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Lysozyme and Mucin cDNAs as Tools for the Study of Serous and Mucous Cell Differentiation*

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The human respiratory tract is a system of tubes leading air from outside the body to a site of close contact with capillary beds where O_2 and CO_2 exchange occurs. While passing through the conducting airways, air is warmed, humidified, and cleansed by contact with a mucus layer lining the inner surface of the airways. The mucus layer is formed by the composite secretions of mucous cells in the epithelium lining the airway lumen and submucosal gland cells. The glands are, by volume, the major component of the secretory system.[1]

Glands lie in the submucosa of all cartilaginous airways and communicate with the airway lumen through ducts. Microscopic analysis reveals two gland cell types: serous cells and mucous cells.[2] Whereas serous cells secrete bactericidal proteins such as lysozyme and lactoferrin, mucous cells primarily secrete mucin.[3]

During fetal development, the airway glands develop by downgrowth of the epithelium into submucosal connective tissue.[4,5] The gland buds first consist of relatively undifferentiated cells containing abundant glycogen and no secretory granules.[6,7] Within a few days, serous and mucous granules appear,[6,7] reflecting the acquisition of mature phenotype. Thus, from a common stem cell, two subpopulations emerge: one producing lysozyme and lactoferrin and the other, synthesizing mucin. We are interested in the molecular events mediating the commitment of stem cells to each of these two pathways. These events are not only significant in the context of development, but also play a role in maintaining a balanced proportion of cell types throughout adult life. It is important to understand the mechanisms underlying serous and mucous cell differentiation because disease-related abnormalities in the functioning of these mechanisms could have critical effects on the rheology and antibacterial properties of the secretion.

To understand the molecular events mediating serous and mucous cell differentiation, we have focused on the regulation of phenotype-specific proteins: lysozyme (for serous cells[8]) and mucin (for mucous cells[9]). The rationale behind this approach is that phenotype is a reflection of cell-specific protein synthesis. Therefore, to understand the mechanisms underlying the establishment of phenotype, it is necessary to understand the mechanisms regulating the synthesis of phenotype-specific proteins.

One of the most important mechanisms regulating specific protein synthesis occurs at the level of mRNA transcription.[10] Through appropriate receptors and signal transduction pathways, environmental stimuli trigger the synthesis or activation of transcription factors in specific cell types.[11] Once activated, transcription factors can enter the nucleus and interact with regulatory DNA sequences on specific genes to upregulate or downregulate transcription. Our objective was to analyze the regulatory DNA sequences and corresponding transcription factors for lysozyme and mucin genes to shed light on the molecular events mediating serous and mucous cell-specific gene expression. To do this, it was necessary to first isolate cDNAs encoding lysozyme and mucin, and subsequently use them to isolate genomic clones containing DNA regulatory sequences.

Isolation and Characterization of Lysozyme cDNAs

To isolate lysozyme cDNAs, we screened a cow tracheal cDNA library with a cDNA probe encoding the "stomach 2" form of cow lysozyme.[12] We obtained 3 cDNAs, each encoding the entire lysozyme coding region, 3′ untranslated regions (2 with poly A+ tails), and 5′ untranslated regions of various sizes.[13] Within the coding region, the 3 clones showed approximately 90% homology. The 3′ untranslated regions were dissimilar, indicating that each cDNA is related to a different gene.

Clone 7A is 1,060 bp (base pairs) in length. The 3′ untranslated region and lysozyme coding region of clone 7A correspond exactly to that of the cDNA from stomach (stomach 2). Due to the tendency for untranslated regions of genes to diverge after duplication, the identity between the 3′ untranslated regions of the tracheal (7A) cDNA and the stomach 2 cDNA indicates that both are products of the same gene. In view of this, the substantial extension of the 5′ end of the tracheal 7A cDNA beyond that of the stomach 2 cDNA (determined by primer extension to be full length) denotes the occurrence of tissue-specific RNA processing.

Primer extension data also show that the transcription start site of the tracheal 7A cognate mRNA is approximately 220 bp upstream of that of the stomach 2 cognate mRNA. This could occur by one of two mechanisms. First, stomach 2 and tracheal 7A cognate mRNAs may have two different transcription start sites in exon 1. If so, genomic DNA would be identical to 7A, up to and including the "extra" 5′ sequence not present in stomach 2 cDNA. Alternatively, 7A may span an intron, with its transcription start site occurring in a previously unrecognized upstream exon. In that case, genomic DNA would correspond to 7A sequence up to a point, but diverge at the intron-exon boundary. Comparison of 7A and genomic sequences reveal that the latter is the case, since the 5′ end of 7A consists of 11 bp not present in a 3 kB genomic clone spanning this region. The interface between the 11 bp and the remainder of the 7A cDNA has the general properties of a splice site. This suggests that the 7A cognate mRNA contains at least one additional exon as compared to the stomach 2 cognate mRNA. Further, the presence of a TATA box approximately 30 bp upstream of the transcription start site of stomach 2 (within the 5′ untranslated region of 7A) suggests that an alternate promoter drives expression of this gene in the trachea. This promoter could mediate phenotype-specific lysozyme synthesis.

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As mentioned above, we have isolated two additional lysozyme cDNAs from the cow trachea. The cDNA 5A is 1,020 bp in length and cDNA 14D is 1,258 bp in length. Start and stop codons demarcate coding sequences much like those reported for other lysozymes. *In situ* hybridization studies are being performed to determine the cellular distribution of the expression of each of these lysozyme genes in the cow trachea.

Only a single lysozyme gene has been identified in the human genome.[14,15] If the cow trachea can be considered a model of that in man, it can be anticipated that human airway lysozyme transcription and processing occurs in an airway-specific manner. Analysis of upstream elements may reveal mechanisms permitting developmental and disease-related control of lysozyme synthesis.

Isolation and Characterization of Mucin cDNAs

Our interest in mucin cDNAs was originally piqued by considering the adverse consequences of mucus overproduction in the airways of humans afflicted by asthma, chronic bronchitis, and cystic fibrosis. Developmental issues aside, it seemed important to understand the mechanisms controlling mucin gene transcription as a first step toward understanding how mucin is overproduced in diseased airways.

Our strategy has been to induce mucus hypersecretion in rats by infection or irritation, or both, then monitor mucin mRNA in the airways of these rats vs specific pathogen-free control animals.[16] We wondered whether mucin mRNA would increase as a function of irritation or infection, and if so, whether this might be an early step in the development of mucus hypersecretion.

Based on the similar amino acid compositions reported for mucins from the airways[17,18] and the intestine,[19] we used a human intestinal mucin cDNA (SMUC-41;[20]) to screen a human airway cDNA library. By this procedure, we isolated a human airway mucin cDNA, HAM-1, showing 96% homology to SMUC-41.[21] Southern blots verified that HAM-1 and SMUC-41 encoded mRNAs transcribed from a single common gene. Although we had initially thought that a human airway mucin cDNA would be more favorable than one from the intestine for monitoring mucin mRNA in the rat airways, the identity between SMUC-41 and HAM-1, paired with size considerations, led us to use SMUC-41 in our studies in the rat.

In southern blots, independent of hybridization conditions, SMUC 41 hybridized to a single approximately 8 kb (kilobase) major fragment in Hinf 1-digested and 1 major fragment at 6.5 kb in Sau 3A-digested human genomic DNA. The DNA from dog, cow, and rat, digested with the same enzymes, yielded multiple bands. Pursuing studies in the rat, we performed northern blots using RNA from the intestine, airways, and heart of specific pathogen-free (SPF) rats. As expected from the positive southern blot, rat RNA hybridized strongly with SMUC-41. In the SPF rats, intestinal RNA produced an intense, polydisperse signal, (maximum size 13 kb), whereas that from the airways and heart was negative. The airway negativity is consistent with the fact that mucous cells are rare in SPF rats.[22,23]

The negative hybridization result in the airways of SPF rats was reversed in rats that lived in the animal colony for 1 to 3 weeks. Serum analysis revealed that some rats spontaneously became infected with Sendai virus, Corona virus, or CAR bacillus. All rats with significant antibody titers for Sendai virus showed mucin hybridization signals. The SPF rats, as well as those infected by other pathogens, did not. Mucin hybridization signals obtained from airway RNA of Sendai virus-infected rats that were also exposed to SO_2 showed signals more intense than those from rats with infection alone.[24]

These data indicate that infection and irritation increase mucin mRNA steady state in rat airways. This does not seem to occur secondary to mucous cell mitosis because mucous cells (containing electron lucent secretory granules) do not incorporate ^3H thymidine during the period of mucin mRNA induction.[25] Further, mucous cells have not been observed to incorporate ^3H thymidine during the period of rapid mucous cell increase following cigarette smoke exposure.[26] In the absence of mucous cell mitosis (as reflected by lack of DNA synthesis), it seems likely that the observed increases in mucin mRNA reflect environmental stimulation of mucin gene transcription, possibly an early event in mucous cell differentiation and hypersecretion.

Based on this interpretation, we are now attempting to obtain rat airway mucin cDNAs and genomic clones containing the mucin promoter. Screening a cDNA library made from the airway RNA of rats infected with Sendai virus and exposed to SO_2, we have isolated several candidate clones. Clones RAM-3 and RAM-7 contain inserts encoding threonine-rich polypeptides. RAM-7 encodes threonine/isoleucine repeats.[27] Both cDNAs hybridize to airway RNA from Sendai virus-infected, SO_2-exposed rats, but not to that from SPF rats. If further characterization confirms their identity as mucin cDNAs, we will use them to isolate genomic clones containing the mucin promoter. Transfection analysis of mutagenized genomic clones should reveal those DNA sequences responsible for upregulation and downregulation of transcription. Transcription factors activated by injury to airway epithelium may eventually be found to mediate the abrupt increases in mucin mRNA steady state we have observed.

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A Novel Antimicrobial Peptide from Mammalian Tracheal Mucosa*

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Extracts of the bovine tracheal mucosa contain an abundant, novel peptide with potent antimicrobial activity. The 38 amino acid peptide, which we have named tracheal antimicrobial peptide (TAP), was isolated by a combination of size-exclusion, ion-exchange, and reverse-phase chromatographic fractionations using antimicrobial activity as a functional assay. The yield was approximately 2 μg/g of wet mucosa. The complete peptide sequence was determined by direct peptide sequence analysis and from a cloned cDNA. Mass spectral analysis of the isolated peptide was consistent with the sequence and indicated the participation of 6 cysteine residues in the formation of intramolecular disulfide bonds. The size, basic charge, and presence of 3 intramolecular disulfide bonds are similar to, but clearly distinct from the defensins, a well characterized class of antimicrobial peptides from mammalian circulating phagocytic cells. The putative TAP precursor is predicted to be relatively small (64 amino acids), and the mature peptide resides at the extreme carboxy-terminus and is bracketed by a short putative propeptide region and an inframe stop codon. The mRNA encoding this peptide is more abundant in the respiratory mucosa than in whole lung tissue.

The purified peptide had antibacterial activity *in vitro* against *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. In addition, the peptide was active against *Candida albicans*, indicating a broad spectrum of activity.

This peptide appears to be, based on structure and activity, a member of a newly emerging group of cysteine-rich cationic antimicrobial peptides found in animals, insects, and plants. The isolation of TAP from the mammalian respiratory mucosa may provide new insight into our understanding of host defense of this vital tissue.

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Initial Characterization of Tektins in Cilia of Respiratory Epithelial Cells*

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The ciliated airway epithelial cell provides essential clearance of airway secretions and inhaled matter through the beating movement of cilia. This predominant cell type of the conducting airways is rendered susceptible by its location to various forms of injury, arising from pollutants, microbes, inflammatory cells, and their media-

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