The iron-sulfur cluster is essential for DNA binding by human DNA polymerase ε

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DNA polymerase ε (Polε) is a key enzyme for DNA replication in eukaryotes. Recently it was shown that the catalytic domain of yeast Polε (PolεCD) contains a [4Fe-4S] cluster located at the base of the processivity domain (P-domain) and coordinated by four conserved cysteines. In this work, we show that human PolεCD (hPolεCD) expressed in bacterial cells also contains an iron-sulfur cluster. In comparison, recombinant hPolεCD produced in insect cells contains significantly lower level of iron. The iron content of purified hPolεCD samples correlates with the level of DNA-binding molecules, which suggests an important role of the iron-sulfur cluster in hPolε interaction with DNA. Indeed, mutation of two conserved cysteines that coordinate the cluster abolished template:primer binding as well as DNA polymerase and proofreading exonuclease activities. We propose that the cluster regulates the conformation of the P-domain, which, like a gatekeeper, controls access to a DNA-binding cleft for a template:primer. The binding studies demonstrated low affinity of hPolεCD to DNA and a strong effect of salt concentration on stability of the hPolεCD/DNA complex. Pre-steady-state kinetic studies have shown a maximal polymerization rate constant of 51.5 s⁻¹ and a relatively low affinity to incoming dNTP with an apparent \( K_D \) of 105 µM.

Abbreviations

hPolε    Human DNA polymerase ε
hPolεCD   Catalytic domain of human DNA polymerase ε
P-domain Processivity domain
EMSA Electrophoretic mobility gel shift assay
TCEP Tris(2-carboxyethyl)phosphine

DNA polymerase ε (Polε) is one of the main eukaryotic replicases responsible for leading-strand synthesis during genome duplication1–3. It belongs to the B-family of DNA polymerases, which also contains Polδ, Polα, and Polζ. Human Polε (hPolε) consists of four subunits: the catalytic subunit (p261) and the accessory subunits p59, p17, and p12, listed in order from bigger to smaller. The p261 is composed of two duplicated exonuclease/polymerase domains, the first of which is active, while the second is inactive and plays an important structural role4–6. In the recently reported structure of the yeast Polε holoenzyme, the small subunits p17 and p12 are tethering the two lobes of p2617. Instability of hPolε holoenzyme results in replication stress, tumorigenesis, and developmental abnormalities8,9.

The Polε catalytic subunit contains three cysteine motifs: CysA, CysB, and CysX10,11. The first two are located at the extreme C-terminus, which interacts with a B-subunit (p59 in humans). The recently discovered CysX motif is located in the N-terminal domain and responsible for DNA polymerase and exonuclease activities. The first report about the presence of a [4Fe-4S] cluster in yeast Polε is dated to 2011, when the cluster location was attributed to a CysB motif12. This cluster position was not confirmed by subsequent work of our group13, which is consistent with the crystal structure of the human Polε subcomplex p59-p261 (2142–2286) showing zinc in CysA and CysB14. In 2014, Jain et al. discovered the CysX motif and demonstrated its importance in the [4Fe-4S] cluster coordination and in DNA polymerase activity of yeast Polε15. Later, the presence of a iron-sulfur
cluster in the CysX motif of yeast Polε was confirmed using a structural approach. Recently it was shown that the iron-sulfur cluster of yeast Polε is redox active and its oxidation affects DNA polymerase activity. Despite these advances, the mechanism of DNA synthesis regulation by the iron-sulfur cluster is still unclear and its role in Polε interaction with DNA has not been studied so far.

In this work, we showed that the [4Fe-4S] cluster is critical for template:primer binding by human Polε. Two point mutations that disrupt cluster coordination abrogate hPolε interaction with DNA, resulting in the loss of DNA polymerase and exonuclease activities. We also analyzed the outcome of the protein expression system on the level of iron and DNA-binding properties of hPolε samples. In addition, we conducted pre-steady-state kinetic and binding studies of hPolε at near-physiological salt concentration.

Results
The iron-sulfur cluster is important for hPolε interaction with DNA. The catalytic domain of human DNA polymerase ε (hPolεCD; residues 28-1194) containing only the CysX motif was expressed in Escherichia coli and purified to near homogeneity (Fig. 1). The concentrated sample displayed a brownish color, a characteristic of proteins containing an iron-sulfur cluster. Interestingly, the same protein overexpressed in insect cells and purified in the same way (purity is shown in Fig. 1) was almost colorless. Analysis of the iron content revealed that the hPolεCD sample expressed in insect cells has an 8.6-fold-lower iron level than the one expressed in E. coli (Table 1). This result indicates that, upon using the routine protocol, insect cells cannot efficiently incorporate the iron-sulfur cluster into a heterologous protein during its overexpression.

For both hPolεCD samples, we analyzed the level of molecules possessing DNA-binding activity by using an electrophoretic mobility shift assay (EMSA; Fig. 2). All reactions contained varying concentrations of hPolεCD and 0.5 µM DNA duplex composed of a 15-mer primer annealed to a 20-mer template (Table 2). After 5 min of incubation at room temperature, the products were resolved by a 5% native gel. This approach allows to separate DNA molecules from protein/DNA complexes and calculate the percentage of complexed DNA for each protein concentration. We found that hPolεCD samples obtained after expression in E. coli and insect cells have 51% and 8.5% of active molecules, respectively (Table 1). The correlation between the iron content and the level of DNA-binding molecules suggests that the iron-sulfur cluster is important for the interaction of hPolε with DNA. All data described below were obtained using the active enzyme concentration unless otherwise indicated.

Table 1. DNA-binding activity of hPolεCD samples correlates with their iron content. *The iron content of four atoms per protein molecule was taken for 100%. bThe molecules showing DNA binding activity. Data are presented as mean ± SD.

Expression system | Iron content (%) | Active molecules (%) |
--- | --- | --- |
insect | 7.3 ± 0.93 | 8.3 ± 0.7 |
E. coli | 63 ± 7.1 | 51.2 ± 4.1 |

Figure 1. Analysis of purity of hPolεCD samples. Samples were separated by 8% SDS-PAGE and stained by Coomassie Brilliant Blue R-250. M–markers; lane 1–hPolεCD(insect); lane 2–hPolεCD (E.coli).
hPolεCD mutant deficient in the [4Fe-4S] cluster displays no DNA interaction and is inactive on the primed template. In order to confirm that the [4Fe-4S] cluster is required for interaction with a template:primer, we obtained the hPolεCD mutant (hPolεCD\textsuperscript{M}) where two of four conserved cysteines (654 and 663) coordinating the cluster were mutated to serines. Interestingly, upon elution from a Heparin HiTrap HP column by a gradient of sodium chloride, the mutant was eluted at a lower salt concentration compared to the intact hPolεCD (SI Fig. S1). Heparin HiTrap is considered an affinity column for DNA-binding proteins because its sulfate groups mimic phosphates of DNA; therefore, the earlier mutant elution from this column indicates a compromised interaction with DNA. Of note, hPolεCD\textsuperscript{M} is contaminated with a proteolysis product of a molecular mass of ~120 kDa, due to reduced affinity to the Heparin column.

Purification of hPolεCD\textsuperscript{M} by a size-exclusion column revealed significant level of aggregates that were eluted in the void volume (SI Fig. S2). Of note, a slightly increased tendency to aggregate was mentioned for the yeast PolεCD mutant deficient in the [4Fe-4S] cluster\textsuperscript{10}. Thus, the size-exclusion column is an important step for hPolεCD\textsuperscript{M} purification, allowing removal of aggregated molecules. The concentrated hPolεCD\textsuperscript{M} sample was colorless, and almost iron-free (iron content of 2.8 ± 0.9%), as was previously shown for yeast Polε\textsuperscript{M} mutants\textsuperscript{10,11}.

Analysis of the DNA-binding activity by EMSA revealed that the interaction with DNA is significantly compromised in the case of the mutant (Fig. 3). Even at four-fold molar excess over DNA, hPolεCD\textsuperscript{M} had no effect on the mobility of single-stranded (ss) and double-stranded (ds) DNA. In contrast, addition of hPolεCD resulted in a significant mobility shift for both DNA substrates, with 72% of dsDNA and 51% of ssDNA in the complex with a protein added at two-fold molar excess (Fig. 3).

Next, we analyzed the DNA polymerase and exonuclease activities of hPolεCD and the mutant using a Cy3-labeled 15-mer primer annealed to a 25-mer template. Both activities require the presence of magnesium ions.
In addition, a dNTP mix was added to the DNA polymerization reactions. Upon a 1–2 min incubation of the primed template with hPolεCD, almost all 15-mer primers were extended to longer products in the presence of dNTPs or degraded to smaller products in the absence of dNTPs (Fig. 4A). A disruption of the iron-sulfur cluster coordination by a C654S/C663S mutation dramatically affected exonuclease and especially DNA polymerase activity, which was not detected even after 80 min incubation at 35 °C (Fig. 4B).

Analysis of the exonuclease activity on the primer alone also showed that hPolεCD \text{M} is significantly less active than the protein with an intact CysX motif (Fig. 5). It is known that DNA polymerases demonstrate stronger exonuclease activity on ssDNA versus dsDNA because only the primer 3'-end enters the exonuclease active site after partial duplex melting. Thus, the absence of a [4Fe-4S] cluster severely affects DNA binding as well as DNA-polymerase and exonuclease activities of hPolε on the primed template.
Binding and kinetic studies of PolεCD. The analysis of PolεCD interaction with DNA was conducted using an Octet K2, which allows for extraction of the rate constants of complex formation ($k_{on}$), dissociation ($k_{off}$), and the dissociation constant ($K_D$), which is inversely proportional to affinity. A 23-mer DNA template with biotin at the 5′-end (Table 2) was primed by an 11-mer DNA primer and loaded on a streptavidin-coated sensor. In the presence of 0.1 M NaCl, hPolεCD samples obtained from E. coli and insect cells have shown similar $K_D$ values close to 200 nM (Table 3). A previous DNA-binding study of hPolεCD conducted in the absence of salt reported a $K_D$ value of 79 nM 16.

Notably, an increase of NaCl concentration from 0.1 to 0.15 M reduced affinity ~ 20-fold, which is mainly due to the 13-fold reduction in $k_{off}$ value (Table 3). We observed a similar effect of salt concentration on affinity to DNA for PolαCD 17 and for a different hPolεCD construct, deficient in exonuclease activity18. Thus, hPolεCD binds a DNA duplex with relatively low affinity at near-physiological salt concentration (Table 3, $K_D$ = 3.3 µM). The obtained $k_{off}$ value of 0.54 s$^{-1}$ indicates that the half-life of the hPolεCD/DNA complex is ~ 1.3 s on average. It was not possible to conduct binding studies for hPolεCD (insect) at 0.15 M NaCl with acceptable accuracy due to the low level of active molecules, which demands very high protein concentration in reaction.

The pre-steady-state kinetic approach was employed in order to assess hPolεCD activity in DNA primer extension and its affinity to incoming dNTP at conditions used for binding studies. Kinetic studies were conducted in the presence of 0.1 M NaCl to allow most of the DNA to complex with hPolεCD before the reaction. Single-nucleotide incorporation experiments were conducted under single-turnover conditions. This assay provides the maximal polymerization rate ($k_{pol}$) and the apparent dissociation constant ($K_D$) for the incoming nucleotide.

hPolεCD (0.8 µM) was incubated with a Cy3-labeled DNA (0.4 µM) and quickly mixed with varying dTTP concentrations under rapid chemical quench conditions. For each dTTP concentration, the fraction of extended primer was plotted against time (Fig. 6A) and the data were fit to a single-exponential equation (Eq. 1). The obtained rate constant values were plotted against dTTP concentration (Fig. 6B) and fit to (Eq. 2), resulting in a $k_{pol}$ of 51.5 ± 1.8 s$^{-1}$ and an apparent $K_D$ of 105 ± 9.8 µM. A $K_D$ value of 31 µM was reported previously for the hPolεCD/exo-)/dTTP complex by another group 16. The 3.4-fold difference with our data might be attributable to the absence of salt in reaction in that study. As shown above (Table 3), salt significantly affects the hPolεCD/DNA complex. The same effect is expected for the hPolεCD/dNTP complex because their interaction interface is mainly electrostatic. An apparent $K_D$ of 105 µM for dTTP indicates that hPolεCD might not be saturated with dNTPs in vivo to achieve the maximal polymerization rate, given the fact that the average concentration of each dNTP in human mitotic cells is approximately 50 µM19 or even lower20. On the other hand, it is possible that the local concentration of dNTPs near the replication fork is significantly elevated. hPolεCD (insect) was not suitable for this assay because of its low level of active molecules. In another study, we have shown that the exonuclease deficient variant of hPolεCD extends the primer at a rate of 62.4 s$^{-1}$ in the presence of 1 mM dTTP 18. Interestingly,

| Expression system | [NaCl] mM | $k_{on}$ mM$^{-1}$ s$^{-1}$ | $k_{off} \times 10^{-3}$ s$^{-1}$ | $K_D$ a nM |
|------------------|-----------|-----------------|-----------------|-----------|
| Insect           | 100       | 162 ± 2.5       | 34.2 ± 6.3      | 213 ± 43  |
| E. coli          | 100       | 252 ± 26        | 41.9 ± 6.7      | 167 ± 24  |
| E. coli          | 150       | 168 ± 29        | 542 ± 15        | 3258 ± 386|

Table 3. Interaction of hPolεCD with DNA. a $K_D$ values are obtained by dividing $k_{off}$ by $k_{on}$. Data are presented as mean ± SD.
a $k_{pol}$ value of 248 s$^{-1}$ was obtained for hPolεCD at 20 °C, which might be due to the absence of salt in reaction. Noteworthy, the catalytic domain of human Polα showed the maximal rate of DNA polymerization of 33.8 s$^{-1}$ in the presence of 0.1 M NaCl.

**Discussion**

The recently discovered [4Fe-4S] cluster, located at the base of the P-domain (Fig. 7) and coordinated by four conserved cysteines, is unique to Polε. Mutation of these cysteines in yeast Polε led to a loss of iron and DNA polymerase activity but not exonuclease activity, as was shown for holoenzyme as well as a separate catalytic domain. The role of the [4Fe-4S] cluster in the DNA-binding properties of Polε has not been studied so far. Our data indicate that the iron-sulfur cluster is critical for interaction of human Polε with DNA. Probably, abrogated DNA binding is responsible for the loss of both activities in hPolεCD. Substrate binding by any enzyme is an initial step that precedes catalysis. It is difficult to imagine how the absence of the distantly located iron-sulfur cluster can affect both catalytic centers and especially the exonuclease one. Structural data indicate that the [4Fe-4S] cluster may stabilize the P-domain that helps Polε to enclose a DNA duplex (Fig. 7). This suggests that in the absence of the cluster, the P-domain changes its conformation and/or position and blocks entrance into the DNA-binding pocket for a template:primer.

It is quite possible that inactivation of DNA polymerase activity of yeast Polε by mutation of cluster-coordinating cysteines is also due to abrogated DNA binding and not catalysis. Intriguingly, the exonuclease activity of yeast Polε was not affected upon disruption of the [4Fe-4S] cluster. Moreover, the DNA polymerase activity slightly recovered when dNTPs concentration was increased ten-fold, pointing to compromised dNTP binding. We cannot exclude the possibility that elimination of the cluster has a slightly different effect on the structure and activity of yeast and human Polε. For example, in cluster-deficient mutants of yeast Polε, the template:primer-binding cleft might be blocked by the P-domain at the smaller level than in hPolεCD.

The biological role of the [4Fe-4S] cluster in regulating DNA-binding properties and activity of Polε requires further investigation. As previously proposed, due to its peripheral position and potential sensitivity to oxidation, the cluster might be a target for oxygen reactive species during oxidative stress. Recently it was demonstrated that the [4Fe-4S] cluster of yeast Polε is redox active and its reversible oxidation affects DNA polymerase activity. We propose that the main role of the P-domain in hPolε is controlling access to the DNA-binding pocket, which might depend on intracellular signals. Even a subtle change in the P-domain position would be enough to prevent template:primer binding (Fig. 7). The concept of the interplay between the P-domain and the [4Fe-4S] cluster in the function of Polε is supported by the fact that they both exist only in the Polε catalytic domain and not in the catalytic domains of other DNA polymerases. On the other hand, we cannot exclude a possibility that the [4Fe-4S] cluster also controls or stabilizes the conformation of the other subdomains like fingers and even of the entire hPolεCD.

This work revealed that insect cells do not efficiently incorporate the [4Fe-4S] cluster into heterologous proteins during overexpression. This may be due to low capacity and/or high specificity of the enzymatic machinery, which is responsible for cluster incorporation. In contrast, the corresponding machinery of bacteria is more robust and non-specific. Previously we have shown that the [4Fe-4S] cluster was erroneously incorporated at a significant level into a zinc-binding domain located at the C-terminus of the catalytic subunit of hPolε. Fortunately, the cluster-containing molecules were unable to make a stable complex with an accessory subunit of hPolε and were separated during purification. Thus, only the Polε catalytic domain contains the iron-sulfur
This study underscores the importance of measuring the iron level in Polε samples, especially when the enzyme is overexpressed in insect cells. Notably, all previous functional studies of hPolε CD were conducted with a recombinant protein expressed in *E. coli*, where the iron content was not analyzed16,23–25.

**Materials and methods**

**Cloning, expression, and purification.** hPolε CD was cloned into pASHSUL-1 plasmid26 to produce the corresponding proteins tagged with N-terminal His-Sumo. The mutant with cysteines 654 and 663 changed to serines was obtained by side-directed mutagenesis. hPolε CD (insect) with a cleavable N-terminal His-Tev tag was cloned into pFastBac-1 plasmid (Invitrogen). hPolε CD and hPolε CD(exo-) were expressed in *E. coli* strain Rosetta-2 (DE3) at 18 °C for 16 h following induction with 0.2 µg/ml anhydrotetracycline. Afterward, cells were harvested by centrifugation at 4000 g for 15 min, washed with PBS, aliquoted, and kept at −80 °C. A high-titer virus stock for hPolεCD (insect) was obtained by using the Bac-to-Bac Baculovirus Expression System from Invitrogen. 1.8 × 10^9 Sf21 cells in 1 L shaking culture were infected with the recombinant virus at a multiplicity of infection of 2 and cultivated at 27 °C for 56 h. Cells were harvested by centrifugation at 200 g for 5 min and frozen.

All samples were purified according to the same protocol, including chromatography on a Ni-IDA column (Bio-Rad), His-tag digestion during overnight dialysis, and chromatography on a Heparin HP HiTrap column (Cytiva) and on a size-exclusion column Superose 12 10/300 GL (Cytiva) in buffer containing 25 mM Tris-HEPES (pH 7.8), 0.15 M NaCl, 1% glycerol, and 2 mM tris(2-carboxyethyl)phosphine (TCEP). Finally, samples were concentrated to 30–60 µM and flash-frozen in aliquots. The purity of obtained samples was analyzed by 8% SDS-PAGE (Fig. 1). Protein concentrations were estimated by measuring the absorbance at 280 nm and using extinction coefficients of 157 mM⁻¹ cm⁻¹; the extinction coefficients were calculated with ProtParam27. The iron content in purified protein samples was determined with use of chromogen ferrozine as described in22.

**Electrophoretic mobility shift assay.** Reactions containing 0.5 µM DNA (Table 2) and varying amount of protein were incubated in 10 µl for 5 min at room temperature in buffer containing 20 mM Tris-Hepes (pH 7.8), 100 mM...
NaCl, 2% glycerol, 2 mM TCEP, and 0.2 mg/mL BSA; 5 µl was then loaded on 5% native PAGE. Samples labeled with Cy3-dye were visualized using a Typhoon 9410 imager (Cytiva) and quantified using ImageJ software (version 1.45 s, National Institutes of Health).

**Binding studies.** Analysis of binding kinetics was done at 23 °C on an Octet K2 (Sartorius AG). This device uses Bio-Layer Interferometry technology to monitor molecular interactions in real time. A template with a biotin-TEG at the 5′-end was annealed to the primer (Table 2) and immobilized on a streptavidin-coated biosensor (SAX, Sartorius AG). The primer was added at two-fold molar excess over the template and contained 3′-dideoxy cytidine. SAX sensors were loaded with oligonucleotide-biotin at 50 nM concentration for 7 min at 500 rpm. Then sensors were blocked by incubating for 2 min in 10 µg/ml biocytin. In the first row of a 96-well microplate (Greiner Bio-One), the first six wells contained the buffer, consisting of 30 mM Tris-Hepes, pH 7.8, 0.1 M NaCl, 2 mM TCEP, and 0.002% Tween 20. The next six wells contained the two-fold dilutions of hPolεCD in the same buffer. All wells in the adjacent row contained only the buffer for reference. Data Analysis HT software (ver. 11.1, Sartorius AG) was used for calculation of binding constants ($k_{on}$, $k_{off}$, and $K_D$) by using the global fitting. The average value and standard deviation were calculated from three independent experiments.

**Pre-steady-state kinetic studies.** Kinetic studies were performed on a QFM-4000 rapid chemical quench apparatus (BioLogic, France) at 35 °C. Reactions contained 0.4 µM hPolεCD (active molecules), 0.2 µM DNA, varying concentrations of dTTP, 25 mM Tris-HEPES, pH 7.8, 0.1 M NaCl, 5 mM MgCl₂, 2 mM TCEP, and 0.2 mg/mL BSA. hPolεCD was incubated with a Cy3-labeled 15-mer primer annealed to a 25-mer DNA template (Table 2), to allow formation of the binary complex, and rapidly mixed with dTTP and MgCl₂ followed by quenching with 0.3 M EDTA. Products were collected in a tube containing 15ul 100% formamide and separated by 20% Urea-PAGE. The Cy3-labeled products were visualized by a Typhoon FLA 9500 (Cytiva) and quantified by ImageJ, version 1.5.3 (NIH). The extended primer fraction was calculated by dividing the amount of extended primer by the amount of primer added in reaction. For each dTTP concentration, the percent of extended primer was plotted against time and the data were fit to a single exponential equation:

$$\frac{[\text{product}]}{A \times (1 - e^{-k_{\text{obs}}t})}$$  \hspace{1cm} (1)

where $A$ is the amplitude, $k_{\text{obs}}$ is the observed rate for dNTP incorporation, and $t$ is the time. The $k_{\text{obs}}$ was plotted against dTTP concentration and the data were fit to the hyperbolic equation:

$$k_{\text{obs}} = \frac{k_{\text{pol}} \times [\text{dNTP}]}{K_D + [\text{dNTP}]}$$  \hspace{1cm} (2)

using GraphPad Prizm software to obtain $k_{\text{pol}}$, the maximum rate of nucleotide incorporation, and $K_D$ the apparent dissociation constant for the incoming nucleotide.

**DNA polymerase and exonuclease assay.** Exonuclease reactions were conducted in 10 µl at 35 °C and contained 0.05 µM PolɛCD, or its mutant (total protein concentration), 0.2 µM DNA (Cy3-labeled 15-mer primer annealed to a 25-mer DNA template), 25 mM Tris-HEPES, pH 7.8, 0.1 M NaCl, 5 mM MgCl₂, 2 mM TCEP, and 0.2 mg/mL BSA. DNA polymerase reactions were performed in a similar way except using 10 nM protein and adding 50 µM dNTPs. The reactions were stopped by addition of 20 µl stop solution (96% formamide, 50 mM EDTA, 0.1% bromophenol blue) and heated to 95 °C for 1 min. The products were separated on a 20% denaturing polyacrylamide gel and visualized by a Typhoon FLA 9500 (Cytiva).

**Data availability** The data that support the findings of this study are included in the Supplementary Information file or available from the corresponding author on request.

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Author contributions
A.E.L., A.G.B., and L.M.M. purified hPolεCD samples. A.E.L. performed functional studies. L.M.M., N.D.B., and E.I.S. prepared the plasmids encoding different hPolε constructs and analyzed their expression. A.G.B. and T.H.T. initiated and supervised the project. A.G.B. and T.H.T. wrote the manuscript, with contributions and critical comments from the other authors.

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