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Purification, crystallization and preliminary X-ray crystallographic analysis of the CIDE-N domain of Fsp27

Fsp27, a member of the CIDE protein family which is selectively expressed in adipocytes, has emerged as a novel regulator for unilocular lipid droplet (LD) formation, lipid metabolism, differentiation of adipocytes and insulin sensitivity. An LD is a subcellular compartment that is used by adipocytes for the efficient storage of fats. The CIDE-N domain of Fsp27 functions as a recruitment platform that induces the correct configuration of the Fsp27 CIDE-C domain to facilitate LD fusion. This study reports the high-yield expression of the mouse Fsp27 CIDE-N domain in *Escherichia coli*; a two-step purification protocol with high efficiency was established and crystallographic analysis was performed. The purity of the recombinant Fsp27 was >95% as assessed by SDS-PAGE. Crystals were obtained at 291 K using 28% polyethylene glycol 4000 as a precipitant. Diffraction data were collected to 1.92 Å resolution and the crystal belonged to space group $P6_5$, with unit-cell parameters $a = b = 63.3$, $c = 37.4$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$. The components of the crystal were identified by ion-trap LC/MS/MS spectrometric analysis. The structure has been solved by molecular replacement and refinement is in progress.

1. Introduction

Obesity, diabetes and liver steatosis are a class of diseases that feature metabolic abnormalities and disturbed energy homeostasis; they have become the most common chronic disorders throughout the world. As has been demonstrated previously, adipose tissue plays critical roles in regulating systemic metabolic balance and glucose homeostasis in addition to its fundamental function in storing and utilizing triacylglycerol (TAG; Rajala & Scherer, 2003).

The CIDE family of proteins CIDEA, CIDEB and CIDEA (also designated Fsp27 in mice), which were originally identified based on homology to the N-terminus of DFF 40/45 (Liang *et al.*, 2003; Inohara *et al.*, 1998), are mainly expressed in brown adipose tissue (BAT, an energy-consumption organ), liver and white adipose tissue (WAT, an energy-storage organ) (Zhou *et al.*, 2003; Li *et al.*, 2007; Nishino *et al.*, 2008; Toh *et al.*, 2008; Gesta *et al.*, 2007). CIDE protein deficiency-sensitized animals have a lean phenotype with higher energy expenditure and improved insulin sensitivity and are protected from a high-fat diet (Gong *et al.*, 2009; Li *et al.*, 2007; Zhou *et al.*, 2003), demonstrating that this group of proteins play a key role in fatty-acid metabolism.

It has been shown that Fsp27 is highly and selectively expressed in WAT (and moderately in BAT; Danesch *et al.*, 1992; Toh *et al.*, 2008). In white adipocytes, excess energy is stored by the accumulation of TAG in the form of distinct large lipid droplets (LDs), which are often characterized as solitary cytoplasmic organelles. The unilocular LDs are composed of a core of neutral TAG and the core is covered by a phospholipid monolayer studded with proteins which regulate LD formation and mobilization (Wolins *et al.*, 2006; Tauchi-Sato *et al.*, 2002; Miura *et al.*, 2002). Fsp27 is one of the LD-associated proteins and is essential for the formation and maintenance of unilocular LDs (Nishino *et al.*, 2008). The sequestration of fat by large LDs also prevents fatty-acid overload, which impairs insulin signalling. The underlying mechanism responsible for unilocular LD formation in WAT remains elusive (Zweytick *et al.*, 2000; Murphy & Vance, 1999). In *in vitro* studies, overexpression of Fsp27 in 3T3-L1 pre-adipocytes

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led to LDs of increased size (Matsusue *et al.*, 2008), while depletion of Fsp27 in fully differentiated adipocytes stimulated lipolysis and resulted in uniformly dispersed LDs with smaller structures than those in control cells (Puri *et al.*, 2007). Furthermore, it was reported that CIDEC expression was increased along with the differentiation of pre-adipocytes and that CIDEC knockdown in pre-adipocytes could block differentiation (Li *et al.*, 2010). These facts demonstrate that Fsp27 acts as a novel regulator of the formation of unilocal LDs, lipid metabolism, adipocyte differentiation and insulin sensitivity.

Homology analysis revealed that the members of the CIDEC protein family contain two domains (designated CIDEC-N and CIDEC-C). The CIDEC-N domains of CIDEA, CIDEB and CIDEC (Fsp27) share 39%, 29% and 38% amino-acid identity with the CAD domain in the N-terminus of Dffa/Dff45/ICAD, respectively. The CIDEC-C domain is unique to the CIDEC family; the CIDEC-C domains of CIDEA and CIDEB share homology (54% and 53% identity, respectively) with that of CIDEC (Fsp27) (Inohara *et al.*, 1998). Signature sequences of the CIDEC-N and CIDEC-C domains have been defined by sequence comparison: the unique penta-amino-acid signature RPXRV was observed within the evolutionarily conserved 37 amino-acid residues that surround the EDGT sequence in the CIDEC-N domain, and a highly conserved XARXTFDXYXXNPXDXXGXLNKVATXYXX-YSXSXD signature was identified in the CIDEC-C domain (Wu *et al.*, 2008). Previous reports suggested that the CIDEC-N domains of CIDEC proteins could regulate killing activity, perhaps by the formation of homophilic dimers (Inohara *et al.*, 1998), while the CIDEC-C domains were necessary and sufficient for apoptotic activity and LD localization (Inohara *et al.*, 1998; Liu *et al.*, 2009).

Recently, a new study demonstrated a novel molecular mechanism responsible for unilocal LD formation for the first time: Fsp27 is highly concentrated at LD-LD contact sites (LDCS) to mediate the formation of fusion pores through which net neutral lipids diffuse from the smaller to the larger LD and ultimately lead to a unique LD fusion. Furthermore, perilipin (Plin), another LD-associated protein, was identified as cooperating with Fsp27 *in vivo* through direct interaction, resulting in significant acceleration of Fsp27-dependent lipid diffusion and LD fusion. It was shown that the Fsp27 CIDEC-N domain plays a modulatory role, functioning as a recruitment platform for the formation of an Fsp27 CIDEC-N/CIDEC-N homodimer or an Fsp27/Plin heterodimer (or both); the recruitment interactions can induce the correct configuration of the Fsp27 CIDEC-C domain or stabilize it to enhance the formation/expansion of the fusion pore(s) and facilitate LD fusion (Gong *et al.*, 2011). However, the detailed structural characteristics of the Fsp27 CIDEC-N domain remained unknown.

In this study, the expression and purification of the CIDEC-N domain of mouse Fsp27 in *Escherichia coli* are reported; crystallization and preliminary X-ray crystallographic analysis were carried out and the crystals obtained diffracted to high resolution (1.92 Å).

2. Materials and methods

2.1. Materials

The enzymes and the PCR amplification kit used for plasmid construction were all obtained from New England Biolabs. A mini plasmid kit and a DNA quick purify/recover kit were obtained from Omega Co. All other chemicals were of analytical grade.

Plasmid Fsp27-GFP was a generous gift from Professor Peng Li of Tsinghua University.

Table 1

Buffers used for Fsp27 CIDEC-N domain purification.

Lysis buffer	25 mM Tris-HCl pH 7.7, 500 mM NaCl, 20 mM imidazole, 1 mM PMSF
Buffer A	25 mM Tris-HCl pH 7.7, 500 mM NaCl, 20 mM imidazole
Buffer B	25 mM Tris-HCl pH 7.7, 500 mM NaCl, 500 mM imidazole
Buffer C	10 mM Tris-HCl pH 7.7, 150 mM NaCl, 2 mM DTT

2.2. Construction of plasmid pRSFDuet-1-Fsp27 39–119

The primers used for the Fsp27 CIDEC-N domain (residues 39–119) were designed based on the published nucleotide sequence of *Mus musculus* CIDEC (GenBank Accession No. BC099676.1). *FseI* and *AscI* sites (shown in bold), which provide convenience for subcloning into the pRSFDuet-1 vector, were introduced into the 5' ends of the primers. The forward and reverse primers were 5'-**GGCCGGCC**-ACCGAGGGCCAGGCC-3' and 5'-**GGCGCGCCTC**AGGGCTTC-CACTTCTGC-3', respectively.

PCR amplification was performed with a DNA Thermal Cycler (Eppendorf) using standard PCR settings with the plasmid Fsp27-GFP as template.

The PCR product Fsp27 39–119 was isolated on a gel, purified using a DNA recover kit and subjected to *FseI/AscI* digestion for 5 h. The cleaved Fsp27 39–119 coding sequence was subcloned into a modified pRSFDuet-1 vector (Novagen) containing an N-terminal 6×His tag and *FseI* and *AscI* restriction-enzyme sites.

2.3. Expression and purification of the Fsp27 CIDEC-N domain

Positive colonies transformed with the plasmid pRSFDuet-1-Fsp27 39–119 were grown in LB medium containing 100 µg ml⁻¹ ampicillin at 310 K; when the culture density approached an OD₆₀₀ of ~0.8, the temperature of the shaker-incubator was reset to 289 K to allow gradual cooling of the cultures. After IPTG induction (0.5 mM), the cultures were incubated in the shaker-incubator for a further 12–16 h and collected by centrifugation. The cell pellets were resuspended in lysis buffer and lysed by sonication (Table 1). The homogenized product was clarified by centrifugation (15 000g, 45 min, 277 K).

All of the following procedures for Fsp 39–119 purification were carried out at 277 K. The clarified lysate was loaded onto a 5 ml HisTrap HP column (GE Healthcare) coupled to an ÄKTApurifier system (GE Healthcare) and the contaminant proteins were washed

Figure 1
Elution profile of the CIDEC-N domain of Fsp27 on Superdex 75 and SDS-PAGE (inset) of the eluted fractions. The left lane contains molecular-mass markers (labelled in kDa).

out with wash buffer (buffer A; Table 1). The His-tagged recombinant Fsp27 was eluted with a 90 ml linear gradient of 20–500 mM imidazole (buffer A to buffer B; see Table 1). Fractions containing the recombinant protein were collected and concentrated using an Ultrafree 10K filter (Millipore) before being applied onto a Superdex 75 column (GE Healthcare) using buffer C (see Table 1). The purity of the recombinant Fsp27 was assessed by SDS–PAGE.

2.4. Crystallization

The purified Fsp27 CIDE-N domain was concentrated to 30 mg ml⁻¹. Protein concentrations were measured by UV–Vis spectroscopy with a NanoVue Plus spectrophotometer (GE Healthcare). The protein solution was clarified by centrifugation at 13 000g for 20 min at 277 K prior to searching for initial crystallization conditions. The screening process was carried out in 24-well plates at

291 K by the hanging-drop vapour-diffusion technique using Index, Crystal Screen and Crystal Screen 2 reagent kits from Hampton Research as described previously (Zhang *et al.*, 2010; Jancarik *et al.*, 1991). The initial conditions were optimized by changing the PEG molecular weight, buffer pH and protein concentration as well as by the use of additives.

2.5. Ion-trap LC/MS/MS spectrometry analysis

Several crystals were washed with reservoir solution, submitted to SDS–PAGE and stained with Coomassie Brilliant Blue, allowing the visualization of a single 14 kDa band. We excised the protein band, chopped it into smaller portions and digested them in-gel using sequencing-grade trypsin at 310 K overnight (Promega). The sample was centrifuged and the supernatant was concentrated for ion-trap LC/MS/MS spectrometric analysis with an Agilent 6300 Ion Trap LC/

Figure 2

Characterization of the components of the Fsp27 crystals by ion-trap LC/MS/MS spectrometric analysis. (a) Matched peptides are indicated in light grey; the identity of the protein was verified with a sequence coverage of 27.62% (amino acids 56–112). (b) Ion-trap LC/MS/MS spectra arranged according to the peptide score. (1) LC/MS/MS spectrum of the m/z 991.2 ion corresponding to the peptide with amino-acid residues 78–103 of Fsp27; (2) LC/MS/MS spectrum of the m/z 565.3 ion corresponding to amino-acid residues 104–112; (3) LC/MS/MS spectrum of the m/z 720.9 ion corresponding to amino-acid residues 57–69; (4) LC/MS/MS spectrum of the m/z 784.9 ion corresponding to amino-acid residues 56–69; (5) LC/MS/MS spectrum of the m/z 1071.6 ion corresponding to amino-acid residues 76–103; (6) LC/MS/MS spectrum of the m/z 715.5 ion corresponding to amino-acid positions 70–75.

Table 2

Purification summary of His-Fsp27 CIDE-N domain.

Purification step	Volume (ml)	Protein concentration (mg ml ⁻¹)	Total protein (mg)	Yield (%)
Soluble cell fraction	60	22.28	1336.8	100
Ni-affinity	26	0.81	20.96	1.57
Superdex 75	11	1.64	18.04	1.35

MS System (Agilent Technologies); the peptide masses were submitted to the database-analysis software *Spectrum Mill* (Agilent Technologies).

2.6. X-ray crystallographic studies

To prepare for X-ray analysis, crystals were incubated with a cryoprotectant solution (crystallization solution plus 15% glycerol), mounted in a nylon-fibre loop and cryocooled at 100 K in a liquid-nitrogen gas stream. Diffraction data collection was performed with single Fsp27 crystals on the BL17U beamline (wavelength 0.9792 Å) of the Shanghai Synchrotron Radiation Facility (SSRF) using a MAR CCD 225 detector. The exposure time was 0.8 s per frame, with a 0.5° oscillation angle; the crystal-to-detector distance was 150 mm. The *HKL*-2000 programs *DENZO* and *SCALEPACK* were employed to index, integrate and scale the intensity data (Otwinowski & Minor, 1997).

3. Results and discussion

3.1. Cloning of the CIDE-N domain of Fsp27

Using PCR amplification, a DNA fragment of 260 bp containing the coding sequence for the Fsp27 CIDE-N domain was obtained and subjected to *FseI*/*AscI* digestion. The digested product was subcloned into the modified pRSFDuet-1 vector cleaved with *FseI* and *AscI* at 289 K for 3–5 h. The ligation product was transformed into the Rossetta strain of *E. coli*. The positive colony containing the plasmid pRSFDuet-1-Fsp27 39–119 was identified by PCR and *FseI*/*AscI* digestion and was verified by nucleotide sequencing.

3.2. Efficient expression and purification of the Fsp27 CIDE-N domain

The CIDE-N domain of Fsp27 N-terminally fused with a 6×His tag was solubly expressed in *E. coli* with a yield of more than 6 mg from 1 l culture medium. The molecular weight of Fsp27 39–119 was

Table 3

Data-collection and processing statistics.

Wavelength (Å)	0.9792
Space group	<i>P</i> 6 ₅
Unit-cell parameters (Å, °)	<i>a</i> = <i>b</i> = 63.3, <i>c</i> = 37.4, α = β = 90, γ = 120
Solvent content (%)	48.9
<i>V</i> _M (Å ³ Da ⁻¹)	2.41
Resolution (Å)	50–1.92
No. of observed reflections	26278
No. of unique reflections	6642
Completeness (%)	99.7 (99.8)
Multiplicity	4.0
Mean <i>I</i> /(<i>I</i> + <i>σ</i> (<i>I</i>))	13.77 (3.7)
<i>R</i> _{merge} † (%)	9.3 (35.5)

$$\dagger R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}.$$

calculated to be approximately 11.9 kDa using the *ProtParam* characterization tool on the Expasy server. After a two-step purification consisting of affinity and gel-filtration chromatography (see Table 2 and Fig. 1), the purity of the protein was more than 95% as assessed by SDS-PAGE visualized by Coomassie R-250 staining (Fig. 1). The molecular mass of the purified product identified by size-exclusion chromatography followed by static laser light-scattering (data not shown) showed that Fsp27 may form a monomer.

3.3. Mass-spectrometric analysis of the Fsp27 CIDE-N domain

The ion-trap LC/MS/MS spectra of the gel-excised band are shown in Fig. 2(b). The list of peptide masses was submitted to the data-analysis software *Spectrum Mill* (Agilent Technologies) and the identity of the protein was verified with a coverage of 27.62% (amino acids 56–112; Fig. 2a) and a score of 1421.30. Thus, the components of the crystal were fully identified.

3.4. Crystallization and data collection of single Fsp27 crystals

Small prolate crystals were observed after one week in several conditions from the Crystal Screen and Index kits (Hampton Research), all of which contained PEG as a precipitant. Optimizations of these initial conditions were carried out as described above, rendering crystals of larger size that diffracted to high resolution (Fig. 3). The best crystals were obtained at 291 K in sitting-drop mode by dispensing droplets comprising 1.5 µl protein solution (20 mg ml⁻¹) and 1.5 µl crystallization buffer (0.2 M sodium acetate trihydrate, 28% PEG 4000, 0.1 M Tris-HCl pH 8.5). The largest crystals were immersed in an optimized cryoprotectant (crystallization buffer plus 15% glycerol) for data collection.

Figure 3

Typical crystals of the CIDE-N domain of Fsp27. (a) An initial crystal. (b) The best crystal grown using the optimized conditions.

Figure 4
Diffraction image of the CIDE-N domain of Fsp27. The crystal diffracted to 1.92 Å resolution.

A complete 1.92 Å resolution data set was obtained from a crystal belonging to space group $P6_3$, with unit-cell parameters $a = b = 63.3$, $c = 47.4$ Å (see Fig. 4 and Table 3). The Matthews coefficient was $2.41 \text{ Å}^3 \text{ Da}^{-1}$ (solvent content of 48.9%), with one Fsp27 molecule per asymmetric unit. Our gel-filtration results also indicate that Fsp27 39–119 may exist as a monomer in solution.

Structure solution was performed by molecular replacement using the structure of human cell-death activator CIDE-A (PDB entry 2eel; RIKEN Structural Genomics/Proteomics Initiative, unpublished work) as a search model, and refinement is in progress.

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