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Molecular Heterogeneity and Alternative Splicing of Human Lactoperoxidase

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Abstract

Human lactoperoxidase (LPO) exists as two distinct molecules independent of glycosylation. The N-terminus of one form is blocked and has not been identified while the other is proteolytically processed at the N-terminus similar to myeloperoxidase. Our analysis identified alternatively spliced human LPO mRNAs that may explain the observed molecular heterogeneity of LPO. Two mRNAs omit propeptide encoding exons while retaining the 5′ exon encoding the secretion signal, consistent with the heterogeneity and suggesting a possible functional role for the propeptide. Two LPO forms were expressed using baculovirus and both showed similar enzyme activity. LC/MS/MS analysis of trypsin digested, partially purified, salivary LPO confirmed the larger unprocessed LPO is present in saliva. To compare variant expression patterns, antisera were raised against purified recombinant (rhLPO) as well as against an antigenic peptide sequence within the exons encoding the propeptide region. Immunohistochemistry demonstrated proLPO was differently localized within gland cells compared to other forms of LPO. The data suggested splice variants may contribute to LPO molecular heterogeneity and its regulation by intracellular compartmental localization.

Keywords

human lactoperoxidase; mRNA splicing; propeptide; airways; submucosal glands

Introduction

Lactoperoxidase, a member of the mammalian heme peroxidase family, uses hydrogen peroxide (H_2O_2) to catalyze the oxidation of thiocyanate (SCN^-) and produce hypothiocyanite ($OSCN^-$) a biocidal compound. LPO acts against bacteria [1], viruses [2–6] and fungi [7–9] and is important in the prevention of mucosal infection. The LPO system has been identified in a variety of mucosal glandular tissues including salivary, mammary, and lacrimal glands, as well as in tracheal and bronchial submucosal glands.

Comparison of amino acid sequences obtained from purified LPO to sequences of cloned cDNAs and to mRNA transcripts predicted from the LPO gene suggests that LPO is proteolytically processed after synthesis to remove both a signal peptide and a propeptide similar to myeloperoxidase (MPO) biosynthesis [for review see 10,11]. Edman degradation of LPO, purified from milk, saliva and tracheal secretions, shows that much of the protein has a blocked N-terminus [12–14]. Although, the sequences obtained suggest that LPO is

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proteolytically processed at the N-terminus, it is possible that unprocessed LPO is also present with a blocked N-terminus. Heterologous expression of human LPO following cDNA transfection also results in truncated heterogeneous, N-terminal LPO sequences [14]. The function of LPO proteolytic processing is not known, however similar processing of MPO apparently does not play a role in regulating activity of the enzyme [for review,11].

LPO expression appears to be upregulated in lactating mammary tissue since LPO is found in colostrum and milk. Peroxidase in rat tracheal glands was reported to be increased by *Mycoplasma pulmonis* exposure of animals maintained in pathogen free conditions [15]. In contrast, LPO appears to be constitutively present in saliva of several species [10] and in sheep and human airways [13,16,17]. The mechanisms that regulate the expression and activity of LPO in response to the needs of epithelial host defense appear to vary among different tissues and little is known about factors regulating its expression and activity.

To date, no *in vitro* cell culture systems have been described that synthesize and secrete endogenous LPO in the amounts expected from its levels in milk, saliva, or airway secretions and thus hampering study of endogenous LPO biosynthesis. In this study we used primary airway epithelial cell cultures that expressed LPO mRNA. The data showed the existence of at least three alternatively spliced LPO mRNA variants expressed in several tissues.

Materials and methods

Materials

Unless otherwise noted all materials were obtained from Sigma Chemical Company (St. Louis, MO).

Cell culture

Airway epithelial cells were isolated from organ donor's lungs that were rejected for transplant. IRB approved consents were obtained by the Life Alliance Organ Recovery Agency and met requirements of the Declaration of Helsinki. Isolated cells were cultured on plastic to expand numbers and then plated on to human collagen IV coated two chamber inserts and redifferentiated at the air-liquid interface (ALI) as described previously [18–20]. Differentiation was monitored by the appearance of mucus and cilia on the apical surface of cultures. All experiments used fully differentiated ALI cultures.

Amplification and cloning of LPO Sequences

The entire LPO coding sequence was amplified from a human tracheal cDNA library [17] using oligonucleotide primers described by Shin et al. [21] and products were cloned (TOPO TA Cloning kit, Invitrogen, Carlsbad, CA) and sequenced. Specific oligonucleotide primers were also designed flanking the exons 3 and 4 (sense, 5′ TTCCCTCATCTTGCTTCAGG; antisense 5′ GCAGTCTCCCGTAATGGTG′). RNA was extracted from airway epithelial ALI cultures using TRIzol (Invitrogen, Carlsbad, CA) and cDNA was made using SuperScript First Strand Synthesis System for RT/PCR (Invitrogen Carlsbad, CA). RNA integrity was confirmed using RNA 6000 Labchips and a bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA) by the University of Miami DNA Microarray Facility. LPO cDNA was amplified by 35 cycles of 30 sec at 94°C, 30 sec at 60°C. and 45 sec at 72°C and followed by a final 5 min elongation at 72°C. PCR products were cloned using pGEM-T Easy Vector system (Promega, Madison, WI) and sequenced using the ABI Prism 3100 Genetic Analyzer (Applied Biosystems) at the Cardiovascular Genetics Lab Sequencing Facility at the University of Miami.

Production of Antibodies to Human LPO

Full length LPO cDNAs were expressed in a baculovirus-insect cell system as described by Shin et al [21]. Hi-Five insect cells were infected with recombinant baculovirus and cultured in suspension in the presence of ∂-aminolevulinic acid for 2 days at 28°C with stirring at 100 RPM. Culture medium was collected and centrifuged at 1000 RPM for 5 min at 4°C. Recombinant human LPO (rhLPO) was purified from culture supernatant as described by Shin et al. [21] and LPO enzyme activity was followed by TMB assay as described previously [17]. rhLPO was excised from SDS gels and utilized for the immunization of rabbits by Covance Laboratories (Hazelton, PA). The identity of purified rhLPO was confirmed following trypsinization and LC/MS/MS at the Protein Chemistry Core Facility at the University of Florida (Gainesville, FL). Antibodies were affinity purified using either native rhLPO or SDS denatured rhLPO coupled to agarose.

The LPO amino acid sequence MSSETPTSRQLSEYLK from exon 4 was synthesized, coupled to KLH and utilized to immunize rabbits by Covance Laboratories. Antibodies (anti-Ex4p1) were affinity purified using peptide coupled to beads with the SulfoLink Kit (Pierce Biotechnology, Rockford, IL) following the manufacturer's instructions.

Immunoblotting

Human samples were obtained using a protocol approved by the University of Miami Institutional Review Board. Tracheal aspirates were collected from patients undergoing general anesthesia for elective surgery indicated for non-pulmonary reasons, as previously described [22]. Saliva samples were collected from normal healthy individuals 2 hours after food intake. Prior to collection, mouth was rinsed with water and approximately 3ml of saliva was collected. Samples were concentrated by TCA precipitation and electrophoresed in Ready Gel SDS 7.5% polyacrylamide gels (Biorad, Hercules, CA). Gels were transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) and blocked overnight with 1% gelatin in 0.05% Tween 20 in Tris pH 7.6 buffered saline (T-TBS). Primary antibodies were used at 1 μg/ml in 10% heat inactivated human serum in T-TBS for 1 hour. Second antibody was alkaline phosphatase-conjugated affinity purified goat anti-rabbit IgG Fc fragment specific (Jackson Immunoresearch Laboratories, West Grove, PA) used at 0.02 μg/ml in 10% heat inactivated human serum in T-TBS. The blot was developed utilizing 5-bromo-4chloro-3-indoyl phosphate and nitroblue tetrazolium.

Immunocytochemistry

Normal human tracheas were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned by the Histology Laboratory at the University of Miami Hospital and Clinics, Sylvester Comprehensive Cancer Center. Autofluorescence of de-paraffinized sections was reduced by treatment with 5 mg/ml sodium borohydride in PBS. Sections were incubated in 10 mM citrate buffer (pH 6) for 15 min at 80°C, then blocked in 1% BSA in PBS, 1 h at room temperature and incubated overnight with 5 μg/ml anti-rhLPO or anti-Ex4p1 in blocking solution. Either non-immune rabbit IgG or peptide competed affinity purified anti-Ex4p1 were used as negative controls. Visualization was achieved using Alexa 555 labeled anti-rabbit IgG (Molecular Probes-Invitrogen, Carlsbad, CA). Sections were then labeled with either mouse anti- secretory leukocyte protease inhibitor (SLPI) (HyCult Biotechnology (Uden, The Netherlands) or mouse anti-Muc5AC (Chemicon, Temecula, CA) and visualized with Alexa 488 labeled anti-mouse-IgG (Molecular Probes-Invitrogen, Carlsbad, CA). Fluorescent images were obtained at the University of Miami Analytical Imaging Core Facility.

Results and Discussion

Identification of splice variants

PCR of a human tracheal cDNA library [17] (Fig. 1) using LPO specific primers (arrowheads, Fig. 2) that flank the previously identified coding sequence (exons 2 and 12, GenBank reference sequence **NM 006151.1**), produced three products of different sizes. Cloning and sequencing of these amplimers revealed that the largest (2.4 kb) and most abundant amplimer comprised the expected full-length coding region of LPO and is referred to as variant 1 (V1). The two smaller, less abundant amplimers were alternatively spliced mRNAs that were missing exon 3 (Variant 2, V2) or both exons 3 and 4 (Variant 3, V3), based on GenBank accession no. **NM 006151.1** (Fig. 2). RT-PCR with mRNA isolated from primary airway epithelial cells, (Fig. 3) and using specific oligonucleotide primers flanking exons 3 and 4 (arrows, Fig. 2) identified the same three splice forms of LPO mRNA. Sequence analysis of additional bands isolated from the gel (arrow heads, Fig. 3) showed mixed sequences with both forward and reverse primers; i.e. two nucleotide peaks at each position over a portion of the sequence, with a unique sequence at 5′ and 3′ ends. Comparison of these mixed sequences and their reverse complement to those obtained from the cloned amplimers (Fig. 1) showed the double peaks could be explained by heteroduplexes of V1, 2 and 3. Anomalous migration on the gels could be due to single stranded loops produced during re-annealing of the variant amplimers following the final cycle of amplification. V2 was previously identified through EST screening (GenBank accession nos. **BX486530**, **DB208370**, **CD722179**) while V3 represented a novel transcript (GenBank accession no. **EF579964**).

Examination of the predicted amino acid sequence of V2 showed that it encodes the translation start codon and predicted secretion signal peptide (exon 2), but skips exon 3 that shifts the reading frame and leads to termination immediately following the secretion signal peptide. On the other hand initiation at the next ATG down stream from the splice site would result in a truncated protein (652 aa) without a predicted signal peptide. On the other hand, V3, like V2, encodes the start codon and predicted signal peptide, however skips exons 3 and 4 that results in an in-frame deletion of the region that others have suggested are the propeptide based on N-terminal sequences detected by Edman degradation [14].

Thus, variant transcripts from the LPO gene appear to be alternatively spliced by skipping exons to generate mRNA that lacks the LPO propeptide but retains the 5′ untranslated region and peptide. In the case of V3, skipping exons is predicted although not shown to result in synthesis of mature LPO similar without proteolytic processing beyond removal of the secretion signal.

To determine whether these variant LPO mRNAs are tissue specific, mRNA was isolated from other LPO expressing tissues: salivary and mammary glands, and redifferentiated airway epithelial cell cultures as well as from liver and testis, as negative controls (Fig. 4). All three variants were present in the glandular tissues where LPO is known to be expressed, but V1 and V2 were also seen in testis. A search of Genbank showed testis-derived EST clones (**AA400148**) although the presence of the LPO protein in this tissue has not been reported. Liver did not contain LPO mRNA as expected. The presence of the LPO splice variants in other tissues where LPO is expressed showed that these variants are not specific to the airway. The relative quantities of the three variant mRNAs appeared to differ between these mRNAs, although V1 was always the predominant form. The relative intensity of the forms also varied among cell cultures from different individuals suggesting that regulation of splicing could be an important regulatory step (data not shown).

Previously reported salivary LPO polymorphisms are most likely unrelated to the splice variants described here since the former depend on disulphide bonding to other salivary

proteins [23]. Alternative splice forms of thyroid peroxidase [24,25] and MPO [26] have been reported. However, unlike the LPO variants, the two alternative splice forms of MPO are not believed to produce functional proteins as one exhibits an early stop codon and the other deletes the secretion signal needed for translocation into the secretory pathway [26].

Characterization of variant protein expression by Western blot analyses and immunocytochemistry

The exact function of the MPO and eosinophil peroxidase propeptide is not understood [11]. Unprocessed MPO is active and thus propeptide removal is not necessary to generate a functional enzyme [27]. LPO V1 and V3 were expressed in insect cells using baculoviruses. Both variants were secreted and were active although V1 was completely processed (see below). V1 and V3, were purified to near homogeneity as described by Shin et al. [21]. When compared to a standard curve using commercially prepared bovine LPO, Vi and V3 had similar enzyme activities equal to 0.14 and 0.34 ng of bovine LPO per ng of purified variant, respectively. V1 was used for antiserum production in rabbits.

N-terminal sequence analysis of LPO, purified from milk, saliva and tracheal secretions, shows the purified enzyme contains a proteolytically processed form with the new N-terminus (XTAIRN) encoded near the 3′ end of exon 4 [14,28], thus effectively removing the propeptide sequence. However, purified LPO typically shows a significant fraction of the enzyme has a blocked N-terminal amino acid preventing analysis of the majority of the protein [12,29]. The N-terminus of V1 expressed in insect cells appeared to be proteolytically processed as no difference in apparent MW was observed when compared to V3 on SDS gels.

In addition, no reactivity was observed with antiserum raised against a peptide from exon 4 (see below) and the most N-terminal peptide from baculovirus expressed V1 detected by LC/MS/MS (NGVWEESLKR) was located near the 3′ end of exon 4 overlapping the N-terminal peptide determined in LPO isolated from human milk [14]. Thus, proteolytic processing effectively prevented assessment of enzyme activity of proLPO in our recombinant expression system.

The absence of the propeptide in V3 suggested that these N-terminal coding exons may serve a specific function. To determine whether unprocessed, i.e. propeptide containing, LPO is present in secretions, antisera directed against a peptide in exon 4 (anti-Ex4p1) was used to compare with antiserum against entire recombinant human LPO (V1, anti-rhLPO). Human tracheal secretions, human saliva, purified rhLPO, human MPO and bovine LPO were examined by Western blot (Fig. 5). A single immunoreactive band was recognized in human tracheal aspirates by both anti-Ex4p1 and rh-LPO antibodies as reported previously with anti-sheep LPO antibodies [30]. Anti-rhLPO identified a major band (~80 kDa) and a slightly faster migrating faint band in human saliva. Anti-Ex4p1 identified the same immunoreactive bands in human saliva as well as several immunoreactive bands of high MW_{app} that could be competed with antigenic peptide (data not shown). These bands were restricted to saliva samples and although competed with peptide may be unspecific.. These results were similar to previous findings in which more than one band were observed in saliva utilizing antibodies against LPO [10,29,31,32]. As expected, anti-rhLPO detected the purified recombinant antigen (Fig. 5, lane 3), as well as bovine LPO (lane 4). Neither anti-rhLPO or anti-Ex4p1 recognized purified human MPO (lane 5, generous gift of Dr. Roger Fenna, University of Miami) even when three times more MPO was loaded compared to LPO indicating the antibody's specificity. All immunoreactive bands were still present after deglycosylation treatment and only an increase in their migration rate was observed (data not shown). Crossreactivity was not observed with purified rhLPO when anti-Ex4p1 was utilized (Fig. 5). Blots performed on saliva and human tracheal aspirates with either antisera show that the band patterns differ slightly from one individual to another in agreement with other reports [17,30]. These data showed that

a significant portion of immunoreactive LPO in secretions contains the exon 4-peptide and thus was at least partially unprocessed, in contrast to the reported N-termini for human LPO from milk determined by Edman degradation [14]. This observation was confirmed by LC/MS/MS analysis of purified human salivary LPO that demonstrated a peptide (QLSEYLK) overlapping with the peptide in exon 4 used for antisera production (data not shown).

The inconsistency between this data which suggested the presence of unprocessed proLPO with the reported processed N-termini of human [14] and bovine [28] LPO, may reflect the portion of LPO with blocked N-termini [12,14] in purified fractions that prevents Edman degradation of the longer form. Thus, the N-terminus detected by Edman degradation may represent only a portion of LPO in saliva and airway secretions.

Immunolocalization of proLPO

Recombinant MPO synthesized without the propeptide is less likely to be delivered to granules and more likely to be degraded, depending on the cell type used for expression [33,34] and thus the MPO propeptide may play a role in correct folding and maturation. For this reason we examined the subcellular localization of proLPO as compared to distribution of all LPO forms. Since V3 has no unique sequence it is not possible to distinguish V3 from processed V1 (proLPO) thus only unprocessed V1 can be specifically localized relative to all other LPO forms.

Human bronchi were used as these were readily available from our organ procurement organization. As seen previously [17], LPO was observed primarily in airway submucosal glands and localized to the basal region of gland acinar cells that appeared to be serous in nature based on hemotoxylin-eosin staining. To confirm and extend these earlier observations, anti-rhLPO was used simultaneously with mouse anti-SLPI antibody as a marker for serous cells [35] and with anti-Mucin5AC, a marker for mucous secreting cells. LPO was co-expressed with SLPI, confirming that LPO is expressed in serous submucosal cells (Supplemental Material) and in the majority of the lungs studied, LPO was not co-expressed in cells labeled with Muc5AC suggesting that LPO is not normally expressed in mucous acini (Supplemental Material). LPO was also detected in airway surface epithelial cells (Supplemental Material). Both anti-rhLPO and anti-Ex4p1 antibodies showed some cross-reactivity with smooth muscle cells and chondrocytes that was blocked by antigen competition. This cross-reactivity was not explored further and may be unspecific..

The vast majority of the anti-rhLPO antibody signal (Fig. 6B) was in the basolateral aspect of the cells whereas anti-Ex4p1 showed that proLPO was homogeneously distributed throughout the cells (Fig. 6*F*). Thus, LPO retaining exon 4 appears to be differently localized suggesting that it may also play a role in targeting LPO to a different intracellular pathway.

Conclusions

Reverse transcription of human LPO mRNA and amplification of cDNA unexpectedly demonstrated alternative splicing. Comparison of the alternative transcripts showed that exons encoding the predicted propeptide were skipped while retaining the secretion signal peptide and suggested that the propeptide may have an important function. Localization of propeptide containing LPO in airways suggested that it is differently distributed within cells. Although these studies do not increase our understanding of propeptide function, the data further support an important role for the LPO propeptide.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

LPO
Lactoperoxidase

rhLPO
recombinant human lactoperoxidase

SLPI
secretory leukocyte inhibitor

SCN^-
thiocyanate

$OSCN^-$
hypothiocyanate

MPO
myeloperoxidase

ALI
air liquid interface

LC/MS/MS
Liquid Chromatography/Mass Spectrometry/Mass Spectrometry

KHL
keyhole limpet hemocyanin

Ex4p1
exon 4 peptide

TCA
trichloroacetic acid

TBS
Tris buffered saline

T-TBS
0.5% Tween-20 Tris buffered saline

SLPI
secretory leukocyte protease inhibitor

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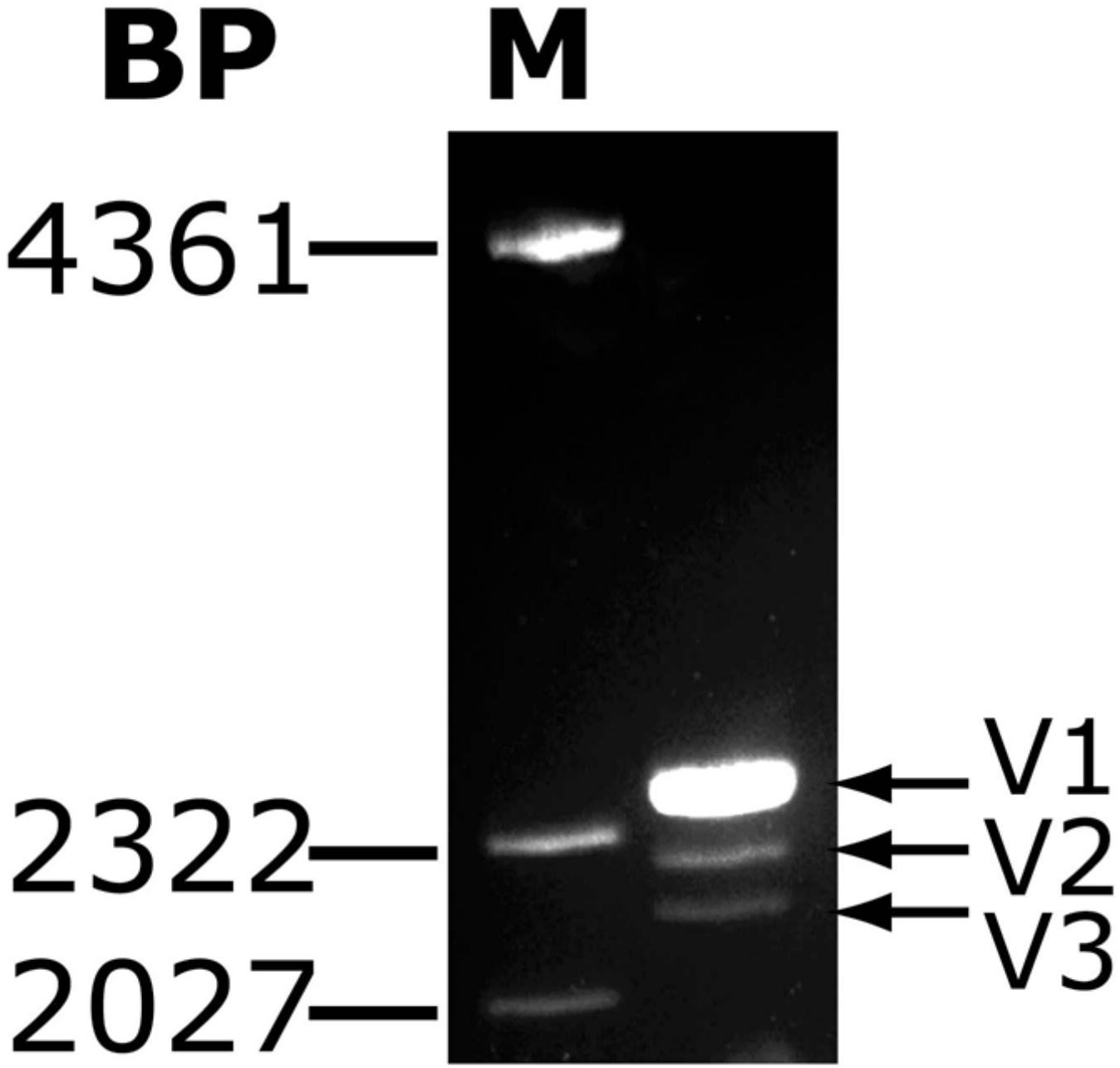

Figure 1.
PCR Identification of LPO splice variants in human airway. PCR with primers flanking the entire LPO coding region (see Figure 2) identified three variants in a human tracheal cDNA library (arrows). Lane M contains DNA size markers in base pairs (BP).

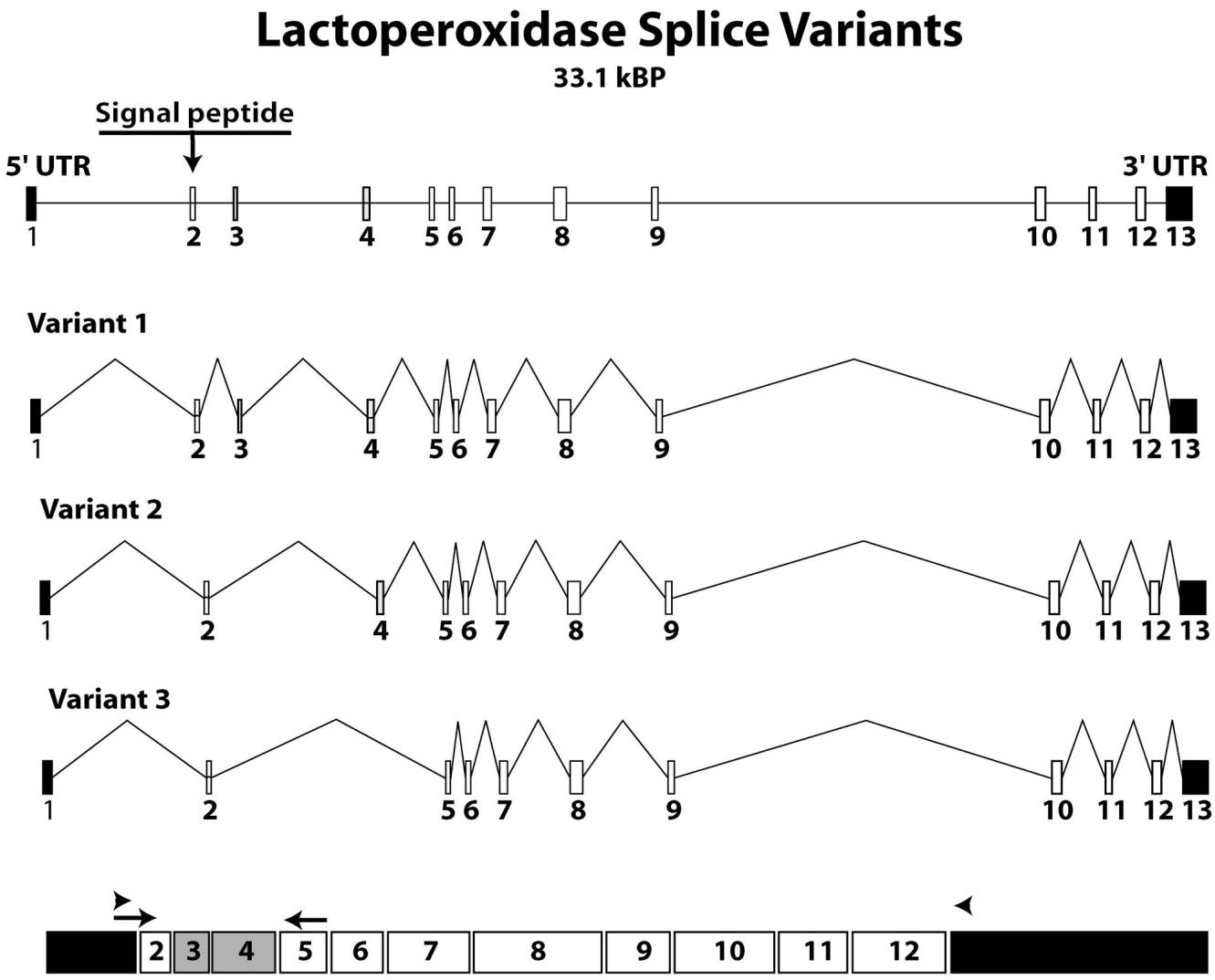

Figure 2.

Diagram of LPO gene exon/intron structure and exon skipping in mRNA splice variants. The LPO transcription unit is shown in the top line. Untranslated exons are shown as solid boxes, invariant exons as open boxes and alternatively used exons as lightly shaded boxes. The ATG initiation codon and entire signal peptide are contained in a single exon, exon 2, (vertical arrow). Exon 3 is removed in V2, while removal of coding exon 3 and 4 generates V3 and removes one potential glycosylation site that may contribute to the complexity of the published sizes of human LPO in milk, saliva, and airway secretions. Proteolytic processing of LPO in milk generates an N-terminus near the 3′ end of exon 4. Primers designed to amplify the full coding region are shown as arrowheads. Primers flanking Exons 3 and 4 are shown as horizontal arrows.

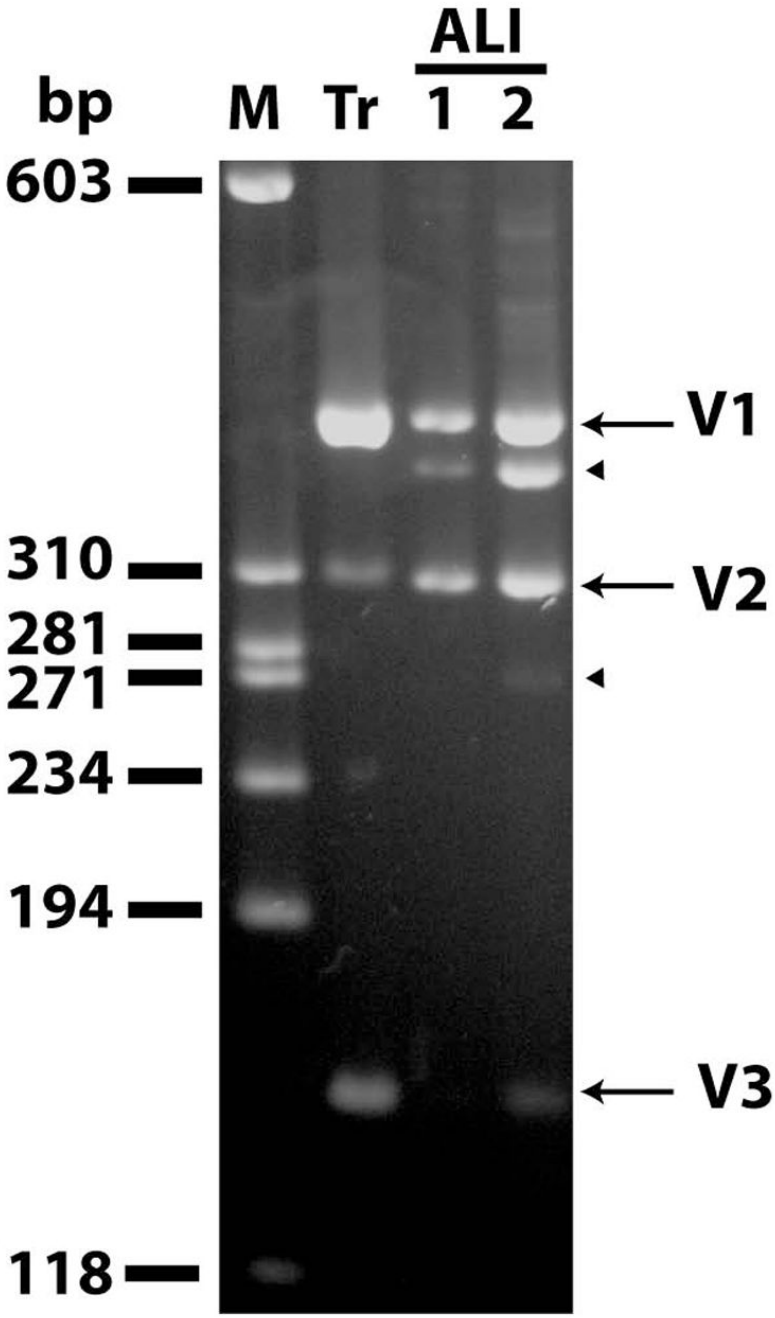

Figure 3.
LPO splice variants in human airway epithelia. PCR with primers located in exons 2 and 5 that flank the spliced regions identified three bands (401, 312, and 128 bp) in a human tracheal cDNA library (lane Tr) and in airway epithelial cell cultures (lanes ALI: 1 and 2, two separate lung donors). Additional bands at ~380 bp and 275 bp were observed (arrowheads) that likely correspond to heteroduplexes of V1, V2 and V3. DNA size markers (lane M) were Hae III digested φX174.

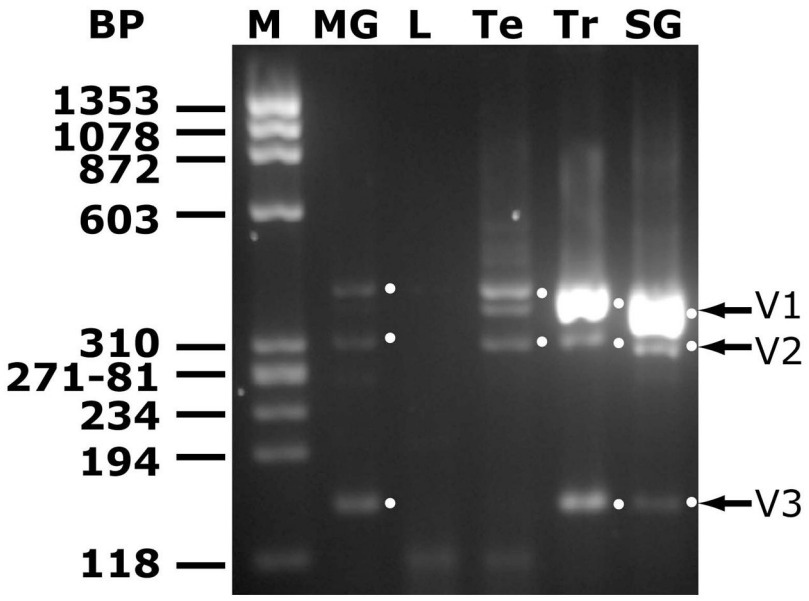

Figure 4.

LPO mRNA variants are present in other tissues. RT-PCR analysis (35 cycles) utilizing primers flanking the spliced regions identified three bands (401, 312, and 128 bp) in a human tracheal cDNA library (lane Tr), mammary gland (lane MG), and salivary gland (SG) RNA. Bands of similar size were also observed in testes (lane Te) but not in liver RNA (lane L). Markers (lane M) were Hae III digested φX174. Lanes containing no RT controls showed no amplimer and were removed from gels. Dots mark the position of the variants.

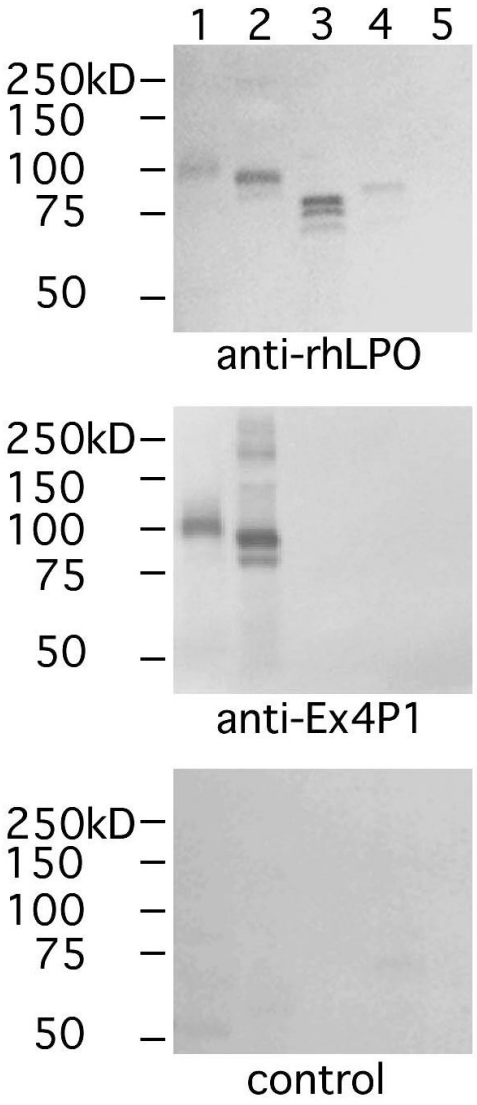

Figure 5.
Western blot analysis of proLPO in secretions. Antibodies against LPO (Anti-rhLPO); LPO propeptide (Anti-Ex4p1) and control (non-immune IgG), were used to probe human tracheal aspirates (100 μg, lane 1), human saliva (30 μg, lane 2), recombinant human LPO (0.1 μg, lane 3), bovine LPO (0.1 μg, lane 4), human MPO (0.3 μg, lane5). Blots were visualized using alkaline phosphatase conjugated goat anti-rabbit Fc fragment antibody.

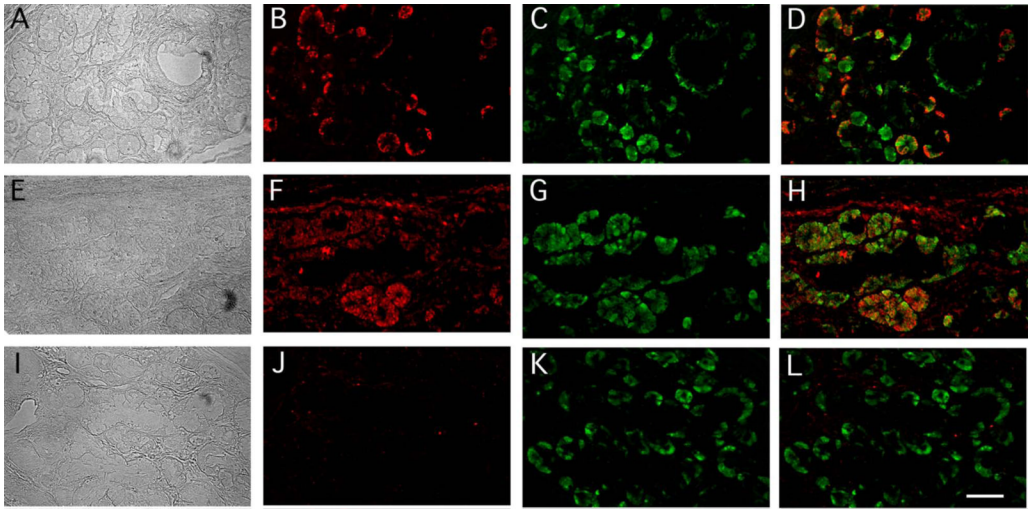

Figure 6.

Subcellular distribution of proLPO. Panels A, E, and I show phase contrast images of human tracheal sections double labeled with antibodies against rhLPO (Panel B) or anti-Ex4p1 (Panel F) and mouse anti- SLPI, (Panels C and G). Peptide competed anti-Ex4P1 (Panel J) was the negative control followed by either anti-rabbit IgG coupled to AlexaFluor555 (red) or anti-mouse IgG coupled to Alexa488 (green). SLPI was used as a marker for serous cells. Anti rhLPO detected localized to the basal region of gland acinar cells (Panel B). When anti Ex4p1 antibody was used, LPO was observed homogeneously localized throughout the glands (Panel F). Both antibodies detected LPO coexpressed with SLPI (panels D and H). Panels D, H and L show the merge of Alexa 555 (SLPI) and Alexa 488 (LPO) labeling. Panels A–L (Bar = 100 μm).