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Research paper

Structural basis for ATP loss by Clp1p in a G135R mutant protein

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ABSTRACT

Pcf11p and Clp1p form a heterodimer and are subunits of the Cleavage Factor IA (CF IA), a complex that is involved in the maturation of the 3′-end of mRNAs in *Saccharomyces cerevisiae*. The role of Clp1p protein in polyadenylation remains elusive, as does the need for ATP binding by Clp1p. In order to obtain structural details at atomic resolution of point mutants of Clp1p, we solved the crystal structure of Clp1-1p (G135R) point mutant complexed with Pcf11p (454–563) domain. The Clp1-1p–Pcf11p structure provides the atomic details for ATP loss while the point mutation preserves intact the Pcf11p interaction surface of Clp1p. This provides a rationale for the absence of phenotype in the yeast *clp1-1* strain. Additionally, the structure allows for the description of an extended binding interface of Pcf11p with Clp1p which is likely to be *S. cerevisiae* specific.

1. Introduction

Polyadenylation is a co-transcriptional event that occurs at the 3′-end of nearly all eukaryotic mRNA precursors with the exception of histone mRNAs [1,2]. This essential maturation process starts with cleavage of the elongating pre-mRNA in the 3′-untranslated region (3′-UTR) followed by the addition of a poly(A) tract at the 3′-hydroxyl end of the upstream cleavage product, the 3′ fragment being degraded by Rat1p/hXrn2 [3,4]. Pre-mRNA processing and transcription termination rely on the recognition of *cis*-elements by the polyadenylation machinery [1,5,6].

In yeast, the polyadenylation machinery comprises two large multi-protein complexes: the Cleavage/polyadenylation Factor IA (CF IA) [7] and the Cleavage and Polyadenylation Factor (CPF). In addition other factors are required to complete the process: the RNA-binding protein Hrp1p/Nab4p [8,9] and the poly(A)-binding proteins Pab1p and Nab2p, the latter being involved in the control of poly(A) tail synthesis and subsequent export of the mature mRNA [7,10–13]. The four CF IA subunits are required to fulfil CF IA function in 3′-end mRNA processing [14]. In addition to its role in the 3′-end mRNA maturation process, CF IA is also involved in transcription termination [15–17] and reinitiation [18]. CF IA consists of Rna14p, Rna15p, Pcf11p and Clp1p. Rna14p is a TPR-containing protein that acts a scaffold protein organizing the overall architecture of CF IA [14]. It binds Rna15p through its C-terminal monkey tail region [19]. Through its homodimerization, Rna14p most probably attracts two copies of Rna15p [20–22]. Rna15p is a modular protein containing at its N-terminus a classical RBD [23] displaying high affinity for U-rich sequences but specifically binding A-rich sequences upstream to the poly-A site, in the presence of Hrp1p [23–26]. The middle region of Rna15p, also called Hinge domain, binds tightly to Rna14p [19] through a co-folding process and the C-terminus of Rna15p binds to Pcf11p [27]. Pcf11p is required for both pre-mRNA 3′-end processing and transcription termination. The CTD-interacting domain (CID) of Pcf11p is dispensable for 3′-end processing *in vitro* but it is essential to avoid transcriptional read-through *in vivo* [17,28]. Indeed, the Pcf11p CID interacts specifically with the Ser2-phosphorylated form of the carboxyl-terminal domain (CTD) of the largest subunit of RNA polymerase II (Pol II) [17,29], while the central and C-terminal regions of Pcf11p contact Rna14p/Rna15p and Clp1p, respectively [28,30]. Finally, Clp1p is an ATP binding protein bearing classical Walker motif [30]. The association of Clp1p to the remainder of CF IA subunits is mainly restricted to a tight interaction with Pcf11p [30]. The heterotetramer of Rna14p–Rna15p associates with only one copy of the Pcf11p–Clp1p heterodimer. The molecular basis for such asymmetry has not been addressed so far.

Even though Clp1p binds ATP, the precise role of this protein in 3′-end processing remains elusive. In higher eukaryotes and archaea, Clp1 proteins are kinases that phosphorylate 5′ hydroxyl ends of RNA [31,32]. In humans, the loss of its kinase activity is connected to progressive motor-neuron loss and connected to tRNA processing rather than 3′-end mRNA maturation alteration [33]. However, this kinase activity is restricted to higher eukaryotes and archaea, while no similar activity could be detected for yeast Clp1p

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[30–32]. It has been shown that addition of ATP stimulates 3′-end mRNA processing and that the cleavage reaction itself can proceed in the presence of CTP and 3′ dATP [9,34,35].

On the basis of recent studies that report extensive and independent mutational analysis of Clp1p [36–38], we have undertaken a structural characterization of some of these point mutations. From the different experiments (Clp1-1p (G135R), Clp1-5p (G135R/G335S), Clp1-6p (G335S), Clp1-19p (L341S), Clp1-16p (G135R/L341S)) altering either ATP-binding site and/or the Pcf11p binding site [37], we could only determine the structure of Clp1-1p (G135R) point mutant complexed with Pcf11p (454–563), while the other samples led to non-diffracting crystals (Clp1-19p) or unstable complexes (Clp1-6p, Clp1-5p and Clp1-16p). The structure provides the atomic details for ATP-loss in Clp1-1p. No ATP is seen in the remodelled binding pocket while Pcf11p interaction is preserved. We also describe an extended binding interaction of Pcf11p with Clp1p. Due to the poor quality of the electron density map in this area the exact nature of the sequence is difficult to assign. A likely assignment is proposed.

2. Material and methods

2.1. Protein preparation and crystallization

The yeast DNA sequence encoding Pcf11p (454–563) protein fragment and full-length Clp1p were amplified by PCR and inserted into a pET-derived (Novagen) plasmid [39] to produce an N-terminal 6His-tag fused Pcf11 protein fragment and a native Clp1p. Point mutants of Clp1p were generated by PCR and further verified by sequencing. Clp1p mutant proteins and Pcf11p were co-expressed in *Escherichia coli* BL21(DE3) and were further purified to homogeneity by an initial cobalt-affinity chromatography step against the His-tag performed in 25 mM Tris–HCl pH 7.5, 150 mM NaCl (Buffer A) and eluted with Buffer A supplemented with 250 mM imidazole. A Hi-Q Sepharose chromatography column was further performed with Buffer A as loading buffer. Elution was performed by a gradient from 150 mM to 1000 mM NaCl in 25 mM Tris–HCl pH 7.5. The His-tag was removed by an overnight incubation with 1/100 (w/w) TEV protease in a Buffer A supplemented with 0.5 mM EDTA and 1 mM DTT. A final size exclusion chromatography HR26/60 75 pg (GE Healthcare) in 25 mM Tris–HCl pH 7.5, 150 mM NaCl was applied to exchange the buffer and remove excess Pcf11p (454–563) and cleaved His-tag. The same procedure was applied for Clp1p point mutants G135R (Clp1-1p), G335S (Clp1-6p), G135R/G335S (Clp1-5p), G135R/L341S (Clp1-16p), L341S (Clp1-19p).

When possible, the protein samples were concentrated up to 10 mg/ml and crystallized at 20 °C by the hanging drop method with a ratio of 1:1 sample protein versus well with 0.5 μL drops. The best Clp1-1p–Pcf11p (454–563) protein crystals were initially obtained after one day from a solution containing 12% PEG 4000 (w/v), 0.1 M HEPES pH 7.5 or Tris–HCl pH 8.5, 0.1 M–0.2 M $MgCl_2$.

Clp1-1p–Pcf11p (454–563) complex crystals were cryo-protected in a solution containing 0.1 M Tris–HCl pH 8.5, 25% PEG3350 (w/v), 5% PEG 400 (w/v) and flash-cooled in liquid nitrogen.

2.2. Structure determination

Crystals belonged to the space group $P2_12_12_1$ with two molecules of heterodimer per asymmetric unit (R.m.s.d. of 1.0 Å on Cα atoms over 428 residues). Datasets were reduced using XDS [40]. We carried out an initial rigid body refinement using Phenix and the Clp1p coordinates from 2NPI PDB prior to further cycles of refinement with Buster and manual model building [41–43]. Statistics of data collection, processing and refinement are summarized in Supplemental Table 1.

3. Results

3.1. Structure of a mutant Clp1p G135R–Pcf11p complex

The wild type and the G135R Clp1p–Pcf11 protein complexes (residues 454–563) yielded well-diffracting crystals and X-ray diffraction data were collected up to 2.77 Å and 2.70 Å respectively (Supplemental Table 1). The structure were obtained using the 2NPI PDB as a starting model and refined to an Rfree/Rfactor of 17.86%/21.30% and 19.06%/22.30%. Clp1p adopts the structure, as described by Noble and co-workers, with a N-terminal domain formed by residues 1-101 (Fig. 1, NtD in red), a C-terminal domain composed of residues 342–445 (CtD, in yellow) arranged around a middle larger domain (Middle, in green) [30]. The first 18 N-terminal residues are also missing in the structure. Overall our Clp1p–Pcf11p (454–563) structure superimposes well on the 2NPI structure with an R.m.s.d. of 0.47 Å on Cα atoms over 428 residues (Fig. S1). Upon structure refinement a non-attributed extra density appeared located around the C-terminal domain of Clp1p (Figs. 1 and 2). The overall quality of the data and the limited resolution in this area of the electron density map made difficult the unambiguous assignment of the sequence. From an initial OMIT map (Fig. 2a) a sequence of 5 consecutive residues could be build as a Cα chain. Upon model refinement a total of 9 residues was built and attributed to residues 460–469 of Pcf11p (Fig. 2b). The rationale for this assignment sits in an overall better agreement with X-ray data for this 9-residue stretch in comparison to other trials on the one hand and in the proximity with residue 475 of Pcf11p on the other hand. These residues are probably providing a mild contribution to the interaction with Clp1p, as their deletion does not alter Clp1p pull-down in comparison to wild-type Pcf11p (454–563) fragment (Fig. S2a). This observation is in agreement with the poor residues conservation of this stretch (Fig. S2b). Altogether, the modelled Pcf11p fragment encompasses residues 460–469 and 475–499.

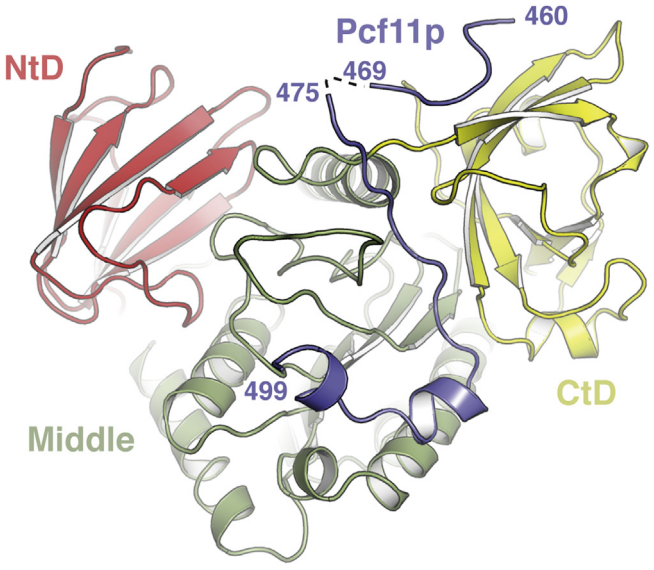

Fig. 1. Structure of Clp1-1p–Pcf11p (454–563). The N-terminal domain (NtD, pink), Middle domain (green) and C-terminal domain (CtD, yellow) are shown as described in Noble et al. [30]. Pcf11p is depicted in blue and the protein boundaries are indicated.