Cloning of comparative gene identification-58 gene in avian species and investigation of its developmental and nutritional regulation in chicken adipose tissue

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ABSTRACT: Adipose triglyceride lipase (ATGL) is the rate-limiting enzyme of lipolysis in chicken adipose tissue. Its regulation is not fully understood. Recent studies suggest ATGL may be regulated by physical protein-protein interactions. Comparative gene identification 58 (CGI-58) has been identified as an activator of ATGL in mice. The purpose of the current study was to clone and sequence the CGI-58 gene in avian species and to investigate its regulation during development, fasting, and refeeding. Here, we report the cloning and sequencing of the complete coding sequence of CGI-58 and the deduced AA sequences for the domestic chicken, turkey, and Coturnix quail. The CGI-58 protein is a 343-AA protein in the chicken and quail, and a 344-AA protein in the turkey. Sequence comparisons with the human and mouse show that the CGI-58 gene is highly conserved among avian and mammalian species, with complete identities at the predicted lipid-binding site. Cell fractionation of chicken fat cells and stromal-vascular cells revealed that CGI-58 is expressed primarily in mature adipocytes (P < 0.01). When compared in multiple organs and tissues, avian CGI-58 is expressed predominantly in the adipose tissue (P < 0.001), similar to ATGL. To understand CGI-58 expression during adipose tissue development, its mRNA expression was measured along with ATGL and stearoyl CoA desaturase (SCD-1) mRNA, an adipogenic marker, in embryos and adults. Messenger RNA expression of CGI-58 increased (P < 0.05) immediately after hatching, concurrent with peak ATGL expression. It is interesting that CGI-58 remained somewhat increased at posthatch d 11 and 33 as SCD-1 mRNA expression increased (P < 0.05). To evaluate the response of CGI-58 to nutritional status, chickens and quail were fasted for 24 h and subsequently refed. After the fasting period, CGI-58 mRNA was induced (P < 0.05) for both chickens and quail and was returned to control levels upon refeeding. The ATGL mRNA responded similarly, increasing dramatically after fasting and quickly decreasing with refeeding. The direct relationship between CGI-58 and ATGL mRNA expression indicates a role for CGI-58 in activating ATGL-mediated lipolysis in avian species.

Key words: adipose tissue, adipose triglyceride lipase, chicken, comparative gene identification-58, lipolysis

INTRODUCTION

Lipolysis is a complex process involving several proteins and their interactions with one another. Adipose triglyceride lipase (ATGL) is the rate-limiting enzyme in adipose tissue lipolysis (Villena et al., 2004; Zimmermann et al., 2004). The mechanism of ATGL regulation is not fully understood. Recent studies suggest that interactions with regulatory proteins may modify ATGL activity. Comparative gene identification-58 (CGI-58) has been shown to greatly increase ATGL activity in vitro through protein-protein interaction (Lass et al., 2006).

Although it belongs to the esterase/lipase subfamily of proteins containing α/β hydrolase folds, CGI-58 exhibits no intrinsic lipolytic activity (Lass et al., 2006; Granneman et al., 2011). At basal conditions, CGI-58 is tightly associated with lipid droplets and becomes increasingly colocalized with ATGL during stimulated lipolysis (Subramanian et al., 2004; Granneman et al., 2007). This suggests an important role for CGI-58 in lipolysis.
We have previously cloned the ATGL gene in avian species (Lee et al., 2009). A study of ATGL expression during fasting and refeeding in chickens and quail showed that ATGL mRNA and NEFA in blood increase dramatically after a 24-h fast and return quickly to basal levels after refeeding (Serr et al., 2009). The ATGL protein is increased by fasting, but unlike its mRNA, decreases gradually after refeeding. The discrepancy between ATGL protein expression and NEFA in the blood suggests that the activity of residual ATGL protein may be attenuated by some posttranslational modification, possibly through regulatory proteins. Comparative gene identification-58 has been identified in chickens (Saarela et al., 2008); however, a BLAST (Basic Local Alignment Search Tool) search in the National Center for Biotechnology Information database provides 2 differently sized proteins for chickens (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The purpose of this study was to clone and sequence the CGI-58 gene in the chicken, turkey, and quail, and to investigate its regulation by development and nutritional status.

**MATERIALS AND METHODS**

All animal care and procedures were approved by The Ohio State University Animal Care and Use Committee.

**Experimental Animals**

The ages of animals and sources of tissues have been described previously (Lee et al., 2009). All animals were euthanized via CO₂ inhalation before tissue collections. To determine the tissue distribution of CGI-58 expression, tissue samples were collected from subcutaneous and abdominal adipose depots, thigh and pectoral muscles, heart, lung, liver, and kidney. Additionally, adipose tissue was sampled randomly from mixed-sex chickens at various points of development: embryonic days (ED) 15 and 17, and posthatch days (PD) 1, 5, 11, and 33. Treatments for chicken and quail in the posthatch period (PD) 1, 5, and 11, and 33. Treatments for chicken and quail in the posthatch period (PD) 1, 5, 11, and 17. We performed experiments on animals at these different points of development to determine the tissue distribution of CGI-58 in the chicken, turkey, and quail.

**Cloning and Sequencing of CGI-58 in the Chicken, Turkey, and Quail**

Tissues were homogenized using a Tissue-emulsifier (Fisher Scientific, Pittsburgh, PA) in Trizol reagent (Invitrogen, Carlsbad, CA) to isolate total RNA according to the manufacturer’s protocol. The quality and relative quantity of RNA were assessed by gel electrophoresis and normalized accordingly. Reverse transcription was performed to generate cDNA by using 1 µg of total RNA and Moloney murine leukemia virus reverse transcriptase (Invitrogen). Conditions for each reverse transcription reaction were as follows: 65°C for 5 min, 37°C for 52 min, and 70°C for 15 min. The cDNA generated was used as templates to amplify the portion of the gene that included the entire coding sequence for CGI-58 in the chicken, turkey, and quail. A set of forward (5’-CCA TGG CCG AGG AGG A-3’) and reverse (5’-GAA GTC TTC AGG CTG ATC AGC G-3’) primers were designed according to assembled chicken CGI-58 (gCGI-58) sequences found in The National Center for Biotechnology Information database (EU419873 and NM_001006365). The full-length CGI-58 genes were amplified by PCR using Platinum DNA Taq polymerase (Invitrogen) under the following conditions: 94°C for 2 min, 40 cycles of 94°C for 15 s, 55°C for 60 s, and 72°C for 60 s, and additional extension at 72°C for 10 min. The PCR products were separated on a 1% agarose gel, and cDNA products of the expected size (~1 kb) were excised. The DNA was extracted from the gel fragment by using a Qiagen Gel Extraction Kit (Valencia, CA). The DNA fragment was ligated into the pCR2.1 vector by using a TOPO TA Cloning Kit (Invitrogen). The pCR2.1 vector containing CGI-58 was transformed into TOP10 chemically competent cells (Invitrogen) and plated on Luria-Bertani ampicillin agar with X-gal. Positive colonies (n = 2 per species) were isolated using a QIAprep Spin Miniprep Kit (Qiagen) and sent to The Ohio State University Sequencing Core Facility for sequencing using an Applied Biosystems 3730 DNA analyzer (Foster City, CA).

**Stromal-Vascular and Fat Cell Fractionation**

Chicken stromal-vascular (SV) and fat cell (FC) fractionation was performed as described in our previous reports (Deiuliis et al., 2006, 2008). Abdominal adipose tissue (2 to 3 g) was obtained from a 21-d-old broiler chicken, washed with PBS, and minced with a razor blade. To separate individual cells, the minced tissues were incubated at 37°C for 1 h in a shaking water bath (180 rpm) with 3.2 mg/mL of collagenase II (Sigma-Aldrich, St. Louis, MO). The digested cells were passed through a 100-µm nylon cell strainer (BD Falcon, Franklin Lakes, NJ) to remove undigested cells. The filtered cells were centrifuged at 200 × g for 5 min at room temperature to separate the FC (top layer) and SV pellets, which were immediately collected for RNA isolation by using Trizol reagent (Invitrogen). The total RNA was used to generate cDNA by using Moloney murine leukemia virus reverse transcriptase (Invitrogen) as described above, which was used as a template for quantitative real-time PCR (qPCR).

**qPCR**

To evaluate relative gene expression, qPCR was performed. Primer sequences for chicken ATGL (gATGL), chicken stearoyl-CoA desaturase-1 (gSCD-1), chicken delta-like homolog 1 (gDLK1), and chicken β-actin have been described in our previous report (Lee et al.,...
and Statistical Analysis

Bioinformatics, Sequence Alignment, and Statistical Analysis

Gene homology analysis was performed using the BLAST tool of the National Center for Biotechnology Information. Alignment and comparison of the chicken, turkey, and quail CGI-58 cDNA and AA were done using ClustalX multiple alignment software (Thompson et al., 1994) in BioEdit Sequence Alignment Editor (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

Differences among developmental ages or fasting and refeeding time points were compared by 1-way ANOVA. For P-values <0.05, Tukey’s test for individual comparisons of means between groups was performed. Comparison of gene expression between the FC and SV fractions was accomplished by Student’s t-test. Statistical analysis for the tissue distribution of CGI-58 expression in the chicken, turkey, and quail was performed by a mixed ANOVA model (2 adipose tissues vs. other tissues) followed by Fisher’s protected least significant difference test. All statistical analyses were performed using the SAS software system (SAS Inst. Inc., Cary, NC).

RESULTS

To clone and sequence the CGI-58 gene for the chicken, turkey, and quail, reverse transcriptase PCR was used to amplify the CGI-58 gene. Sequencing of the resulting cDNA showed the complete coding sequence of CGI-58 to be 1,032 bp in the chicken and quail, and 1,035 bp in the turkey. The additional 3 bp in the coding sequence of the turkey were due to an insertion of 1 codon at nucleotide position 15 (Supplemental Figure 1). Sequences from the chicken, turkey, and quail have been deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank) under accession numbers HQ896422, HQ896423, and HQ896424, respectively. The CGI-58 gene among these avian species is highly conserved; the chicken nucleic acid sequence has 98% homology with that of the turkey and 97% with that of the quail. The turkey and quail have 97% nucleotide sequence similarity (Supplemental Figure 1).

Computer generation of the AA sequence from the nucleic acid sequence revealed open reading frames of 343 AA in the chicken and quail, and 344 AA in the turkey (Figure 1). The chicken and turkey proteins exhibited 99% similarity, only because of the insertion of 1 AA in the turkey at position 3. The quail AA sequence showed 98% homology with the chicken. When compared with the deduced AA sequences of both the human and mouse CGI-58, the chicken proteins were 78% homologous to the human and mouse proteins. The gCGI-58 protein was 6 and 8 AA shorter than the human and mouse proteins, respectively, with deletions occurring at the N-terminal region at positions 1 to 3, 6, and 17 to 20 (Figure 1). It is important that the 3 tryptophan residues (positions 21, 25, and 29), which are thought to be important for ATGL and lipid droplet interaction, were conserved in avian CGI-58. Additionally, the canonical lipase motif area of human and mouse CGI-58 (residues 153 to 157) was conserved among the chicken, turkey, and quail.

To investigate gCGI-58 expression in preadipocytes vs. adipocytes, we compared mRNA expression between the FC and SV cell fractions, of which the SV consisted mostly of preadipocytes. We measured the mRNA expression of gATGL and stearoyl-CoA desaturase-1 (SCD-1) as markers for adipocyte differentiation. Kaestner et al., 1989; Lee et al., 2003, 2009). As determined by qPCR, gATGL and gSCD-1 expression was dramatically greater (P < 0.01) in FC than in SV (Figure 2A and 2B). The expression of DLK1, a preadipocyte marker, was also measured to further confirm differentiation (Deulilis et al., 2006). Expression of gDLK1 mRNA was decreased (P < 0.01; Figure 2C) in the FC vs. the SV fraction. We concluded, based on the expression of gATGL, gSCD-1, and gDLK1 in the FC and SV fractions, that the separation of adipose fractions was successful and that it measured the mRNA
expression of gCGI-58. In FC cells, relative gCGI-58 expression was increased by about 7-fold ($P < 0.01$; Figure 2D) compared with that in SV cells.

Relative quantification in multiple tissues revealed that CGI-58 mRNA was detectable in all tissues of the chicken, turkey, and quail, but was greatest in the subcutaneous and abdominal adipose tissues ($P < 0.001$; Figure 3).

The relative expression of gCGI-58, gATGL, and gSCD-1, as measured by qPCR during in vivo adipose development, is shown in Figure 4. From ED 15 to 17, CGI-58 mRNA expression decreased slightly, followed by a significant increase ($P < 0.05$) at PD 1 and PD 5. At PD 11, gCGI-58 mRNA expression was still somewhat increased and tapered off by PD 33. Expression of gATGL mRNA was also increased ($P < 0.05$) at PD 1. The expression of gSCD-1 mRNA remained very low until PD 5 and increased further ($P < 0.05$) at PD 11, declining only slightly at PD 33.

To examine the response of CGI-58 mRNA expression to nutritional status, adipose tissue was collected from chickens and quail that had been fasted for 24 h and subsequently refed for various lengths of time. After the fasting period, CGI-58 mRNA expression increased greatly ($P < 0.05$) in both the chicken and quail (Figures 5 and 6, respectively). Upon 4 h of refeeding in the chicken and 2 h of refeeding in the quail, CGI-58 mRNA expression returned to the levels observed before fasting. Concurrently, we measured the expression of ATGL mRNA, which exhibited the same pattern of increase after a 24-h fast ($P < 0.05$ and $P < 0.01$ in the chicken and quail, respectively), and returned to basal levels after refeeding in both species.

**DISCUSSION**

Avian CGI-58 is a highly conserved protein among the chicken, turkey, and quail, as well as among mammalian human and mouse proteins (78% homologous). The α/β esterase/lipase subfamily of proteins is characterized by α/β hydrolase folds containing a catalytic triad, GXSXG, through which they exert their activity (Ollis et al., 1992). In CGI-58, the active serine residue is replaced by asparagine, preventing it from any inherent lipolytic activity (Lass et al., 2006). This alteration is seen in the human and mouse, and is also present in the chicken, turkey, and quail proteins (residues 153 to 157). This may suggest some evolutionary pressure for maintaining this motif to assist the activity of existing lipases, rather than CGI-58 itself acting as a triglyceride hydrolase.
The chicken and quail proteins are 6 bp shorter than the human protein, and the turkey is 5 bp shorter because of deletions occurring in the first 20 residues. Recently, Gruber et al. (2010) demonstrated that the full sequence of mouse CGI-58 is necessary for the stimulation of ATGL activity in vitro. Truncations at the N-terminal region from position 5 reduced ATGL stimulation, with no apparent stimulatory effect from position 30. The N terminus of mouse CGI-58 has a region encoding 3 tryptophan residues at positions 21, 25, and 29; point mutations to alanine at each of these residues are increasingly unable to localize with lipid droplets and stimulate ATGL (Gruber et al., 2010). The aromatic residues, especially tryptophans, are crucial for binding proteins to membranes (Gelb et al., 1999; Han et al., 1999). It is therefore expected that the tryptophan-rich area may serve as the lipid droplet-binding region, and that it is critical to CGI-58 func-

**Figure 2.** Relative mRNA expression in preadipocytes and adipocytes separated by cell fractionation. Messenger RNA expression of the (A) adipose triglyceride lipase (*ATGL*), (B) stearoyl-CoA desaturase-1 (*SCD-1*), (C) delta-like homolog 1 (*DLK-1*), and (D) comparative gene identification-58 (*CGI-58*) genes in fractionated chicken adipose cells was measured by quantitative real-time PCR (n = 4). β-Actin was used as a normalization gene. Bars represent mean ± SEM **P < 0.001. SV = stromal vascular cell fraction, FC = fat cell fraction.
Cloning and expression of avian CGI-58 genes

Figure 3. Tissue-specific mRNA expression of comparative gene identification-58 (CGI-58) in the chicken, turkey, and quail. Total RNA was isolated from the subcutaneous (SQ) and abdominal adipose tissue (AF), thigh muscle (T. Mus) and pectoralis major muscle (P. Mus), heart, lung, liver, and kidney. (A) Chicken CGI-58 mRNA expression was measured by quantitative real-time PCR (n = 4), using β-actin as a normalization gene. Messenger RNA expression of CGI-58 in (B) turkey (n = 3) and (C) quail (n = 4) tissues was measured by quantitative real-time PCR using ribosomal protein S13 (RPS13) as a housekeeping gene. Bars represent mean ± SEM. ***P < 0.001 (2 adipose tissues vs. other tissues).
Figure 4. Temporal mRNA expression in chickens during development. Total RNA was isolated from the subcutaneous fat of chickens (n = 4) at embryonic days (ED) 15 and 17, and on posthatch days (PD) 1, 5, 11, and 33. Messenger RNA expression of (A) comparative gene identification-58 (CGI-58), (B) adipose triglyceride lipase (ATGL), and (C) stearoyl-CoA desaturase 1 (SCD-1) was measured by quantitative real-time PCR. Bars represent mean ± SEM. *bP < 0.05.
tion. This region is conserved in the chicken, turkey, and quail. The C-terminal truncations that were tested (removals from the C-terminus ranging from 16 to 26 AA in length) resulted in the inability of purified CGI-58 to promote ATGL-mediated FFA release (Gruber et al., 2010). There were no deletions or noteworthy alterations from mouse and human proteins at the C-terminal regions of the avian CGI-58. These sequence data demonstrate that there may be some biological and evolutionary advantages to maintaining the conserved regions of CGI-58 across species.

The ability of CGI-58 to localize with lipid droplets and activate ATGL may also be determined by alternative splicing in some species. A recent in vitro study of mouse CGI-58 characterized a short isoform of CGI-58 lacking exons 2 and 3 (Yang et al., 2010). The short variant exhibited predominantly cytosolic localization and greatly reduced ATGL stimulation, whereas the full-length protein present on the lipid droplet was able to stimulate FFA release through ATGL. Search of the GenBank database and the BLAST search in The National Center for Biotechnology Information database provided 2 nucleotide sequences for the chicken with differently sized open reading frames (GenBank EU419873, 343 AA; GenBank NM_001006365, 208 AA). The shorter version

Figure 5. Expression of comparative gene identification-58 (CGI-58) and adipose triglyceride lipase (ATGL) mRNA in fasted and refeď chickens. Total RNA was extracted from subcutaneous fat at various time points: control (Con), 24-h fasting (24h-F), and after 4 (4h-RF), 8 (8h-RF), 12 (12h-RF), and 24 h (24h-RF) of refeeding. The mRNA expression of (A) CGI-58 and (B) ATGL was measured by quantitative real-time PCR (n = 6), with β-actin used as a normalization gene. Bars represent mean ± SEM. \(^{a,b}P < 0.05.\)
appeared to be missing 154 bp, corresponding with exon 4; therefore we considered the possibility of alternative splicing in the chicken. Using primers designed to amplify the entire region of *CGI-58* between the start and stop codons, we amplified the *CGI-58* gene from cDNA of chickens, turkeys, and quail. Expecting that the appearance of alternative variants of the gene might be affected by an induced lipolytic state, we used cDNA from both control ad libitum-fed and 24-h-fasted chickens. Visualization of the PCR products on a 1% agarose gel revealed only 1 major product of 1,032 bp in length. On the basis of this experiment, we did not predict any alternative splicing variants of *CGI-58* among these avian species.

**Figure 6.** Comparative gene identification-58 (*CGI-58*) and adipose triglyceride lipase (*ATGL*) mRNA expression in quail during fasting and refeeding. Total RNA was extracted from subcutaneous fat at various time points: control (Con), 24-h fasting (24h-F), and after 2 (2h-RF), 4 (4h-RF), and 8 h (8h-RF) of refeeding. The expression of (A) *CGI-58* and (B) *ATGL* mRNA was measured by quantitative real-time PCR (*n* = 6), and β-actin was used as a control. Bars represent mean ± SEM. *a,b* *P* < 0.05.
The CGI-58 protein normally exhibits a tight association with lipid droplets (Subramanian et al., 2004). Cell fractionation revealed that gCGI-58 was predominantly expressed in mature adipocytes when compared with preadipocytes in the SV fraction, which contained very small amounts of lipids. It is important that gATGL was also mainly expressed in adipocytes and that expression was quite low in SV cells (Lee et al., 2009). As an activator of ATGL, it can be expected that CGI-58 will be expressed similarly in different cell types. Quantification of CGI-58 expression in various tissues of the chicken, turkey, and quail showed the greatest amounts in subcutaneous and abdominal adipose tissues, similar to ATGL (Lee et al., 2009). The association of CGI-58 and ATGL gene expression with adipose tissues allows access to the triacylglycerol substrate contained within the lipid droplets of adipocytes.

Temporal expression of lipolytic and adipogenic genes provided insight into adipose tissue development during embryonic and posthatch growth. The SCD-1 gene, a Δ9-desaturase involved in the synthesis of unsaturated fatty acids, was used as a marker for adipogenesis. Expression of SCD-1 mRNA is upregulated during adipocyte differentiation in vitro and in vivo (Kaestner et al. 1989; Lee et al., 2003). Its expression was predictably very low during embryonic time points and PD 1 because the embryo had been using the yolk sac for energy for growth. After the initial stresses of hatching, gSCD-1 expression was increased, indicating adipocyte development with age. Messenger RNA expression of gATGL was considerably heightened at PD 1, concordant with our previous report (Lee et al., 2009), possibly to provide energy after the strain of hatching and before the first access of the chick to food. Expression of gCGI-58 mRNA was also induced at PD 1 but remained relatively high until PD 11. Although we would expect CGI-58 to mirror the attenuated ATGL mRNA expression with increased adipogenesis, it is difficult to say what factors might affect CGI-58 transcription at such an early age when rapid development occurs.

Previously, we reported the induction of the ATGL gene in the chicken and quail by fasting (Serr et al., 2009). We concurrently observed a greater amount of NEFA in the blood. Upon the reintroduction of feed, mRNA expression of ATGL and NEFA quickly decreased to amounts seen in control animals. It was surprising that the amount ATGL protein was still remarkably increased, decreasing gradually over 24 h. We suspected that the protein was somehow inactivated to account for the reduced blood NEFA concentrations; perhaps ATGL enzyme activity was diminished by dissociation from an activator protein. Messenger RNA expression of CGI-58 in fasted and re-fed chickens and in quail was directly related to that of ATGL mRNA. The tapered amount of CGI-58 mRNA after refeeding may result in an insufficient amount of CGI-58 protein to activate the ATGL enzyme to continue NEFA release. In a recent study, we also demonstrated the possibility that an inhibitor of ATGL protein activity, G(0)/G(1) switch gene 2, may have a role in regulating NEFA release after refeeding (Oh et al., 2011). Expression of G(0)/G(1) switch gene 2 mRNA is decreased after fasting, potentially allowing for ATGL enzyme activity, and may inhibit NEFA release when its expression increases during refeeding. We predict it may be the coordinated actions of CGI-58 and G0S2 proteins that allow the ATGL enzyme to respond quickly and efficiently to altered nutritional conditions.

In summary, the CGI-58 gene was cloned and the full-length cDNA sequences were determined for the chicken, turkey, and quail. The CGI-58 AA sequence was very highly conserved among these species, and was very similar to that of humans and mice. Avian CGI-58 was highly expressed in adipose tissue, similar to its target protein, ATGL. The expression patterns of CGI-58 and ATGL mRNA after hatching and during fasting and refeeding suggest that the CGI-58 protein is important in the activation of the ATGL enzyme in avian species.

**LITERATURE CITED**


