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Developmental regulation of chicken surfactant protein A and its localization in lung

Weidong Zhang [a], Tryntsje Cuperus [a], Albert van Dijk [a], Karsten Skjødt [b], Søren Hansen [b], Henk P. Haagsman [a], Edwin J.A. Veldhuizen [a, *]

[a] Department of Infectious Diseases and Immunology, Division of Molecular Host Defence, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands
[b] Department of Cancer and Inflammation Research, Institute of Molecular Medicine, University of Southern Denmark, DK-5000, Odense, Denmark

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ABSTRACT

Surfactant Protein A (SP-A) is a collagenous C-type lectin (collectin) that plays an important role in the early stage of the host immune response. In chicken, SP-A (cSP-A) is expressed as a 26 kDa glycosylated protein in the lung. Using immunohistochemistry, cSP-A protein was detected mainly in the lung lining fluid covering the parabronchial epithelia. Specific cSP-A producing epithelial cells, resembling mammalian type II cells, were identified in the parabronchi. Gene expression of cSP-A markedly increased from embryonic day 14 onwards until the time of hatch, comparable to the SP-A homologue chicken lung lectin, while mannan binding lectin and collectins CL-L1 and CL-K1 only showed slightly changed expression during development. cSP-A protein could be detected as early as ED 18 in lung tissue using Western blotting, and expression increased steadily until day 28 post-hatch. Our observations are a first step towards understanding the role of this protein in vivo.

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1. Introduction

Surfactant Protein A (SP-A) is a lung specific collagenous C-type lectin; a group of proteins referred to as collectins (Veldhuizen et al., 2011). Collectins are structurally characterized by the presence of 4 basic units: an N-terminal segment with one to three cysteine residues; a collagen-like region containing multiple Gly-X-Y repeats; an α-helical neck region and a carbohydrate recognition (lectin) domain (CRD). The neck region of collectin monomers is thought to initiate trimerization, which is stabilized by further non-covalent inter-chain interactions in the collagen-like region. Higher oligomeric forms (depending on the specific collectin) are formed from these trimers, stabilized by disulfide bridges located in the N-termini. These oligomeric forms give rise to an ordered orientation of multiple lectin domains, which is the active functional domain in many aspects of collectin function. For SP-A this oligomeric form resembles a so-called bouquet of flowers, an octadecamer of 6 trimers in which all lectin domains are clustered.

In mammals, SP-A has been well studied. One of its main functions is to facilitate the formation of tubular myelin, a highly ordered lipid/protein precursor structure from which a pulmonary surfactant surface film is formed at the air-liquid interface in the lung. This surface film prevents alveoli from collapsing at the end of exhalation. In addition to this biophysical function, SP-A has a growing number of innate immunity functions. For example, it can bind and aggregate bacterial, viral and fungal pathogens; thereby preventing dissemination; it can stimulate phagocytic activity of macrophages, and it can modulate immune responses of innate immune cells. For a more extensive description of SP-A functions the reader is referred to some reviews (Haagsman et al., 2008; Waters et al., 2009; Whitsett, 2005).

The information on chicken SP-A (cSP-A) is scarce, though there are some interesting differences between mammals and birds which necessitate a deeper understanding of the avian SP-A orthologue. Firstly, cSP-A possesses a significantly truncated collagen region (only 3 Gly-X-Y repeats are present), while a second orthologue of SP-A, named chicken lung lectin (cLL) completely lacks this collagen region. Although no structural studies have been performed on cSP-A or cLL, it seems plausible that these SP-A orthologues, without a significant collagen region, cannot form the characteristic octadecamer structure. On the contrary, other

* Corresponding author. Department of Infectious Diseases and Immunology, Division of Molecular Host Defence, Faculty of Veterinary Medicine, Utrecht University, P.O. Box 80.165, 3508TD, Utrecht, the Netherlands.
E-mail address: E.J.A.Veldhuizen@uu.nl (E.J.A. Veldhuizen).

chicken collectin orthologues such as mannan binding lectin (cMBL), Collectin Liver 1 (cCL-L1) and Collectin Kidney 1 (cCL-K1) do not have altered collagen regions. Secondly, and this is possibly linked to the first aspect, birds have tubular lungs that do not contain alveoli. The air capillaries of the avian tubular lung are aerated during inspiration and expiration via a highly distinct system of parabronchi and air sacs through which air flow is directed in a single direction, resulting in a highly efficient gas exchange system (Kuethe, 1988; Scheid, 1979). This rigid structure does not require an extreme reduction in surface tension, which could be reflected through a functionally altered pulmonary surfactant system.

In this study, lung and liver tissues were collected at 11 different time points starting at embryonic day (ED) 12 to day 28 post-hatch. The developmental gene expression of cSP-A, cLL and 3 other collectins in the lung and liver were analyzed by real-time quantitative PCR. In addition, cSP-A localization in the lung was studied using immunohistochemistry (IHC) and the developmental expression of cSP-A protein expression in the lung was further shown by Western blotting. We demonstrated that gene and protein expression of cSP-A is clearly developmentally regulated, that cSP-A is present in the lung as an extracellular component of the lung lining fluid of the tertiary bronchi of the lung, and is produced by specific lung epithelial cells.

2. Materials and methods

2.1. Animals and sample collection

Fertilized eggs of a chicken broiler line (Ross 308) were obtained from a local commercial hatchery (Lagerwey BV, Barneveld, The Netherlands). The eggs were incubated at 37.8 °C and a relative humidity of approximately 55%. After hatch, chickens were housed in one group in a ground stable under controlled hygienic conditions. Water and commercial feed were provided ad libitum during the entire experiment. Chickens (n ≥ 4) were euthanized by cervical dislocation at embryonic day (ED) 12, 14, 16, 18, 20 and 1, 4, 7 days after hatching. Chickens at day 14, 21, 28 were euthanized by electrocution followed by exsanguination. Lung and liver samples for gene expression and protein analysis were collected and frozen immediately in liquid nitrogen, and stored at −80 °C, while samples for immunohistochemistry were stored in 4% formalin. All experiments were conducted in accordance with protocols approved by the Dutch experimental animal committee (DEC).

2.2. RNA isolation and cDNA synthesis

Lung and liver samples were removed from the −80 °C freezer and kept in liquid nitrogen until cut by sterilized blades. Approximately 50 mg of tissue was homogenized using a MagNA Lyser with MagNA Lyser Green Beads (Roche) at a speed of 6500 × g, 50 s. Total RNA was isolated using the High Pure RNA Tissue Kit (Roche) and treated with DNase I (Roche) according to the manufacturer's protocol. Presence of contaminating genomic DNA was checked by PCR. The RNA concentration and integrity was measured using a NanoDrop ND 100UV and cDNA was generated using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories) according to the manufacturer's instructions.

2.3. Real-time quantitative RT-PCR

Quantitative detection of the gene products of five chicken (col) lectins, cSP-A, cLL, cMBL, cCL-L1 and cCL-K1, plus two reference genes, GAPDH and 28S was performed using quantitative real time PCR using iQ Supermix (Bio-Rad Laboratories). The following

cycling protocol was used: 7 min at 90 °C (denaturation) followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Primers and probes for each gene are shown in Table 1. Quantitative expression of collectin genes relative to the two reference genes was calculated using the qBase + analysis software (Biogazelle, Zwijnbeke, Belgium) (Vandesompele et al., 2002).

2.4. Protein isolation

In brief, 50 mg frozen tissue was homogenized using MagNA Lyser technology with ice-cold RIPA buffer (150 mM NaCl, 50 mM Tris-HCL, 1% Triton X-100, 0.5% sodium deoxycholic acid, 0.1% SDS, pH = 8). Protease Inhibitor Cocktail Tablet (cOmplete™, Mini, EDTA-free, Roche) was added in RIPA buffer to prevent proteolytic degradation. The homogenate was lysed by incubation for 2 h at 4 °C with gentle shaking. The lysate was transferred into new Eppendorf tubes and centrifuged for 20 min at 12,000 rpm in a pre-cooled micro-centrifuge to remove cell debris. Supernatants were collected and stored at −80 °C until assayed. The protein concentration of the samples was measured using the BCA protein assay kit (Thermo Scientific), according to the manufacturer's instructions. The absorbance was read at 562 nm using the Fluostar Omega microplate reader (BMG LABTECH, Inc).

2.5. Production of monoclonal anti cSP-A antibodies

Monoclonal antibodies (mAbs) were essentially produced by the principles described by Köhler and Milstein (Köhler and Milstein, 1975) in outbred NMRI mice. Briefly, a fusion protein representing the CRD of cSP-A conjugated to an N-terminal 6× HisTag was produced in Escherichia coli using pBad as a vector (Life-Technologies). The fusion protein was isolated as inclusion bodies, denatured with urea, refolded and purified on a Nickel chelate column according to the manufacturer's instructions (Life-Technologies). Positive clones were identified by ELISA using microtiter plates coated with the fusion protein and on ELISA plates coated with lung lavage of chicken. Cells from the positive wells were cloned at least four times by limiting dilution. mAbs were purified by means of affinity chromatography using a HiTrap Protein G HP column (GE Healthcare) under previously described conditions (Åkerström and Björck, 1986).

2.6. Western blotting

Proteins (100 μg per lane) denatured with sample buffer (1:1) were separated by SDS-PAGE using 4–15% precast gels (Mini-Protean TGX, Bio-Rad Laboratories), and transferred onto 0.2 μm

Table 1
Real−time qRT-PCR primers and probes for chicken collectins.

Name	Sequence (5′- 3′)
cSP-A Forward	GGAATGACAGAAGGTGCAATCAG
Reverse	GCAATGTTGAGTTTATTAGCTACAAATG
Probe	(FAM)-CCGCCTTGTTGTCTGCCAGTTTTAGTGC-(BHQ)
cMBL Forward	TCCAGCCATCTTCTGTTTCAATTC
Reverse	CCACAGATCAGCACCTTTACCT
Probe	(FAM)-TGGCTGTCAGGAAAGCCAATG-(BHQ1)
cCL-L1 Forward	ATTGTCAAAGAAGAGAAGAATTACAGAG
Reverse	GAGGAGATGTAATCAGCAAGCAG
Probe	(FAM)-CGTTGGTCACCTCATCTTTAGGCATGGC-(BHQ)
cCL-K1 Forward	GGGAGCCCAACAATGCCTATG
Reverse	GTAATATGACATGCAACATCATTCCAC
Probe	(FAM)-TGCCACCATCTCCACACAGTCCTCCTC-(BHQ)
cLL Forward	CTTACAAGGGAAGATAACAAAGTCTGG
Reverse	CATTCCTTGGAGATGCAATACACC
Probe	(FAM)-CTCCTGGCACATTTTCACCGTAGCATGG-(BHQ)

Nitrocellulose membranes (*Trans*-Blot Turbo Mini Nitrocellulose Transfer Packs, Bio-Rad Laboratories) using the *Trans*-Blot Turbo system (Bio-Rad Laboratories). Membranes were incubated with TTBS buffer (20 mM Tris–HCL, 0.5 M NaCl, 0.05% Tween-20, pH = 7.4) containing 3% non-fat milk overnight at 4 °C to block non-specific binding. After a 5 min wash with TTBS buffer, the membranes were incubated with mouse monoclonal *anti*-chicken SP-A antibody (clone 99-61-1) at a dilution of 1: 1000 in blocking buffer for 1 h at room temperature. The membranes were washed three times for 10 min with TTBS at room temperature. Membranes were then incubated with horse radish peroxidase conjugated Goat-anti-Mouse IgG (Sigma-Aldrich) as secondary antibody at a dilution of 1:5000 in TTBS buffer containing 3% nonfat milk for 1 h at room temperature. The membranes were washed twice with TTBS buffer and once with TBS buffer (20 mM Tris-HCL, 0.5 M NaCl, pH = 7.4) for 10 min with shaking at room temperature. Blots were developed using the ECL Blotting Reagents (GE Healthcare) according to the manufacturer's instructions. Finally, protein bands were visualized by Image Lab software (Bio-Rad).

2.7. Deglycosylation of cSP-A

Proteins isolated from 28-day old chicken lung, muscle and trachea (100 μg) were incubated with PNGase F (New England BioLabs) at 37 °C for 3 h according to the manufacturer's instruction. Deglycosylated protein was analyzed under reducing condition by SDS-PAGE and Western blotting as described above.

2.8. Immunohistochemistry (IHC)

Chicken tissues were fixed in 4% formalin and then embedded in paraffin. Paraffin sections were cut at 4 μm, transferred onto Superfrost Ultra Plus slides (Thermo Scientific) and incubated at 60 °C overnight. Paraffin-embedded sections were deparaffinized by placing them in two separate xylene baths for 2 min each and then rehydrated in a graded series of alcohol (100%, 100%, 96%, 70%) for 2 min each. Antigens were retrieved by heating these sections in citrate buffer (10 mM Trisodium citrate, 0.05% Tween-20, pH = 6) in the microwave for 10 min. The sections were allowed to cool for 15 min at room temperature and were washed three times with PBS/Tween buffer (137 mM NaCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.05% Tween 20, pH = 7.4) for 5 min each. Endogenous peroxidase activity was blocked by incubating with 1% H_2O_2 in methanol for 30 min. The sections were blocked with 10% normal goat serum and 2.5% BSA in PBS for 1 h. Next, sections were incubated with monoclonal anti chicken SP-A antibody diluted in 1% BSA (1:50 for mAb 99-61-1; 1:10 for mAb 99-61-9) for 1 h. The sections were washed three times with PBS/Tween buffer for 5 min each, then incubated with secondary antibody, biotinylated Goat-anti-Mouse IgG (1:250 dilution; Vector Laboratories) for 30 min. The sections were then incubated with avidin/biotin complex reagent (Vectastain Elite ABC Kit (standard), Vector Laboratories) for 30 min followed by incubation with diaminobenzidine solution (Sigma-Aldrich) for 10 min in the dark. The sections were washed with aqua dest for 5 min and colored with hematoxylin (Merck) for 1 min, dehydrated in a graded series of alcohol (70%, 96%, 100%, 100%) for 2 min each, cleared in xylene (Merck) twice for 2 min each, and mounted for imaging. Representative images were taken on a Zeiss Axioskop Microscope with an Olympus DP25 camera.

2.9. Statistical analysis

Statistical analysis of collectin gene expression was performed using SPSS Version 20 for Windows. All data were analyzed by one-way ANOVA with a Bonferroni multi comparison post-hoc test.

Significant differences between means were defined as p < 0.05.

3. Results

3.1. Collectin gene expression in lung and liver

In this study mRNA was isolated from lung and liver of chickens ranging in age from embryonic day 12–28 days post-hatch. Gene expression analysis of cSP-A, and 4 other (col)lectins showed that cSP-A and cLL were highly expressed in the lung, cCL-L1 and cCL-K1 had intermediate expression, while for cMBL no or only minimal gene expression was detected. For cSP-A and cLL, a comparable gene expression pattern was observed during development. Both genes were strongly expressed from ED 14 onwards, increased 300–500 fold between ED 14 and hatch and remained relatively stable thereafter. The gene expression pattern of cCL-L1 and cCL-K1 was clearly different from cSP-A and cLL. A modest increase in cCL-L1 expression (5- to 10-fold) was observed between ED 14 and hatch, whereas cCL-K1 expression levels increased by a mere 2-fold in this period. (Fig. 1).

In the liver, expression of all five collectin genes was detected but at very different levels. cMBL was highly expressed, intermediate levels were found for cCL-L1 and K1 and very low levels for cSP-A and cLL. Gene expression of cSP-A and cLL in liver increased after hatch (5- to 15-fold) but remained low in absolute terms compared to the lung or to the gene expression level of the other collectins. Expression levels of cMBL in the liver were highest during embryonic development and declined from ED 14 onward to hatch, after which cMBL expression levels stabilized. However, differences in gene expression were never higher than 3-fold, indicating relatively subtle differences in gene expression throughout development.

Finally, no large differences were observed for the gene expression of cCL-L1 and cCL-K1 in liver during development. Both genes showed a slight decrease in gene expression just after hatch but this only reached significant differences between some pairs of individual days, similar to the results in lung (Fig. 2).

3.2. Localization of cSP-A in lung

Localization of cSP-A was determined by IHC on paraffin embedded lung sections. To optimize the procedure and to obtain a general picture of cSP-A protein production in the chicken lung, day 28 tissues were stained first. cSP-A was mainly found in its secreted form in the epithelial lining fluids of the tertiary bronchi, mainly in the atria, and in some small segments in the infidibulum (Fig. 3A). Intracellular staining of cSP-A was found as well in some individual epithelial cells, which likely reflect avian epithelial type II cell homologues (Fig. 3B). In some cases, small amounts of cSP-A were observed in the lumen of the air capillaries (Fig. 3C). Comparison of cSP-A expression throughout development showed that specific staining was detected from ED 20 at the epithelial surface of the parabronchi. Interestingly, increased staining in the parabronchi was observed during development with maximum levels at the last day sampled, day 28 (Fig. 3D).

3.3. Developmental expression of cSP-A in lung

In order to determine protein expression levels of cSP-A in the lung during development, the same amount of total protein (100 μg) from homogenized lung samples of each age was used for Western blotting. cSP-A was undetectable from ED 12 to ED 18 on blot. Faint reactive bands of cSP-A (approx. 26–32 kDa) were detected at ED 20 and day 1. After hatch, the intensity of the cSP-A bands increased and reached a maximum at day 28 (Fig. 4), similar
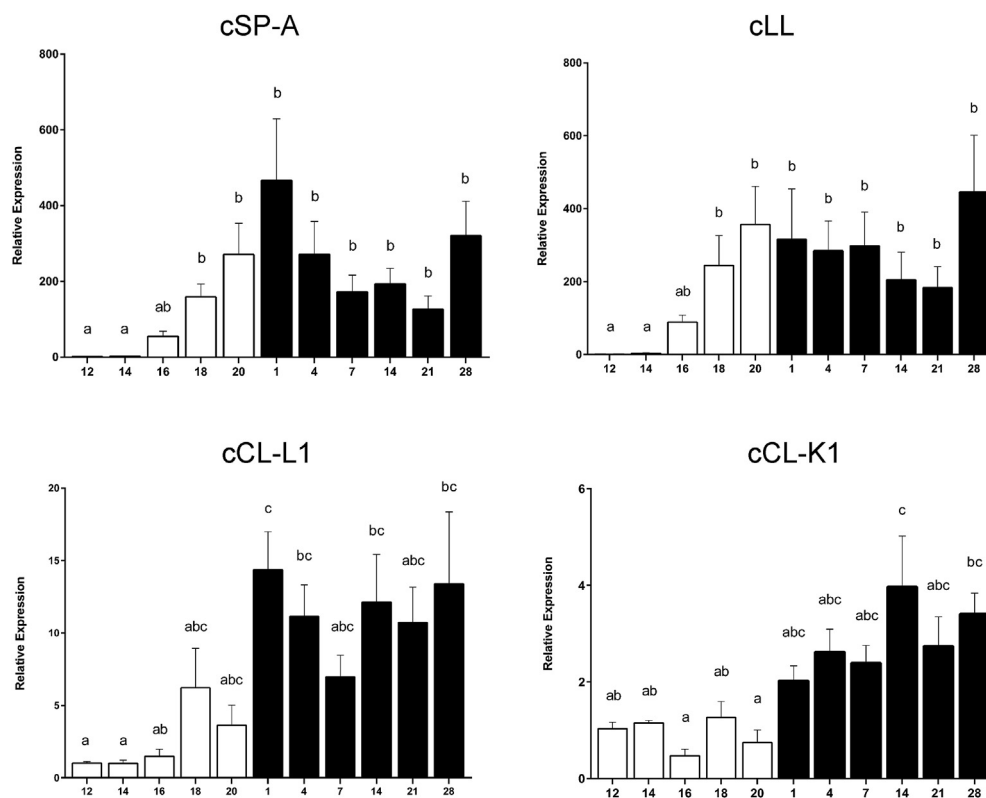
Fig. 1. Gene expression of cSP-A, cLL, cCL-L1 and cCL-K1 in the chicken lung during development. Average ± SEM are shown for four (ED 12, ED 14 and ED 16) or five samples. Data points being significantly different from each other are denoted by different letters (p < 0.05). White and black bars are shown for embryonic and post-hatching days respectively.

to the IHC results. This indicates that, although gene expression does not change much after hatch, the actual protein level of extracellular cSP-A in the lung increases over time.

3.4. Tissue distribution of cSP-A at the protein level

Total protein isolation was performed on a set of tissues all obtained from 28-day old chicken and analyzed for cSP-A expression using Western blotting. In lung, the specific cSP-A double band of 26–32 kDa was again intensively stained. In other tissues also some other, less intensive, bands at higher molecular weight were observed, which, based on molecular weight do not represent cSP-A. Interestingly, a single band at approximately 26 kDa, close to the calculated mass of cSP-A was detected in trachea and, surprisingly, in muscle (Fig 5). When isolated lung proteins were subjected to PNGase treatment, a clear reduction in size to a single band of approximately 26 kDa was observed, indicating that lung cSP-A is heterogeneously glycosylated (Fig 6). The same PNGase treatment on the tracheal or muscle immunoreactive band (Fig 6 and Fig. S1) did not result in a size shift on the gel, indicating that the protein is not a glycosylated truncated form of cSP-A. IHC on trachea and muscle tissues could not detect cSP-A, while western blotting using a second cSP-A antibody (clone 99-61-9) also failed to detect this 26 kDa band in these tissues (while lung cSP-A was still stained; Fig. S2). Based on these results it currently seems most likely that out of the tissues tested, cSP-A is exclusively present in lung tissue.

4. Discussion

The fundamentally different respiratory system of chickens and the lack of a substantial collagen region in chicken SP-A indicate that this protein has likely different structural and functional characteristics compared to its mammalian counterparts. As a first step towards understanding the function of avian SP-A homologues, we determined the gene and protein expression of cSP-A and gene expression of cLL and 3 other collectins during (embryonic and early post-hatch) development.

In the lung, gene expression of cSP-A and cLL was detectable from ED 14 onward and significantly increased until hatching. For cSP-A, expression at such an early age has been detected before using Northern blots (Johnston et al., 2000). The similar expression profile of both genes and the assumption that cLL arose through gene duplication of cSP-A (Hamzić et al., 2015) suggests that both genes might be similarly regulated. At the protein level, cSP-A was detectable in the lung homogenate of ED 20 chicken embryos. The intensity of the cSP-A band increased after hatch up to day 28, showing that a net increase in cSP-A protein level occurred after hatching, probably due to accumulation of secreted protein within the lung. Studies in mammals described a very similar gene expression profile of SP-A; usually at 50–75% of gestation SP-A mRNA is first detectable and expression increases towards birth after which it remains relatively stable. However, the large increase in SP-A protein levels after birth is not observed in these studies. In one study, a 4-fold increase after birth in rat lungs was described, but other studies in baboons, rats and rabbits showed relatively stable SP-A levels up to adulthood (Mendelson et al., 1986; Schellhase et al., 1989; Seidner et al., 1996; Shimizu et al., 1991; Tan et al., 1999). It would be interesting to determine whether the SP-A expression profile in chicken is reflecting a general avian developmental regulation of SP-A and what the consequences are for innate immune protection especially in the early stages of life.

The availability of monoclonal antibodies against cSP-A enabled us to study cSP-A localization and expression in more detail. Antibody clone 99-61-1 was used throughout the study as this antibody
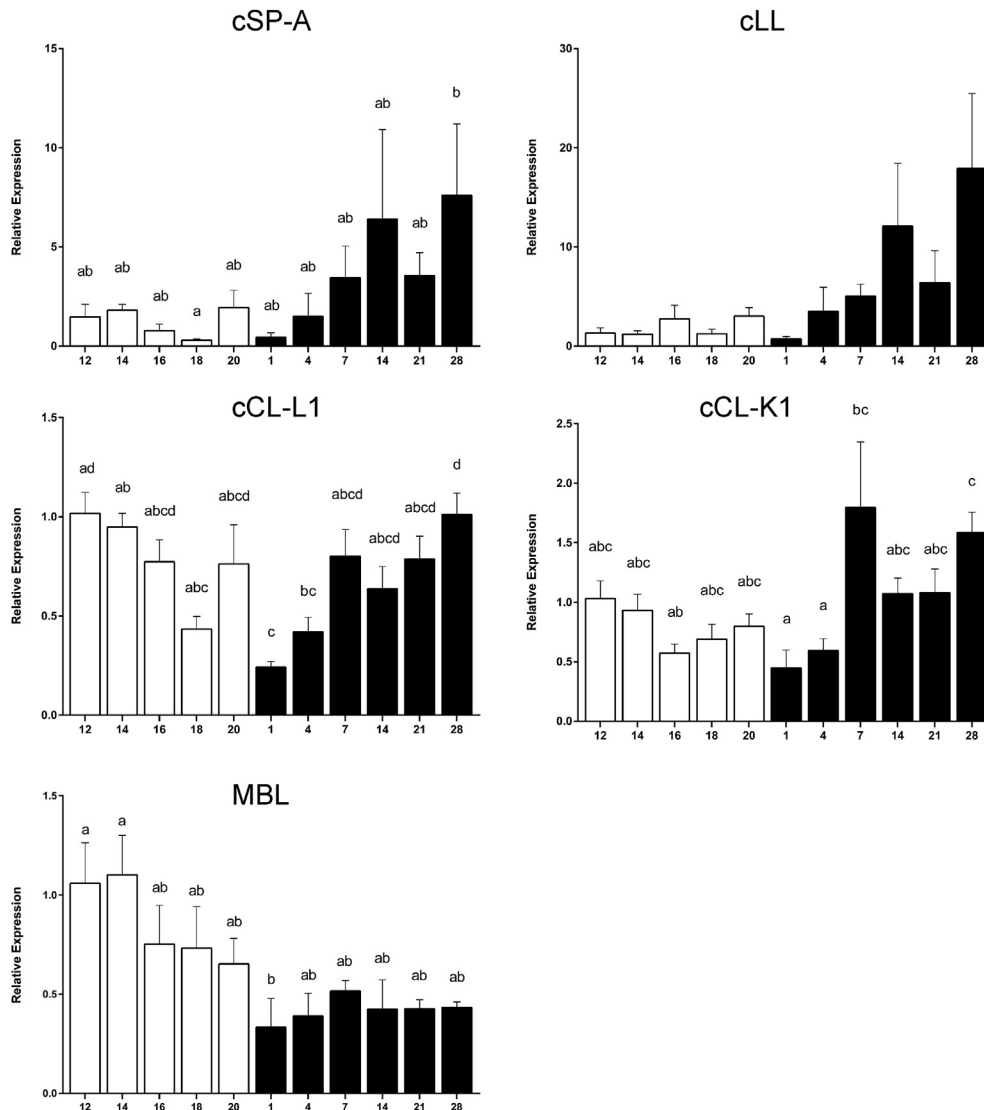
Fig. 2. Gene expression of cSP-A, cLL, cCL-L1, cCL-K1 and cMBL in the liver during development. Average ± SEM are shown for four (ED 12, ED 14 and ED 16) or five samples. Data points being significantly different from each other are denoted by different letters (p < 0.05). White and black bars are shown for embryonic and post-hatching days respectively.

had highest sensitivity in Western blotting and IHC, while clone 99-61-9 was used to verify specificity of the antibody in the experiments. Despite a relatively high sequence homology of cSP-A with cLL, (recombinantly expressed) cLL protein was not recognized by the monoclonal antibody on Western blotting (data not shown). Immunohistochemistry of lung tissues showed that cSP-A protein could not be detected at ED 12, 14, 16, or 18, but specific staining of the lung epithelial surface was seen at ED 20. After hatch, the intensity of cSP-A staining increased with age, reaching a maximum by day 28 in accordance with the Western blotting data. Most of the positive staining was observed at the top of the epithelial surface of parabronchi and atria, indicative of secreted protein in the surface lining fluid, and comparable to mammalian localization of SP-A (Madsen et al., 2003). In 28-day old chicks, a small amount of immuno-reactivity was also detected in the air capillaries of the lung. Some intracellular staining was observed for cells in the parabronchus and this reactivity likely represents surfactant producing cells in the chicken comparable to mammalian epithelial type II cells. A similar staining pattern was observed when antibody clone 99-61-9 was used (Supplementary file S3). Since a number of different lung epithelial cell types seems to be present in the *Aves*

branch, there is some controversy in the literature on the existence of true type II cells in the avian lung. An epithelial granular cell present in the parabronchus produces and secretes lamellar bodies into the lumen, and this is often considered the avian type II cell (Bernhard et al., 2001; Smith et al., 1986). However, another cell type, a squamous non-granular atrial cell, also produces and secretes a trilaminar substance that covers the lung epithelium (Scheuermann et al., 1997, 2000), while a more recent study only distinguishes one type I and one type II pneumocyte in the chicken lung (Bjørnstad et al., 2014).

A few studies have reported the ability to detect cSP-A using IHC and Western blotting techniques. Zeng and coworkers showed cSP-A in the mesobronchi but not in atria and air capillaries of chicken lung using *anti*-rat-SP-A antibody (Zeng et al., 1998). By use of Western blotting, another study failed to detect cSP-A in chicken lung lavage (Bernhard et al., 2001), while Sullivan et al. detected cSP-A in lung lavage with a 2-fold larger mass than expected under reducing conditions (Sullivan et al., 1998). However, all these studies used polyclonal *anti*-mammalian SP-A antibodies of which the identity of the reacting protein was not investigated. It is possible that a non-SP-A related protein was detected and denoted
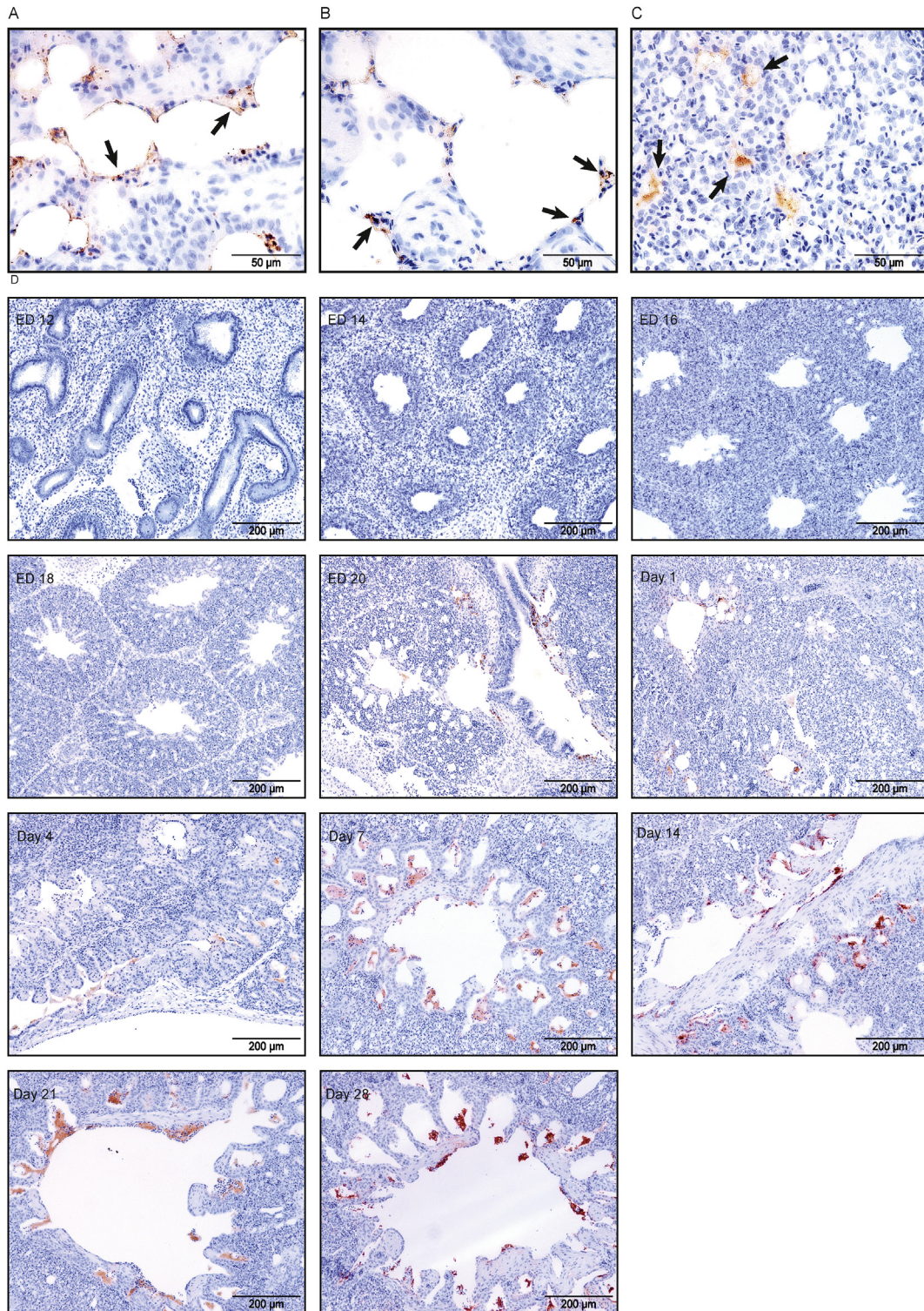
Fig. 3. Immunohistochemistry of 28-day old chicken lung. Representative pictures (Magnification 400× for 3A-C; 100× for 3D) of multiple samples are shown using mAb 99-61-1 (1:50). A) cSP-A staining is observed in the extracellular epithelial lining fluid. B) clear intracellular staining in specific parabronchial epithelial cells can be observed (arrow) C) cSP-A staining is observed in air capillaries D) overview of cSP-A staining in lung tissue from day ED 12 − day 28 post-hatch.

as SP-A (Bernhard et al., 2004).

Although localization of cSP-A is similar to mammalian SP-A, functional consequences of the structural differences have not been properly addressed. Studies on total chicken pulmonary surfactant have shown that it lacks the ability to form tubular myelin

in vivo, linking some of the structural features of cSP-A to adapted biophysical properties (Bernhard et al., 2001). In addition, chicken pulmonary surfactant also failed to reduce surface tension to the same extent as mammalian surfactant did *in vitro* (Bernhard et al., 2004). However, other factors such as lipid composition or absence
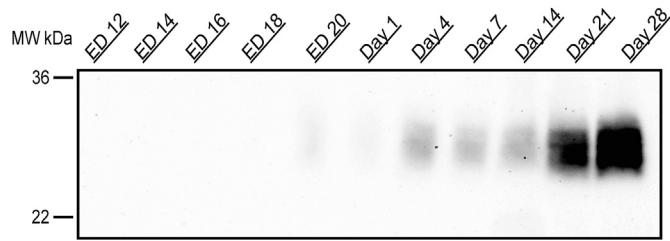
Fig. 4. Western blotting showing expression of cSP-A in lung tissue from ED 12 to day 28 post-hatch. 100 μg of lung tissue of each developmental stage was applied per lane. mAb 99-61-1 was used as primary antibody at a 1:1000 dilution.

of other pulmonary surfactant proteins, SP-B or SP-C, possibly also contributed to this lack of surface tension reducing activity. Since no information is available on the innate immune function of cSP-A, one can only speculate to what extent it resembles mammalian SP-A. Many functions of SP-A are initiated through calcium-dependent binding of glyco-conjugates on pathogens, but also on receptors of immune cells. Since the CRD of mammalian SP-A contains a high homology with cSP-A, including the glycan-binding site and other specific C-type lectin characteristics (Hogenkamp et al., 2006), it seems likely that cSP-A would be functionally active in these processes. Its localization in the extracellular lining liquid enables cSP-
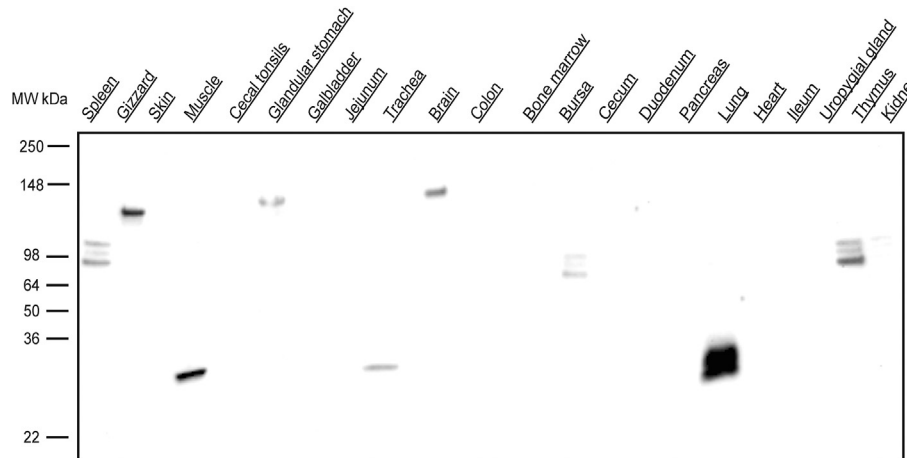
Fig. 5. Western blotting showing tissue distribution of cSP-A in a set of tissues from 28-days old chickens. Each lane represents 100 μg protein of the specified tissue. mAb 99-61-1 was used as primary antibody at a 1:1000 dilution.
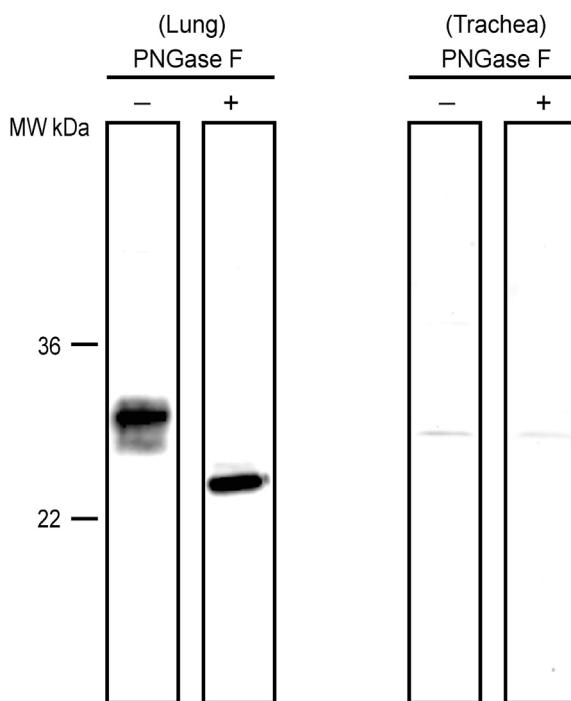
Fig. 6. Western blotting showing lung and tracheal tissue samples treated with PNGase. Only the cross-reactive cSP-A band in lung is glycosylated. mAb 99-61-1 was used as primary antibody at a 1:1000 dilution.

A to interact immediately with incoming pathogens. However, some interactions of mammalian SP-A with innate immune receptors such as SPR-120, signal inhibitory regulatory protein α and the calreticulin-CD91 complex (receptors initiating phagocytosis and modulating cellular immune cell functions) are mediated through the collagen region of mammalian SP-A (Jakel et al., 2013). It will be intriguing to discover to what extent and through what adaptations homologous immune functions of cSP-A are present and to what extent cSP-A contains species-specific activity.

Finally, the role of cLL, as a second SP-A homologue needs similar attention. Localization and regulated expression indicate a link between the two proteins. One possibility that needs to be addressed is whether cLL and SP-A are actually two different proteins or whether they are both part of a heterologous trimeric complex. In humans (and primates, but not in rodents), two SP-A genes are present which code for 2 closely related, but different SP-A monomers (4 amino acids difference, all in the collagen-like domain) (Floros and Hoover, 1998; Katyal et al., 1992). It was shown that the trimeric form of hSP-A can be built up from one SP-A1 and two SP-A2 chains. It was also described that both SP-A1 and SP-A2 are needed for tubular myelin formation *in vivo* (Wang et al., 2010). Similarly, the serum collectins CL-L1 and CL-K1 were shown to form heterologous trimers indicating that this could be a general concept for collectins to add functional flexibility to the oligomeric protein (Henriksen et al., 2013).

In conclusion, our results provide the first description of transcriptional and translational levels of cSP-A during development in both embryonic and post-hatch animals, which will provide fundamental data for future studies. Moreover, this study reports the tissue distribution and localization of cSP-A, which could

provide a first step towards understanding the function of this collectin (and possibly cLL) in relation to its altered structure compared to mammalian SP-A.

Acknowledgements

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.dci.2016.03.010.

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