Control of complex formation of DNA polymerase α–primase and cell-free DNA replication by the C-terminal amino acids of the largest subunit p180

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Abstract DNA polymerase α–primase is a heterotetrameric complex essential for simian vacuolating virus 40 (SV40) DNA replication. We show that the C-terminal 67 amino acid residues of the human p180 subunit are essential for SV40 DNA replication as they are required for binding of the p68 subunit and play a role in the interaction with the primase subunits, p48 and p58. Furthermore, we demonstrate that exchanging these residues to those of mouse origin can only partially rescue the SV40 DNA replication activity of DNA polymerase α–primase.

Key words: DNA polymerase α; Enzyme activity; Protein–protein interaction; Simian vacuolating virus 40 DNA replication

1. Introduction

The evolutionarily highly conserved heterotetrameric DNA polymerase α–primase complex initiates DNA replication in eukaryotes ranging from yeast to vertebrates (for review see [1–4]). Of the constituent subunits the largest, p180, has DNA polymerase activity and is alternatively known as DNA polymerase α [1,4,5]. The smallest subunit, p48, is a primase [2,6–10] (for review see [1,2,11]). The remaining subunits, p68 and p58, have no known enzymatic activities but evidence exists that p68 fulfills a regulatory role [1,12–16] and in vitro studies indicate that p58 stabilises the primase activity of p48 and influences the primer length [9,17]. DNA polymerase α–primase has been the subject of much research as detailed knowledge of its function is fundamental to understanding the mechanism of eukaryotic DNA replication. A great contribution in this respect was the establishment of cell-free DNA replication systems based on simian vacuolating virus 40 (SV40) [18,19]. This polyomavirus is dependent on the host DNA replication machinery and contributes to the replication process only one essential virally encoded protein, large T antigen (TAg), a DNA helicase which also acts in recognition of the replication origin and in the recruitment of host DNA replication factors including DNA polymerase α–primase [18,20].

The catalytic regions of human DNA polymerase α are located centrally in the protein, roughly between amino acid residues 400 and 1100 [21]. The functions of other parts of the protein have only been partially resolved. An N-terminal region, spanning residues 195–313, was shown to be involved in binding SV40 TAg [22]. The C-terminus is involved in interaction with the DNA template and studies with murine DNA polymerase α showed that the three smaller subunits of the DNA polymerase α–primase complex also bind to C-terminal regions [21]. Previously it was found that C-terminal elements of DNA polymerase α are likely to play a role in functional interactions with SV40 TAg [23]. In order to define more closely the regions necessary for these interactions we investigated the activity of a C-terminal truncation mutant of human DNA polymerase α in a cell-free SV40 DNA replication assay. We show that deletion of the 67 extreme C-terminal amino acid residues leads to a total loss of DNA replication activity and in an inability to bind the p68 subunit as well as a reduced affinity for the primase subunits. Our results expand on data of Mizuno et al. [21] concerning the polymerase α sequences required to bind p68.

2. Materials and methods

2.1. Construction of baculoviruses expressing chimeric p180 proteins

For the construction of recombinant baculoviruses we used the Bac-to-Bac® system (Invitrogen, Heidelberg, Germany), according to the manufacturer's protocol (see also [14,23]). pFB/ΔC67-Hp180 was established by digesting pFB/Hp180 with *Age*I, filling in the overhanging ends with *Escherichia coli* DNA polymerase I Klenow fragment and religating. This results in a four amino acid frameshift followed by a stop codon.

2.2. Proteins

SV40 TAg and the DNA polymerase α–primase complex (p180-p68-p58-p48) were purified from baculovirus-infected insect cells as described [24–27]. RPA was bacterially expressed and purified as outlined before [28,29].

The protein concentration was determined according to Bradford [30] using a commercial reagent with bovine serum albumin as a standard (Bio-Rad, Munich, Germany). SDS gel and Western blotting electrophoresis were carried out as described [26]. Enzyme assays were performed as presented earlier [8,31].

2.3. Preparation of S100 extracts and replication of SV40 in vitro

S100 extracts were prepared from logarithmically growing FM3A cells as previously described [26,32]. The replication of SV40 DNA in

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Abbreviations: SV40, simian vacuolating virus 40; TAg, large T antigen

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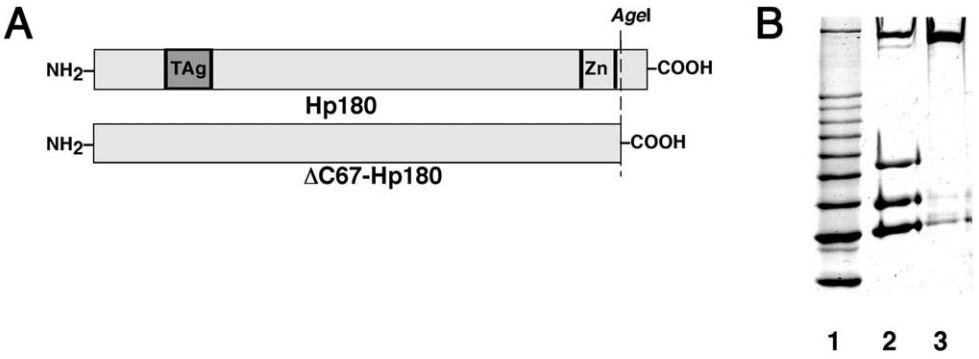

Fig. 1. A: DNA polymerase α mutant created in this study. Top: wild-type DNA polymerase α showing the SV40 TAg binding site mapped by Dornreiter et al. [22] as a shaded box marked 'TAg' and the location of two essential zinc finger motifs as two bold vertical lines marked 'Zn'. The restriction site used to make the truncation mutant is shown at the corresponding position on the protein map. Bottom: ΔC67-Hp180. B: SDS–polyacrylamide gel electrophoresis of purified DNA polymerase α–primase complexes consisting of human or mutant p180 subunits and human p68, p58 and p48. Approximately 4 μg of each protein complex was loaded and the gel was stained with Coomassie brilliant blue. Lane 1: 10 kDa protein markers (Invitrogen); lane 2: wild-type DNA polymerase α–primase; lane 3: $(\Delta C67\text{-}Hp180)H_2$.

vitro was performed as previously described [24,25]. DNA polymerase α–primase was added as indicated. The incorporation of radioactive dNMP was measured by acid precipitation of DNA and scintillation counting [33].

3. Results

3.1. Truncation of the C-terminal 67 amino acid residues of DNA polymerase α prevents binding to p68

We constructed a mutant human DNA polymerase α polypeptide with a truncation of the 67 C-terminal amino acids (ΔC67-Hp180; Fig. 1A) which we could purify by immunoaffinity. When we tried to purify this protein in complex with the three smaller human subunits we found that despite several attempts we were able only to purify a heterotrimeric complex consisting of ΔC67-Hp180, p58 and p48 ($(\Delta C67\text{-}Hp180)H_2$, Figs. 1B and 2). The primase subunits were present at much lower levels than in the wild-type complex and accordingly we found the mutant complex to have very much reduced primase activity on poly(dT) (specific primase activity: 55 U/mg) compared with the wild-type complex (specific primase activity: 500 U/mg). Western blot analysis showed the p68 subunit to be completely absent from the purified mutant complex although it was efficiently expressed by the baculovirus in the insect cells (Fig. 2).

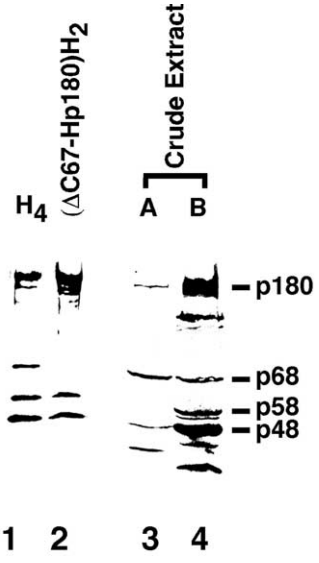

Fig. 2. Western blot analysis of purified $(\Delta C67\text{-}Hp180)H_2$. Lane 1: 4 μg of purified wild-type DNA polymerase α–primase; lane 2: 4 μg of purified $(\Delta C67\text{-}Hp180)H_2$. Lanes 3 and 4: crude extract from insect cells infected with four baculoviruses encoding the subunits ΔC67-Hp180, p68, p58 and p48 before (B) and after (A) incubation with phosphocellulose showing that most p68 remains unbound. The scan shown here is at a high level of sensitivity showing the total absence of p68 from the purified $(\Delta C67\text{-}Hp180)H_2$ complex. Due to the high sensitivity the detection of p48 and p58 is out of linear range and both polypeptides appear more abundant in $(\Delta C67\text{-}Hp180)H_2$ than in Fig. 1B although the proteins presented here are from the same purification.

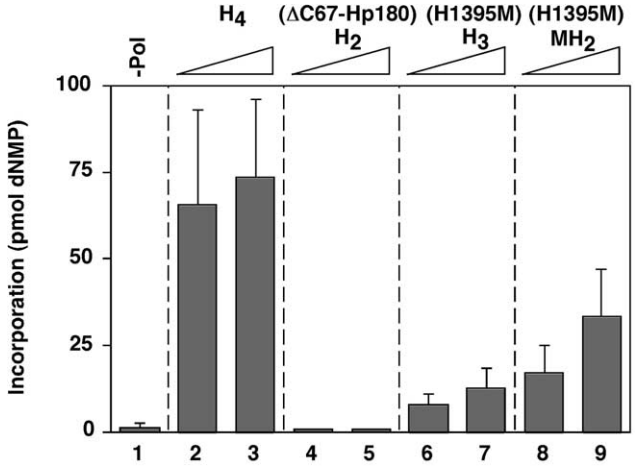

Fig. 3. In vitro SV40 DNA replication assay with 0.5 and 1.0 DNA polymerase units of the indicated DNA polymerase α–primase complexes. The various enzyme complexes were incubated at 37°C and after 1 h samples were taken and the incorporation of acid-insoluble dNMPs was measured (H_4 denotes wild-type DNA polymerase α–primase; H_2 stands for human p58 and p48; H_3 stands for human p68, p58 and p48; MH_2 denotes murine p68 and human p58 and p48). Enzyme activities were determined beforehand with a DNA polymerase assay on activated calf thymus DNA. The DNA replication experiments were repeated four times. The mean of the obtained incorporation values is shown and standard deviations are indicated as error bars. Lane 1: control reaction lacking DNA polymerase α–primase.

3.2. SV40 DNA replication with the C-terminal truncation mutant of DNA polymerase α

In the SV40 DNA replication assay the complex (ΔC67-Hp180)H_2 showed no activity (Fig. 3). We previously reported the construction of a chimeric DNA polymerase α polypeptide, H1395M, containing a murine protein sequence C-terminal of amino acid residue 1395 [23]. This effectively replaces the sequences absent from ΔC67-Hp180 with the murine DNA polymerase α C-terminus. H1395M forms a complex with the three smaller human subunits and has both specific DNA polymerase and primase activities in the range of those of the wild-type. In the SV40 DNA replication assay this complex has approximately 16% of the activity of the wild-type human DNA polymerase α–primase complex (Fig. 3). Substituting murine p68 for human p68 in the complex with H1395M enhances activity approximately two-fold, which supports the finding that interactions with p68 require a region C-terminal of amino acid residue 1395 of DNA polymerase α (Fig. 3). The time-dependent incorporation of the chimeric human–mouse enzyme complexes was similar to that of the wild-type complexes but significant dNMP incorporation was only detectable after 1 h of incubation since the chimeric enzymes were several fold less active than the wild-type one. However, longer incubation times yielded an incorporation of acid-insoluble dNMPs by the wild-type enzyme that was no longer linear (data not shown).

4. Discussion

Mizuno et al. [21] showed that a core region from amino acid residues 330 to 1279 of murine DNA polymerase α is minimally required to carry out DNA synthesis. They also reported that a C-terminal region of murine DNA polymerase α, from amino acid residue 1235, binds the p68 subunit and that two highly conserved zinc finger motifs within this region are essential for this interaction [21]. Our data support the fact that the C-terminus of DNA polymerase α is essential for the interaction with p68 (Figs. 1 and 2). Furthermore, our findings indicate that the zinc finger motifs are insufficient for binding the p68 subunit as these motifs are present in our truncation mutant ΔC67-Hp180 which nevertheless fails to bind p68 (Figs. 1 and 2), implying that there is an additional p68 binding or regulatory site beyond amino acid residue 1395. Fig. 3 shows an SV40 DNA replication assay with a chimeric DNA polymerase α mutant in which the C-terminus beyond amino acid residue 1395 is of murine origin [23]. This mutant binds the three smaller human DNA polymerase α–primase subunits, p68, p58 and p48, to the same degree as does the wild-type [23]. This mutant complex shows limited activity in a DNA replication assay which can be increased by substituting murine p68 for its human counterpart (Fig. 3; [23]). This augmentation of activity is not seen when the same substitution is made in a complex containing wild-type human DNA polymerase α [23,32]. Therefore, we conclude that the protein sequence beyond amino acid residue 1395 contains one or more elements which control the structure of DNA polymerase α–primase, the initiation complex or both, very likely, but not necessarily exclusively, through an interaction with the p68 subunit.

ΔC67-Hp180 shows a diminished association of the primase subunits, p48 and p58 (Fig. 1). This is again consistent with previous findings that the DNA polymerase α C-terminus binds to these subunits [21]. Our results indicate that the sequences beyond amino acid residue 1395 are required for an efficient interaction with primase but that other sequences are necessarily involved as our mutant clearly binds primase to some degree (Fig. 2). An alternative explanation of our results is that primase binds to DNA polymerase α in a p68-dependent fashion. This is, however, inconsistent with the finding that mutation of the C-terminal zinc finger motifs in DNA polymerase α abolishes binding of p68 but not of the primase subunits [21] and with the successful purification of a complex consisting of DNA polymerase α and primase in the absence of p68 [26,34]. Such a heterotrimeric complex proved devoid of activity in an SV40 DNA replication assay [34], consistent with the inactivity of (ΔC67-Hp180)H_2 (Fig. 3) and with the essential nature of p68 in vivo [35].

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