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Chicken Lung Lectin is a functional C-type lectin and inhibits haemagglutination by Influenza A Virus

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Abstract

Many proteins of the calcium-dependent (C-type) lectin family have been shown to play an important role in innate immunity. They can bind to a broad range of carbohydrates, which enables them to interact with ligands present on the surface of micro-organisms. We previously reported the finding of a new putative chicken lectin, which was predominantly localized to the respiratory tract, and thus termed chicken Lung Lectin (cLL). In order to investigate the biochemical and biophysical properties of cLL, the recombinant protein was expressed, affinity purified and characterized. Recombinant cLL was expressed as four differently sized peptides, which is most likely due to post-translational modification. Crosslinking of the protein led to the formation of two high-molecular weight products, indicating that cLL forms trimeric and possibly even multimeric subunits. cLL was shown to have lectin activity, preferentially binding to α-mannose in a calcium-dependent manner. Furthermore, cLL was shown to inhibit the haemagglutination-activity of human isolates of Influenza A Virus, subtype H3N2 and H1N1. These result how that cLL is a true C-type lectin with a very specific sugar specificity, and that this chicken lectin could play an important role in innate immunity.

Key words: Chicken Lung Lectin; Innate Immunity; Influenza A Virus; C-type Lectin

1. Introduction

The innate immune system provides a first line defense against potential pathogens, bridging the interval between exposure to the pathogen and the specific response of the adaptive immune system. The effectiveness of the innate immune system highly depends on the recognition of pathogens. For this purpose, the innate immune system relies on pattern recognition molecules which are capable of binding to regular patterns of carbohydrates present on the surface of pathogens. Within this group of pattern recognition molecules, the calcium-dependent (C-type) lectins represent a family of proteins which are found throughout the animal kingdom (Day, 1994). These proteins share a structural homology in their carbohydrate recognition domains (CRD) but differ with respect to their carbohydrate specificity. We recently reported the discovery of a chicken lectin which was designated chicken Lung Lectin (cLL) due to its predominant expression in the chicken respiratory system (Hogenkamp et al., 2006). Based on sequence homology, cLL was identified as a C-type lectin, and although it could not be classified as a collectin due to its lack of a collagen domain, the sequence of its CRD was found to be most homologous to that of the collectin Surfactant Protein A (SP-A) (39% - 45% similarity, depending on the species, (Hogenkamp et al., 2006). To our knowledge, no other C-type lectins with more structural similarities to cLL have been identified to date. Collectins and several other C-type lectins have been identified as important molecules in innate immunity. For example, SP-A plays an important role in innate defense against invading bacterial pathogens in the lung (reviewed in Wright et al., 2001 and Hogenkamp et al., 2007), as well as viruses, including Influenza A Virus (IAV). Other lectins such as RegIIIγ (Narushima et al., 1997), and its human homologue

72 HIP/PAP (Lasserre et al., 1992), affect the gut flora composition and have been
73 shown to possess direct antimicrobial properties (Cash et al., 2006). Tetranectin is a
74 secreted C-type lectin (Berglund and Petersen, 1992; Sørensen et al., 1995) which
75 has also been found in chicken (unpublished data, GenBank accession no.
76 **AJ277116**). Its function is not quite clear yet, but it has been suggested to play a role
77 in cellular immunity (Stoevring et al., 2005). In this study, we expressed recombinant
78 cLL to investigate the structural and functional characteristics of this protein. The
79 carbohydrate-specificity of cLL was tested, and the putative protective role of cLL
80 was studied by investigating haemagglutination-inhibition and neutralization of viral
81 infectivity of various IAV strains.

2. Materials and Methods

2.1. RNA extraction and cDNA synthesis

Total cellular RNA from lung tissue from healthy female Ross 308 broiler chicken was extracted using TRIzol© (Invitrogen, Carlsbad, CA) and Magnalyser Green Beads (Roche Diagnostics GmbH, Mannheim, Germany Diagnostics GmbH, Mannheim, Germany). The cDNAs used as templates in PCR were synthesized using 1 µg DNase I treated RNA with M-MLV-RT and 500 µg/ml oligo dT12-18 primers (Invitrogen, Carlsbad, CA) in a 20 µl reaction volume with incubation at 37°C for 50 min.

2.2. Polymerase chain reaction and amplified DNA fragment isolation

To amplify cDNA of the mature peptide sequence of cLL, a PCR reaction was performed using FastStart DNA Taq-polymerase (Roche Diagnostics Gmbh, Mannheim, Germany), cLL-specific forward primer 5'-GGATCCAAACCAACACAGATTTTTCC-3', and cLL-specific reverse primer 5'-GCGGCCGCAAACTGGCAGACAACAAG-3' (Hogenkamp et al., 2006). The forward primer contained the BamHI restriction site sequence, and cLL reverse primer contained the NotI restriction site sequence. Amplification comprised of initial denaturation at 95°C for 2 min, followed by 40 cycles consisting of 95°C for 30s, 49°C for 30s, 72°C for 1 min and a final extension at 72°C for 7 min. PCR-products were analyzed by agarose gel electrophoresis and purified with a QIAEX agarose gel extraction kit (Qiagen, Valencia, CA).

2.3. Cloning and sequencing of the PCR products

The purified PCR fragments were ligated into a pCR® 4-TOPO® plasmid vector (Invitrogen, Carlsbad, CA). Ligated plasmids were transfected into TOP10 Escherichia coli cells by heatshock. Clones were selected by growth on Luria-Bertani broth (LB)-plates containing 100 µg/ml kanamycin. Positive clones were screened with PCR for correct product size and sequenced. Sequence reactions were performed using an ABI PRISM BigDye Terminator v3.0 Ready Reaction Cycle Sequencing kit (Applied Biosystems, Foster City, CA). All reactions were carried out in both directions using the T7 and T3 primer sites and separated on an ABI PRISM 3100 fluorescent DNA sequencer (Applied Biosystems, Foster City, CA). cLL-inserts were isolated from positive clones using BamHI and NotI restriction sites and ligated into a pABC-cystatin-hisN vector with a cystatin signal peptide and an in-frame N-terminal His-tag.

2.4. Transfection

HEK293-EBNA cells (ATCC CRL10852) were grown in 90% Freestyle (GIBCO; Invitrogen, Carlsbad, CA) and 10% Ca^{2+}-free Dulbecco's modified Eagle's medium (DMEM) (GIBCO; Invitrogen, Carlsbad, CA), containing 5% FCS (Invitrogen), 1% pluronic (Sigma-Aldrich, St.Louis, CA), 10 mM HEPES, 4 mM L-glutamine, 200 U/l penicillin G, 0.1 mg/l streptomycin and 50 µg/ml geneticin. Cells were maintained in exponential growth using Erlenmeyer flasks at 120 rpm on an orbital shaker mounted in a Reach-In CO_2 incubator (Clean Air Techniek, Woerden, The Netherlands). HEK293-EBNA cells were transfected using DNA-PEI (Polysciences, Warrington, PA) according to Durocher et al (2002). Briefly, 24 hrs before transfection, cells were seeded at 2.5 x 10^5/ml in medium without FCS. The next day DNA-PEI complexes were formed by a 10 min incubation of plasmid DNA at 20

µg/ml with PEI at 40 µg/ml in Optimem (GIBCO; Invitrogen, Carlsbad, CA), 25 µl of this mixture was used for each ml of cell culture to be transfected. Small scale transfections (4 ml) were performed in 6 well plates, large scale transfections were performed in a 3L Erlenmeyer flask (Corning, NY).

2.5. Purification of cLL

The supernatant of transfected HEK293-EBNA cells was collected after 6 days and concentrated to a final volume of approximately 250 ml using a hollow fiber column (molecular-mass cut-off 10 kDa, Amersham Biosciences, Uppsala, Sweden). Purification of cLL was performed by affinity chromatography (adapted from van Eijk et al., 2002). Briefly, 1 ml (bed-volume) mannan-sepharose (Sigma, St. Louis, MO), equilibrated in 50 mM Tris-HCl, 5 mM $CaCl_2$, and 0.05% (vol/vol) Tween-80, pH 7.4 was added to the supernatant and $CaCl_2$ was added to a final concentration of 5 mM. The mixture was stirred overnight at 4°C. Sepharose beads were washed with 25 ml washing buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, 500 mM NaCl, 0.05% Tween-80, pH 7.4). This washing procedure was repeated with 25 ml washing buffer without Tween-80. cLL was eluted with 50 mM Tris-HCl, 5 mM EDTA, pH 7.4. The eluted protein was concentrated using Amicon Ultra centrifugal filter units with a molecular weight cutoff of 10 kDa (Millipore, Billerica, MA), after which the buffer was changed to 5 mM Tris-HCl, 150 mM NaCl, pH 7.4 by repetitive washing. Purified cLL was stored in aliquots at -20°C.

2.6. Electrophoresis and Western Blot Analysis

Proteins (0.1-1 µg/lane) were analyzed by SDS-PAGE as described by Laemmli (1970) using 10% polyacrylamide gels. Protein bands were visualized by Coomassie

staining. For immunoblot analysis, proteins (0.1-1 µg/lane) were transferred electrophoretically from the gels onto nitrocellulose membrane. Immunostaining was performed using mouse anti-His_6 monoclonal antibody (Roche Diagnostics Gmbh, Mannheim, Germany). Primary antibodies were detected by peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) (Sigma, St. Louis, MO).

2.7. Enzymatic deglycosylation of cLL

N-deglycosylation treatment of cLL was performed using N-glycanase® PNGase F (ProZyme, Inc., San Leandro, CA) according to the manufacturer's instructions. Briefly, 45 µl cLL (230 µg/ml) was mixed with a 10 µl incubation buffer (0.25 M sodium phosphate, pH 7.0) 2.5 µl denaturation solution (2% SDS and 1 M β-mercaptoethanol). The sample was heated to 100°C for 5 min, after which the sample was cooled and 2.5 µl detergent solution (15% NP-40) was added. 2 µl of N-glycanase (5 U/ml) was added, and after 16 hr of incubation at 37°C, samples were concentrated using Microcon centrifugal filter units (MWCO 10 kDa) (Millipore, Billerica, MA). Samples were immediately processed for further analysis. O-deglycosylation treatment was performed using 1 µl 0.5 mU/µl O-Glycosidase (Roche Diagnostics Gmbh, Mannheim, Germany) according to the protocol used for N-glycanase-PNG-ase. However, for O-glycosidase treatment the cLL storage buffer was changed to phosphate buffer (pH 7.0) using Microcon centrifugal filter units (MWCO 10 kDa) (Millipore, Billerica, MA). Deglycosylation by both N-glycanase-PNG-ase and O-glycosidase was carried out similarly to O-deglycosylation, but in this case both enzymes were added simultaneously.

2.8. DIG-Glycan detection

To assess possible glycosylation of cLL and enzymatically treated cLL, the DIG-Glycan detection kit (Roche Diagnostics Gmbh, Mannheim, Germany) was used according to the manufacturer's instructions. Briefly, samples containing cLL were loaded onto gel and subsequently transferred from the gel onto nitrocellulose membrane.

The presence of glycoconjugates was assessed by labeling oxidized sugars with DIG-0-3-succinyl-ε-aminocaproic acid hydrazide, which was subsequently detected using anti-digoxigenin-AP and staining with NBT/X-phosphate.

2.9 Bis (Sulfosuccinimidyl) suberate (BS)-crosslinking of cLL

Crosslinking of cLL using Bis (Sulfosuccinimidyl) suberate (BS^3) (Pierce, Rockford, IL) was performed according to the manufacturer's instructions. Briefly, the cLL storage buffer was changed to 10 mM HEPES buffer, pH 7.5 containing 3 mM EDTA using Microcon centrifugal filter units (MWCO 10 kDa) (Millipore, Billerica, MA). Final concentration of cLL was approximately 170 µg/ml. BS^3 (50 mM) was added to the protein solution and incubated at room temperature for 30 min. The reaction was stopped by adding SDS-PAGE sample buffer to the mixture, which was subsequently heated to 100°C and loaded onto the gel for further analysis.

2.10. MALDI TOF-TOF

After protein separation on 12% SDS-PAGE and fixation in 50% methanol and 7% acetic acid, cLL was visualized using GelCode Blue Stain reagent (Pierce, Rockford, IL). The four visible bands were cut from the gel individually and subjected to in-gel tryptic digestion as previously described by van Balkom et al. (2005). Subsequently, these bands were identified by matrix-assisted laser desorbtion/ionization (MALDI)

TOF-TOF analysis. The samples, dissolved in 0.1% acetic acid, were concentrated using μC18-ZipTips (Millipore) and eluted directly on the target plates in 1 μl of a saturated solution of R-cyano-4-hydroxycinnamic acid in 50% (v/v) acetonitrile. Data were acquired on a MALDI TOF-TOF instrument (Applied Biosystems 4700 Proteomics Analyzer) in positive reflectron mode at a laser intensity of 3800 and a bin time of 0.5 ns.

2.11. Carbohydrate specificity

Polyacrylamide (PAA)-coupled glycoconjugates (~20% substitution; Lectinity, Lappeenranta, Finland) were coated (5 μg/ml) in 0.2 M sodium cacodylate buffer (pH 9.2) on NUNC maxisorb plates (NUNC, Roskilde, Denmark) overnight at 4°C. Plates were blocked with 1% ELISA-grade BSA (Fraction V, fatty acid free; Calbiochem, San Diego, USA) in TSM (20 mM Tris-HCL pH 7.4, 150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$) and cLL was added (10 μg/ml) for 2-3 hrs at 37°C in the presence or absence of 10 mM EDTA. After washing with TSM containing 0.1% Tween-20, 1 μg/ml anti-His_6 antibody (Roche Diagnostics Gmbh, Mannheim, Germany) was added for 1 hr at room temperature. Binding was detected using a peroxidase-labeled anti-mouse IgG antibody (Jackson, West grove, PA, USA) and measured in a Benchmark Microplate Reader using the Microplate Manager software (both from Biorad, Hercules, CA).

2.12. Testing the activity of cLL against human isolates of IAV

2.12.1. Virus preparations

IAV was grown in the chorioallantoic fluid of 10-day-old chicken eggs and purified on a discontinuous sucrose gradient as described previously by Hartshorn et al. (1988). Virus stocks were dialyzed against PBS, aliquoted, and stored at -70°C. A/Phillipines/82 (H3N2) (Phil), and its bovine serum β inhibitor-resistant variant Phil/BS, were provided by Dr. E. M. Anders (Department of Microbiology, University of Melbourne, Melbourne, Australia). A/Puerto Rico/8/34 (H1N1) (PR-8), which lacks the high-mannose glycans on the haemagglutinin molecule, was provided by Dr. J. Abramson (Department of Pediatrics, Bowman Gray School of Medicine, Wake Forest University, Winston Salem, NC).

2.12.2. Neutralization of infectivity

Madin-Darby canine kidney (MDCK) cell monolayers were prepared in 96-well plates and grown to confluence. These layers were then infected with a PBS^{++} (PBS with 1 mM calcium and 0.5 mM magnesium; Gibco BRL, Grand Island, NY) - diluted IAV preparation (Phil strain) which was preincubated for 30 min at 37°C in the presence or absence (control) of increasing amounts of cLL. After exposure of the MDCK cells to the IAV or IAV/cLL mixture for 30 min at 37°C, the cells were washed three times in serum-free DMEM (Gibco BRL, Grand Island, NY) containing 1% (w/v) penicillin-streptomycin and subsequently incubated for 7 hrs at 37°C. Next, the monolayers were washed, fixed, and FITC-labeled for IAV nucleoprotein as described previously by Hartshorn et al. (2002), after which fluorescent foci were counted.

2.12.3. Haemagglutination-inhibition assay

Haemagglutination (HAA)-inhibition was measured by serially diluting cLL preparations in round-bottom 96-well plates (Serocluster U-Vinyl plates; Costar, Cambridge, MA) using PBS^{++} as diluent (25 µl per well). After adding 25 µl of IAV solution, giving a final concentration of 40 HAA U/ml or 4 HAA U/well, the IAV/cLL mixture was preincubated for 15 min, followed by the addition of 50 µl of human erythrocyte suspension in PBS^{++}. The entire procedure was performed at room temperature. The minimal concentration of cLL, required to fully inhibit the HAA caused by the virus, was determined by reading the plates after 2 hrs. HAA was detected as the formation of a pellet of erythrocytes.

2.13. Statistical analysis

Statistical analysis was performed using SPSS version 12.0.1 for Windows. Analysis of mean values between groups was carried out using Levene's Test for Equality of Variances. When equality of variance was assumed, mean values between groups were compared with an independent t-test, in which $p<0.05$ was considered to indicate statistical significance.

3. Results

3.1. Production and characterization of recombinant cLL

Preliminary results from the small scale transfections (not shown) showed that cLL was most effectively secreted using the mature peptide sequence ligated into the pABC-cystatin-hisN vector with a cystatin signal peptide and an in-frame N-terminal His-tag. Therefore, this vector was selected for large-scale (1 liter) transfections. The predicted amino-acid sequence of cLL expressed in this vector is shown in **Figure 1**. Affinity purification of the recombinant protein using mannan-sepharose yielded approximately 500-800 µg of protein per 1L batch (**Figure 2a**). Analysis of the purified product on SDS PAGE showed that four different bands varying in size between approximately 22 kDa and 27 kDa were present. Western Blot analysis using anti-His_6 antibody showed a positive signal for all four bands (**Figure 2b**) in the untreated cLL-sample (Lane 1) indicating that all four bands were recombinant products . To identify whether different glycosylation patterns of the protein could account for the observed multiplet, a DIG-glycan staining was performed. Results of this DIG-glycan detection are shown in **Figure 2c** and indicate that all of the 4 recombinant protein bands contained sugar moieties. However, enzymatic treatment with N-glycanase / PNGase or O-glycanase of cLL did not result in a size shift (lane 2-6), that would indicate loss of sugar moieties. The multimeric state of cLL was investigated by crosslinking experiments using Bis (Sulfosuccinimidyl) suberate. SDS PAGE analysis of the crosslinked cLL showed that monomeric cLL bands were not present anymore while two distinct high-molecular weight bands appeared upon BS^3 treatment of cLL (**Figure 3**).

296 *3.2. Analysis by MALDI-TOF-TOF*

297 MALDI-TOF-TOF analysis was performed in order to investigate the cause of
298 appearance of four bands. However, the spectra of the trypsin-digested protein of all
299 four bands were similar, and could not explain the size difference observed on SDS-
300 PAGE. Several peaks in the spectra could be assigned to sequences in the
301 recombinant protein, including the N- and C-terminus which were retrieved for all
302 four bands (data not shown).

303

304 *3.3. Carbohydrate specificity*

305 Carbohydrate specificity of cLL was tested by use of PAA-coupled glycoconjugates.
306 cLL was observed to preferentially bind to α-mannose-PAA and trimannose-PAA
307 (Man3), but not to any of the other glycoconjugates (**Figure 4**). Binding to α-
308 mannose-PAA and Man3-Paa was also tested in the presence of EDTA, which
309 significantly reduced the binding, indicating that cLL binding to α-mannose and
310 Man3 is calcium-dependent.

311

312 *3.4. Testing the activity of cLL against human isolates of IAV*

313

314 *3.4.1. Neutralization of infectivity*

315 In order to investigate whether cLL was capable of protecting MDCK cell
316 monolayers from infection by Phil-strain, Phil/BS-strain or PR-8 strain IAV, virus
317 preparations were preincubated with increasing concentrations of cLL prior to adding
318 them to the cells. No differences were observed in the number of fluorescent foci,
319 indicating that cLL does not interfere with infection of these cells (data not shown).

320

321 *3.4.2. cLL mediated HAA-inhibition*

322 In the HAA-inhibition-assay, cLL was capable of inhibiting HA-activity of all three
323 human isolates of IAV tested. The mean concentrations at which erythrocytes were
324 no longer agglutinated by the virus are depicted in **Figure 5**. Phil/BS was inhibited at
325 a much lower concentration of cLL than its parent strain Phil. Haemagglutination-
326 activity of PR-8 was inhibited at a concentration comparable to that of Phil.

4. Discussion

We previously reported the finding of cLL which, based on sequence homology, was predicted to be a C-type lectin (Hogenkamp et al., 2006). The sequence of its CRD is most similar to SP-A, but cLL does not contain a collagenous domain and therefore could not be classified as a collectin. To our knowledge, this protein lacks further similarity to other C-type lectins and should therefore be regarded as a novel type of lectin (Hogenkamp et al., 2006).

In this study, recombinant cLL was used to further characterize structural and functional properties of this lectin. It was found that cLL appeared as four differently sized products on SDS-PAGE gel (**Figures 2a and b**). No potential GPI-modification sites were found in the sequence, and since the sequence of cLL does not contain any potential N- glycosylation sites, it is highly unlikely that differential N-glycosylation could account for the appearance of four products instead of one. However, a positive signal was observed for all bands using the DIG-glycan detection kit (**Figure 2c**). Aspecific DIG-labeling or staining may explain this result. Accordingly, enzymatic treatment with N-Glycanase and/or O-glycosidase did not result in a size shift (**Figures 2b and c**), and no difference in intensity of DIG-glycan staining was observed between enzymatically treated samples. However, it cannot be excluded that cLL contains sugar moieties that are insufficiently removed by enzymatic treatment. As for O-linked glycans, it is known that these glycans are highly heterogeneous while in general O-glycosidases show a restricted specificity for subclasses of O-linked sugars. Therefore, we can not exclude the possibility that O-linked glycans are indeed present and result in heterogeneity of the cLL polypeptide chains as illustrated by results presented in **Figure 2**.

352 The four bands obtained by SDS-PAGE analysis on purified cLL were
353 subjected to MALDI-TOF-TOF and resulted in identical spectra in which the C- and
354 N-terminal fragments could be retrieved for all four bands, identifying all bands as
355 (isoforms of) cLL.
356 This method is rather stringent, therefore it is possible that posttranslational
357 modifications are removed during the procedure. Further analysis using 2D gel
358 electrophoresis combined with liquid chromatography coupled to tandem mass
359 spectrometry (LC-MS/MS) may reveal what causes the differences in mass. As this
360 concerns a recombinant protein, it would be very interesting to find out whether the
361 same size differences are present in native cLL.
362 In mammalian species, collectin monomers are known to organize into higher
363 order multimers (Crouch et al., 1994; King et al., 1989). Crosslinking cLL led to the
364 formation of two high molecular weight products (**Figure 3**), suggesting that this
365 protein is capable of forming higher order subunits. The presence of heptad repeats
366 in the cLL-amino acid sequence could allow for the formation of trimeric subunits
367 (Hogenkamp et al., 2006), characteristic for all C-type lectins. Furthermore, it is
368 known that the carbohydrate-binding activity of C-type lectins is quite weak when
369 they are in their monomeric form (Kishore et al., 1996). During the procedure of
370 affinity-purifying cLL by use of mannan-sepharose beads, the beads were washed
371 twice with a relatively large volume of washing buffer. These conditions would most
372 likely be too stringent if carbohydrate-binding by cLL were weak, making it more
373 likely that this protein is present as a trimer or a higher multimeric form.
374 Analysis of the carbohydrate-specificity of cLL revealed that the protein
375 binds to α-mannose and Man3 in a calcium-dependent manner, but other
376 carbohydrates tested (including galactose) were not bound by cLL (**Figure 4**). It is

possible that the presence of a Glu-Pro-Ser motif in the CRD of cLL accounts for the relatively high specificity of cLL. Most other mannose type-collectins contain a Glu-Pro-Asn motif (Drickamer, 1992) and most SP-As contain a Glu-Pro-Arg motif, whilst retaining their preference for mannose over galactose (McCormack et al., 1994). However, Drickamer (1992) showed that substitution of one amino acid is enough to change the sugar binding specificity of C-type lectins. It would be interesting to determine if construction of a similar Glu-Pro-Ser motif in other C-type lectins would result in a comparable specificity for mannose.

The activity of cLL against human isolates of IAV was tested in viral inhibition assays. In HAA-inhibition assays cLL showed strong activity against the Phil/BS strain (**Figure 5**). Two other strains tested, Phil strain and PR-8, were less susceptible to cLL inhibition. The infection of MDCK cells by IAV could not be inhibited by cLL which may be explained by the, compared to collectins, relative weak interaction of cLL with these IAV strains. The change in equilibrium between cLL-bound and free virus particles could result in a constant supply of free IAV as MDCK cell infection draws free virus particles from the medium. This may explain the discrepancy between the results from the MDCK cell infections and the HAA-inhibition assays.

It is not yet clear what mechanism underlies the susceptibility of the different IAV strains with respect to the inhibition of haemagglutination by cLL. The Phil/BS strain (subtype H3N2) differs from the parent Phil strain, in that the high-mannose oligosaccharide overlying the sialic acid receptor-binding site of the HA molecule is absent (Hartley et al., 1992). It is possible that this reveals targets for cLL binding that are not available in the parent strain, resulting in increased binding. The PR-8 strain also lacks high mannose glycans on the HA molecule (Schwarz and Klenk,

1981), but since this concerns a H1N1 subtype of IAV it is possible that differences in targets available on the HA molecule or the neuraminidase may result in decreased binding. It will be interesting to see what mediates cLL binding to IAV, since binding of SP-A (to which cLL is most similar) is thought to occur via binding of the sialic acid receptor of the virus to sialylated N-linked oligosaccharide present in the CRD of SP-A (Benne et al., 1995). It is possible that cLL, similar to SP-D (Hartshorn et al., 2000), binds to HA and neuraminidase in a C-type lectin-like manner, but the exact mechanism remains to be elucidated.

Conclusion

The chicken lung lectin was successfully expressed in HEK293-EBNA cells. The purified protein proved to be a C-type lectin, as predicted from its sequence. Analysis of its carbohydrate specificity revealed that this protein has an unusual high preference for binding α-mannose and trimannose. The results of the viral inhibition assays showed that cLL has moderate HAA-inhibition activity against the human IAV strains Phil and PR-8 and a strong inhibitory activity against Phil/BS. These results indicate that cLL could play an important part in the innate immune system of chickens.

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Figure Captions.

Figure 1. Predicted amino acid sequence of recombinant cLL.

1: extra Gly-Ser-residues added after signal peptide; **2**: extra Gly-Ser-residues added as a result of inserting the BamHI restriction site into the sequence; **3**: extra Alanine-residues added as a result of inserting the NotI restriction site into the sequence

Figure 2. Production and structural characterization of cLL.

(**A**) Coomassie staining of cLL eluted from mannan-sepharose beads. (**B**) Western Blot-analysis of the eluted cLL and enzymatically treated cLL using anti-His6. (**C**) DIG-glycan detection for both Sham-treated and enzymatically treated cLL. *(B&C): 1: untreated cLL, 2: Sham-treated N-Glycanase-PNGase, 3: N-Glycanase-PNGase-treated cLL, 4: N-Glycanase-PNGase and O-glycosidase-treated cLL, Lane 5: O-glycosidase-treated cLL, Lane 6: Sham-treatment O-glycosidase.*

Figure 3. BS-crosslinking of cLL.

Left lane: untreated cLL; Right lane: crosslinked cLL.

Figure 4. Carbohydrate specificity of cLL.

cLL binds to α-mannose and Man3 in a calcium-dependent manner. *Man3: trimannose; LeX: Lewis X; LeY: Lewis Y; LeA: Lewis A; H-type 2: Lewis H-type 2; H-type 3: Lewis H-type 3; LN: Gal-β-GlcNAc; LDN: GalNAc-beta-GlcNAc; chi-3: GlcNAc-GlcNAc-GlcNAc*

Figure 5. Haemagglutination activity-inhibition by cLL

Minimal concentration of cLL (μg/ml) necessary to inhibit HA-activity of human isolates of IAV. Values are mean concentration ± s.d. of three separate experiments.

<-------------------- **Signal peptide** ----------><-**1**-><-- **HIS-tag** ---><-**2**-><----------
M-A-R-P-L-C-T-L-L-L-L-M-A-T-L-A-G-A-L-A-G-S-H-H-H-H-H-H-G-S-K-P-T-Q-I-F-P-

---------- **N-terminal region** ----------><---------- **Neck Domain** ----------
V-P-G-F-K-A-E-R-G-I-S-Q-A-Y-L-P-G-F-P-S-V-A-G-S-E-M-D-D-A-V-L-Q-L-K-D-R-I-S-

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K-L-E-G-V-L-Y-L-Q-G-K-I-T-K-S-G-G-K-I-F-A-T-S-G-K-T-A-D-F-H-A-T-V-K-M-C-Q-E-

---------- **Carbohydrate Recognition Domain** ----------
A-G-G-C-I-A-S-P-R-N-A-D-E-N-A-A-I-L-H-F-V-K-Q-F-N-R-Y-A-Y-L-G-I-K-E-S-L-I-P-

----
G-T-F-Q-F-L-N-G-G-E-L-S-Y-T-N-W-Y-S-H-E-P-S-G-K-G-E-E-E-C-V-E-M-Y-T-D-G-T-

----------><-**3**-->
W-N-D-R-R-C-N-Q-N-R-L-V-V-C-Q-F-A-A-A