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A Novel Polymorphism In The Chicken Adipocyte Fatty Acid-Binding Protein Gene (*FABP4*) That Alters Ligand-Binding and Correlates With Fatness

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Abstract

Similar to the mammalian *FABP4* gene, the chicken (*Gallus gallus*) *FABP4* gene consists of four exons separated by three introns and encodes a 132 amino acid protein termed the adipocyte fatty acid binding protein (AFABP). In the current study, a novel G/A polymorphism in exon 3 of the chicken *FABP4* gene was identified associated with different chicken breeds that lead to either Ser or Asn at amino acid 89 of the AFABP protein. The Baier chicken averages $0.89 \pm 0.12\%$ abdominal fat and expresses the G allele (Ser 89 isoform) while the Broiler chicken typically has $3.74 \pm 0.23\%$ abdominal fat and expresses the A allele (Asn 89 isoforms). cDNAs corresponding to the two AFABP isoforms were cloned and expressed in *Escherichia coli* as GST fusions, purified by using Glutathione Sepharose 4B chromatography and evaluated for lipid binding using the fluorescent surrogate ligand 1-anilinonaphthalene 8-sulphonic acid (1,8-ANS). The results showed that AFABP Ser89 exhibited a lower ligand binding affinity with apparent dissociation constants (Kd) of $7.31 \pm 3.75 \mu\text{M}$, while the AFABP Asn89 isoform bound 1,8-ANS with an apparent dissociation constant of $2.99 \pm 1.00 \mu\text{M}$ ($P=0.02$). These results suggest that the Ser89Asn polymorphism may influence chicken AFABP function and ultimately lipid deposition through changing the ligand binding activity of AFABP.

Keywords

AFABP; chicken; ligand binding; lipid; polymorphism; fatty acid; FABP4

INTRODUCTION

Fatty acid-binding proteins (FABPs) belong to a supergene family of hydrophobic ligand binding proteins expressed widely in invertebrates and vertebrates. FABPs exhibit high affinity and selectivity for long chain fatty acids (FA) (Ockner et al., 1972) and have been implicated in intracellular transport, growth and differentiation, signal transduction, gene transcription, modulation of enzyme activity and protection of enzymes from the detergent-like effects of

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free FA (Tipping and Ketterer, 1981; Grinstead et al., 1983; Glatz and Veerkamp, 1985; McArthur et al., 1999; Zimmerman and Veerkamp, 2002).

In mammals, the intracellular or cytoplasmic FABPs form a group of at least nine distinct proteins that exhibited unique expression patterns and were anecdotally named according to the tissue in which each was first identified: L (liver), I (intestinal), H (heart), A (adipocyte), E (epidermal), IL (ileal), B (brain), M (myelin) and T (testis) (Hertzel and Bernlohr, 2000; Chmurzynska, 2006). Their molecular mass ranges from 14- to 15-kDa and all FABP subtypes show similar structural features containing a helix-turn helix domain and ten antiparallel β strands (β A- β J) that form a β barrel. The bound ligand is found within the barrel in a central internal water-filled cavity. The overall gene organization for the vertebrate FABP gene family is described by four exons and three introns and although the intron length is variable among the members, they bisect the coding region at essentially the identical location in each gene (Bernlohr et al., 1997; Hertzel and Bernlohr, 2000; Zimmerman and Veerkamp, 2002; Storch and Corsico, 2008).

The chicken (*Gallus gallus*) *FABP4* gene has been cloned and found to be organizationally similar to the mammalian *FABP4* gene. The chicken *FABP4* gene consists of four exons separated by three introns and encodes a 132 amino acid protein expressed in the fat tissue (Fisher et al., 2001; Wang et al., 2004). Recently, a *FABP4* polymorphism was reported that may be linked to chicken body fat content. Wang et al. (2006) detected a C/T substitution in the exon 1 of chicken *FABP4* gene that did not change the coding region (both encode phenylalanine) but statistic analysis indicated that the substitution was correlated with chicken abdominal fat content in F2 recourse populations. Independently, Luo et al. (2006) reported that the same substitution mutation in exon 1 was also significantly correlated with abdominal fat, subcutaneous fat and intramuscular fat content of pectoralis major muscle in Beijing-YOU chickens.

In the current study, a novel G/A polymorphism in exon 3 of the chicken *FABP4* gene has been identified that is also associated with fatness of different chicken breeds and changes the AFABP coding region of amino acid 89 to either a serine or asparagine. Moreover, the mitation at exon 3 was statistically linked to the previously reported silent substution mutation in exon 1. To understand the ligand binding properties of the protein linked to this polymorphism, cDNAs corresponding to the two AFABP isoforms were cloned and the corresponding proteins expressed and purified. We report herein that the two isoforms exhibit statistically different ligand binding properteis that may underlie the fat deposition in the chicken.

MATERIALS AND METHODS

Animals

Broiler chickens (*Gallus gallus*) were derived from the Arbor Acres commercial population. The Baier chicken is a Chinese local egg-laying breed which has yellow feathers, beak and feet but white ears. The adult body mass of the Baier chicken averages 1,450 g for cocks and 1,190 g for hens. All birds were raised at the Northeast Agricultural University poultry farm. Commercial corn-soybean-based diets that met all NRC requirements (National Research Council, 1994) were utilized in the study. From hatch to 3 wk of age birds received a starter feed (3,000 kcal ME/kg and 210 g/kg CP) and from 3 to 12 wk of age birds were fed a grower diet (3,100 kcal ME/kg and 190 g/kg CP). 52 Broiler chickens and 86 Baier chickens were sacrificed at 12 wk of age, the abdominal fat pad recovered, weighed and reported as a percentage of total body weight.

Identification of Chicken FABP4 Gene Polymorphism

To identify polymorphisms in the chicken *FABP4* gene, total RNA was isolated from adipose tissue samples of three Broiler and three Baier chickens using the Trizol reagent. The reverse transcription reactions were carried out using the TaKaRa RNA PCR Kit Ver.2.1. Primers for amplifying a fragment of 435 bp from the *FABP4* cDNA were; forward, 5'-AGA CTG CTA CCT GGC CTG ACA-3'; and reverse, 5'-AAG ACG GCT TCC TCA TGC-3'. The PCR components contained 10 mM Tris-HCl, 50 mM KCl, and 1.5 mM MgCl₂, pH 8.3, 200 μM of each deoxynucleotide triphosphate (dNTP), 0.25 μM of each primer, 2 μL of cDNA, and 1 U of *Taq* DNA polymerase in a final volume of 25 μL. The thermal cycling conditions were 94 °C for 5 min followed by 30 cycles of 94 °C for 50 s, 55 °C for 1 min, and 72 °C for 1 min. The PCR products were purified using a Gel Extraction Mini Kit and sequenced in forward and reverse directions with an ABI 3730 sequencer. Polymorphisms in these sequences were scanned by the DNAMAN package Version 4.0.

Genotyping of Chicken FABP4 Gene

Genomic DNA was isolated from venous blood samples of 120 Broiler chickens and 120 Baier chickens (60 birds of each sex) with phenol/chloroform and dissolved in 200 μL of 10 mM Tris-HCl, and 1 mM EDTA, pH 8.0. A genomic fragment of 141 bp corresponding the polymorphism (Ser89Asn) at *FABP4* exon 3 was amplified and analyzed with the WAVE DNA fragment analysis system (Transgenomic). Heteroduplex formation was induced by heat denaturation of PCR products at 94 °C for 5 min followed by gradual reannealing from 94 °C to 25 °C over 45 min. Gradient parameters were determined based on size and G-C content of the amplicon. In this study, the PCR product was detected within a total time of 8.5 min in a linear acetonitrile gradient with buffer A: 0.1 M triethylammonium acetate (TEAA), 0.025% acetonitrile (ACN); and buffer B: 0.1 M TEAA, 25% ACN with a flow rate of 0.9 mL/min at a mobile-phase temperature of 57.3 °C. Each run included a DNA loading and linear separation step (increasing buffer B from 46.6% to 55.5% over 5 min), peak elution step (54.8–55.3% buffer B over 1 min), a wash step with buffer D (75% acetonitrile over 1 min) and an equilibration step with 41.5% buffer B over 1.5 min. Genotyping analysis was carried out using a three-step procedure. Firstly, 5 μL of each product was subject to denaturation and annealing to separate the heterogeneous duplex's from the homogeneous duplex's. Secondly, the homogeneous PCR products were mixed in a 1:1 ratio with an aliquot of PCR amplicon from a known homogeneous genotype sample (AA genotype), denatured, annealed and separated as indicated. In this step, the two types of duplex's can be indentified: the heterogeneous GG genotype and the homogeneous AA genotype. Finally, in order to check the genotype, 3 samples of each genotype identified by WAVE were amplified, purified, and sequenced with a automated ABI 3730 DNA sequencing device as described previously.

Expression, Purification and Characterization of AFABP Isoforms

Chicken cDNAs corresponding to the two AFABP isoforms were cloned into PGEX 4T-1, transformed into *E.coli*. BL21(DE3) and protein expression induced using 0.4 mM IPTG. The glutathione S-transferase (GST) fusion proteins were purified by glutathione Sepharose 4B chromatography and dialyzed into 50 mM sodium phosphate pH 8.0 overnight at cold room, and its concentration estimated from the absorbance at 280 nm. Previous studies on a series of GST-AFABP mutants have shown that the binding properties of the *E. coli* derived fusion protein are indistinguishable from that of the native protein (Sha et al., 1993) likely due to the N-terminus of the protein (where the GST fusion is placed) being ~ 180° from the portal opening where ligand entry/exit is controlled (Ory et al., 1997).

Ligand binding to AFABP was measured in a fluorescence based assay using the probe 1-anilinonaphthalene 8-sulphonic (1,8-ANS) as described by Kane and Bernlohr (1996). In this assay, 1,8-ANS was dissolved in absolute ethanol, diluted to 500 nM in 50 mM sodium

phosphate buffer pH 8.0 (final ethanol less than 1%) and its concentration determined using the extinction coefficient ($\epsilon_{372\text{nm}}$) of $8000 \text{ cm}^{-1} \text{ M}^{-1}$. Concentrated protein in 50 mM sodium phosphate buffer pH 8.0 is added in 5 μL aliquots to 500 μL of 1,8-ANS, mixed for 1 min under dim light and the fluorescence recorded at 472 nm.

Statistical Analysis

Data for abdominal fat content in the two chicken breeds was analyzed by t test using SAS 6.12 software (SAS Institute Inc., Cary, NC, USA). Analysis of allelic frequencies between Broiler and Baier chickens was evaluated using Chi-square test. Binding constants for AFABP 1,8-ANS interaction were calculated using Prism software from at least five independent assays for each protein and analyzed using Student's *t*-test.

RESULTS

Identification of FABP4 Polymorphisms and Genotype Analysis

Sequence alignment and comparison of the amplified chicken *FABP4* sequences revealed the existence of a two polymorphisms. One polymorphism had previously been described by Wang (2006) was a silent C/T substitution located in the exon 1. The second polymorphism was identified in exon 3 of the *FABP4* gene and corresponded to a novel A to G substitution resulting in conversion of asparagine 89 to serine; referred to as Ser89Asn (Figure 1). Also shown in Figure 1 is a comparison of the chicken AFABP sequence to other AFABP sequences at position 17 and 89. The Ser89Asn polymorphism was evaluated in 240 birds and the frequency of individual genotypes (AA, AG and GG) in the two breeds determined (Table 1). The allele frequency was significantly different between the two chicken breeds ($P < 0.01$) with the A allele derived mainly from Broiler chickens and the G allele from Baier chickens. Importantly, there is a significant difference in abdominal fat content between the Broiler and Baier chickens. The Baier chicken exhibited an average abdominal fat content of 0.89% while the Broiler chicken typically exhibited an average abdominal fat content of 3.74% (Table 1). The differences in allelic frequency and fat deposition were evident in both male and female chickens of both breeds.

Expression, Purification and Characterization of AFABP Isoforms

cDNA for each of the two FABP4 isoforms was cloned into bacteria and the corresponding protein expressed as N-terminal fusion proteins with glutathione S-transferase. Evaluation of the cell culture supernatants and debris pellet by SDS-polyacrylamide gel electrophoresis confirmed that each GST-AFABP isoforms was soluble (data not shown). The supernatants containing the soluble GST-AFABP fusions were purified using Glutathione Sepharose 4B chromatography and were estimated to be greater than 95% homogeneous based on SDS-polyacrylamide gel electrophoresis (Figure 2). The yield of purified protein was 5–10 mg/liter of cell culture. Isothermal binding reactions with 1,8-ANS were performed on each of the two AFABP isoforms and the corresponding progress curves are displayed in Figure 3. The results demonstrate that the two AFABP isoforms have differing binding affinities for 1,8-ANS. AFABP Ser89 exhibited a lower ligand binding affinity with an apparent dissociation constant (Kd) of $7.31 \pm 3.75 \mu\text{M}$, while the Asn89 isoform bound 1,8-ANS with an apparent dissociation constant of $2.99 \pm 1.00 \mu\text{M}$ ($P=0.02$).

DISCUSSION

The chicken *FABP4* gene was investigated as a possible genetic factor in determining variance of abdominal fat content between the Broiler and Baier chicken. Previously, a silent substitution mutation in exon 1 (C/T) was reportedly linked to fat deposition (Luo et al., 2006; Wang et al., 2006) however the molecular basis for this obscure in the absence of any change in protein

sequence. As a consequence of those finding, additional polymorphisms were searched for and as a result, this work identifies a previously unknown substitution (Ser89Asn) in the coding sequence that is associated with different chicken breeds. The Baier chicken (Ser89 form) averages of 0.89% abdominal fat while the Broiler chicken (Asn89 form) typically has 3.74% abdominal fat at 12 weeks of age. This suggested that the Asn89 allele might be associated with higher fat deposition. It remains unknown whether the exon 3 G/A substitution in the chicken *FABP4* gene provides a molecular explanation for the previously reported linkage between a silent mutation in exon 1 of the gene and chicken abdominal fat, subcutaneous fat and intramuscular fat contents (Luo et al., 2006; Wang et al., 2006). However, linkage disequilibrium analysis showed that the G allele of the Ser89Asn polymorphism associated with the T allele of the silent C/T substitution, and the A allele of Ser89Asn mostly linked with the C allele of the C/T substitution in chickens from the Northeast Agricultural University F2 population ($D'=0.85$; $r^2=0.43$). A comparison of the different chicken AFABP sequences with selected other AFABP sequences at position 17 and 89 is shown in Figure 1B. Interestingly, while position 17 is an invariant Phe, position 89 is more flexible. Of note is the Ser89 in the domestic goose sequence while the common duck and atlantic salmon contain an Asn at position 89.

Aside from changes in the coding region, the absolute level of AFABP expression may affect fat deposition. Li et al. (2008) recently reported that the expression of *FABP4* significantly correlated with abdominal fat, breast intramuscular fat and thigh intramuscular fat and was highly expressed in the Beijing-YOU chicken compared to the Jing Xing chicken. However, in different Broiler chicken populations, the expression of AFABP protein did not generally correlate with fatness at most weeks of age¹.

Analysis of the ligand binding properties of the two AFABP isoforms was undertaken to determine if the propensity to accumulate adipose tissue was possibly linked to the ligand binding activity of AFABP. The fluorescent molecule 1,8-ANS is frequently used as a surrogate ligand and has been used to characterize fatty acid binding activities for several proteins in the FABP family (Kane and Bernlohr, 1996). Given the large degree of conservation in both primary sequence and tertiary structure between mammalian and chicken FABPs (Wang et al., 2004), we hypothesized that 1,8-ANS should bind with similar properties and can be used to assess the affinity of ligands. Figure 3 reveals that AFABP Ser89 exhibited an apparent dissociation constant (K_d) of $7.31 \pm 3.75 \mu\text{M}$, while the Asn89 isoform bound the ligand with an apparent dissociation constant of $2.99 \pm 1.00 \mu\text{M}$ ($P=0.02$). It should be noted that the 1,8-ANS binding assay generally yields apparent binding affinities that are considerably weaker than those measured using other assays such as acrylodan-derivatized intestinal fatty acid binding protein (Storch and Corsico, 2008). Additionally, the 1,8-ANS binding affinity for FABPs mirrors that for natural fatty acids supporting its use as a simple analytical tool for analysis of ligand association. Figure 4 shows a ribbon diagram of the murine AFABP structure with position 89

Shi, H., Wang, Q., and Li, H., manuscript in preparation modeled as a serine residue (PDB: 1ADL). Aside from the residues that form the salt-bond to the fatty acid carboxylate (Arg 106, Arg126 and Tyr 128) previous studies had implicated the helix-turn-helix domain (Liou et al., 2002) and Phe 57 (Ory et al., 1997; Simpson and Bernlohr, 1998) as key determinants in membrane association and ligand binding. Position 89 in the chicken AFABP protein is predicted to reside within a turn between βF and βG and would represent a novel site for control of ligand binding. Also shown in Figure 4 in space-filling mode is the position of bound oleic acid. Ser89 is distant from the bound lipid and not likely to affect the affinity for ligands via direct interaction with the acyl chain. Moreover, the side chain of Ser89 extends externally from the ligand-binding domain and therefore is more likely to be involved in surface associations. An alternate possibility is that Ser89 or Asn89 differentially associate with the

GST fusion partner and that interaction is sufficient to induce a conformational change in the AFABP polypeptide to subtly change binding affinity. Further work will be required to define the mechanism by which the Ser89Asn polymorphism affects lipid association.

Interestingly, a G to A transition at codon 54 of the mammalian intestinal FABP gene results in an amino acid substitution (Ala54Thr) and is linked to a variety of metabolic conditions in some humans. The Thr54 form exhibits a ~ 2-fold increase in fatty acid binding and may affect the development of human disease. For example, the Thr54 isoform is prevalent in the Pima Indian population and is linked to the development of insulin resistance and type 2 diabetes (Pratley et al., 2000). Moreover, the Thr54 polymorphism is associated in several populations world wide with obesity and increased cardiovascular risk (de Luis et al., 2007). As such, small changes in FABP lipid binding affinity may have profound influences on energy homeostasis.

Similar to the mammalian *FABP4* gene, the chicken *FABP4* gene is also expressed in fat tissue, where it is extremely abundant (Fisher et al., 2001; Wang et al., 2004). AFABP-null mice exhibit reduced basal and hormone-stimulated lipolysis (defined as the regulated release of fatty acids from the cell) both in situ and in vivo, but demonstrate rates of fatty acid influx identical to those of wild type mice suggesting that the protein facilitates trafficking of fatty acid from the site of lipid hydrolysis to the plasma membrane during lipolysis (Coe et al., 1999; Baar et al., 2005).

Importantly, AFABP-null mice accumulated intracellular free fatty acid (Coe et al., 1999) suggesting that lipolysis was attenuated while intracellular triglyceride hydrolysis was not decreased. These results may be explained by a second role for AFABP in lipolysis. Indeed, AFABP with a bound fatty acid serves a regulatory role by associating with the activated, phosphorylated hormone-sensitive lipase (HSL) on the surface of the lipid droplet (Smith et al., 2007). Such interaction between AFABP and HSL may result in the reduction of lipid hydrolysis via either delivering a fatty acid for feedback inhibition or via direct protein-protein interaction such that the loss of the AFABP may result in increased lipid hydrolysis (Smith et al., 2007). The data presented here has shown that the Asn 89-containing protein has a 2.4-fold greater affinity for 1,8-ANS than does the Ser89-containing protein and is associated with chicken of higher abdominal fat. Such increased fatty acid binding by the Asn89 isoform may result in greater association with HSL and reduced lipid hydrolysis. As such, the Broiler chicken with AFABP Asn89 may exhibit reduced lipid hydrolysis and result in deposition of more body fat than the Baier chicken with AFABP Ser89. An alternate possibility is that the Ser89Asn polymorphism affects membrane association. Studies by Storch and colleagues (Liou et al., 2002) have shown that AFABP interacts with biological membranes and that such interaction is mechanistically linked to fatty acid association/dissociation. If the Ser89Asn substitution differentially affects membrane association, the rate at which fatty acids exchange on and off the protein may be affected sufficiently to alter triglyceride metabolism. Future experiments will focus on testing these hypotheses.

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A

		Phe 17		Asn 89
Broiler cDNA	ATGT----CTAGTGAAAAC	TTT	GAGGACTAT-----	AGACAATGGCACA
Baier cDNA	ATGT----CTAGTGAAAAC	TTT	GAGGACTAT-----	AGACAGTGGCACA
		Phe 17		Ser 89

B

<u>AFABP Isoform (accession)</u>		<u>Pos. 17</u>	<u>Pos. 89</u>
Human	CAG3318	Phe	Gly
Rat	AAH84721	Phe	Gly
Mouse	CAJ18597	Phe	Gly
Pig	ABK58161	Phe	Gly
Goose	AF472610	Phe	Ser
Cow	NP776739	Phe	Glu
Sheep	ABY25854	Phe	Glu
Salmon	ACN12235	Phe	Asn
Duck	ABC96712	Phe	Asn

Figure 1. Sequence of chicken AFABP isoforms

A; Partial sequence of the Broiler and Baier AFABP cDNA containing the initiating ATG codon, the silent C/T substitution (from exon 1) encoding Phe17 and the A/G polymorphism (from exon 3) encoding either Asn or Ser at position 89. **B;** Comparison of chicken AFABP isoforms to other AFABP isoforms.

Figure 2. Purification of AFABP fusion protein from bacterial lysates

Lane 1, *E. coli* crude soluble extract containing Ser 89 form; Lane 2, AFABP Ser 89 purified protein; Lane 3, crude soluble *E. coli* extract containing Asn 89 form; Lane 4, AFABP Asn 89 purified protein. Numbers to the left of the gels show the molecular mass of the protein standards (Std) in kilodaltons.

Figure 3. 1,8-ANS binding by AFABP isoforms

Increasing concentrations of Ser89 (squares) or Asn89 (triangles) AFABP in 50 mM sodium phosphate, pH 8.0 were incubated with 500 nM 1,8-ANS and the increase in fluorescence at 472 nm obtained. The apparent dissociation constant is displayed in the inset table. Data were taken from a single experiment and is representative of at least five independent experiments and reported as the averages $\pm$ standard deviation. The apparent dissociation constants were significant ($P=0.02$) between the Asn89 and Ser89 isoforms.

Figure 4. Ribbon diagram of the FABP fold showing the positions of Ser 89 relative to the bound fatty acid.

Table 1
Abdominal fat composition and genotypic analysis of Broiler and Baier chickens

Sex	Breeds	Abdominal fat (%)	Genotype (n)		Number	Allele frequency		χ^2 (P) ²
			AA	AG		A	G	
Male	Broiler	2.63±0.24 ^A (26) ^I	24	33	60	0.67	0.33	12.03 (0.001)
	Baier	0.16±0.054 ^B (39)	5	31	60	0.34	0.66	
Female	Broiler	4.81±0.24 ^A (26)	32	27	60	0.76	0.24	25.25 (0.001)
	Baier	1.46±0.17 ^B (47)	4	26	60	0.28	0.72	
Male and Female	Broiler	3.74±0.23 ^A (52)	56	60	120	0.72	0.28	37.64 (0.001)
	Baier	0.89±0.12 ^B (86)	9	57	120	0.31	0.69	

^I Numbers showed in bracket are the individuals selected.
^{A, B} Means within a row with no common superscript are different by t test ($P < 0.01$). Abdominal fat are expressed as percentage of body weight at 12 wk of age.
² Chi-square test between allele frequencies and breeds.