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Role of Lynx1 and related Ly6 proteins as modulators of cholinergic signaling in normal and neoplastic bronchial epithelium

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ABSTRACT

The ly-6 proteins are a large family of proteins that resemble the snake three finger alpha toxins such as α-bungarotoxin and are defined by their multiple cysteine residues. Multiple members of the ly-6 protein family can modulate nicotinic signaling including lynx1, lynx2, slurp-1, slurp-2 and prostate stem cell antigen (PSCA). Consistent with the expression of multiple nicotinic receptors in bronchial epithelium, multiple members of the nicotinic-modulatory ly-6 proteins are expressed in lung including lynx1 and lynx2. We studied the role of lynx1 as an exemplar of the role of ly-6 proteins in lung. Our data demonstrates that lynx1 acts as a negative modulator of nicotinic signaling in normal and neoplastic lung. In normal lung lynx1 serves to limit the ability of chronic nicotine exposure to increase levels of nicotinic receptors and also serves to limit the ability of nicotine to upregulate levels of GABAA receptors in lung. In turn this allows lynx1 to limit the ability of nicotine to upregulate levels of mucin which is mediated by GABAergic signaling. This suggests that lynx1-mimetics may have potential for treatment of asthma and COPD. In that most lung cancer cells also express nicotinic receptor and lynx1 we examined the role of lynx-1 in lung cancer. Lynx1 levels are decreased in lung cancers compared to adjacent normal lung. Knockdown of lynx1 by siRNAs increased growth of lung cancer cells while expression of lynx1 in lung cancer cell decreased cell proliferation. This suggests that lynx1 is an endogenous regulator of lung cancer growth. Given that multiple small molecule negative and positive allosteric modulators of nicotinic receptors have already been developed, this suggests that lynx1 is a highly druggable target both for development of drugs that may limit lung cancer growth as well as for drugs that may be effective for asthma or COPD treatment.

1. Introduction

Nicotinic acetylcholine receptors (nAChRs) are widely expressed in lung as part of a non-neuronal paracrine and autocrine network as well as for targets of traditional sympathetic and parasympathetic signaling. Acetylcholine is synthesized by bronchial epithelial cells and pulmonary neuroendocrine cells from which it is released to feedback on itself (autocrine), on neighboring cells (paracrine) or to be circulated in blood to effect distal cells (endocrine) [1,2]. Many aspects of non-neuronal acetylcholine are similar to neuronal acetylcholine signaling; indeed it is likely that cholinergic signaling evolved in simple epithelial structures prior to being borrowed for synaptic signaling [3–5]. Non-neuronal cholinergic signaling uses the same nAChRs as does cholinergic signaling and the nAChRs in non-neuronal networks are modulated by ly-6 family members just as neuronal nAChRs are.

The ly-6 proteins are a large family of small proteins related to snake α-neurotoxins [6,7] such as the α7 nAChR antagonist α-bungarotoxin. Ly-6 proteins are also known as 3 finger proteins because of their conserved structure of 3 fingers anchored by cysteine bonds surrounding a hydrophobic core. In humans more than 25 genes encoding ly-6 proteins have been identified, most with multiple forms produced by alternate splicing [7–9]. Most of the ly-6 proteins are membrane bound, anchored by a GPI linkage; however some of family members lack the GPI linkage and are secreted. The GPI linkage can be cleaved by phospholipases so it is likely that some of the family members can exist in both membrane-bound and secreted forms.

Consistent with the similarity of structure of the mammalian ly-6 proteins to α-bungarotoxin, many of the ly-6 proteins modulate nAChR signaling. Miwa et al. [10,11] used similarity to α-bungarotoxin to identify a mammalian α-bungarotoxin-like protein in mouse brain which they named lynx1 and then demonstrated that it was a negative allosteric regulator of α4β2 and α7 nAChR receptors. Subsequently lynx1 has also been shown to modulate α6-containing nAChR [12] as well as shifting nAChR stoichiometry from high sensitivity (alpha4)2 (beta2)3 to low sensitivity (alpha4)3 (beta2)2 nAChR through interaction as a chaperone in the endoplasmic reticulum [13]. In addition to lynx1, Miwa and co-workers also identified a second ly-6 protein in brain, lynx2 and showed that it too was a negative regulator of α7

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nAChR [14]. Of note, the cDNA for lynx2 had in fact been previously described twice before, though not in the context of nAChR regulation, and named both lypd1 and PHTS which is indicative of the nomenclature problems surrounding the ly-6 proteins, in which many of the family members have been described multiple times and given multiple names.

Lynx1 and lynx2 are not the only ly-6 proteins that regulate nAChR. The ly-6 protein, slurp-1 was initially characterized for its role in the skin disease Mal de Maleda [15,16] and is a positive regulator of α7 nAChR [17,18]. Slurp-1 differs from most of the ly-6 proteins as it lacks a GPI linkage and is secreted. Slurp-2 is encoded by an alternate transcript of the *LYNX1* gene and has been reported to be a negative nAChR regulator [19]. Slurp-1, like slurp-2, lacks a GPI-anchor and is secreted. Prostate stem cell antigen (PSCA) has been shown by Nishi and co-workers to be yet another ly-6 protein that negatively regulates α7 nAChR [20]. The Pates (named for prostate and testis expression, the site of their original characterization) are another group of ly6 proteins, of which human Pate B has been shown to be a positive regulator of α7 [21]. Thus lynx1, lynx2, Slurp-1, Slurp-2, PSCA and Pate-B are ly-6 proteins that have been demonstrated to modulate nAChR activity but it is likely that other members of the ly-6 family will also turn out to modulate nicotinic signaling.

As shown in Fig. 1A, the *LYNX1* gene gives rise to both the lynx1 and slurp-2 proteins through alternate splicing. Such alternate splicing is typical of the ly-6 family, with most of the ly-6 genes giving rise to multiple proteins. Another characteristic of the ly-6 genes is that many of the genes fall in gene clusters. The 10-gene cluster on chromosome 8 that encompasses *LYNX1* is shown in Fig. 1B. Another ly-6 gene cluster encodes the PATE genes on chromosome 11. Lynx2 however (encoded by the *LYPD1* gene) occurs by itself on chromosome 2.

Given the expression of multiple ly-6 modulators of nAChR activity in brain, our previous characterization of multiple nAChR subtypes in airway epithelium [22,23] raised the question of whether ly-6 proteins are similarly expressed in normal lung to modulate nicotinic signaling in lung and the potential role of such ly-6 proteins in lung cancer. In this review, we discuss data on the roles of nAChR-modulatory ly-6 proteins in normal and neoplastic lung.

2. Materials and methods

2.1. Drugs and epithelial cell cultures

All animal procedures were approved by the Oregon National Primate Research Center Institutional Animal Care and Utilization Committee. Bronchial epithelial cell cultures were established from lungs obtained from rhesus macaques (newborn to 2 years old) undergoing necropsy from protocols not expected to alter lung function as described by Wu et al. [24,25] with modifications as previously described by Fu et al. [3]. All drugs used for the study were obtained from Sigma (St. Louis, MO).

2.2. Immunohistochemistry

Single and dual immunohistochemistry shown in Fig. 2 was performed after Proskocil et al. [1]. The antibody used for lynx1 was as described and validated by Sekhon et al. [26]. Localization of expression of lynx1 in pulmonary neuroendocrine cells was determined by colocalization with serotonin as described by Fu and Spindel [27]. The antibody used for α7-nAChR staining was rat monoclonal 219 generously provided by Jon Lindstrom [28] as previously described by Sekhon et al. [22] and validated by comparison to fluorescently-labeled α-bungarotoxin toxin binding. Other antibodies used were sc-135296 for lynx2 (goat anti-lypd1, Santa Cruz Biotechnology, Dallas TX) and sc-98140 for slurp1 (goat anti-slurp1, Santa Cruz Biotechnology). These antibodies were validated by RT-PCR detection of target molecules in same tissues.

2.3. qPCR and siRNA knockdown

Quantitative PCR on lynx1, nAChR, GABA receptors and mucin in bronchial epithelial cells was performed using primers and probes as described by Fu et al. [29]. ON-TARGET plus siRNAs for lynx1 and negative control siRNA were purchased from Dharmacon (Lafayette, CO). siRNAs were transfected at a concentration 100–150 nM with DharmaFECT 1 according to the manufacturer's instruction. Forty-eight hours after transfection, cells were harvested for real-time PCR or for Western blotting. The lynx1 siRNA (TCAGCAACATCGAGAACTT) was chosen to be distinct from the other transcripts of the lynx1 gene and was 100% homologous to NM_001266622. The lynx1 siRNA caused a 75% decrease in lynx1 siRNA levels as measured by qPCR and confirmed by western blotting. Quantification of lynx1 RNA levels in human tumors was as described by Song et al. [30]. All human samples were deidentified prior to receipt.

2.4. Lentiviral transduction of lynx1

The human lynx1 cDNA was subcloned into the lentiviral shuttle vector pLVI-IRES which contains a CMV promoter to drive cDNA expression and a bicistronic-expressed GFP marker. Lentivirus was prepared and titrated as previously described [31]. A549 cells were infected at a

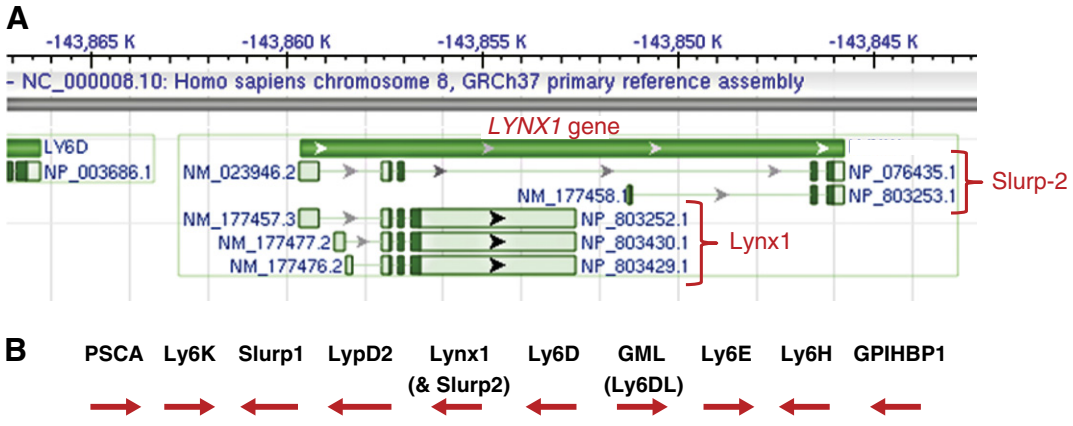

Fig. 1. Chromosomal structure of the *LYNX1* gene and locus. A. The gene is named *LYNX1*, and alternate splicing gives rise to the lynx1 protein and to two slurp-2 proteins. Slurp-2 exists in a short and long form and both forms of slurp-2 lack the exon of *lynx1* which encodes the GPI linkage thereby resulting in the secretory nature of Slurp-2. The shorter form of slurp-2 has no amino acids in common with the lynx1 protein while the longer form of slurp-2 shares the second coding exon of the *lynx1* gene with the lynx1 protein. B. The *LYNX1* gene occurs in a cluster of 10 ly-6 proteins spanning approximately 650,000 bases.