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The screening of expression and purification conditions for replicative DNA polymerase associated B-subunits, assignment of the exonuclease activity to the C-terminus of archaeal pol D DP1 subunit

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Abstract

The B-subunits of replicative DNA polymerases belong to the superfamily of calcineurin-like phosphoesterases and are conserved from Archaea to humans. Recently we and others have shown that the B-subunit (DP1) of the archaeal family D DNA polymerase is responsible for proofreading 3′–5′ exonuclease activity. The similarity of B-subunit sequences implies a common fold, but since the key catalytic and metal binding residues of the phosphoesterase domain are disrupted in the eukaryotic B-subunits, their common function has not been identified. To study the structure and activities of B-subunits in more detail, we expressed 13 different recombinant B-subunits in *Escherichia coli*. We found that the solubility of a protein could be predicted from the calculated GRAVY score. These scores were useful for the selection of proteins for successful expression. We optimized the expression and purification of *Methanocaldococcus* (*Methanococcus*) *jannaschii* DP1 of DNA polymerase D (MjaDP1) and show that the protein co-purifies with a thermostable nuclease activity. Truncation of the protein indicates that the N-terminus (aa 1–134) is not needed for catalysis. The C-terminal part of the protein containing both the calcineurin-like phosphoesterase domain and the OB-fold is sufficient for the nuclease activity.

Keywords: DNA polymerase; Exonuclease; Archaeal DP1; B-subunit; Recombinant protein

To achieve accurate transmission of the genetic information from mother to daughter cells all living organisms encode DNA polymerases (pols)[4] that fulfil various replicative and repair functions. Since these mechanisms are very important for the survival of the cell, nature has evolved safety systems by employing different pols for similar functions.

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[4] *Abbreviations used:* pol, DNA polymerase; His-tag, a tag with six histidines; HAT-tag, histidine affinity tag; IPTG, isopropyl-β-D-thiogalactopyranoside; CBB, Coomassie brilliant blue; fr., fraction; aa, amino acid.

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Replicative pols are enzymes whose main function is to copy bulk chromosomal DNA. Interestingly, the members of three different families perform the same replicative function indicating a complex evolutionary history. In the copying process, bacteria and eukaryotes utilize the family C and B enzymes, respectively, while two different classes of pols, B and D, have been implicated in the third kingdom of life, the Archaea [1].

All replicative pols, with the exception of archaeal pol B, possess a multisubunit structure. For example, the eukaryotic family B members, pols α, δ, and ε have mainly four subunits, the A-subunit being responsible for the polymerase and exonuclease activities [reviewed in 2]. Although their C- and D-subunits are specific for the enzyme, the B-subunits are conserved from Archaea to humans [3,4].

Interestingly, archaeal B-subunits do not associate with the family B DNA polymerases, but instead are tightly bound to the family D enzymes [5]. Furthermore, the polymerase associated B-subunits belong to the large calcineurin-like phosphoesterase superfamily defined by an active site harbouring two divalent metal ions that are involved in catalysis [3,6,7].

The B-subunit of archaeal pol D, DP1, is associated with a large DP2-subunit [5]. Its amino acid sequence has no homology to other pols with the exception of putative zinc-finger regions found also in the family B enzymes [5,8,9]. Although only very limited polymerase activity has been detected for DP2; it has been suggested to be responsible for the polymerase activity [8]. The mutation of two key aspartate residues abolished the polymerase activity of the holoenzyme leaving the proofreading exonuclease activity intact [9].

The conservation of the B-subunits of different pols argues for a common function. It has been proposed that the B-subunits are required for the regulation of DNA polymerase activity, for guiding the polymerases to the replication fork, or for forming a scaffold for protein–protein interactions [4].

The seven residues involved in metal coordination and catalysis in the members of the calcineurin-like phosphoesterase superfamily have been conserved in archaeal DP1 of family D pols, but disrupted in their eukaryotic counterparts [3,10]. High conservation suggests that archaeal DP1 is a 3′–5′ exonuclease, whereas the absence of key residues in the eukaryotic B-subunits reflects loss of this activity [10]. Indeed, *Methanocaldococcus* (*Methanococcus*) *jannaschii* and *Pyrococcus horikoshii* DP1s were recently shown to execute a 3′–5′ exonuclease activity [11,12]. However, the proposed common function for all B-subunits has not yet been identified.

With this goal in mind, we screened through the expression of 13 different recombinant B-subunits from eukaryotic pols δ and ε, as well as from archaeal pol D. Although the B-subunits were mainly expressed poorly or as insoluble proteins in *Escherichia coli*, we were able to express the DP1 of *M. jannaschii* pol D (MjaDP1) in a soluble form and purify it to apparent homogeneity. We show that the C-terminal part of the protein executes a 3′–5′ exonuclease activity.

Materials and methods

Materials

HiTrap Chelating HP, HiTrap Q Sepharose Fast Flow, Chelating Sepharose Fast Flow, Superdex 200 10/300 GL, and Heparin Sepharose CL-6B were from Amersham Biosciences. DNA oligonucleotides were either synthesized by an Applied Biosystems 394 DNA/RNA synthesizer, or ordered from Amersham Biosciences, Gibco Life Technologies or Sigma Genosys. The *E. coli* expression strains BL21 (DE3)pLysS, BL21 Star (DE3)pLysS, and BL21-Codon plus (DE3)-RIL were from Novagen, Invitrogen, and Stratagene, respectively, whereas the expression vectors pET3a and 3d were purchased from Novagen. Complete Mini, EDTA-free Protease Inhibitor Cocktail Tablets were from Roche Diagnostics GmbH. SDS–PAGE gels were stained with Bio-Safe Coomassie (Bio-Rad). The sequences for the 6× His- and HAT-tags were obtained from the vectors of Novagen and BD Clontech, respectively. Restriction enzymes and T4 DNA ligase were purchased from Amersham Biosciences, MBI Fermentas, and New England Biolabs. PCR amplification was performed either with DyNAzyme EXT (Finnzymes) or *Pfu* DNA polymerase (Stratagene).

Prediction of hydrophilicity

The sequences of the B-subunits obtained with BLAST [13] were analysed by ProtParam of the Swiss Institute of Bioinformatics [14] to predict hydrophilicity of the proteins. The hydrophilicity was given as GRAVY (Grand Average of Hydropathicity) score, which is calculated as the sum of hydropathy values of all the amino acids, divided by the number of residues in the sequence [15].

Expression constructs for the B-subunits of replicative DNA polymerases

The B-subunits of replicative pols expressed in this study are shown in Table 1. The expression constructs were cloned either into a pET3a or a 3d vector using PCR amplification and restriction enzyme digestions. When necessary, vectors were modified by ligating oligonucleotides containing additional restriction enzyme recognition sites. The ends of inserts were trimmed to avoid the incorporation of extra amino acids into the constructs except for the N-terminal His-tag constructs in