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An iron-sulfur domain of the eukaryotic primase is essential for RNA primer synthesis

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Abstract

Primases synthesise the RNA primers that are necessary for replication of the parental DNA strands. Here we report that the heterodimeric archaeal/eukaryotic primase is an iron-sulfur (Fe-S) protein. Binding of the Fe-S cluster is mediated by an evolutionarily conserved domain at the C terminus of the large subunit. We further show that the Fe-S domain is essential to the unique ability of the eukaryotic primase to start DNA replication.

De novo synthesis of RNA primers by primases is essential for cellular and viral DNA replication1,2. Archaeal and eukaryotic primases are heterodimeric enzymes with a small (PriS) and a large (PriL) subunit2. Although the catalytic activity resides within PriS, the PriL subunit is necessary to primase function as disruption of the PriL gene in yeast is lethal3. Reported roles for PriL include stabilisation of PriS, participation in initiation of RNA primer synthesis, determination of product size and transfer of the primer to DNA polymerase α4-11. A recent crystallographic model of the heterodimeric primase from the archaeon *Sulfolobus solfataricus* provided the first description of the large subunit but did not include its C-terminal domain (PriL-CTD)12. The presence of four conserved cysteines in archaeal and eukaryotic PriL-CTD sequences suggests that the PriL-CTD might be a metal-binding domain (Supplementary Figure 1).

We set out to characterise the biochemical and biophysical properties of the PriL-CTD. Freshly purified samples of *S. solfataricus* PriL-CTD expressed in bacteria as glutathione S-transferase (GST) fusion protein consistently displayed a yellow-brown colour, which turned darker upon concentration of the sample (Supplementary Figure 2). The absorption spectrum of the *S. solfataricus* GST-PriLCTD showed a broad shoulder around 400 nanometers (nm), next to the expected protein peak at 280 nm (Figure 1a). Fading of the colour under aerobic conditions and a decrease in absorption at 400 nm over time indicated the presence of a chromophore in the PriL-CTD, which is sensitive to air oxidation. Purified *Saccharomyces cerevisiae* PriL-CTD fused to a maltose-binding protein (MBP) displayed a similar colour and increased absorption at 400 nm as the *S. solfataricus* GST-PriLCTD (Supplementary

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Author Contributions

SK and LP conceived the project and designed the experiments; SK prepared the recombinant proteins and performed the biochemical experiments; JH performed the EPR analysis; JDM first suggested that the chromophore in the PriL-CTD might be a Fe-S cluster and performed the CD analysis; TK assisted with the primase assays; SK and LP interpreted the data and wrote the paper.

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Figure 2 and Figure 1a). However, the colour of the *S. cerevisiae* MBP-PriLCTD sample did not fade over time and there was no decrease in the absorption at 400 nm, suggesting that the chromophore was stable under aerobic conditions. Gel filtration chromatography of proteolysed *S. cerevisiae* MBP-PriLCTD demonstrated that the chromophore co-migrated with the PriL-CTD (Figure 1b).

The absorption profiles of the PriL-CTD fusion proteins prompted us to investigate the presence of iron by biochemical means. Spectrophotometric analysis using the iron-specific indicator bathophenantroline confirmed the presence of iron in the PriL-CTD samples (data not shown). Variable stoichiometric ratios between iron and protein were obtained for the *S. solfataricus* GST-PriLCTD, likely due to chromophore instability in aerobic conditions. However, the assay yielded a reproducible stoichiometric ratio of ~3:1 iron to protein for the *S. cerevisiae* MBP-PriLCTD, which was later refined to 3.3:1 iron to protein (standard deviation of the ratio is 0.055; Supplementary table 1) for the full-length heterodimeric primase. The presence of iron in the PriL-CTD samples was confirmed by proton-induced X-ray emission (see Supplementary Figure 3). No other metal such as copper, nickel or molybdenum, which can be cofactors of metallo-cluster enzymes, was detected.

Electron paramagnetic resonance (EPR) is commonly used for the study of Fe-S cluster proteins, as each type of cluster yields a characteristic spectroscopic signal under the appropriate experimental conditions. EPR analysis of the *S. solfataricus* GST-PriLCTD gave a strong indication of the presence of an Fe-S cluster (data not shown). However, obtaining a reproducible EPR signal proved difficult, most likely due to cluster instability or misincorporation in the recombinant protein. We therefore focused on the EPR analysis of the more stable *S. cerevisiae* MBP-PriLCTD. No EPR signal was observed for freshly purified MBP-PriLCTD or upon addition of the reducing agent, sodium dithionite (data not shown). However, treatment with the oxidizing agent potassium hexacyanoferrate III, $[Fe(CN)_6]^{3-}$, gave rise to a strong EPR signal (Figure 1c). EPR analysis of the full-length heterodimeric primase in the presence of $[Fe(CN)_6]^{3-}$ produced an identical spectrum to that of the MBP-PriLCTD. The oxidised EPR spectrum is most consistent with the presence of a $[3Fe\text{-}4S]^{1+}$ cluster as the major species, together with variable amounts of $[4Fe\text{-}4S]^{3+}$ clusters. Thus, EPR analysis and the presence of four conserved cysteine ligands suggest that the PriL-CTD is capable of ligating a [4Fe-4S] cluster, and that one of the Fe-centres is labile and either not fully incorporated during protein expression, lost during purification or expelled from the cluster upon oxidation to the $[4Fe\text{-}4S]^{3+}$ state. Taken together, the evidence demonstrates the presence of an Fe-S cluster in the large subunit of archaeal and eukaryotic primases. Surprisingly, the presence of iron has remained unnoticed since initial reports of a primase activity in eukaryotic cells appeared over two decades ago13-15.

In order to investigate the role of the Fe-S cluster in primase activity, we generated a series of mutants of the *S. cerevisiae* primase, where the four putative cysteine ligands (Cys 336, Cys 417, Cys 434, Cys 474) were replaced by alanine. The effect of the substitutions on the Fe-S cluster stability was assessed by measuring the relative iron incorporation and 400 nm absorption of wild-type and mutant primase. All four single-point mutants showed a moderate reduction in iron incorporation and decreased absorption at 400 nm (Figure 2a, b). A double mutation of cysteines 336 and 474 resulted in a severe disruption of the Fe-S cluster, as demonstrated by a drastically reduced amount of iron incorporation and baseline 400 nm absorption, similar to the effect seen with the removal of the entire PriL-CTD (Figure 2a, b). Double substitutions of Cys 336 and Cys 417, as well as Cys 336 and Cys 434, destabilised the large subunit to an extent that prevented the purification of the enzyme.

Primases initiate DNA synthesis by assembling short RNA primers from individual nucleotides. The primase mutants were tested for their ability to synthesise RNA primers on

a single-stranded poly(dT) template, in the presence of ATP. On this template, the wild-type enzyme synthesises RNA primers that are 8-12 nucleotides in length and multiples thereof16. Each of the four single-point Cys-to-Ala mutants was capable of initiating RNA synthesis in a manner indistinguishable from the wild-type enzyme (Figure 2c). However, the double C336A, C474A primase mutant showed clearly reduced activity and a diminished preference for synthesis of oligoribonucleotides of defined size (Figure 2d). A truncated version of the yeast enzyme lacking the PriL-CTD (ΔCTD primase) was incapable of RNA primer synthesis.

In addition to the ability to initiate RNA synthesis, primases can also extend an existing primer-template substrate. We therefore sought to determine whether mutations that disrupt the Fe-S cluster rendered the enzyme completely inactive or affected only its ability to initiate synthesis. To this purpose, we compared the ability of wild-type and mutant primase to elongate a 15-nucleotide oligoA primer annealed to the poly(dT) template. Under these conditions, the wild-type primase demonstrated both RNA primer initiation and extension activities (Figure 2e). In contrast to their impaired initiation activity, the double C336A, C474A mutant and ΔCTD primase demonstrated a level of activity comparable to that of the wild-type enzyme in the extension assay. We conclude that the essential role of the Fe-S cluster is specific to the initiation of RNA primer synthesis.

Circular dichroism (CD) analysis of the *S. cerevisiae* primase indicated that the PriL-CTD is predominantly alpha helical in structure (Supplementary Figure 4 and Table 2). The double C336A, C474A mutation induced a small decrease in alpha helical content of the primase, corresponding to a loss of 28 out of the 123 PriL-CTD residues predicted by CD to be in alpha helical conformation. Thus, CD analysis indicates that disruption of the Fe-S cluster by the double C336, C474A mutation causes a partial, rather than a complete, unfolding of the PriL-CTD. The observation that the double C336A, C474A mutation has a severe negative effect on RNA primer synthesis suggests that the Fe-S cluster is spacially contiguous to a functionally important site in the PriL-CTD. Furthemore, a specific configuration of the Fe-S cluster does not appear to be required for primase activity, as the EPR spectra of the wild-type and the equally active C336A primase mutant are clearly different (Supplementary Figure 5).

In the current paradigm of primase function, the enzyme possesses two nucleotide-binding pockets, the initiation and elongation sites, that need to be simultaneously occupied for formation of the initial dinucleotide17. Previous work has shown that virtually all aspects of primase activity, including initiation, translocation and processivity of the enzyme, are influenced by the large subunit5,7-9. Our finding that the Fe-S domain is essential for synthesis of the RNA primer but not for its elongation points to a specific role of the PriL-CTD in dinucleotide processing. Taken together, these observations suggest a model of primase activity in which residues from both PriS and PriL-CTD contribute to efficient nucleotide binding at the initiation site. This model rationalises biochemical evidence showing that PriL stimulates catalytic activity5,8,9 and can bind DNA7,10, that different regions of PriL contact PriS5,10, that both subunits participate in the formation of the catalytic site4 and that single-point mutations in PriL can drastically reduce the amount of RNA primer synthesised by the primase8 (Supplementary Figure 6).

Supplementary Material

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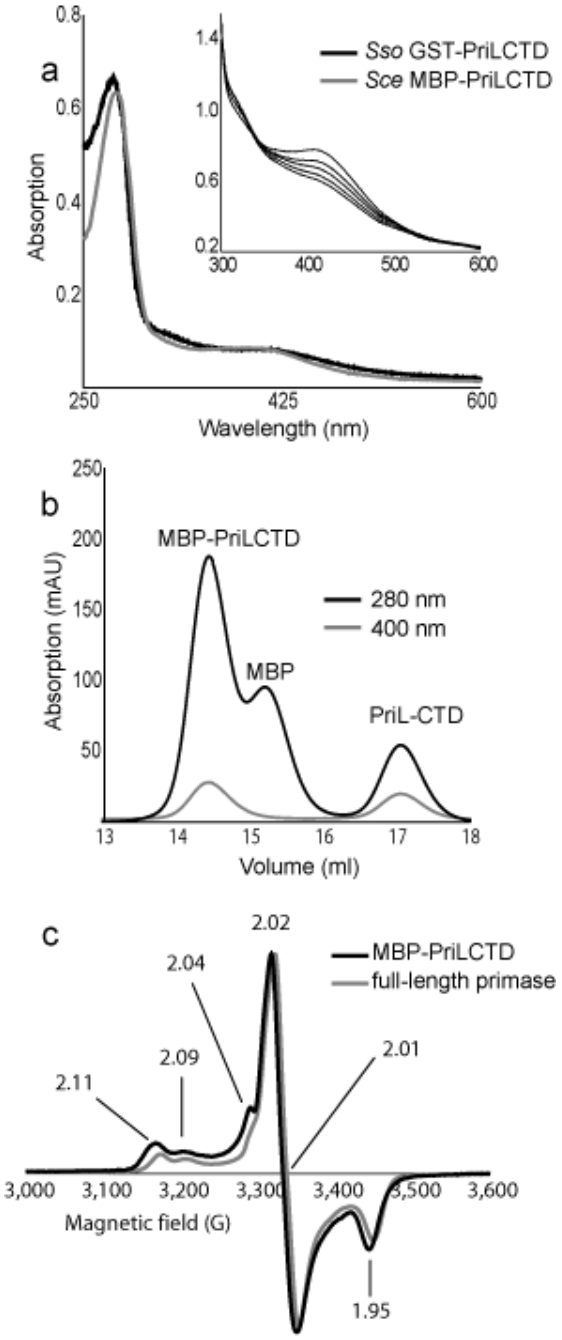

Figure 1.

The archaeal/eukaryotic primase is a Fe-S protein. (**a**) 250-600 nm absorption spectra of the archaeal/eukaryotic PriL-CTD fusion proteins (*Sso; S. solfataricus; Sce: S. cerevisiae*). The inset shows the hourly decrease in the 400 nm absorption of the *S. solfataricus* PriL-CTD over a period of four hours. (**b**) Gel filtration analysis of partially cleaved *S. cerevisiae* MBP-PriLCTD fusion protein. (**c**) Overlay of the EPR spectra for *S. cerevisiae* PriL-CTD and full-length primase, after treatment with $[Fe(CN)_6]^{3-}$. The g-values for the salient features of the spectra are indicated in the figure.

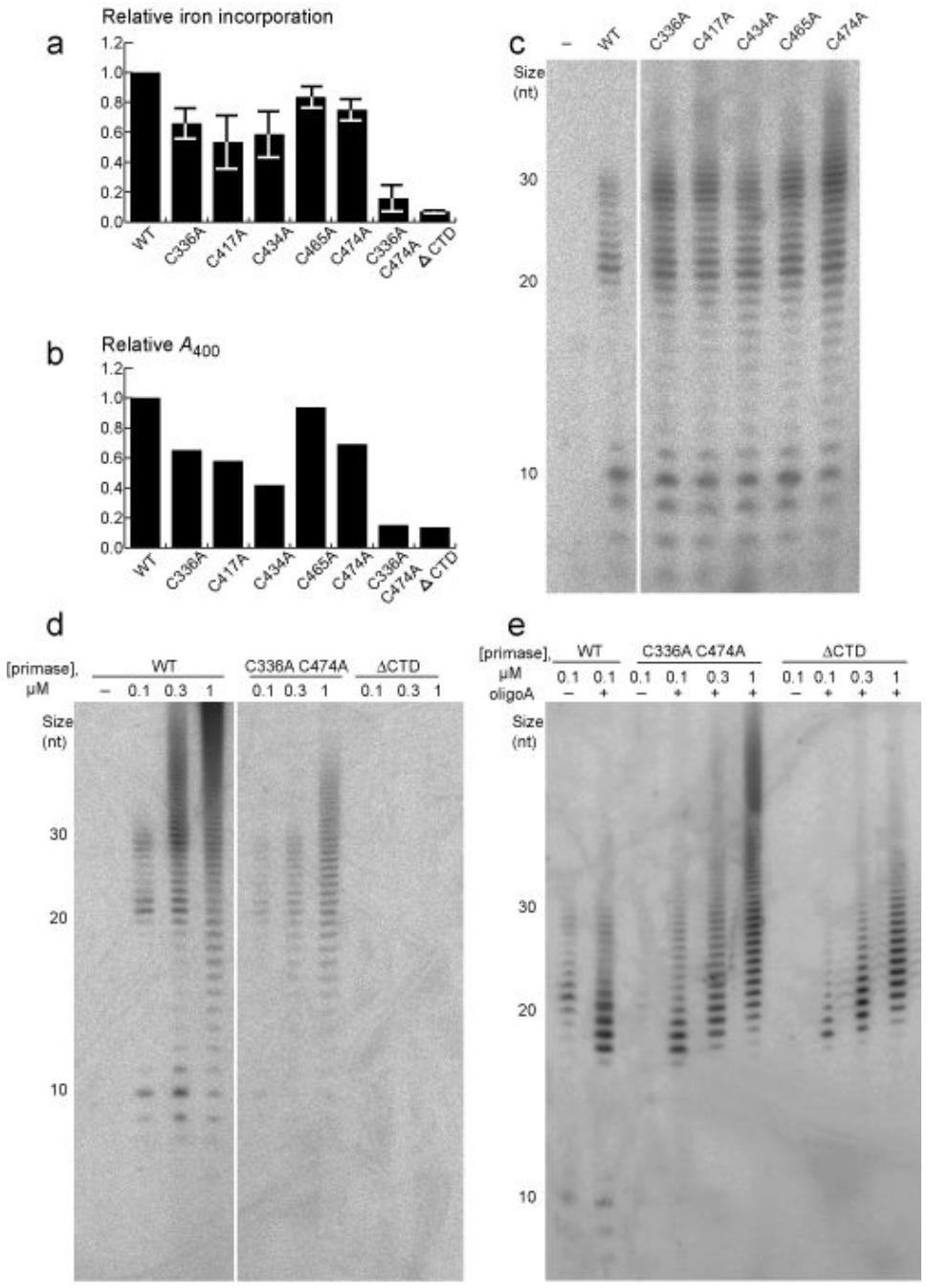

Figure 2.

Functional analysis of the *S. cerevisiae* primase. (**a**) Relative iron incorporation and (**b**) 400 nm absorption of wild-type and mutant yeast primases. Error bars report s.d. (**c**) RNA primer synthesis on a poly(dT) template by the wild-type primase and four single-point (Cys 336, Cys 417, Cys 434, Cys 474) primase mutants. A version of the primase harbouring a mutation to cysteine 465 (C465A), which is only present in yeast primase, was used as positive control. Size of reaction products are indicated for 10-, 20- and 30mer oligoribonucleotides. (**d**) RNA primer synthesis on a poly(dT) template in the presence of increasing amounts of wild-type, double C336A, C474A mutant and ΔCTD primase. ΔCTD

refers to the truncated primase lacking the PriL-CTD. (**e**) Elongation of an oligoA_{15} primer/poly(dT) template substrate by increasing amounts of wild-type, double C336A, C474A mutant and ΔCTD primase.

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