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Expression Characterization, Polymorphism and Chromosomal Location of the Porcine Calsarcin-3 Gene

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ABSTRACT : Calcineurin is a calmodulin dependent protein that functions as a regulator of muscle cell growth and function. Agents capable of interacting with calcineurin could have important applications in muscle disease treatment as well as in the improvement of livestock production. Calsarcins comprise a family of muscle-specific calcineurin binding proteins which play an important role in modulating the function of calcineurin in muscle cells. Recently, we described the first two members of the calsarcin family (calsarcin-1 and calsarcin-2) in the pig. Here, we characterized the third member of the calsarcin family, calsarcin-3, which is also expressed specifically in skeletal muscle. However, unlike calsarcin-1 and calsarcin-2, the calsarcin-3 mRNA expression in skeletal muscle kept rising throughout the prenatal and postnatal development periods. In addition, radiation hybrid mapping indicated that porcine calsarcin-3 mapped to the distal end of the q arm of pig chromosome 2 (SSC2). A C/T single nucleotide polymorphism site in exon 5 was genotyped using the denaturing high performance liquid chromatography (DHPLC) method and the allele frequencies at this locus were significantly different among breeds. (**Key Words :** Expression, Localization, Polymorphism, Calsarcin-3, Porcine)

INTRODUCTION

Calcineurin, a calcium/calmodulin-dependent serine threonine phosphatase, is an important signaling molecule in skeletal muscle, as it promotes differentiation, the slow-fiber phenotype and possibly also muscle fiber hypertrophy. Calcineurin binds to the calsarcins, a family of muscle specific proteins of the sarcomeric Z-disc, which is a focal point in the regulation of contraction both in skeletal and cardiac muscle. Calsarcin-1, -2 and -3 all interact with calcineurin and the Z-disc proteins α-actinin, γ-filamin, myotilin, telethonin and cipher (Faulkner et al., 2000). The expression of calsarcin-1 (CS-1) is restricted to slow-twitch skeletal muscle fibers, whereas that of both calsarcin-2 (CS-2) and calsarcin-3 (CS-3) is enriched in fast-twitch fibers (Frey et al., 2000; Takada et al., 2001; Frey et al., 2002). Several studies have shown that calcineurin controls the skeletal muscle fiber type by stimulating slow muscle gene promoters and slow fiber differentiation both in cultured cells and *in vivo* (Chin et al., 1998; Schulz et al., 2004). In addition, CS-1 knockout mice showed enhanced calcineurin signaling and an excess of slow skeletal muscle fibers, indicating that CS-1 negatively modulates the function of calcineurin (Frey et al., 2004). The calsarcins may not only have a structural role in Z-disc assembly via their ability to bind different Z-disc proteins, but also have a possible involvement in calcineurin signaling pathways that are activated via their binding to calcineurin.

These findings indicate that the calsarcins may interact with calcineurin to control the muscle fiber type. In livestock production, the meat quality is affected greatly by the proportions of muscle fiber type (Fonseca et al., 2003). So, these indications also raised the possibility that calsarcins may play a role in the calcineurin signaling pathway. Thus, they may be useful for improvement of pork quality in agricultural applications. Recently, we have identified and characterized the first two members of the calsarcin family (CS-1 and CS-2) in pig (Wang et al., 2006a). Here we describe the third member, porcine calsarcin-3 (CS-3), the mRNA expression pattern, chromosome assignment, polymorphism and the protein location in C2C12 cells.

MATERIALS AND METHODS

Source of animals and tissues

Pig tissue samples employed in the gene expression

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Table 1. Primers employed in these experiments

Gene name	Primer name	Primer and probe sequences (5'–3')	Binding region	PCR (Tm)	Size (bp)
Calsarcin-3	cDNAPL[a]	CGCTCATCCGTTCGCTCAGT	Exon1	62	1,226
	cDNAPR	GTATAAGCTAGAGCACCGGA	3'-UTR		
	Intron5PL[b]	GCTGCCAACAGCCCCGAG	Exon5	60	520
	Intron5PR	TCCTTCAGTGGCTCGGCAT	Exon6		
	SNPPL[c]	CCATGTCTCCCTGGAGTCGC	Exon5	60	116
	SNPPR	GGATGTGAGGAGGCCACTTA	Intron6		
	CDSPL[d]	CCACTCGAGCTATGATCCCCAAGGAGCAGAA	Exon2	58	758
	CDSPR	CTGAAGCTTGCAGCTCCTCAGACTC	Exon7		
	Real-time PL	CCGAGTCTGAGGAGCTGTAG	Exon7	60	167
	Real-time PR	GGACTGCTGAACTTGGTGAC	Exon7		
	Taqman probe	FAM-TTCGGGCACCATCTGGAGACAG-TAMRA	Exon7		
GAPDH	Real-time PL	CGTCCCTGAGACACGATGGT	Exon1	60	194
	Real-time PR	GCCTTGACTGTGCCGTGGAAT	Exon3		
	Taqman probe	FAM-CGGAGTGAACGGATTTGGCCGC-TAMRA	Exon1-Exon2		

[a] Primers for isolating targeted cDNA.
[b] Primers for amplifying intron 5.
[c] Primers for SNP genotyping and radiation hybrid mapping.
[d] Primers for constructing the expression vector. The restriction sites were underlined.

analysis were described before (Wang et al., 2006b). In brief, the embryos were collected from pregnant females of Tongcheng pigs during three embryonic periods (33, 55, and 90 day post conception) and three postnatal periods (2, 28-day, and adult), the longissimus dorsi muscle were collected and stored at -80°C. Twelve different tissues were collected from four mature Wuzhishan mini pigs for spatial expression studies. The genetic variability analysis within the porcine CS-3 gene employed genomic DNAs from 189 individuals that represented three Chinese indigenous breeds (Tongcheng, Laiwu and Wuzhishan pigs) and three introduced commercial breeds (Landrace, Yorkshire and Duroc). Pigs (n = 192) for association studies were from two experimental Yorkshire lines originating from a commercial population (Sonesson et al., 1998). The traits collected included Birth weight, Weaning weight, Starting weight (at starting fattening) Body weight 170 days, Backfat thickness, and body weight and backfat thickness after slaughter day (age) correction. The association between genotypes and traits was analyzed by the *t*-test (Wang et al., 2004).

Molecular cloning of porcine CS-3 gene

The human CS-3 gene sequence (GenBank accession no NM_133371) were compared to all sequences available in the pig EST databases using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST). We selected the matched porcine ESTs (DT328161.1, DY424940.1, DT330736.1 and DT325112.1) which shared more than 90% sequence identity to the human gene to assemble a draft cDNA contig of the porcine CS-3 gene. To verify and clone the cDNA sequence of porcine CS-3, RT-PCR was performed using M-MLV reverse transcriptase (Promega), Taq polymerase (TaKaRa) and total RNA as template, which isolated from pig muscle using the Trizol reagent (Invitrogen)(Pan et al., 2003), and specific primers across the coding region of the gene (Table 1). The predominant PCR product was purified and subsequently cloned into the pEGM-T-Easy vector (Promega) prior to sequencing. The sequence of the cDNA clone was deposited in GenBank finally (GenBank accession number DQ1430410).

TaqMan analyses of calsarcin-3 mRNA expression

The amplification primer pairs and Taqman probes were listed in Table 1. The real-time PCR procedure was described previously (Wang et al., 2006a).

Determination of T595C polymorphisms, genotyping and association analysis

After alignment of the pig CS-3 mRNA sequence with the human DNA sequence (GenBank accession number NC_000005.8), the putative exon boundaries appeared. The predicted introns, except intron 4 and 5, were very large make it hard to determine their length by PCR. Primers intended to amplify across the putative intron 5 region was designed based on the pig CS-3 mRNA (Table 1). A T595C polymorphism site located in exon 5 was identified after sequencing and alignment of PCR products originating from different individuals. A 116 bp DNA fragment containing this site was amplified by PCR with another primer pair (Table 1) and then subjected to genotyping by denaturing high-performance liquid chromatography (DHPLC) using an automated HPLC instrument (WAVE, Transgenomic, CA). Samples were run at the 66.5°C as recommended by the software authors and eluted from the column using a linear acetonitrile gradient at a constant flow rate of 0.9 ml/min. The gradient start and end points were adjusted according to the size of the fragment.

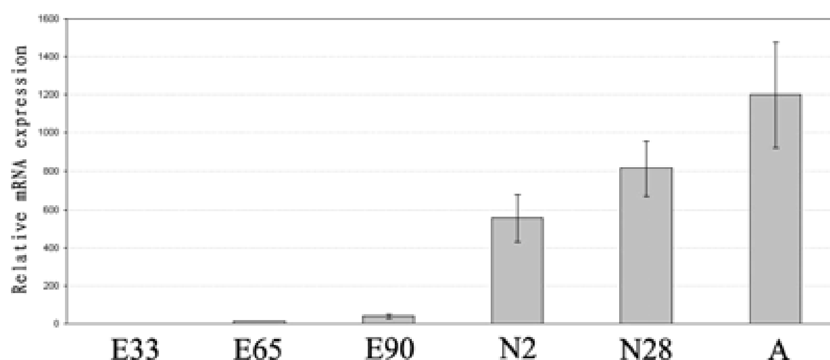

Figure 1. Temporal expression profiles of porcine calsarcin-3 during skeletal muscle development in Tongcheng pigs. Relative levels of calsarcin-3 mRNA were calculated emloying the Comparative Ct method with GAPDH as the reference in each sample (Wang et al., 2006). The value of CS-3 expression in E33 was arbitrarily set to 1. Bars represent the mean±SD (n = 3). E33, E65, E90, N2, N28 and A indicate six stages of skeletal muscle development in Tongcheng pigs, prenatal day-33, -65 and -90, postnatal day-2 and 28 and adult, respectively.

Chromosomal mapping by IMpRH

Radiation hybrid mapping was performed using the INRA-University of Minnesota 7000 rads radiation hybrid panel (IMpRH), consisting of 118 hamster-porcine hybrid cell lines (Yerle et al., 1998). The primer pair used in genotyping the T595C mutation also performed well in the radiation mapping experiment. The mapping process was described previously (Li et al., 2006).

Transient expression of porcine calsarcin-3 in C2C12 cells

Mouse skeletal myoblasts (C2C12) were cultured in DMEM/high glucose supplemented with 20% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin and maintained at 37°C in 5% CO_2. For cellular localization studies, the open reading frame (ORF), encoding porcine calsarcin-3, was amplified from its cDNA clone with PCR (Table 1) and subcloned into the XhoI-HindIII site of the pEGFP-N3 vector (BD Biosciences Clontech) to yield a mammalian expression plasmid pCS-3-GFP, then transfected into C2C12 cells using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen).

RESULTS

Molecular characterization and expression analysis of porcine calsarcin-3 gene

With the primer pair cDNAPL/cDNAPR, we isolated the calsarcin-3 gene from pig muscle. The deduced porcine CS-3 mRNA contained a 738 bp ORF flanked by a 223 bp 5'-UTR and a 458 bp 3'-UTR. This ORF is predicted to encode a polypeptide of 245 amino acids, with an expected molecular mass of 26.5 kDa and pI of 7.3. The sequence of porcine CS-3 was deposited in GenBank (GenBank accession number DQ143041).

Figure 1 shows the expression of CS-3 during the pig muscle development. In the fetal stages of development in Tongcheng pigs, we observed that the CS-3 mRNA expression was up-regulated from 33 to 90 dpc in skeletal muscle. An up-regulation expression pattern was also observed after birth, with dramatic higher expression levels compared to the prenatal stages (Figure 1). The CS-3 mRNA expression level increased by almost 1200-fold and 30-fold in adult muscle compared with the muscle from the 33-day and 90-day of embryos. Taqman analysis was also performed to determine the relative mRNA expression of CS-3 in various pig tissues. However, the amplify curve could be produced with only the muscle tissue cDNA templates, indicate CS-3 gene was absent in the tissues other than muscle.

Polymorphism and association analysis

The T/C substitution at position 595 of the porcine calsarcin-3 gene is a silent mutation. The 116 bp PCR fragment which contains the site was employed for genotyping through the DHPLC equipment. As a result, the T595C was individually analyzed in almost 200 unrelated animals and an experimental population described previously. The genotyping results showed great variation in allele frequencies between Chinese indigenous and introduced commercial breeds (Table 2). However, the association analysis within the experimental population revealed no significant association between this polymorphism site with any of the economic traits investigated (Data not show).

Chromosome assignment

The chromosomal location of porcine CS-3 was assigned by PCR screening of a whole genome porcine/hamster radiation hybrid panel as described in

Table 2. Allele frequencies of different pig breeds showing polymorphism*s* in the porcine calsarcin-3 locus[a].

Breed	Number	Allele frequency	
		C	T
Landrace	25	0.4	0.6
Yorkshire	27	0.471	0.529
Duroc	16	0.5	0.5
Tongcheng	42	0.143	0.857
Laiwu	39	0.231	0.769
Wuzhishan	40	0.062	0.938

[a] SNP at nucleotide 595 of porcine CS-3 mRNA sequence (GenBank accession number DQ143041).

Materials and Methods. The resulting code (10010000001000000100001000000100010000001000000000 0000000000000011000100000000010011010010000101000 00000100000000001000100000) was loaded into the Roslin Radiation Hybrid Database (Milan et al., 2000). Two-point analyses revealed that the porcine calsarcin-3 gene maps close to the DNA marker S0036 (57cR; LOD score 6.92). The marker S0036 has been mapped previously to the distal end of the short arm of pig chromosome 2 (SSC2).

Cellular localization of porcine CS-3 in C2C12 cells

The cellular location of porcine calsarcin-3 was studied by fluorescence and confocal analysis of C2C12 cells transiently transfected with pCS-3-GFP plasmid. The porcine CS-3-GFP fusion protein localized to the cytoplasm and cell membranes at 48 h after transfection (Figure 2). Green fluorescence was detected throughout the control cells, transfected with the GFP vector alone (data not shown).

DISCUSSION

This paper presents the results of an initial study of porcine calsarcin-3, which is exclusively expressed in skeletal muscle. We firstly employed real-time PCR to detect the mRNA expression through six important stages during muscle development in pig. The porcine CS-3 mRNA expression increased constantly during the different stages investigated and the transcript level was significantly higher in the adult than in the prenatal and neonatal periods. Our previous data indicated that porcine CS-1 was down-regulated through the embryonic development while CS-2 was up-regulated (Wang et al., 2006a). The expression pattern of CS-3 in prenatal periods resembled to CS-2 but not CS-1. However, porcine CS-3 showed a progressive increase after birth and reaching a maximum value in adult, but both CS-1 and CS-2 were decreased significantly during that time. Therefore, it is possible that CS-3 may play a more important role in maintenance in adult than the other two members of the calsarcin family.

Several potential polymorphisms within the porcine CS-3 were detected by comparison of genomic DNA fragments from different pig breeds. We genotyped the C595T substitution by using the DHPLC equipment in almost 189 unrelated pigs, representing five indigenous and introduced commercial breeds. The allelic distribution revealed that the Chinese indigenous breeds had higher frequencies of the T allele whereas foreign breeds had higher frequencies of the C allele. We further conducted preliminary association study in an experimental population reported previously. It is a pity that there was no significant association between the calsarcin-3 locus and the traits studied. This C595T substitution does not induce any amino acid alteration. However, it may be linked to other loci controlling the traits of interest. We shall continue to perform association analyses in other larger populations and to further detect valuable SNPs in the porcine CS-3 locus to determine whether it really affects some muscle fiber characteristics.

We have mapped the porcine CS-3 gene to the proximal end of the long arm of pig chromosome 2. The human CS-3 gene maps to human chromosome 5q31 (Frey et al., 2002). Thus, the map location for the porcine CS-3 gene is consistent with the known human-pig comparative map (Goureau et al., 1996). The results of the present study will provide a molecular basis for the function and structure of the porcine CS-3 gene and for further investigations into the expressional regulation of porcine CS-3 and how it modulates calcineurin during muscle development.

Figure 2. Cellular localization of porcine CS-3-GFP fusion protein detected by confocal microscopy. C2C12 cells transfected with the porcine CS-3-GFP construct were observed using laser scanning confocal microscopy 48 h after the transfection. A: dic; B: green fluoresce; C: overlay. (enlargement ×400).

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