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Structure of the kinase domain of Gilgamesh from *Drosophila melanogaster*

The CK1 family kinases regulate multiple cellular aspects and play important roles in Wnt/Wingless and Hedgehog signalling. The kinase domain of *Drosophila* Gilgamesh isoform I (Gilgamesh-I), a homologue of human CK1- γ , was purified and crystallized. Crystals of methylated Gilgamesh-I kinase domain with a D210A mutation diffracted to 2.85 Å resolution and belonged to space group $P4_32_12$, with unit-cell parameters $a = b = 52.025$, $c = 291.727$ Å. The structure of Gilgamesh-I kinase domain, which was determined by molecular replacement, has conserved catalytic elements and an active conformation. Structural comparison indicates that an extended loop between the $\alpha 1$ helix and the $\beta 4$ strand exists in the Gilgamesh-I kinase domain. This extended loop may regulate the activity and function of Gilgamesh-I.

1. Introduction

The casein kinase 1 (CK1) family of serine/threonine protein kinases is evolutionarily conserved within eukaryotes and regulates multiple physiological functions, such as membrane transport, circadian rhythm, cell division and apoptosis (Knippschild, Gocht *et al.*, 2005). The CK1 kinases are key regulators of Wnt and Hedgehog signalling through phosphorylation of a series of substrates, including Dishevelled, LRP6, β -Catenin, APC, Ci and Smo (Price, 2006). The CK1 family kinases have been linked to cancer and neurodegenerative diseases such as Alzheimer's and Parkinson's diseases, and sleeping disorders, and thus have become new therapeutic targets (Knippschild, Wolff *et al.*, 2005; Perez *et al.*, 2011).

Human CK1 kinases have six isoforms: α , δ , ϵ , $\gamma 1$, $\gamma 2$ and $\gamma 3$. The family members have a highly conserved kinase domain, but differ in the N- and C-terminal regions (Knippschild, Gocht *et al.*, 2005; Cheong & Virshup, 2011). In general, the CK1 kinases are in a constitutively active state and do not require phosphorylation in the activation loop. The activity of CK1 kinases can be inhibited by autophosphorylation at the C-terminus, although usually cellular protein phosphatases dephosphorylate and keep them constitutively active *in vivo* (Rivers *et al.*, 1998; Cegielska *et al.*, 1998). The CK1 kinases have similar substrate specificity, and their canonical consensus sequences are pS/pT-X-X-**S/T** or a substitution of the phosphorylated residues by acidic residues such as D/E-X-X-**S/T**, where pS/pT refers to a phosphoserine or phosphothreonine, X refers to any amino acid and the residues in bold refer to the target site (Flotow *et al.*, 1990; Flotow & Roach, 1991).

Drosophila melanogaster has 26 proteins that are homologous to human CK1 in the UniProt database. Based on sequence alignment, among these 26 proteins, CK1- α is similar to human CK1- α , with 77.0% identity, double-time (also named Discs overgrown protein kinase) resembles human CK1- ϵ and δ with 85.0 and 77.0% identity, respectively, and Gilgamesh isoforms (A, B, C, E, G and I) have 66–71% identity with human CK1- γ . Like human CK1, *Drosophila* CK1 is also involved in the Wingless (Wg)/Wnt signalling pathway (Yanagawa *et al.*, 2002; Strutt *et al.*, 2006; Guan *et al.*, 2007; Zhang *et al.*, 2006). Double-time has been shown to modulate *Drosophila* circadian rhythms, and constitutes one feedback loop in the *Drosophila* circadian molecular clock with Period and Timeless (Kloss *et al.*, 1998, 2001; Price *et al.*, 1998; Preuss *et al.*, 2004; Rothenfluh *et al.*, 2000; Cyran *et al.*, 2005; Fan *et al.*, 2009).

Gilgamesh was first identified in glial cells located at the posterior edge of the *Drosophila* eye disc and is involved in temporal control of glial cell migration (Hummel *et al.*, 2002). Further studies have demonstrated that Gilgamesh plays roles in spermatogenesis and olfactory learning, and participates in the Wg/Wnt pathway, in which Gilgamesh phosphorylates the Wg coreceptor Arrow (Zhang *et al.*,

2006; Tan *et al.*, 2010; Nerusheva *et al.*, 2008, 2009). Gilgamesh is also required for planar cell polarity (PCP)-mediated processes to regulate cellular and tissue morphogenesis (Gault *et al.*, 2012). Gilgamesh restricts trichome formation and regulates trichome morphogenesis through directing Rab11–Nuf–Sec15 vesicle localization and trafficking.

Figure 1

Sequence alignment of CK1 kinases from different species including *D. melanogaster* (DROME), *Homo sapiens* (HUMAN), *Mus musculus* (MOUSE), *Xenopus laevis* (XENLA) and *Danio rerio* (DANRE; zebrafish) was performed with *ClustalW2* (Larkin *et al.*, 2007; Goujon *et al.*, 2010). The secondary structure is shown according to the structure of Gilgamesh-I kinase domain. Kinase domains of CK1 kinases are highlighted with a yellow background. Residues different in Gilgamesh-I/G and other CK1 kinases are boxed. Because Gilgamesh isoforms A and E, isoforms B and C, and isoforms C and G have 100% identity, respectively, and isoforms B and I and isoforms C and I have 99.79% identity, respectively, we chose the longer isoforms E, G and I for sequence alignment.

Here, we purified and crystallized the kinase domain of *Drosophila* Gilgamesh isoform I (Gilgamesh-I). The crystal structure of methylated Gilgamesh-I kinase domain with a D210A mutation was solved by molecular replacement. The overall structure of the Gilgamesh-I kinase domain is similar to reported CK1 kinase domains except for an extended loop between the $\alpha 1$ helix and $\beta 4$ strand.

2. Materials and methods

2.1. Gene cloning and protein expression

The DNA sequence of the *Drosophila* Gilgamesh-I kinase domain (amino acids 56–360) was inserted into plasmid HT-pET28a, which was modified from pET28a and has an N-terminal 6 $\times$ His tag following a TEV protease cleavage site. To enhance the homogeneity of the protein, we mutated Asp210, which is critical for recognizing one of the ATP-bound Mg²⁺ ions, to alanine (D210A) to inactivate Gilgamesh-I. The recombinant plasmid HT-pET28a-Gilgamesh-I was validated by sequencing.

The HT-pET28a-Gilgamesh-I plasmid was transformed into *Escherichia coli* BL21(DE3) CodonPlus competent cells. The cells were incubated in 750 ml Terrific Broth medium with 30 $\mu\text{g ml}^{-1}$ kanamycin and 34 $\mu\text{g ml}^{-1}$ chloramphenicol at 310 K until the absorbance reached 1.0 at a wavelength of 600 nm. The temperature was then decreased to 293 K and isopropyl β -D-1-thiogalactopyranoside was added to the medium to a final concentration of 0.5 mM

to induce protein expression. The culture was then incubated at 313 K for 18 h.

2.2. Protein purification

The following procedures were performed at 277 K. The *E. coli* cells were collected and centrifuged at 6000g for 10 min and suspended with five times the volume of lysis buffer consisting of 20 mM HEPES pH 7.5, 500 mM NaCl, 20 mM imidazole, 5% glycerol, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF). The cells were then broken by three passes through a High Pressure Homogenizer (JNBio) at 1200 bar (1 bar = 100 kPa). The debris was removed by centrifugation for 40 min at 20 000g. The supernatant was mixed with pre-equilibrated Ni Sepharose (GE Healthcare) for 1 h and the beads were then washed with lysis buffer without PMSF. The proteins were eluted with 300 mM imidazole in lysis buffer. The sample was desalted to 20 mM HEPES pH 7.5, 250 mM NaCl, 5% glycerol, 1 mM DTT. The 6 $\times$ His-tagged Gilgamesh-I was digested with TEV protease at 293 K for 2 h. The 6 $\times$ His tag and TEV protease were then removed using Ni Sepharose again. The protein was concentrated using a 10 kDa cutoff Amicon Ultra-15 (Millipore) and applied onto a HiLoad 16/60 Superdex 75 column (GE Healthcare) with 20 mM HEPES pH 7.5, 100 mM NaCl, 1 mM DTT. The purity of proteins was monitored by SDS-PAGE. Purified Gilgamesh-I was concentrated to 6 mg ml⁻¹.

2.3. Lysine methylation

The lysine-methylation reaction was performed before gel filtration with protein concentrations of 1 mg ml⁻¹ in 20 mM HEPES pH 7.5, 250 mM NaCl, 5% glycerol, 1 mM DTT. The method followed Walter's protocol (Walter *et al.*, 2006). 20 μl 1 M dimethylamine-borane complex (ABC) and 40 μl 1 M formaldehyde were added per ml protein solution, and the mixture was incubated at 277 K for 2 h. A further 20 μl ABC and 40 μl formaldehyde were then added and incubation continued for 2 h. Following a final addition of 10 μl ABC, the reaction was incubated overnight at 277 K. The reaction was quenched by adding 1 M Tris-HCl pH 7.5 to a final concentration of 20 mM in the reaction mixture.

2.4. Crystallization

Crystallization trials were carried out at 291 K by the sitting-drop vapour-diffusion method. The 2 μl sitting drops consisted of 1 μl protein solution and 1 μl reservoir solution and were equilibrated against 100 μl reservoir solution. Crystals were optimized using sitting-drop and hanging-drop vapour diffusion. Crystals were soaked in reservoir solution plus 25% glycerol and flash-cooled in liquid nitrogen.

2.5. Data collection, structure determination and refinement

Diffraction data were collected on beamline BL17U at the Shanghai Synchrotron Radiation Facility (SSRF), China and processed using *HKL-2000* (Otwinowski & Minor, 1997). The structure of the Gilgamesh-I kinase domain was solved by molecular replacement with *Phaser-MR* in the *PHENIX* suite (McCoy *et al.*, 2007; Adams *et al.*, 2010) using human CK1- $\gamma 3$ (PDB entry 2izr, G. Bunkoczi, E. Salah, P. Rellos, S. Das, O. Fedorov, P. Savitsky, J. E. Debreczeni, O. Gileadi, M. Sundstrom, A. Edwards, C. Arrowsmith, J. Weigelt, F. Von Delft, S. Knapp, unpublished work) as a search model. A solution with one Gilgamesh-I molecule in the asymmetric unit was found. The structure was refined using *phenix.refine* with

Figure 2
Elution profile of unmodified (a) and methylated (b) Gilgamesh-I kinase domains from a HiLoad 16/60 Superdex 75 column. Inset, SDS-PAGE.

Table 1

Data-collection and refinement statistics for Gilgamesh-I.

Values in parentheses are for the highest resolution shell.

Data collection	
Space group	$P4_32_12$
Unit-cell parameters (Å)	$a = b = 52.025$, $c = 291.727$
Wavelength (Å)	0.97915
Resolution range (Å)	50.0–2.85 (2.90–2.85)
Total reflections	63868
Unique reflections	9124
Completeness (%)	90.7 (100.0)
Multiplicity	7.0 (8.8)
$R_{\text{merge}}^{\dagger}$	0.185 (0.955)
$\langle I/\sigma(I) \rangle$	16.2 (4.5)
Mosaicity (°)	0.300
Refinement	
Resolution (Å)	36.79–2.86
No. of reflections	9074
$R_{\text{work}}/R_{\text{free}}^{\ddagger}$	0.254 / 0.296
No. of atoms	
Protein	2335
Water	26
R.m.s. deviations	
Bond lengths (Å)	0.003
Bond angles (°)	0.751
Average B factor (Å ²)	85.69

$\dagger R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$. $\ddagger R_{\text{work}} = \frac{\sum_{hkl} ||F_{\text{obs}}| - |F_{\text{calc}}||}{\sum_{hkl} |F_{\text{obs}}|}$. R_{free} was computed identically except all reflections belonged to a test set consisting of a 10% random selection of the data.

TLS restraints, and model building was performed in *Coot* (Adams *et al.*, 2010; Emsley *et al.*, 2010; Afonine *et al.*, 2012).

2.6. Structure deposition

The coordinate file and structure factor for the crystal structure of the Gilgamesh-I kinase domain were deposited in the RCSB Protein Data Bank under accession code 4nt4.

3. Results and discussion

3.1. Determination of the structure of Gilgamesh-I kinase domain

The kinase domains of *Drosophila* Gilgamesh isoforms are highly conserved with CK1- γ from human to zebrafish (Fig. 1). However, compared with CK1- γ 1 and Gilgamesh-E, both Gilgamesh-G and Gilgamesh-I contain four additional residues, 118-Asn-Ala-Pro-Asp-

121, in the loop between the α 1 helix and the β 4 strand. We purified the kinase domain of Gilgamesh-I using Ni-affinity chromatography and gel filtration (Fig. 2*a*). The protein eluted as a monomer from a Superdex 75 column and showed a single band on SDS-PAGE with a molecular weight close to the theoretical value of 36.3 kDa (Fig. 2*a*). Crystals of the Gilgamesh-I kinase domain appeared in several conditions: (i) 0.1 M ammonium citrate pH 7.0, 12% PEG 3350, (ii) 0.2 M ammonium sulfate pH 6.0, 20% PEG 3350, (iii) 0.2 M ammonium phosphate dibasic pH 8.0, 20% PEG 3350 and (iv) 0.2 M ammonium phosphate monobasic, 0.1 M Tris-HCl pH 8.5, 50% 2-methyl-2,4-pentanediol. However, these crystals were twinned or had low diffraction resolution. We then prepared and crystallized methylated protein. The methylated protein showed similar properties to the unmethylated protein in gel filtration and SDS-PAGE (Fig. 2*b*). Crystals of methylated protein appeared in a condition consisting of 1% tryptone, 50 mM HEPES pH 7.0, 9% PEG 3350. A crystal of methylated protein diffracted to 2.85 Å resolution and belonged to space group $P4_32_12$, with unit-cell parameters $a = b = 52.025$, $c = 291.727$ Å (Table 1). The relatively low overall completeness (90.7%) of the data is due to a high background in the resolution range around 3.6–3.7 Å, where the completeness is low. The structure of the Gilgamesh-I kinase domain was determined by molecular replacement using the human CK1- γ 3 structure (PDB entry 2izr) as a search model. There is one molecule of the Gilgamesh-I kinase domain present in the asymmetric unit. The structure was modelled and refined with a final R_{work} of 0.254 and an R_{free} of 0.296. Details of the refinement statistics are summarized in Table 1.

3.2. Overall structure of Gilgamesh-I kinase domain

The structure of the Gilgamesh-I kinase domain contains nine α -helices (α 1–9) and eight β -strands (β 0–7) (Fig. 3). Like other protein kinases, the Gilgamesh-I kinase domain is comprised of N- and C-lobes. It contains conserved subdomains and elements for catalysis, such as the partially disordered glycine-rich loop between the β 1 and β 2 strands for binding ATP, the C-helix (α 1 helix) where a conserved glutamate (Glu105) forms a conserved salt bridge to Lys91 in the β 3 strand, the catalytic loop between the α 3 helix and the β 6 strand containing the Tyr-Arg-Asp (YRD) motif (the His-Arg-Asp or HRD motif commonly found in protein kinases), the Asp-Phe-Gly (DFG) motif in which Asp210 is critical for recognizing one of the ATP-bound Mg^{2+} ions, and the activation loop critical for kinase activation

Figure 3

The overall structure of the Gilgamesh-I kinase domain shown as cartoon representations. Key residues are shown in stick representation. The catalytic loop and activation loop are coloured slate and yellow, respectively.

Figure 4

(a), (b) Structural comparison of Gilgamesh-I and human CK1- γ kinase domains. (c)–(e) Detailed view of the extended loop in Gilgamesh-I and its comparison with human CK1- γ .

which forms an ion pair with arginine from the Tyr-Arg-Asp motif (Fig. 3). As seen in reported structures of CK1 kinases, both the activation loop and the Lys91–Glu105 salt bridge of Gilgamesh-I are in the active conformation, although we used the mutation D210A for crystallization and no residue is phosphorylated in our structure.

3.3. Structure comparison of Gilgamesh-I and human CK1- γ kinase domains

The structure of Gilgamesh-I kinase domain is similar to that of reported human CK1- γ (PDB entries 2cmw, G. Bunkoczi, P. Rellos, S. Das, P. Savitsky, F. Niesen, F. Sobott, O. Fedorov, A. C. W. Pike, F. Von Delft, M. Sundstrom, C. Arrowsmith, A. Edwards, J. Weigelt, S. Knapp, unpublished work; 2c47, G. Bunkoczi, P. Rellos, S. Das, E. Ugochukwu, O. Fedorov, F. Sobott, J. Eswaran, A. Amos, L. Ball, F. Von Delft, A. Bullock, J. Debreczeni, A. Turnbull, M. Sundstrom, J. Weigelt, C. Arrowsmith, A. Edwards, S. Knapp, unpublished work; 2chl, G. Bunkoczi, E. Salah, P. Rellos, S. Das, O. Fedorov, P. Savitsky, O. Gileadi, M. Sundstrom, A. Edwards, C. Arrowsmith, E. Ugochukwu, J. Weigelt, F. Von Delft, S. Knapp, unpublished work; and 2izr), with r.m.s.d.s of 0.495–0.773 Å. We performed an in-depth comparison and found that an extended loop between the α 1 helix and the β 4 strand, corresponding to residues 115–His-Ala-Asp-Asn-118, is present in the Gilgamesh-I kinase domain but not in other reported CK1 kinases (Figs. 4a, 4b and 4c). In the Gilgamesh-I kinase

domain, the main chains of residues Ala116 and Asp117 form hydrogen bonds to the guanidine of Arg125 and the main chain of Ile126 (Fig. 4d). In human CK1- γ , a glutamine occupies the position of Arg125 in Gilgamesh-I, and this hydrogen bonding in Gilgamesh-I does not exist in human CK1- γ (Fig. 4e). Since the region near the C-helix (α 1 helix) is important for kinase activity, the extended loop may play a role in regulating the activity and function of Gilgamesh, which need to be further studied. Our structure, together with reported CK1 structures, suggests that CK1 kinases have conserved structure features. Our work provides a structural basis for further functional and evolutionary studies of CK1 kinases.

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