliquots of conditioned medium (3 ml) were analyzed for secreted recombinant LOX proteins. The composition of the medium was adjusted to 150 mM NaCl, 1% Triton X-100, and 50 mM Tris (pH 8.0) and incubated with 5 μl (1.0 μg/μl) anti-V5 antibody (AB3792; Upstate) for 1 h at 4°C with mild shaking. Protein A/G PLUS-agarose (60 μl; Santa Cruz) was added and incubated for 20 h at 4°C with mild agitation. Following centrifugation, three washes in lysis buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris, pH 8.0) and two washes in PBS, proteins were eluted by adding 40 μl 1× SDS–PAGE sample buffer (50 mM Tris–HCl (pH 6.8), 10% glycerol, 2% SDS, 0.1% bromophenol blue, and 100 mM DTT). Samples (35 μl) were loaded onto 12.0% PAGE gels in the presence of SDS. Western blotting using anti-V5 HRP (Invitrogen) was performed as described below. Alternatively, secreted recombinant proteins were detected in the conditioned medium that was concentrated 10-fold in a Centricon Utracel YM-10 filter (Millipore).

WESTERN BLOT ANALYSES

Protein samples, prepared as described above, were resolved by 12.0% SDS–PAGE and electrophoretically transferred to PVDF membranes in transfer buffer (25 mM Tris, 102 mM glycine, 20% methanol) using a Mini Trans-Blot apparatus (Bio-Rad) run at 30 V overnight at 4°C. Equal protein loading was routinely assessed by staining the membranes with 0.1% (w/v) Ponceau S. in 5% acetic acid. The membranes were treated, incubated with the primary antibodies anti-V5-HRP (R961-25; Invitrogen), or anti-CNX (ab13505; Abcam), and developed using chemiluminescence reagents as described [Lucero et al., 2008]. Images were captured using the Molecular Imager Gel Doc XRS (Bio-Rad). The exposure time was set to achieve maximal sensitivity without reaching pixel saturation. As a result, the image in Figure 2B showed a granular background texture without compromising the resolution of the bands in the blot.

INDIRECT IMMUNOFLUORESCENCE

For indirect immunofluorescence, CHO cells were plated (150,000 per well; 2-well Lab Tek II slide). At near confluence, cell layers were processed for indirect immunofluorescence, incubated for 1 h with a 1:500 dilution of monoclonal anti-V5-FITC antibody (Invitrogen) in 10% FBS/PBS at 25°C in the dark and imaged in a Nikon Eclipse 400 microscope as described [Lucero et al., 2008]. The area corresponding to the fluorescent signal in microphotographs was quantitated using the ImageJ v1.40g imaging processing and analysis tools in Java (Wayne Rasband, National Institutes of Health, USA, in the public domain at http://rsbweb.nih.gov/ij/). At least 10 randomly selected fluorescent images from each condition were first converted to grayscale and then to the binary mode, using the automated threshold, to calculate their area (mm^2) by the particle analysis tool.

SUBCELLULAR FRACTIONATION BY EQUILIBRIUM SEDIMENTATION

Four plates of CHO cells expressing each LOX construct were plated (4.0×10^6cell/150 mm plate) and grown to post-confluence. Cell layers were treated, homogenized, freed of nuclei and cell debris, and subjected to Optiprep gradient centrifugation as described [Lucero et al., 2008] Aliquots (30 μl) of each gradient fraction were resolved in 4–15% polyacrylamide gradient gels (Bio-Rad) [Lucero et al., 2008]. Western blotting using HRP-conjugated anti-V5 antibody (R961-25; Invitrogen) or anti-calnexin antibody (ab13505; Abcam) (ER marker) was performed as described above.

ER-ASSOCIATED PROTEIN DEGRADATION STUDIES

CHO cells were plated (75,000 per well; 4-well Lab Tek II slide), and, near confluence, the cell layers were washed twice with PBS and fresh medium containing epoxomicin (0 or 1 μM dissolved in DMSO) was added and cells were incubated for 2 h under standard tissue culture conditions. Control cells received identical volumes of DMSO. Cells were fixed and examined by indirect immunofluorescence as described above. To assess changes in protein accumulation with epoxomicin treatment, cells were plated (900,000 cells/60 mm plate) and similarly exposed to 0 or 1 μM epoxomicin for 1 h at 37°C. Cell layers were collected and SDS–PAGE/Western blotting was performed as described above. Parallel thymidine incorporation assays were performed to assess cell viability with 1 μM epoxomicin treatment.

LYSYL OXIDASE ACTIVITY ASSAY

CHO cells expressing different LOX cDNA constructs, were plated (900,000/60 mm plate) and grown to post-confluence. Cell monolayers were washed three times with 10 ml HBSS and incubated with serum-free and phenol red-free medium (4 ml per 60 mm plate). Secreted LOX activity was determined in samples of conditioned medium that were withdrawn from cultures at different time points. Sample volumes, normalized to 10^6 cells, were clarified by centrifugation at 10,000*g* for 5 min and concentrated 10-fold by ultrafiltration at 4°C in Centricon Ultracel, 10 kDa MW cut off devices (Millipore, Amicon Ultra). Protein concentration in samples was determined by the bicinchoninic acid (BCA) protein assay (Pierce) using bovine serum albumin as standard.

LOX activity was determined fluorometrically in 10 μg/50 μl aliquots by monitoring the production of H_2O_2 through the oxidation of Amplex Red (Invitrogen) using synthetic peptidyl-lysine heteropolymer lysine/tyrosine (4:1) (20–50 kDa, Sigma–Aldrich) at 5 μg/ml as the LOX substrate in the assay mixture as described [Lucero et al., 2008]. We have used this peptidyl-lysyl heteropolymer instead of the commonly used diaminopentane or *N*-hexylamine as a LOX substrate to prevent potential interference by amino oxidase activities. One unit of activity is defined as the amount of enzyme required to produce 50 pmol H_2O_2/min, using purified LOX from bovine aorta [Kagan and Cai, 1995] as a standard. The kinetics of fluorescence change was monitored using a TECAN Infinite M200 microplate reader.

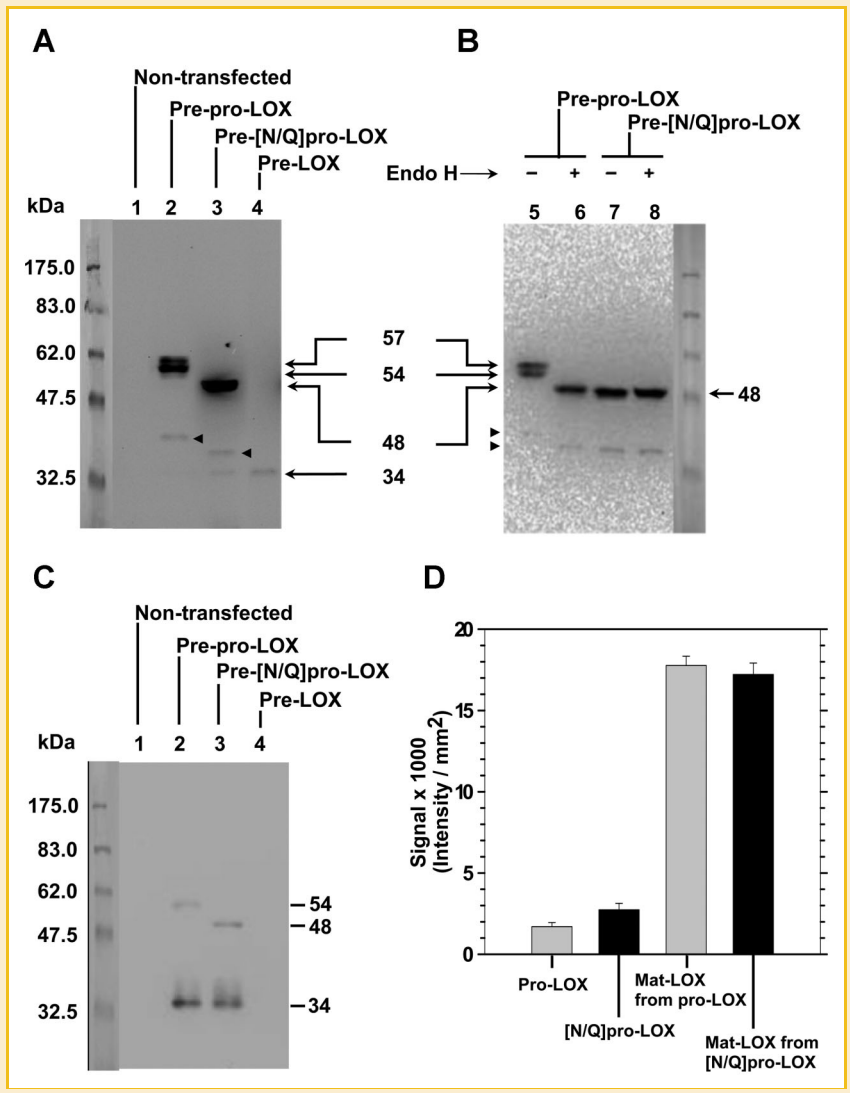

Fig. 2. A: Expression of LOX cDNA constructs in CHO-K1 cell layers were analyzed by Western blotting of the whole cell lysate using anti-V5 antibody. Arrowheads indicate N-terminally truncated fragments of unknown origin. B: Samples treated with EndoH. Note: lanes 3 and 4 in panel A of the original figure were swapped to facilitate the presentation of the results. C: Immunoprecipitation, and detection of LOX proteins in the conditioned medium of cell expressing LOX cDNA constructs. The molecular mass (kDa) of LOX proteins are indicated on the side of the figures. D: Densitometry plot of the Western blot in (C). Error bars are the standard deviation of the mean from three independent determinations. Note: lanes 3 and 4 of the original figure were swapped to facilitate the presentation of the results.

RESULTS

IN VITRO TRANSCRIPTION, TRANSLATION, AND TRANSLOCATION OF LOX cDNA CONSTRUCTS

Pre-pro-LOX and pre-[N/Q]pro-LOX constructs were first tested in an in vitro coupled transcription–translation reaction in the absence and in the presence of microsomes, to assess the production of full-length proteins and their glycosylation status. The pre-pro-LOX construct yields a single 51 kDa protein in the absence of microsomes. In the presence of microsomes two additional proteins of 57 and 54 kDa (faint band) were observed (Fig. 1B; lanes 1 and 2). The predicted molecular weight of the 417 amino acid residues encoded by the full-length LOX transcript fused to the cDNA encoding the V5-HIS tag is 49.5 kDa. Thus, the 57 kDa protein is the triple glycosylated form of full-length LOX that converts into the 54 kDa form after cleavage of the signal sequence. Both, glycosylation and cleavage of signal sequence indicate translocation to the lumen of the ER. It has been shown [Rutkowski et al., 2001] that the addition of microsomes to this in vitro translation assay medium partially inhibits protein synthesis (compare the densities of the 51 kDa protein in Fig. 1B, lanes 1 and 2). It has also been shown [Rutkowski et al., 2001] that the efficiency of in vitro co-translational translocation depends on the constructs used in the assay. This was manifested by the greater amount of the 51 kDa, non-glycosylated form of pre-pro-LOX over that of its 57 kDa glycosylated form (Fig. 1B, lane 2). In vitro translation of the pre-[N/Q]pro-LOX construct yielded a 51 kDa protein in the absence (Fig. 1B, lane 3) and a 48 kDa protein in the presence (Fig. 1B, lane 4) of microsomes, confirming the lack of glycosylation in this glycosylation-null mutant and the successful cleavage of its signal

sequence. The 57 kDa protein produced by the translation of pre-pro-LOX in the presence of microsomes (Fig. 1B, lane 5) is a glycoprotein, since this form and the 54 kDa signal-sequence-cleaved form are not observed upon treatment with endoglycosidase H (Endo H) (Fig. 1B, lane 6). Thus, it can be concluded that the pre-pro-LOX and pre-[N/Q]pro-LOX constructs encode the predicted full-length proteins; that pre-pro-LOX is glycosylated at asparagine residues in the lumen of the ER; that pre-[N/Q]pro-LOX is an *N*-glycosylation-null mutant; and that the signal sequences of these expressed proteins are cleaved.

The pre-LOX construct produced a 37 kDa protein in the absence of microsomes (Fig. 1B, lane 7) and a 34 kDa protein in the presence of microsomes (Fig. 1B, lane 8). This reduction in size corresponds to a loss of the 27 residue signal sequence. EndoH treatment of pre-LOX in the presence of microsomes did not cause further mobility shift, as anticipated by the lack of consensus *N*-glycosylation sites in the mature LOX (not shown).

EXPRESSION AND DETECTION OF LOX PROTEINS WITHIN THE CELL

Although mat-LOX is released into the medium after cleavage of the proenzyme, it can also be associated with fibrous proteins of the extracellular milieu, including collagen [Cronlund et al., 1985], elastin [Thomassin et al., 2005], fibronectin [Fogelgren et al., 2005], and cell surface proteins [Lucero et al., 2008]. Thus, pro-LOX is predictably localized intracellularly, within the secretory pathway, as well as in the extracellular milieu, while mat-LOX is detected in the extracellular compartment either free in the conditioned medium or bound to extracellular matrix proteins. In whole cell extract analysis of post-confluent cultures, it is expected that a certain level of the 34 kDa mature LOX be detected due to its adherence to extracellular matrix proteins.

Whole cell extracts of non-transfected cells lack antigenic reactivity against the anti-V5 antibody, as expected (Fig. 2A, lane 1). Cells expressing pre-pro-LOX display two major LOX precursor proteins of 54 kDa (triple glycosylated pro-LOX) and 57 kDa (triple glycosylated pre-pro-LOX) and a minor band of 40 kDa (arrowhead) (Fig. 2A, lane 2). This result revealed that a fully glycosylated pro-LOX undergoes cleavage of its signal sequence. In contrast, cells expressing pre-[N/Q]pro-LOX show a single band of precursor protein of 48 kDa and two minor bands of 37 kDa (arrowhead) and 34 kDa (mat-LOX) (Fig. 2A, lane 3). Considering that an N-linked glycan residue has an average molecular mass of about 2.0 kDa, the apparent molecular weights of the minor bands migrating as 40 and 37 kDa proteins in extracts of cells expressing pre-pro-LOX and pre-(N/Q)pro-LOX, respectively (arrowheads in Fig. 2A), suggest that these are the mono-glycosylated (lane 2) and non-glycosylated (lane 3) forms of an N-terminus truncated product. Furthermore, both of these forms include the glycosylation site at asparagine 144 (see construct diagram in Fig. 1). The 40 kDa product in cells expressing pre-pro-LOX is readily deglycosylated by Endo-H (Fig. 2B, lanes 5 and 6 arrowheads).

Although the origin of these 40 and 37 kDa fragments are unknown, it provides, by serendipity, evidence pointing to the glycosylation of asparagine 144. Cells expressing pre-LOX (Fig. 2A, lane 4) display only a minor 34 kDa band corresponding to sec-LOX. As indicated above, the 34 kDa band in lane 3 most likely originates in the extracellular milieu. In contrast, the 34 kDa band (Fig. 2A, lane 4) could be of extracellular or intracellular origin depending on whether sec-LOX expressed from the pre-LOX construct is competent for secretion or not. This issue is clarified in the results presented in Figure 2C. The steady-state levels of the precursor pro-LOX (lane 2) and (N/Q)-pro-LOX (lane 3) are visibly higher than the level of sec-LOX (lane 4), indicating that the presence of the propeptide domain, regardless of its glycosylation state, is critical for the optimal accumulation of LOX precursors. Thus, it is clear that sec-LOX fails to accumulate significantly (Fig. 2A, lane 4), suggesting that a degradation process may be involved.

The 54 kDa (triple glycosylated pro-LOX) and the 57 kDa (triple glycosylated pre-pro-LOX) proteins in cells expressing pre-pro-LOX (Fig. 2A, lane 2) were converted to a single 48 kDa protein by treatment with Endo-H (Fig. 2B, lane 6). Treatment of whole cell extracts from cells expressing pre-[N/Q]pro-LOX with Endo-H (Fig. 2B, lanes 7 and 8) did not alter the 48 kDa ([N/Q]pro-LOX) protein band, reinforcing the conclusion that the triple N/Q mutations generated an *N*-glycosylation-null mutant that is fully processed to lose its signal sequence. Interestingly, the lack of glycosylation in the [pre-[N/Q]pro-LOX seems to favor a complete processing of the signal sequence in vitro.

The apparent molecular weights of the precursor LOX proteins expressed by cells which had been transfected with pre-pro-LOX or pre-[N/Q]pro-LOX constructs (Fig. 2A) are consistent with those produced by the translation of these constructs in vitro (Fig. 1B).

These results also indicate that: (1) pre-pro-LOX is fully translocated and glycosylated in live cell cultures as judged by the lack of the 51 kDa protein in cells expressing pre-pro-LOX (compare lane 2 in Fig. 1B and lane 2 Fig. 2A), (2) pre-[N/Q]pro-LOX is not glycosylated, and (3) sec-LOX accumulated to a small extent, possibly within the intracellular compartment.

SECRETION OF RECOMBINANT LOX PROTEINS

Analysis of the cell-free, conditioned medium allows for the detection of only those proteins that are successfully secreted. Thus, proteins expressed and released into the cell-free conditioned medium from non-transfected cells and from cells expressing pre-pro-LOX, pre-[N/Q]pro-LOX, or pre-LOX were monitored by Western blot analysis.

Non-transfected cells displayed no signal (Fig. 2C, lane 1). As reported in other studies [Trackman et al., 1992; Thomassin et al., 2005] cells expressing pre-pro-LOX secrete a glycosylated 54 kDa pro-LOX protein and the 34 kDa mat-LOX (Fig. 2C, lane 2) but not the 57 kDa glycosylated species bearing the signal sequence observed in whole cell extract analysis (Fig. 2A, lane 2). Similarly, pro-LOX and mat-LOX are detected in conditioned medium from cells expressing the *N*-glycosylation-null construct pre-[N/Q]pro-LOX (Fig. 2C, lane 3), with (N/Q)pro-LOX migrating as a 48 kDa species, as expected. Densitometry analysis (Fig. 2D) showed that mat-LOX from pro-LOX and from [N/Q]pro-LOX accumulated in the conditioned media to a similar level (Fig. 2D). The N-terminal truncation products of 40 and 37 kDa observed in whole cell extracts (Fig. 2A, bands marked by the arrowheads) are not secreted. Remarkably, cells expressing pre-LOX cDNA, that is, mature LOX

targeted to the secretory pathway without the propeptide region (referred to as sec-LOX), completely failed to secrete mature LOX (Fig. 2C, lane 4). Thus, pro-LOX is secreted and processed regardless of its glycosylation state. Sec-LOX, without its N-terminal pro-region, fails to be secreted, consistent with a previous report [Thomassin et al., 2005]. Therefore, in the absence of the pro-sequence, the protein corresponding to the physiological mature LOX normally found in the extracellular milieu is targeted to the secretory pathway but fails to secrete.

SUBCELLULAR LOCALIZATION OF LOX RECOMBINANT PROTEINS

The analyses of proteins expressed in whole cell extracts (Fig. 3) suggest that recombinant LOX proteins can populate the secretory pathway within the cell. Indirect immuno-fluorescence (Fig. 3A) reveals that pro-LOX, [N/Q]pro-LOX, and sec-LOX displayed a reticular pattern with a peri-nuclear distribution, both characteristics of ER-localized proteins in CHO cells [Kato et al., 2006]. Additional evidence supporting the localization of LOX expressed proteins in the ER is presented in Figure 5. Pro-LOX and [N/Q] pro-LOX, to a lesser extent, populate the ER beyond the nuclear periphery. Conversely, sec-LOX is more restricted to a region of the ER closer to the nucleus (nuclear envelope ER). A similar subcellular localization pattern is observed when these proteins were expressed in rat fetal lung fibroblasts-6 (RFL-6) cells (not shown). The occupancy of larger ER areas by pro-LOX ($1{,}413.0 \pm 175.8\ mm^2$) and [N/Q]pro-LOX ($968.2 \pm 156.8\ mm^2$) when compared with sec-LOX ($405.7 \pm 74\ mm^2$) (Fig. 3B) is suggestive of higher steady-state levels of these proteins extending to transitional ER domains involved in exiting the ER [Shibata et al., 2006]. Conversely, sec-LOX accumulated mostly at the nuclear periphery where translocation to the ER as well as retro-translocation for degradation in the cytoplasm takes place [Shibata et al., 2006]. The steady-state accumulation of a secretory protein is maintained by the balance between its rate of synthesis and its rate of degradation and/or secretion. Since the steady-state levels of secreted pro-LOX and [N/Q]pro-LOX appear quite similar, and mat-LOX is not secreted at all (Fig. 2C), it is likely that the lesser accumulation of sec-LOX protein in the ER is due to a higher rate of degradation.

EPOXOMICIN AUGMENTS THE ACCUMULATION OF PRE-LOX IN THE ER

Incubation of cells for 1 h with 1 μM epoxomicin, an inhibitor of proteasomal activity [Meng et al., 1999], did not produce a detectable increase in the steady-state level of pro-LOX (Fig. 4A) or

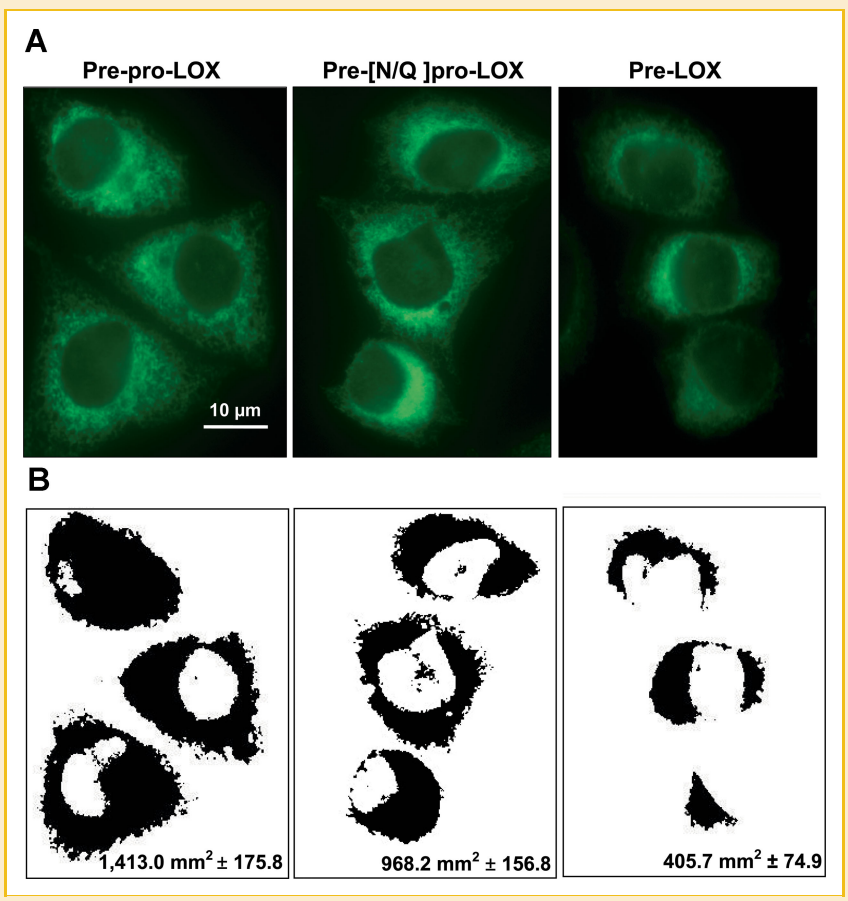

Fig. 3. V5/6xH-tagged, recombinant LOX proteins were detected by immunofluorescence microscopy of cell monolayers. A: The cDNA constructs used to transfect the cells are indicated on the top of the images. B: The areas corresponding to the fluorescent signal, shown in the binary mode. The calculated fluorescent areas and their corresponding standard deviations from 10 randomly selected images are shown in the bottom of the binary images. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

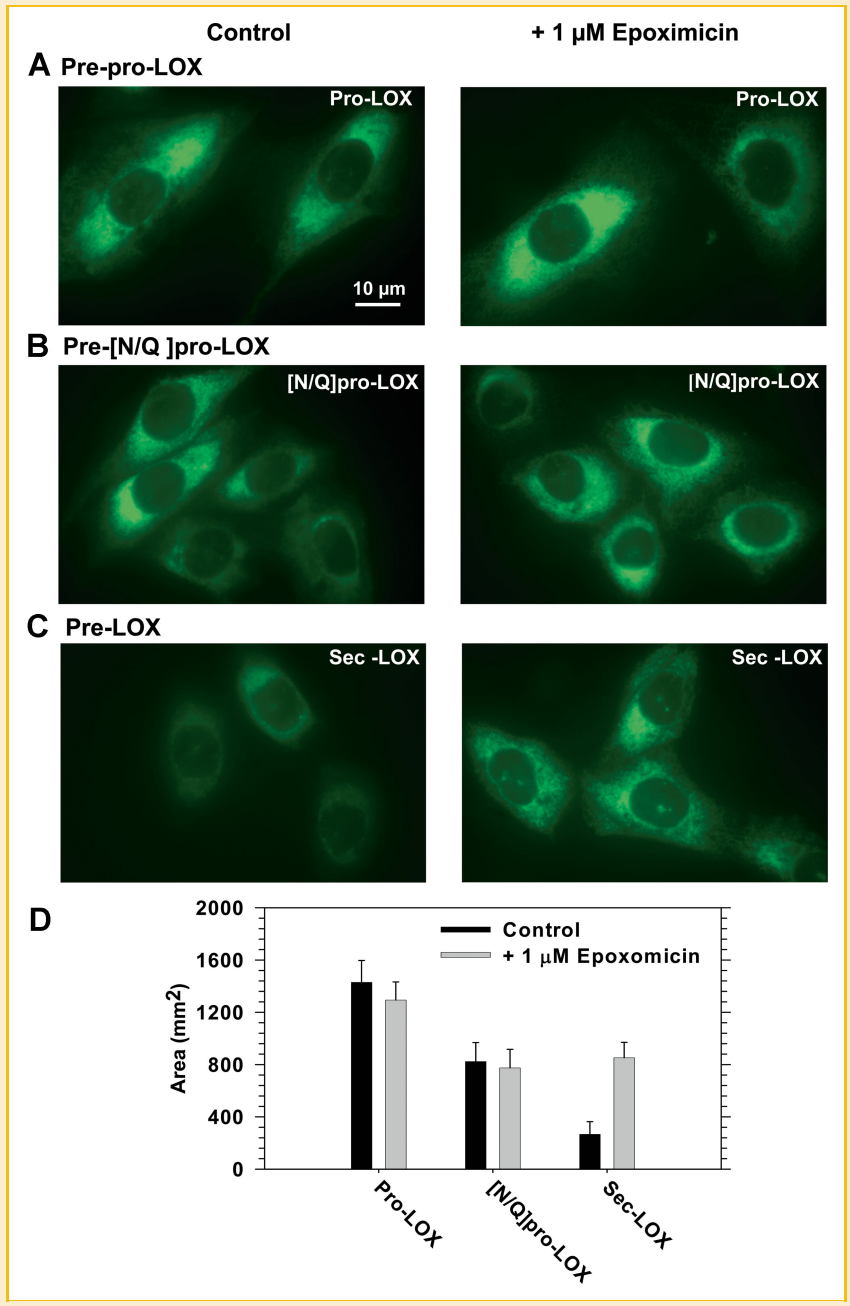

Fig. 4. Cells transfected with pre-pro-LOX cDNA (A), pre-[N/Q]pro-LOX cDNA (B) or pre-LOX cDNA (C) were incubated in the absence (control) or in the presence of 1 μM epoxomicin for 1 h and processed for immunofluorescence microscopy analysis as indicated in the legend of Figure 3. Plots (D) show the calculated occupancy area of the expressed proteins. Error bars are the standard deviations of the mean value obtained from 10 randomly selected images. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

[N/Q]pro-LOX (Fig. 4B). However, the level of sec-LOX was clearly increased by proteosomal inhibition (Fig. 4C). Similar results were observed when lactacystin [Romero et al., 2008] was used as the proteasome inhibitor (data not shown). The increase in sec-LOX steady-state level in the ER, induced by brief inhibition of the proteasome, did not drive the secretion of sec-LOX to the medium (data not shown). These results indicate that sec-LOX is targeted to the ER-associated protein degradation system (ERAD) [Tsai et al., 2002] for degradation by the proteasome [Brodsky and McCracken, 1999]. This finding may explain why sec-LOX accumulates at lower levels than Pro-LOX and [N/Q]pro-LOX. The calculated occupancy areas of expressed LOX proteins in the absence and in the presence of 1 μM epoxomicin are shown in Figure 4D.

LOCALIZATION OF EXPRESSED LOX PROTEINS IN THE ER

The subcellular localization of LOX proteins was further studied using sub-cellular fractionation by equilibrium sedimentation in a wide-range density gradient to resolve plasma membrane, endosomes, Golgi, ER, peroxisomes, and lysosomes (see the Materials and Methods Section). Each of the expressed LOX proteins sedimented

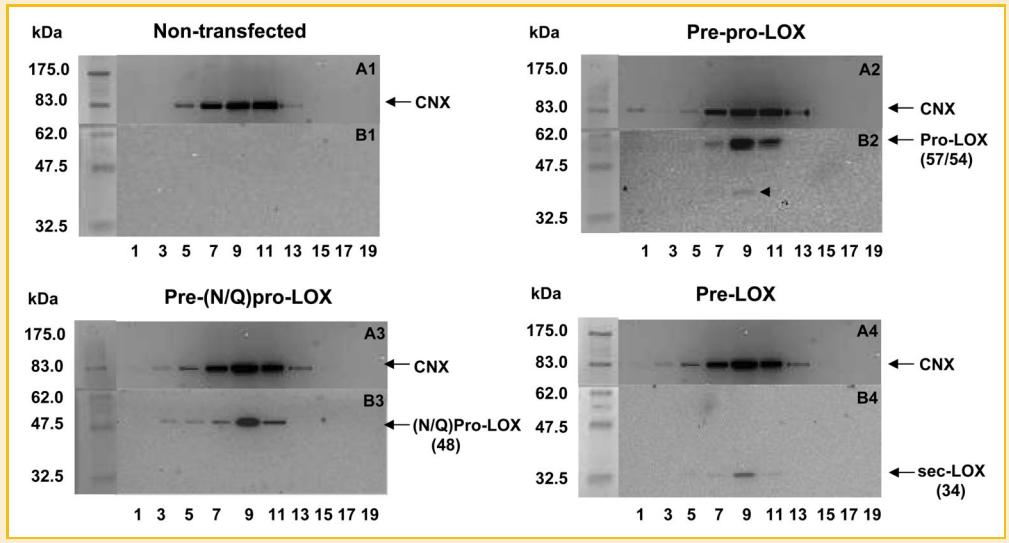

Fig. 5. Subcellular fractions of cells expressing the LOX cDNA constructs are indicated on top of the figures. Samples taken from the top of the gradient were sliced at around the 68–70 kDa mark and the upper portions (A) were probed with anti-calnexin (CNX) antibody to localize the ER fraction and the lower portions (B) were developed with anti-V5 antibody to localize LOX proteins. The ER marker CNX, as well as all the LOX proteins, floated at a density of 1.10 g/ml (fraction number 9), the floatation density of the ER fraction from mammalian cells. Numbers in parenthesis on the side of the figures are the molecular mass (kDa).

sharply around fraction number 9, with an approximate density of 1.10 g/ml, characteristic of the ER compartment in CHO cells [Zhang et al., 1998; Lefterov et al., 2000; Puglielli et al., 2001]. In addition, the identity of the ER fraction in the gradient fractions was confirmed by monitoring endogenous calnexin, a widely used ER fraction marker (Fig. 5, panels A1–4). Pro-LOX appears as a single, major 57 kDa protein (Fig. 5, panel B2) throughout the gradient fractions. Intriguingly, the signal-sequence-cleaved Pro-LOX of 54 kDa observed in whole cell extracts (Fig. 2) is barely detected in these ER gradient fractions. Conversely, the N-terminal truncation product of 40 kDa, first observed in whole cell extracts (Fig. 2A), is observed in the ER fraction (Fig. 5, panel B2 arrowhead). [N/Q]pro-LOX is produced as a single 48 kDa protein (Fig. 5, panel B3). Neither pro-LOX, nor [N/Q]pro-LOX were processed in the ER, as indicated by the lack of the 34 kDa mat-LOX (Fig. 5, panels B2,3). Sec-LOX accumulates in the ER as a single band of 34 kDa in cells expressing the pre-LOX construct (Fig. 5, panel B4). This demonstrates that the signal sequence containing 27 amino acid residues (2.85 kDa) is successfully cleaved after complete translocation of the three LOX constructs expressed. This result, rules out the lack of signal sequence cleavage as the basis for the lack of secretion of sec-LOX from cells transfected with the pre-LOX construct (Fig. 2C, lane 4).

THE RATE OF PRO-LOX SECRETION AND PROCESSING TO MATURE LOX IS INDEPENDENT OF PROPEPTIDE GLYCOSYLATION

The results in Figure 2C strongly suggest that glycosylation of pro-LOX in the pro-region is not essential for steady-state secretion or processing to release mature LOX. The possibility that the rate of secretion and/or processing of the secreted precursor to render mat-LOX could be influenced by glycosylation was investigated by monitoring the accumulation of mat-LOX in the conditioned medium from cells expressing pro-LOX and [N/Q]pro-LOX (Fig. 6). Mat-LOX protein was produced at a similar rate in both cases.

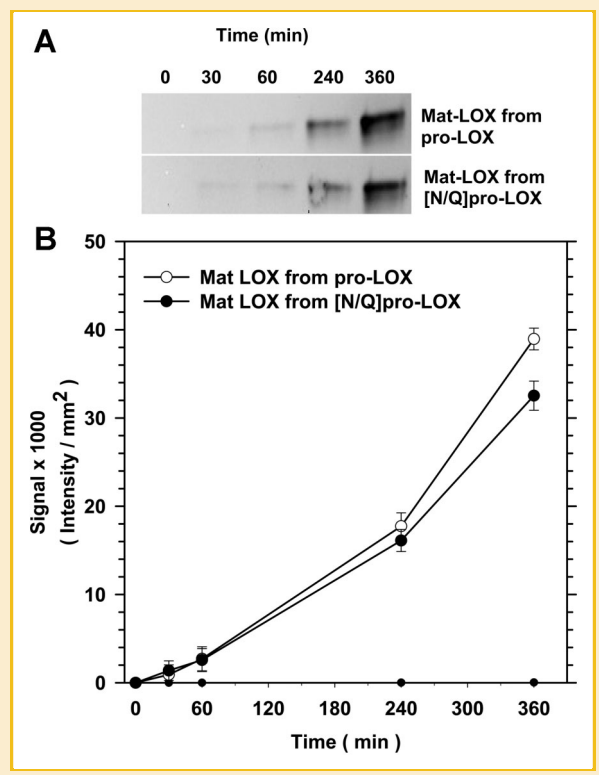

Fig. 6. Mature LOX in the conditioned media was determined by immunoprecipitation at the indicated times after addition of the serum-free medium, using anti-V5 antibody. A: Time course analysis by Western blot of secreted LOX from pro-LOX (glycosylated precursor) and [N/Q]pro-LOX (non-glycosylated) precursor. B: Densitometry analysis of the Western blot signals from (A). Bars are the standard error from three independent determinations.

Densitometry analysis of the signal in Figure 6A showed that the initial rate of appearance (0–60 min) of mat-LOX is not influenced by the glycosylation of the precursor (Fig. 6B). At longer times (>120 min), a slightly higher rate of accumulation is observed in mat-LOX derived from pro-LOX than from [N/Q]pro-LOX.

GLYCOSYLATION OF THE PROPEPTIDE IS REQUIRED FOR OPTIMAL LOX ACTIVITY

The observation that the secretion of LOX requires the propeptide region (Fig. 2C), and is independent of glycosylation (Figs. 2C and 6) led us to investigate the possibility that glycosylation of the pro-region influences the enzymatic activity of mat-LOX. LOX activity, assayed 120 min after adding fresh medium to the cell monolayers, was minimal in non-transfected cells (38.4 ± 18.5 mU/min) and showed its maximal value in cells expressing pro-LOX (255.7 ± 38.2 mU/min; Fig. 7A). On the other hand, LOX activity from cells expressing the glycosylation null mutant [N/Q]pro-LOX was 134.1 ± 33.9 mU/min, i.e., ~40% lower than the activity detected in cells expressing pro-LOX. Predictably, cells expressing sec-LOX failed to produce any activity above the level of non-transfected cells. A time-course analysis of LOX activity in cells expressing pro-LOX and [N/Q]pro-LOX reveals that less LOX activity is derived from the glycosylation-deficient precursor ([N/Q]pro-LOX) when compared to that of the glycosylated precursor (pro-LOX) and that this difference is sustained for up to 6 h (Fig. 7B).

DISCUSSION

Beyond its well-known role in the oxidation of collagen and elastin for maintaining the structure and function of the extracellular matrix [Kagan, 1986], LOX activity has been more recently implicated in normal and pathological processes [Lucero and Kagan, 2006]. Recent evidence has already underlined novel extracellular roles for the LOX-PP. In the extracellular space, the LOX-PP functions to recognize and bind proLOX to elastin, one of its extracellular substrates [Thomassin et al., 2005]. LOX-PP has also been shown to possess *ras* recision function in the extracellular milieu [Palamakumbura et al., 2003, 2004; Wu et al., 2007]. Conversely, the intracellular role of LOX-PP and its glycosylation within the secretory pathway have not been fully ascertained.

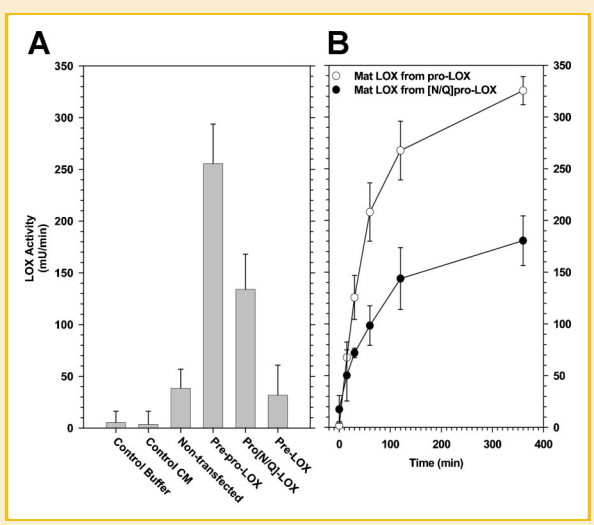

Fig. 7. A: LOX activity (mU/min) in conditioned media that was incubated for 2 h with non-transfected CHO cells and with cells transfected with the cDNA constructs indicated in the abscissa. B: Time course of LOX activity in the conditioned medium from cells expressing pro-LOX and [N/Q]pro-LOX (mU/min). Bars represent the standard deviation from the mean value of four independent determinations.

The optimal activity of a secretory enzyme like LOX depends on the acquisition of a native conformation through its folding pathway within the lumen of the ER and the associated post-translational modifications that occur throughout through the secretory pathway. Propeptides of a variety of secretory proproteins are released by proteolytic cleavage within the secretory pathway or in the extracellular compartment. This event generates mature proteins gaining their biological activities at their destinations. Thus, a key function of LOX-PP is very likely the maintenance of LOX in an inactive state within the secretory pathway [Kagan and Li, 2003]. Propeptides may also function as intramolecular chaperones to facilitate correct folding. For instance, the propeptide of the endosomal aminopeptidase cathepsin E [Yasuda et al., 2005] and pro-hormones [Romero et al., 2008] are essential for the correct folding, maturation, and eventual targeting of these proteins to their destinations.

The in vitro translation/translocation assays thoroughly confirmed that the expressed constructs used in this study (Fig. 1A) yielded the proteins of the expected sizes, which were translocated to the ER lumen where triple glycosylation took place only on the native full-length protein (pre-pro-LOX) but not in the glycosylation-null mutant (pre-pro[N/Q]-LOX) or in the propeptide-deletion mutant (pre-LOX) (Fig. 1B). The cleavage of the signal sequence was clearly detected in pre-LOX (Fig. 1B, lanes 7 and 8). Intracellular analysis of the proteins expressed in CHO cells revealed that all of these recombinant proteins are efficiently translocated into the secretory pathway (Fig. 2) and confirmed the *N*-glycosylation of three consensus *N*-glycosylation sites in pro-LOX as well as the lack of glycosylation in pro[N/Q]-LOX. This result also shows the lesser accumulation of sec-LOX compared to pro-LOX and pro[N/Q]-LOX.

We have previously suggested that the propeptide is not essential for the secretion of active LOX since deletion of the sequence coding for a large portion of the propeptide region did not alter these processes in CHO cells [Kagan et al., 1995]. However, a sequence encoding 30 residues of the C-terminal propeptide domain was retained in the LOX constructs designed in that previous work. Subsequent studies in 3T6-5 fibroblasts [Seve et al., 2002] and RFL-6 cells [Thomassin et al., 2005] in which the entire sequence encoding the propeptide was deleted suggested that the pro-region is required for the secretion of the enzyme. Thus, the propeptide appears to be essential for the secretion of mature LOX in 3T6-5 cells [Seve et al., 2002], RFL-6 cells [Thomassin et al., 2005], and CHO cells (Fig. 2C). This conclusion does not rule out the possibility that the essential role of LOX-PP in the secretion of mature LOX could be determined by the cell type. In fact, a recent report showed that mature LOX was secreted when expressed in human embryonic kidney 293 cells [Atsawasuwan et al., 2008]. It is not known if the secreted mature

LOX in 293 cells [Atsawasuwan et al., 2008] is active. In this context, the use of constructs similar to pre-LOX (Fig. 1A) to study the extracellular functions of the mature LOX, such as its promotion of invasive phenotypes or its oxidation of novel extracellular proteins, may mislead researchers absent of an independent confirmation that mature LOX is in fact secreted and active.

Early studies using tunicamycin to block *N*-glycosylation showed that pro-LOX secretion in normal human skin fibroblasts (GM 05565) is independent of glycosylation [Kosonen et al., 1997]. The extracellular analysis of LOX proteins expressed in CHO cells demonstrated that the pro-LOX and [N/Q]pro-LOX species were secreted and efficiently processed to generate mat-LOX while sec-LOX was not secreted (Fig. 2C). These findings demonstrated that, while glycosylation of LOX-PP is not required for secretion and extracellular processing the propeptide sequence as such is essential for secretion.

Visualization of expressed LOX proteins in the whole cell by indirect immunofluorescence revealed that while secretory pro-LOX

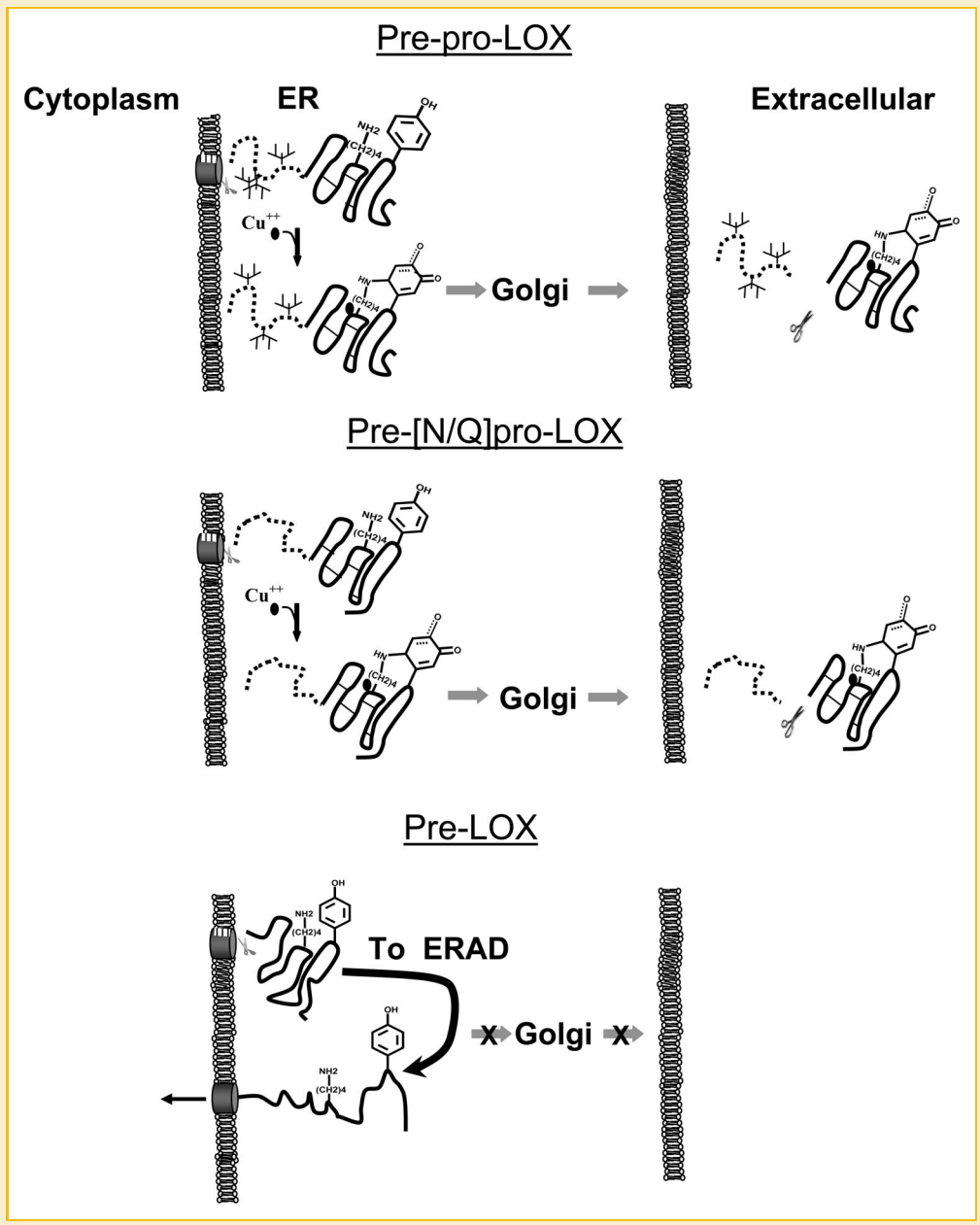

Fig. 8. In the lumen of the ER, pre-pro-LOX acquires three glycans in its propeptide and at least three disulfide bonds in the mature LOX sequence. In addition, Lys 320 and tyrosine 355 are crosslinked and a copper cation is incorporated into the enzyme moiety. Subsequently, pre-pro-LOX exits the ER, moves to the Golgi network, and finally reaches the extracellular compartment where mature LOX is released from the glycosylated propeptide by BMP-1. Pre-[N/Q]pro-LOX undergoes a similar folding pathway except that the lack of glycosylation in the propeptide results in a defective folded structure leading to a mature LOX with reduced activity. Pre-LOX never leaves the ER and is retro-translocated for degradation by the proteasome.

and pro[N/Q]-LOX accumulated throughout the ER, non-secretory sec-LOX was mainly observed in the perinuclear ER (Fig. 3), possibly due to its early retro-translocation for degradation. Evidence that sec-LOX is indeed retro-translocated for degradation in the ER was obtained when cells expressing this protein were treated with epoxomicin, a potent inhibitor of the proteasome (Fig. 4). Although the steady-state accumulation of sec-LOX was enhanced by epoxomicin, this did not result in secretion of sec-LOX (data not shown). Thus, in the absence of the propeptide, sec-LOX fails to leave the ER and is retro-translocated for degradation, presumably due to unsuccessful folding. Another possibility is that sec-LOX folds appropriately but, because of its lack of a propeptide, cannot interact with the COPII complex to exit the ER via vesicle budding and is then disposed out of the ER via retro-translocation. The observation that, in steady-state, pro-LOX and [N/Q]pro-LOX proteins are detected only in the ER (Figs. 3 and 5) is consistent with the retention caused by the various modifications to which these proteins are subjected in this compartment. The lack of sec-LOX in the ER of cells expressing pro-LOX and [N/Q]pro-LOX (Fig. 5) reinforces previous observations that these LOX species are not intracellularly processed [Trackman et al., 1992; Cronshaw et al., 1995; Panchenko et al., 1996] and that the mat-LOX detected in whole cell extracts (Fig. 2A, lanes 2 and 3) derives from the extracellular compartment. Finally, the lack of glycosylation resulted in lower enzyme activity of the mature LOX (Fig. 7). We hypothesize that this impaired activity of mature LOX deriving from a non-glycosylated precursor is due to some defect in the final folded structure of the enzyme. Thus, the propeptide is required for folding and its glycosylation enhances the efficiency of folding presumably through the interaction of its glycans with calnexin and/or calreticulin of the ER folding quality control system for glycoproteins. The non-glycosylated [N/Q]pro-LOX eludes the ER quality control system, but at the cost of lower activity of mature LOX. It is also possible that the rather bulky, highly hydrophilic *N*-glycan groups in LOX-PP help maintaining the polypeptide in solution during the folding process, thus facilitating the acquisition of the native folded state. This role of glycosylation in protein folding and stability was demonstrated in yeast acid phosphatase [Riederer and Hinnen, 1991] and invertase [Schulke and Schmid, 1988].

Remarkably, LOXL-1, the closest family member to LOX, has no predicted glycosylation sites in its propeptide and, contrary to the phenomenon presented here (Fig. 2C), the complete deletion of the LOXL-1 propeptide did not prevent the efficient secretion of mature LOXL-1 [Thomassin et al., 2005] This requirement for the *N*-glycosylation of LOX-PP to provide a mature LOX with optimal activity predicts a functional organismal hierarchy distinguishing LOX from LOXL-1. Indeed, while LOXL-1 knockout animals are viable [Liu et al., 2004], LOX knockouts are perinatal lethal [Mäki et al., 2002]. Another aspect that deserves further examination is how the ER-associated protein degradation operates on non-glycosylated proteins [Denic et al., 2006], exemplified here by the proteasomal-mediated degradation of sec-LOX (Fig. 4C). A model depicting the initial (ER) and final (extracellular compartment) steps of the LOX constructs studied herein is shown in Figure 8.

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