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Research Article

Identification of chicken lysozyme *g*2 and its expression in the intestine

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Abstract. Lysozyme is an important component of the innate immune system, protecting the gastrointestinal tract from infection. The aim of the present study was to determine if lysozyme is expressed in the chicken (*Gallus gallus*) intestine and to characterise the molecular forms expressed. Immunohistochemical staining localised lysozyme to epithelial cells of the villous epithelium along the length of the small intestine. There was no evidence for lysozyme expression in crypt epithelium and no evidence for Paneth cells. Immunoblots of chicken intestinal protein revealed three proteins: a 14-kDa band consistent with lysozyme *c*, and two additional bands of approximately 21 and 23 kDa, the latter consistent with lysozyme *g*. RT-PCR analyses confirmed that lysozyme *c* mRNA is expressed in 4-day, but not older chicken intestine and lysozyme *g* in 4- to 35-day chicken intestine. A novel chicken lysozyme *g*2 gene was identified by in silico analyses and mRNA for this lysozyme *g*2 was identified in the intestine from chickens of all ages. Chicken lysozyme *g*2 shows similarity with fish lysozyme *g*, including the absence of a signal peptide and cysteines involved in disulphide bond formation of the mammalian and bird lysozyme *g* proteins. Analyses using SecretomeP predict that chicken lysozyme *g*2 may be secreted by the non-classical secretory pathway. We conclude that lysozyme is expressed in the chicken small intestine by villous enterocytes. Lysozyme *c*, lysozyme *g* and *g*2 may fulfil complimentary roles in protecting the intestine.

Key words. Lysozyme; chicken; Paneth cell; antimicrobial; innate immunity.

The gastrointestinal tract is a major site for pathogen invasion and an array of innate defence mechanisms including the synthesis of cationic antimicrobial peptides, such as the defensins, and enzymes, such as lysozyme, operate to prevent this [1–3]. Two structurally distinct forms of lysozyme are found in vertebrates, chicken-type (*c*) and goose-type (*g*), with differing sizes, radically different amino acid sequences and distinctive patterns of expression [4]. Lysozymes are antimicrobial proteins which catalyse (1,4-β-N-acetylmurmidase) the hydrolysis of β(1-4) glycosidic bonds between the N-acetylglucosamine and N-acetylmuramic acid repeating unit of peptidoglycan, a constituent of bacterial cell walls. In addition, a more general antimicrobial role for lysozymes, has been proposed, related to the cationic structure, rather than catalytic activity. Peptides relating to the helix-loop-helix domain (amino acids 87–114) of chicken lysozyme *c* have been shown to be active against both Gram-positive and Gram-negative bacteria with the proposed mechanisms involving crossing the outer membrane via self-promoted uptake and causing damage to the inner membrane through channel formation [5].

In the small intestine of humans and mice, lysozyme *c* is synthesised and secreted by specialised Paneth cells [1, 6–8]. These granulated epithelial cells, located at the base of the intestinal crypts, synthesise a plethora of antimicrobial proteins and peptides, in addition to lysozyme

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[1, 8]. Mice differ from humans in possessing two lysozyme *c* genes, lysozyme M, expressed in leukocytes and several epithelial tissues, and lysozyme P, normally restricted to Paneth cells [9]. Transgenic mice in which the lysozyme M gene has been inactivated compensate by expressing Paneth cell lysozyme in their macrophages, indicative of the importance of the protein in host innate defences [10]. The chicken genome, like the human, has a single lysozyme *c* gene, which is highly expressed in the oviduct and macrophages [4, 11]. Regulation of expression in the two tissues is very different: oviduct expression is under the control of steroid hormones while macrophage expression is regulated by lipopolysaccharide (LPS), and involves increased transcript stability [4]. Whether lysozyme *c* is synthesised by cells of the avian intestine is less clear and the presence of Paneth cells in the avian gastrointestinal tract is controversial. While Humphrey and Turk [12] reported the presence of Paneth cells, based on low-power light microscopic studies, in the crypt epithelium of chick intestinal villi, there is no consensus for the presence of Paneth cells in avians [8]. However, the common expression of lysozyme *c* by a variety of other vertebrate cells, including epithelial cells of the trachea and stomach [13, 14], indicates that lysozyme *c* expression by avian intestinal epithelial cells cannot be excluded.

Northern analysis has identified lysozyme *g* expression in the bone marrow and lung of the chicken, although not in the oviduct, hence its absence from chicken egg white [15]. In contrast, lysozyme *g* is present in the egg white of many birds, including geese and swans, which suggests that the pattern of lysozyme *g* expression varies significantly between avian species [16].

In the present study, we have investigated the hypothesis that lysozymes are expressed in epithelial cells of the chicken (*Gallus gallus*) intestine and thus may contribute to the innate immunity of the chicken gut. The studies led to the identification, through *in silico* analyses, of a novel chicken lysozyme *g*2.

Materials and methods

Immunohistochemical and histological procedures

Chick (17-day-old) small intestine was removed immediately after sacrifice and washed with ice-cold phosphate-buffered saline (PBS). The intestine was divided into three sections and each section arbitrarily designated as duodenum, jejunum and ileum. The three sections were embedded in a single block of paraffin wax and slides of 5- to 10-µm-thick sections prepared. Mouse (14-day-old) small intestine was similarly treated. Sections stained with haematoxylin and eosin (H&E) were imaged under incidence fluorescence to identify Paneth cells [17]. Merged images, using both FITC and TRITC channels, were collected using a Leica TSC NT confocal laser scanning microscope.

Sections for lysozyme immunohistochemistry were dewaxed, washed with 0.1 M PBS, fixed in 2% paraformaldehyde, permeabilised for 10 min using 0.05% Triton X-100 in 1% paraformaldehyde, then blocked for 30 min using 10% goat serum in 0.1 M PBS. After washing, the sections were incubated overnight at 4 °C with a 1:100 dilution of primary antibody raised in rabbits against hen egg white lysozyme (Abcam), washed in PBS and goat anti-rabbit FITC-conjugated secondary antibody (Sigma) diluted 1:100 in 0.1 M PBS and 1% goat serum added to enable visualisation. The sections were washed, mounted using DAKO mounting medium and examined using an Olympus DP50 microscope in fluorescent mode.

Western analyses

Protein was extracted from chick (8-day-old) small intestine by homogenisation in lysis buffer (100 mM NaCl, 10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 10% SDS, 0.5% deoxycholate, 1 µg/ml aprotinin, 100 µg/ml PMSF). The homogenate was heated at 100 °C for 10 min and centrifuged at 4 °C. The supernatant was collected and 100 µg protein (Bradford reagent) electrophoresed on a 7.5% SDS polyacrylamide gel and transferred onto a PDVF membrane (Hybond P; Amersham). The membrane was incubated overnight at 4 °C in a blocking solution of PBS, 0.1% v/v Tween 20 and 5% w/v milk powder containing 1:20,000 dilution of the rabbit anti-hen egg white lysozyme antibody (Abcam). The membrane was washed in PBS Tween 20 (0.1%) and incubated for 60 min with horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma) diluted in blocking solution. After washing, bands were visualised by chemiluminescence (ECL Plus; Amersham) and the membrane exposed to X-ray film according to the manufacturer's recommendations.

Lysozyme mRNA analyses

Total RNA was extracted from avian tissues and white blood cells using RNAzol B (Biogenesis) according to the manufacturer's instructions. To reduce degradation, RNAase inhibitor (Invitrogen) was added to each sample (1 U/µg RNA) before storage at –80 °C. All samples were pretreated, before reverse transcription (RT), with DNAase (Promega) at a concentration of 1 U/µg RNA. RT-PCR was achieved using the GeneAmp RNA PCR kit (Perkin Elmer) and oligonucleotide primers synthesised to regions of the target genes. The nucleotide sequences of the chicken lysozyme *c* and *g* primers were as follows: lysozyme *c* (NM_205281) (forward) 5′GACGATGTGAGCTGGCAG 3′exon 2, (reverse) 5′GGATGTTGCACAGGTTCC 3′ exon2; lysozyme *g* (X61002) (forward) 5′CACGCTGGCAAAATACTGAAG 3′ exon 3,

(reverse) 5′TTCCCAACACCAGCATTGTAG 3′exon 5; lysozyme *g*2 (forward) 5′ CATTCCATCTTTGGTTGC 3′exon 3, (reverse) 5′CCACCTTTGAGCTGCTGTTC3′ exon 4. RT was performed at 42 °C for 1 h followed by heat inactivation for 5 min at 95 °C. cDNA was amplified with an initial 1-min denaturation step of 95 °C, 35 cycles of 1 min at 95 °C, 1 min at the annealing temperature 55 °C, 2 min of 72 °C and a final extension step of 72 °C for 10 min. RT-PCR products were resolved by electrophoresis using either 1 or 1.5 % TBE agarose gels, visualised with ethidium bromide and imaged under UV illumination. In control samples, reverse transcriptase was omitted to demonstrate that PCR amplification was not due to contamination with genomic DNA. RT products were verified by DNA sequencing.

Results

Immunolocalisation of lysozyme in the intestine

Lysozyme was localised to the surface epithelium of villi in the small intestine of chickens, revealed with a polyclonal antibody to hen egg white lysozyme. Particularly strong staining was observed at the enterocyte brush-border luminal surface. Crypts, at the base of the villi, were very poorly stained for lysozyme (fig. 1 C). Immunofluorescent staining was observed in duodenal, jejunal and ileal sections, suggesting lysozyme synthesis by epithelial cells along the linear axis of the chicken small intestine. In contrast, analyses of mouse intestinal sections with the same antibody revealed staining localised to crypt cells at the base of the intestinal villi. No staining was identified along the epithelial surfaces (fig. 1 A). H&E sections from mouse intestine demonstrated marked fluorescence particularly evident in the outer muscle layers and along villi, including the surface brush-border. Focused areas of fluorescence were also observed at the base of the crypts (fig. 2 A) identifying granulated Paneth cells [17]. In contrast, chicken intestine showed generally weaker fluorescence of the mucosa, although, again, muscle layers fluoresced strongly. There was no evidence for cells fluorescing at the base of chicken intestinal crypts (fig. 2), consistent with an absence of Paneth cells.

Lysozyme protein in intestinal tissues

Western blot analyses of intestinal proteins extracted from chicken intestine revealed three protein bands (fig. 3). One protein, migrating at 14 kDa, was characteristic of lysozyme *c* and comparable to that isolated in chicken egg white. In addition, two larger protein bands, migrating at approximately 21 and 23 kDa were observed in protein extracted from chicken intestine, but not chicken egg white.

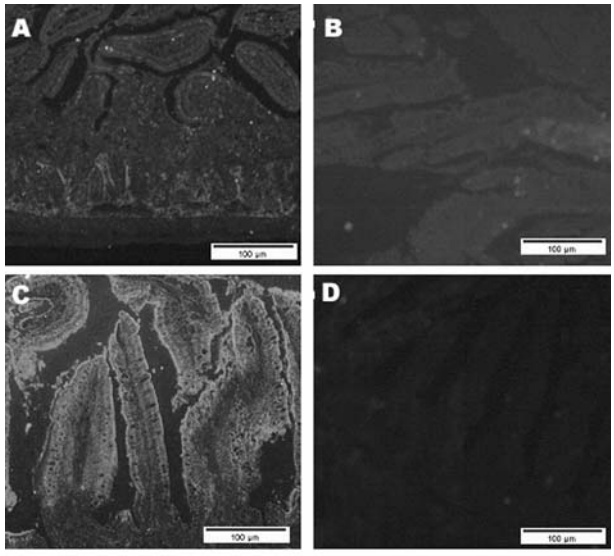

Figure 1. Immunohistochemical localisation of lysozyme *c* in intestine: mouse intestine (14-day-old) (*A*, *B*) and chicken intestine (17-day-old) (*C*, *D*). *A*, *C* Lysozyme *c* localisation. *B*, *D* Control sections with omission of primary (anti-lysozyme) antibody.

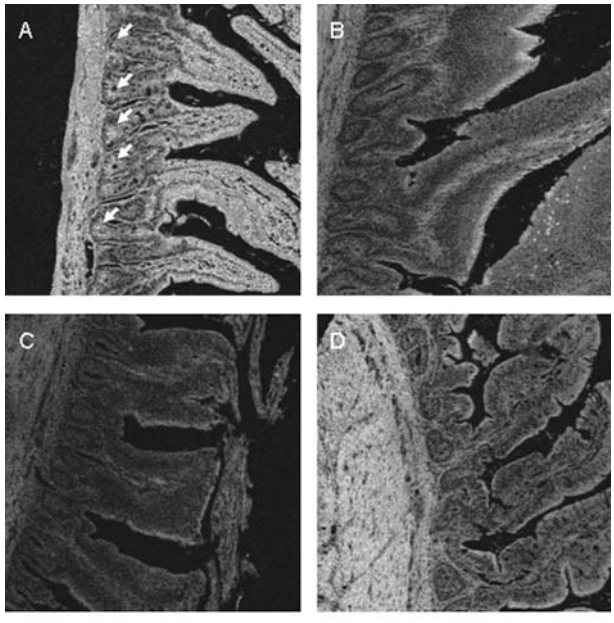

Figure 2. H&E fluorescence of intestinal sections: mouse intestine (*A*) and chicken (17-day-old) duodenum (*B*), jejunum (*C*) and ileum (*D*). Fluorescing Paneth cells at the base of intestinal crypts in the mouse (*A*) are indicated by arrows. All sections imaged with identical settings.

In silico identification of a novel chicken lysozyme *g*2

The Western data indicated two lysozome proteins, in addition to lysozyme *c*, with masses of around 21 and 23 kDa. The 23-kDa form has a molecular mass consistent with that reported for lysozyme *g* [15]. A BLASTn

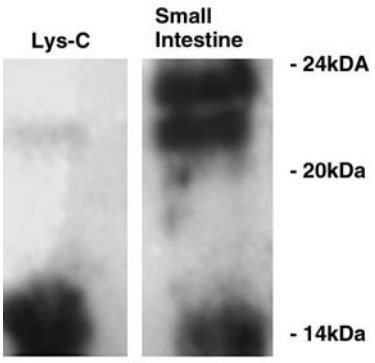

Figure 3. Immunoblot of commercial hen egg white lysozyme *c* and chicken (8-day-old) small intestinal proteins, using rabbit anti-hen egg white lysozyme antibody. The position of marker proteins is indicated.

search of the chicken EST database (http://www.chick.umist.ac.uk [18]) revealed a second novel sequence with DNA homology to the lysozyme *g* gene, encoding a distinct lysozyme *g* product (lysozyme *g*2). The lysozyme *g*2 genomic sequence, like that of lysozyme *g*, is composed of five exons and four introns (fig. 4). Analysis of the chicken genome indicates that both lysozyme *g* and *g*2 map to chromosome 1, lie in a head-to-tail arrangement and are separated by approximately 5.5 kb. The deduced amino acid sequence of lysozyme *g*2 predicts a 21-kDa protein with significant homology to lysozyme *g* (fig. 5). The protein includes the triad of conserved glutamic and aspartic acid residues critical to lysozyme catalytic activity, but the four conserved cysteines involved in disulphide bond formation of the mammalian and bird lysozyme *g* proteins are absent. Chicken lysozyme *g*2 shows considerable homology with type-G lysozymes isolated from fish [19–21] and in particular lacks a predicted signal peptide sequence.

Lysozyme mRNA expression in chicken intestine

To investigate the intestinal expression of lysozyme genes, RT-PCR analyses of RNA extracted from the intestine of 4-, 17- and 38-day birds were performed. In each case, where bands were observed, they were of the predicted size and confirmed by DNA sequencing to be identical to the lysozyme product for the specific primer sets. Lysozyme *c* expression was detected in the intestinal RNA from 4-day-old birds, but lysozyme *c* mRNA was not detected in the intestine of 17- or 38-day birds (fig. 6). Lysozyme *g* expression was observed in the in-

CTGCTGTGTTTGGGTGGGTACATGGAGGCTGCTGGACACTGCCCTTTTCCCATAAAAGCCCTGCCATGAGGGAAGT
GCTAAGGCAGCTGTGCCCACAGCTATAACACACGCACATCTCAAGCAAATGCCCAGAGACAGTGGGGAGTCATC
M S
CTGCAGCAAACATGTCAGG<Intron 1~500bp>
G C S N F Y G N I A N V E T T G A S Q
CTGTTCTAATTTCTATGGGAACATAGCAAATGTTGAAACAACTGGTGCATCACAGAG
R T A K P E G L S Y A
AACTGCGAAGCCGGAAGGTCTGAGCTATGCAGG <Intron 2 ~600bp>
G V A A S E K I A E R D L K N M D K Y
AGTTGCGGCTTCAGAGAAGATTGCTGAAAGAGATTTGAAGAATATGGACAAATATAA
K E T I T K V A N S K C I P P S L V A
AGAAACTATTACAAAAGTGGCCAACAGCAAGTGCATTCCACCATCTTTGGTTGCTGC
A V I S R E S H A G T A L K D G W G D
TGTTATCTCTCGAGAGTCACACGCTGGGACGGCACTGAAGGATGGCTGGGGTGACC
H G N A F G L M Q
ACGGTAATGCATTTGGTTTAATGCAGGT <Intron 3 ~900bp>
V D K R Y H K P H G A W D S E E H I K
TTGACAAACGTACCATAAACCTCATGGGGCATGGGACAGTGAAGAGCACATAAAAC
Q G T D I L C Q S I T D I Q K K F P T
AAGGCACAGACATTTTGTGTCAGTCAATAACCGATATTCAGAAAAAATTCCCAACAT
W S K E Q Q L K G
GGAGTAAGGAACAGCAGCTCAAAGGT <Intron 4 ~740bp>
G I S A Y N A G T R N V R T Y E G
GGTATTTCAGCCTATAATGCAGGAACAAGAAATGTCCGGACCTATGAAGGAAT
M D V G T T H D D Y A N D V V A R A K
GGATGTTGGCACAACACACGACGACTATGCCAACGATGTGGTTGCAAGAGCCAAGTT
F F Q R N G Y *
CTTTCAGAGAAATGGATACTGAGAAGGATATGAGTGATTATACAATACTTACAGCAAATACTTTTACAT
TACCAAAATTGCAAATCGTTAGTCAGTAGGTGATTTTGTTACCAATTTTGTACTGCTGTTAGACACATGCACTGATT
AATAAAATCTAAAGTTGCCCCTGTTAAACAGCATTATGACTCTTCCCTGCAGCAATAAATTGCTGTCTGAGAAACA
ACAAGCAGAGATGAAAACTGCTTGAATCTTTTTGTGAAGTGAAAAAAA

Figure 4. Lysozyme *g*2 genomic sequence determined by in silico analyses. The gene consists of five exons (illustrated) and four introns (summarised). 5′ to the ATG start codon (underlined) is a promoter sequence containing a classical TATAA box (underlined), with the start of transcription G (bold, underlined) 3′ to this. A consenus NF-κB-binding site is indicated (underlined) between the TATAA box and the start codon. 3′ to the fifth exon is a polyadenylation site (underlined).

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                                                  ↓              ↓          ↓
Chicken g    MLGKNDPMCLVLVLLGLTALLGICQGGTGCYGSVSRIDTTGASCRTAKPEGLSYCGVRAS
Chicken g2   ----------------------MSGCSNFYGNIANVETTGASQRTAKPEGLSYAGVAAS
                                   * :  ** :  ::***** ********** ** **
                                             ↓
Chicken g    RTIAERDLGSMNKYKVLIKRVGEALCIEPAVIAGIISRESHAGKILKNGWGDRGNGFGLM
Chicken g2   EKIAERDLKNMDKYKETITKVANSKCIPPSLVAAVISRESHAGTALKDGWGDHGNAFGLM
               ******  *:***  * :*    ** * ::* :********  **:**** ** ****

Chicken g    QVDKRYHKIEGTWNGEAHIRQGTRILIDMVKKIQRKFPRWTRDQQLKGGISAYNAGVGNV
Chicken g2   QVDKRYHKPHGAWDSEEHIKQGTDILCQSITDIQKKFPTWSKEQQLKGGISAYNAGTRNV
             ********  * *: * **:*** **   :  **:*** *:: *************  **

Chicken g    RSYERMDIGTLHDDYSNDVVARAQYFKQHGY
Chicken g2   RTYEGMDVGTTHDDYANDVVARAKFFQRNGY
             *:** **:** **** *******  *   **
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Figure 5. Deduced amino acid sequence of chicken lysozyme *g*2 compared with chicken lysozyme *g*; * indicates identity: indicates similarity. The triad of conserved glutamic and aspartic acid residues critical to lysozyme catalytic activity are highlighted in bold. The four conserved cysteines involved in disulphide bond formation of the mammalian and bird lysozyme *g* proteins (absent in chicken lysozyme *g*2) are indicated by ↓. The N-terminal signal peptide of chicken lysozyme *g* is absent from lysozyme *g*2.

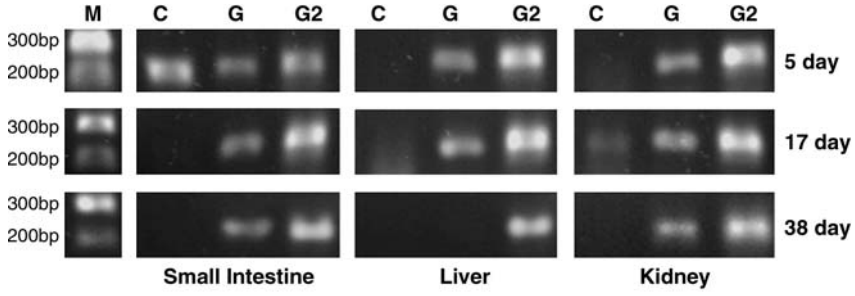

Figure 6. Ethidium bromide-stained gels following electrophoresis of PCR products generated from chicken intestine from 5-, 17- and 38-day-old chicks, using chicken lysozyme *c*-, *g*- and *g*2-specific primers. Products from the kidney and liver are included for comparison.

testinal RNA from birds of all ages. Similarly, lysozyme *g*2 expression was detected in the intestine from chickens of all ages (fig. 6). RNA extracted from chicken liver and kidney revealed expression of lysozyme *g*2 in the animals at each age. Whereas lysozyme *g* expression was in the kidney at all ages, expression was only observed in the liver of younger (4- and 17-day) but not older (38-day) birds. In contrast, lysozyme *c* expression was not detected in the liver or kidney, regardless of age (fig. 6). Consistent with published patterns of expression of chicken lysozyme *c* and *g*, mRNA for all three lysozyme genes was detected in chicken white blood cells (data not shown).

Discussion

Lysozyme is an antibacterial protein, important in innate defence and primarily functioning to protect against microbial attack. In the ruminant stomach, lysozymes have an additional, primary role as digestive enzymes [13, 22]. In the intestine of mice and humans, lysozyme *c* is synthesised by Paneth cells located in the villous crypts, although Paneth cells are not uniformly found in mammalian species [1, 6, 7]. To date the existence of Paneth cells in the avian intestine has only been noted in a single publication using light-microscopic methods [8, 12]. Our own studies provided no evidence for intestinal Paneth cells in the chicken. There was no evidence for characteristically granulated cells, lysozyme localisation or H&E-induced fluorescence, at the base of chicken intestinal crypts, in contrast to the mouse. Thus, while lysozyme is produced by the chicken gut, it is not by specialised Paneth cells. In contrast, the immunohistochemical analyses suggest that avian gut lysozyme is synthesised by intestinal epithelial cells along the length of the villi, consistent with enterocytes.

Lysozyme *c* gene expression was detected in the small intestine of young, up to 8-day-old, birds. However, molecular analyses of the intestinal RNA of older birds (≥ 17-day-old) failed to detect lysozyme *c* mRNA by RT-PCR. This result supports the northern data of Nakano and Graf [15], who also failed to detect lysozyme *c* expression in the small intestine of a 4-week-old bird. As the avian gut

develops, intestinal lysozyme *c* gene expression may be down-regulated.

In addition to lysozyme *c* expression in young birds, lysozyme *g* mRNA was expressed in the intestine and this is the likely source of the lysozyme detected in the intestinal epithelium by immunohistochemical staining. C- and G-type lysozymes, while differing in primary amino acid sequences, exhibit strong common antigenic properties [23]. Thus lysozyme *g* may subsume the role of lysozyme *c* in the adult chicken intestine. Chicken lysozyme *g* has previously been identified in myelomonocytic cells and lung tissue [15]. This same study failed to detect lysozyme *g* in the intestine; this may reflect the insensitivity of the Northern analyses and the relative loading of the gels. Western analyses of the chicken gut proteins using the hen egg white lysozyme antibody identified two protein bands at approximately 21 and 23 kDa, consistent with the synthesis of two lysozyme *g* isoforms. One isoform was predicted to be chicken lysozyme *g* [15] and RT-PCR analyses using specific oligonucleotide primers supported its intestinal expression. Lysozyme *g* is a secreted protein and the lysozyme staining observed in the adult chicken intestine suggests an important role in the innate defence mechanisms of the avian gut.

Mammalian genomes have two lysozyme *g* genes and the proposal has been made that the genes result from early lineage-specific gene duplication [16]. The two human lysozyme *g* genes are located on chromosome 2 and positioned approximately 30 kb apart. Similarly, the mouse genome contains two different but closely linked lysozyme *g* genes, again separated by approximately 30 kb, located on chromosome 1 [16]. Chicken chromosome 1 is also the location of the chicken lysozyme *c* and *g* genes. Our Western data predicted a third lysozyme and BLASTn searching of the BBSRC ChickEST Database identified a second novel chicken lysozyme *g*2 gene. This gene was in close proximity to the lysozyme *g* gene and DNA sequence homology suggests that the two genes probably arose through gene duplication. The encoded amino acid sequence of chicken lysozyme *g*2 predicts a classic C-terminal lytic transglycosylase domain but it does not predict a signal peptide nor does it contain the four conserved cysteine amino acids classically involved in disulphide bond formation.

Fish, in addition to mammals and birds, also synthesise lysozyme *g* [16]. The lysozyme *g* protein synthesised by the Japanese flounder, orange-spotted grouper and common carp shows similarity to the predicted protein sequence for chicken lysozyme *g*2 reported here, in that they lack a signal peptide and the cysteines necessary for disulphide bond formation [19–21]. Chicken *g*2, together with flounder and grouper lysozyme *g*, retains the triad of conserved glutamic and aspartic acid residues critical to lysozyme catalytic activity, and recombinant flounder and grouper lysozyme *g* demonstrate classical lysozyme lytic activity against *Micrococcus lysodeikticus* and other bacteria [19, 20]. Moreover, as observed in the chicken, the flounder and grouper lysozyme *g* genes are expressed in whole blood and by an array of tissues including the kidney, liver and intestine [19, 20]. Interestingly, the flounder lysozyme *g* gene was shown to be up-regulated in response to a bacterial challenge, especially in the intestine, which suggests that this protein plays an important role in fish innate immunity [19]. Whether lysozyme *g* expression in the avian intestine is upregulated in response to infection is not known. However, the presence of putative NF-κB transcription factor-binding sites in the 5′ untranslated region of the lysozyme *g*2 gene strongly suggests a role in the avian innate immune response.

Chicken lysozyme *g* has a 26-amino-acid N-terminal extension presumed to be a signal sequence [15]. In contrast, fish lysozyme *g* does not have predicted signal peptide sequences within the N-terminal sequences [16, 19–21]. Thus, fish lysozyme *g* may not be secreted, but may instead serve an intracellular function [16]. Similarly, chicken lysozyme *g*2 does not have a predicted N-terminal signal peptide sequence (fig. 4). Secretion of proteins without a classical N-terminal signal sequence is recognised and interestingly includes proteins involved in immune responses, such as annexin 1, thioredoxin, interleukins and galnectin [24]. Analysis of chicken lysozyme *g*2 using a sequence-based method for prediction of secretory proteins targeted to the non-classical secretory pathway, SecretomeP [24], confirmed the absence of an N-terminal signal sequence, while the neural network prediction score was 0.620 (above the threshold of 0.5) with odds of 1.6 that the protein is secreted. In contrast, fish lysozyme *g* does not predict signal peptide sequences and has low prediction scores for accessing a non-classical pathway of protein secretion. While not conclusive, particularly since the SecretomeP predictive software is trained on mammalian protein sequences, this raises the interesting possibility that chicken lysozyme *g*[2] may be a secreted protein in the intestine.

That the lysozyme *g* genes are expressed at very low levels in mammals is interesting. The exception appears to be the mouse lysozyme *g* gene which is expressed at relatively high levels in the mouse tongue [16]. To date, there are no reports in mammals of intestinal lysozyme *g* expression which may reflect the expression and synthesis of lysozyme *c* in intestinal Paneth cells. One is tempted to speculate that the lysozyme *g* proteins are the major antibacterials in the chicken small intestine and play important roles in controlling bacterial numbers in this part of the gastrointestinal tract compared to the large intestine and caecum. However, whether synergy exists between these two proteins, and their precise role in intestinal innate immunity remain to be established.

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