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Mutations of rat surfactant protein A have distinct effects on its glycosylation, secretion, aggregation and degradation

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ABSTRACT

Aims: Surfactant protein A (SP-A) plays critical roles in the innate immune system and surfactant homeostasis of the lung. Mutations in SP-A2 of the carbohydrate recognition domain (CRD) impair its glycosylation and are associated with pulmonary fibrosis in humans. We aim to examine how mutations in SP-A that impair its glycosylation affect its biological properties and lead to disease.
Main methods: We generated rat SP-A constructs with two types of mutations that impair its glycosylation: N-glycosylation site mutations (N21T, N207S and N21T/N207S) and disease-associated CRD mutations (G231V, F198S). We transfected these constructs into Chinese hamster ovary (CHO)-K1 cells and assessed biochemical differences in cellular and secreted wild-type and mutant SP-As by western blot, immunofluorescence, and sensitivity to enzymatic digestion.
Key findings: Mutations of the CRD completely impaired SP-A secretion, whereas mutations of N-glycosylation sites had little effect. Both types of mutations formed nonidet p-40 (NP-40) insoluble aggregates, but the aggregates only from CRD mutations could be partially rescued by a chemical chaperone, 4-phenylbutyrate acid (4-PBA). The majority of CRD mutant SP-A was retained in the endoplasmic reticulum. Moreover, both types of mutations reduced SP-A stability, with CRD mutant SP-A being more sensitive to chymotrypsin digestion. Both types of soluble mutant SP-A could be degraded by the proteasome pathway, while insoluble aggregates could be additionally degraded by the lysosomal pathway.
Significance: Our data provide evidence that the differential glycosylation of SP-A may play distinct roles in SP-A secretion, aggregation and degradation which may contribute to familial pulmonary fibrosis caused by SP-A2 mutations.

Introduction

Surfactant protein A (SP-A) is a secretory glycoprotein of the collectin family and is primarily synthesized by alveolar type II pneumocytes. Accumulating evidence indicates that SP-A plays critical roles in surfactant homeostasis and pulmonary immunity (Crouch et al., 2000; Wright, 2005). SP-A is encoded by the *SFTPA* gene and the cDNA sequence from different species shows extensive homology. The rat *SFTPA* gene has been mapped to chromosome 16p14 and encodes a 284 amino acid protein (Gao et al., 1996; White et al., 1985). The monomer of SP-A consists of four core domains (Fig. 1A), which can assemble to form an oligomeric SP-A molecule. SP-A is synthesized in the endoplasmic reticulum (ER) and undergoes several post-translational modifications including N-linked glycosylation. After folding and assembly, SP-A exits the ER and transits through the Golgi apparatus to undergo sialylation, which is a specific modification of N-linked glycans, and other modifications before its maturation and secretion (Osanai et al., 2006). A recent genome-wide association study (GWAS) revealed that human SP-A2 mutations (G231V, F198S) are associated with familial pulmonary fibrosis (Wang et al., 2009). Interestingly, these mutations lead to deficiency in SP-A2 glycosylation, specifically sialylation, and secretion (Song et al., 2012). However, the role of impaired SP-A2 glycosylation in familial pulmonary fibrosis is not known.

Protein glycosylation, one of many important post-translational modifications, regulates protein folding, stability, and secretion, allowing proteins to play diverse roles in cell differentiation, signaling, and immunity (Apweiler et al., 1999; Martin-Rendon and Blake, 2003; Roth et al., 2010; Sola et al., 2007). There are two major classes of protein glycosylation: N-linked and O-linked glycosylation. N-linked

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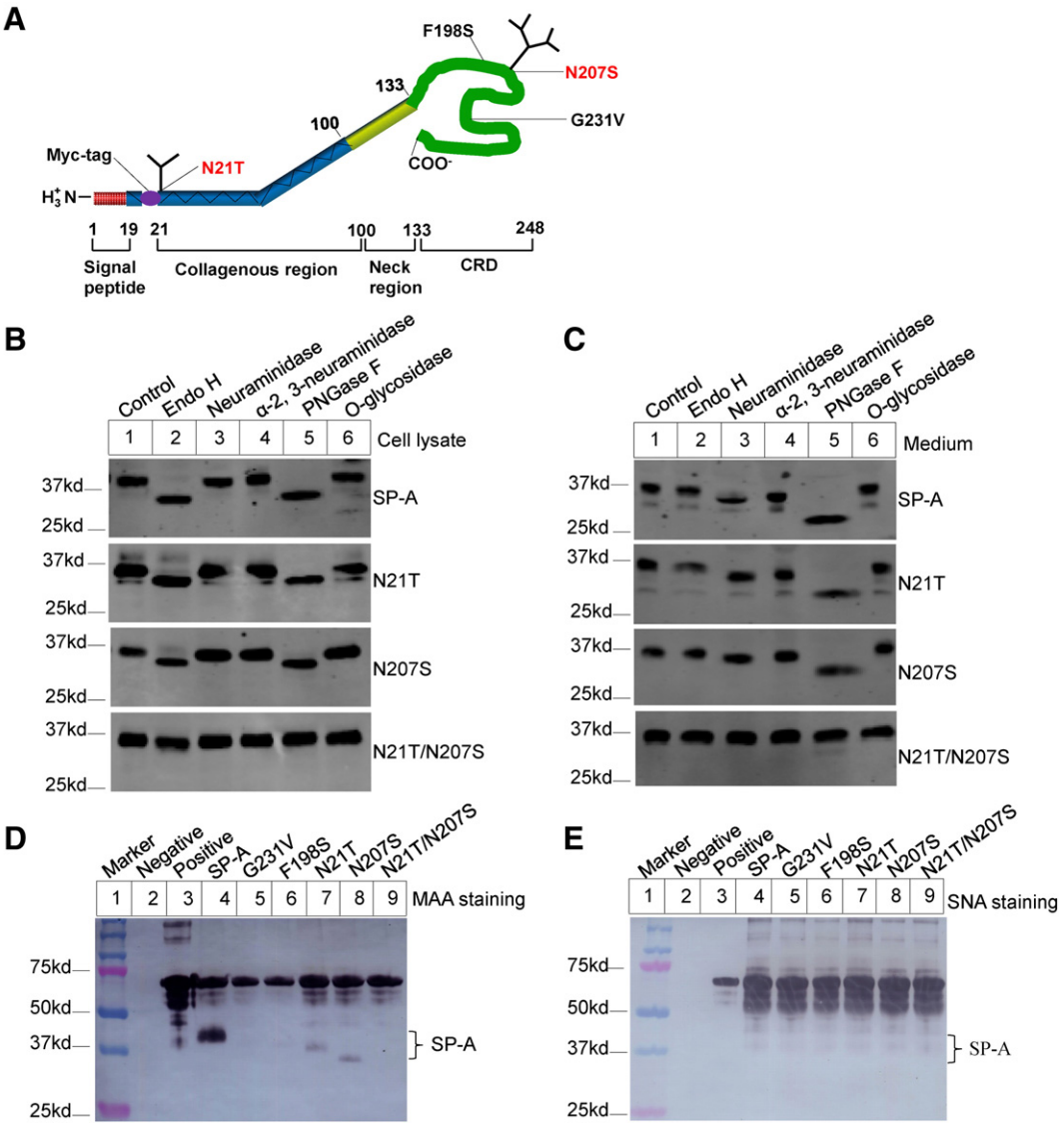

Fig. 1. Generation of glycosylation-defective rat SP-A mutations. (A) Schematic of our constructed rat SP-A with a Myc epitope at amino acid position 21 following the signal sequence. The functional domains of SP-A, including the carbohydrate recognition domain (CRD), and the four amino acid sites that we mutated are indicated. This includes N-glycosylation mutants (N21T, N207S, and N21T/N207S) and CRD mutants (G231V and F198S). (B–C) CHO-K1 cells were transiently transfected 48 h with plasmids encoding Myc-tagged wild-type and N-glycosylation mutants of rat SP-A. Proteins from cell lysates (B) or medium (C) were digested with Endo H, neuraminidase, α-2,3-neuraminidase, PNGase F, or O-glycosidase according to the manufacturer's instructions. Digested products were subjected to 10% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-Myc antibody. (D–E) The presence and linkage patterns of terminally linked sialic acids on both wild-type and mutant SP-As from medium were confirmed by lectin binding assay using *Maackia amurensis agglutinin* (MAA) (D) and *Sambucus nigra agglutinin* (SNA) (E) to specifically bind α-2,3-linked and α-2,6-linked terminal sialic acid, respectively. The positive control glycoproteins (fetuin for α-2,3-linkage and transferrin for α-2,6-linkage) and negative control (PNGase F-treated transferrin) were included.

glycosylation begins in the ER by the direct attachment of a glycan to an asparagine (Asn) residue in the consensus sequence Asn-Xxx-Ser/Thr (where Xxx represents any amino acid except proline) (Burda and Aebi, 1999; Knauer and Lehle, 1999; Leroy, 2006). O-linked glycosylation occurs at a serine (Ser) or threonine (Thr) residue by the attachment of N-acetylgalactosamine (Peter-Katalinic, 2005). Site-directed mutagenesis of glycan linked-amino acids (Asn, Ser or Thr) in a protein reduces its glycosylation and can disrupt its biological functions (Beers et al., 2013). However, mutations in non-glycan-linked amino acids could also lead to glycosylation defects. For example, a common mutation of the cystic fibrosis transmembrane conductance regulator (CFTR), a plasma membrane associated glycoprotein found in cystic fibrosis (ΔF508-CFTR), leads to incomplete glycosylation and reduces its trafficking to the cell surface (Zeitlin, 2000).

In this study, we introduced two types of mutations into rat SP-A: (1) N-glycosylation site mutations (N21T, N207S and N21T/N207S), and (2) carbohydrate recognition domain (CRD) mutations (G231V, F198S). We examined how these different mutations affect SP-A glycosylation/sialylation, secretion, aggregation, stability, and degradation. Our findings enhanced the insight into the relationship between gene mutations, post-translational modifications, and protein functions, which will be useful toward understanding lung health and disease.

Materials and methods

Cell culture

Chinese hamster ovary (CHO)-K1 cell line (Cat#CCL-61) purchased from the American Type Culture Collection was used for expression of rat SP-A. Cells were cultured in Dulbecco's modified Eagle's medium/nutrient F-12 (DMEM/F12) (Cat#SH30023.01B; HyClone) plus 5% fetal bovine serum (FBS) (Thermo Scientific, Rockford, IL) and 1% Penicillin–Streptomycin (Cat#SV30010; HyClone) at 37 °C in an atmosphere of 5% CO_2.