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Sequence analysis

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Herpesviral helicase-primase subunit UL8 is inactivated B-family polymerase

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ABSTRACT

Motivation: Herpesviruses are large DNA viruses causing a variety of diseases in humans and animals. To develop effective treatment, it is important to understand the mechanisms of their replication. One of the components of the herpesviral DNA replication system is a helicase–primase complex, consisting of UL5 (helicase), UL52 (primase) and UL8. UL8 is an essential herpesviral protein involved in multiple protein–protein interactions. Intriguingly, so far no UL8 homologs outside of herpesviruses could be identified. Moreover, nothing is known about its structure or domain organization.

Results: Here, combining sensitive homology detection methods and homology modeling, we found that the UL8 protein family is related to B-family polymerases. In the course of evolution, UL8 has lost the active site and has undergone a reduction of DNA-binding motifs. The loss of active site residues explains the failure to detect any catalytic activity of UL8. A structural model of human herpes virus 1 UL8 constructed as part of the study is consistent with the mutation data targeting its interaction with primase UL52. It also provides a platform for studying multiple interactions that UL8 is involved in. The two other components of helicase–primase complex show evolutionary links with a newly characterized human primase that also has DNA polymerase activity (PrimPol) and the Pif1 helicase, respectively. The role of these enzymes in recovering stalled replication forks suggests mechanistic and functional similarities with herpesviral proteins.

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1 INTRODUCTION

Herpesviruses are a large group of double-stranded (ds) DNA viruses responsible for a variety of human and animal diseases. Therefore, understanding herpesviral DNA replication is of great importance for the development of effective drugs. In addition, many herpesviral DNA replication proteins have functional counterparts in eukaryotes, thereby providing important insights into eukaryotic DNA replication mechanisms.

Human herpesvirus 1 (HHV-1) has seven proteins essential for DNA replication. These include origin-binding protein UL9, DNA polymerase UL30 and its processivity factor UL42, single-stranded DNA binding (SSB) protein ICP8 (UL29) and the tripartite helicase–primase complex consisting of UL5, UL52 and UL8. UL5 is a superfamily I DNA helicase (Weller and Coen, 2012) related to yeast Pif1 and bacterial RecD (Hodgman, 1988). UL52 is a primase belonging to the superfamily of archaeo-eukaryotic primases (Iyer *et al.*, 2005). UL8 does not have any known catalytic activity (Dodson and Lehman, 1991). It appears to be important for the nuclear import of UL5 and UL52 and is known to interact not only with the UL5–UL52 subcomplex, but also with UL30, ICP8 and UL9 (Muylaert *et al.*, 2011). Surprisingly, despite the essential role of UL8 in DNA replication, so far nothing is known about its structure and domain organization. Moreover, there are no known homologs of UL8 outside the herpesviruses (Muylaert *et al.*, 2011).

Using a combination of sensitive homology detection methods and protein structure modeling, we explored the UL8 family and found that the C-terminal half of UL8 is related to B-family polymerases. This finding came as a surprise, because UL8 is devoid of catalytic activity. Our subsequent sequence and structure analysis revealed that UL8 has lost motifs important for the polymerase catalytic activity and has undergone a reduction of DNA-binding motifs. To see whether UL5–UL52–UL8 helicase–primase is evolutionary conserved in herpesviruses, we further explored each of the three components. As a result, we identified 'missing' UL5 helicases in the family *Malacoherpesviridae,* thereby providing further support for the evolutionary conservation of the helicase–primase complex. Interestingly, interactions related to those within the helicase–primase complex have also been observed in eukaryotes.

2 METHODS

2.1 Databases

Non-redundant ('nr') protein sequence database was obtained from NCBI (ftp://ftp.ncbi.nlm.nih.gov/blast/db/). Viral protein sequences were also downloaded from NCBI using the following query: 'http://www.ncbi.nlm.nih.gov/protein/?term=dsDNA+viruses,+no+RNA+stage'.

2.2 Homology searches

Routine sequence similarity searches were performed using PSI-BLAST (Altschul *et al.*, 1997) and Jackhmmer (Eddy, 2011). Searches were run against the nr70 sequence database (the NCBI 'nr' database filtered to 70% identity) till convergence using *E*-value = 1e-03 or a more stringent inclusion threshold. We performed high sensitivity searches with HHpred (Söding *et al.*, 2005). Profiles were built with PSI-BLAST (three iterations, the 1e-03 inclusion threshold). Resulting profiles were then used as queries for searching against profile databases derived from known

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protein structures in PDB (www.pdb.org) or SCOP (Murzin *et al.*, 1995) and from protein families in Pfam (Punta *et al.*, 2012).

2.3 Homology modeling

Initial structural templates and corresponding sequence-structure alignments were obtained from a number of distant homology detection and structure modeling servers: I-TASSER (Roy *et al.*, 2010), RaptorX (Källberg *et al.*, 2012), pGenTHREADER (Lobley *et al.*, 2009), FFAS-3D (Xu *et al.*, 2014), HHPred (Söding *et al.*, 2005) and COMA (Margelevičius and Venclovas, 2010). Consensus approach was then used to select modeling template(s). Sequence-structure alignments were iteratively improved by monitoring the quality of resulting models with ProSA-web (Wiederstein and Sippl, 2007). Models were constructed with Modeller 9v10 (Sali and Blundell, 1993).

2.4 Sequence alignments

Structure-based sequence alignments were derived using DaliLite (Holm and Park, 2000). Multiple sequence alignments were generated with MAFFT (Katoh and Standley, 2013).

2.5 Sequence clustering

UL5, UL52 and their homologs were clustered using CLANS (Frickey and Lupas, 2004). Sequences used for clustering were identified using Jackhmmer/PSI-BLAST searches against nr70 and viral protein databases and supplemented with experimentally characterized cellular homologs. Non-conserved N- and C-termini were removed from sequences before clustering. Clustering with CLANS was performed using the PSI-BLAST option (two iterations against nr70 database with inclusion threshold of 1e-03 and 1e-45 for UL52 and UL5, respectively).

3 RESULTS

3.1 UL8 is inactivated B-family polymerase

Systematic iterative sequence searches using Jackhmmer and UL8 proteins as queries provided an initial hint that the UL8 family, instead of being novel, might be related to B-family polymerases. For example, the search with bovine herpes virus 2 UL8 (gi: 14161473) after four iterations produced a statistically significant match (*E*-value = 0.001) with the B-family polymerase (gi: 150401083) from *Methanococcus aeolicus*. The results of more sensitive profile–profile searches have further substantiated the initial finding. Thus, HHpred readily identified (95% probability) the relationship between HHV-1 UL8 and the B-family DNA polymerase from the archaeon *Thermococcus gorgonarius* (SCOP: d1tgoa2). This newly discovered relationship was also consistently supported by other sensitive homology detection and modeling servers (see Section 2). The identified similarity between UL8 and B-family polymerases is limited to the C-terminal half of UL8 sequences (~393-727 a.a. of HHV-1) (Fig. 1). The relationship is remote as the aligned region of UL8 proteins and B-family representatives shares only 9–12% sequence identity. Our attempts to identify any characterized homolog of the UL8 N-terminal region were unsuccessful. This does not necessarily mean that the region harbors novel structural domain(s). B-family polymerases at their N-terminus typically have the exonuclease domain (Rothwell and Waksman, 2005). Thus, it is conceivable that the UL8 N-terminal region represents an exonuclease-like domain diverged beyond recognition.

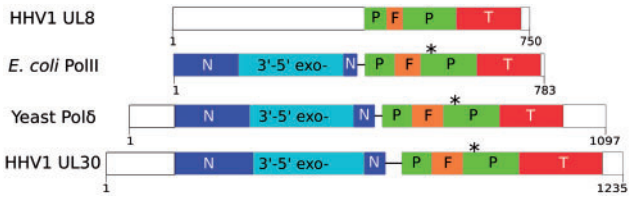

Fig. 1. Domain organization of HHV-1 UL8 and B-family DNA polymerases. N, N-terminal; P, palm; F, fingers; T, thumb. Asterisk indicates the polymerase active site ('DTD' motif)

To further corroborate the identified homology and to obtain insight into the structure of UL8 C-terminal region (UL8c), we constructed homology models for UL8c of HHV-1 (available at http://www.ibt.lt/bioinformatics/models/hhv1_ul8/) and its relatives from the Simplexvirus genus (Supplementary Table S1). Models were constructed using the structure of *Escherichia coli* PolII (PDB: 3k57) (Wang and Yang, 2009) as a template from the optimized sequence-structure alignments. To have reference points, we constructed computational models of several B-family polymerases using the same *E.coli* PolII structure as the modeling template. We chose those B-family polymerases, for which experimentally determined structures were available, and therefore, we could obtain the 'ideal' (structure-based) alignment between them and PolII. We only selected B-family polymerases that were <30% identical to *E.coli* PolII, so as to make the situation more similar to that between UL8 and PolII. Thus, we chose PolB of phage RB69, yeast Pol δ, *Pyrococcus furiosus* PolB and HHV-1 DNA polymerase UL30 and generated models for them using structure-based alignments. Evaluation of models was performed with Prosa. More negative Prosa *Z*-score values imply more energetically favorable structure. Not surprisingly, Prosa *Z*-score values for UL8c models were worse than for the crystal structure of PolII used as a template (−7.48 for HHV-1 UL8c and −8.86 for PolII). Nonetheless, some of UL8c models scored relatively high (Supplementary Table S1). Moreover, UL8c models of HHV-1 and some other herpesviruses scored better than all of the reference models, except for *P.furiosus* PolB. These results strongly suggest that UL8c and B-family polymerases are indeed structurally similar and imply that UL8c models are unlikely to contain serious flaws.

To understand the differences between UL8c and B-family polymerases, we performed a detailed analysis of sequence and structure motifs. A prominent feature of UL8c is the lack of the intact active site motif 'DTD' (Fig. 1 and Supplementary Fig. S1). Only the second aspartate from this motif is conserved in a number of α-herpesviruses. The fingers subdomain, which is important for recognition and binding of the incoming nucleotide (Rothwell and Waksman, 2005), is reduced in UL8c. Furthermore, sequence region preceding fingers subdomain has a deletion in UL8c. In addition, UL8c lacks the 'KKRY' motif, known to play an important role in stabilizing B-form of the DNA (Franklin *et al.*, 2001). Taken together, these features indicate that UL8 is not an active DNA polymerase, consistent with the failure to detect any enzymatic activity in UL8 using experimental approaches (Dodson and Lehman, 1991). Consistent with modifications of UL8c DNA-binding motifs,

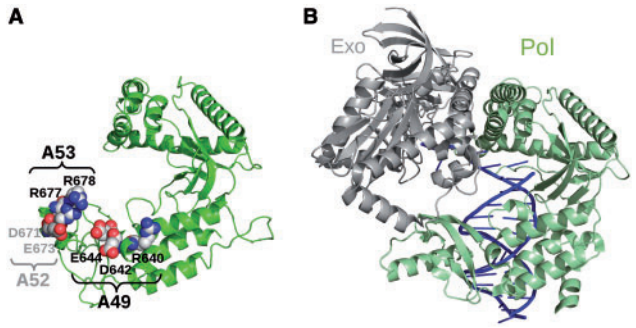

Fig. 2. A model of HHV-1 UL8c compared with *E.coli* PolII. (**A**) UL8c and its mutants that affect binding to UL52. Mutated residues are shown in space-filling representation (carbon atoms, gray/white; nitrogen, blue; oxygen, red) and are labeled. (**B**) Crystal structure of *E.coli* PolII complexed with DNA. Polymerase (Pol) and exonuclease (Exo) domains are colored in green and gray, respectively

UL8 does not exhibit ssDNA or dsDNA binding on its own (Parry *et al*., 1993). However, UL8 appears to modulate DNA binding by UL5-UL52, the other two subunits of helicase–primase complex (Chen *et al*., 2011).

Thus, sequence and structure analysis of UL8 indicates that it lacks motifs necessary for polymerase activity and has undergone a reduction of DNA-binding motifs.

3.2 Protein–protein binding sites in UL8

UL8c structural models enabled us to look at known and putative binding sites mediating interactions with other proteins. Recently, systematic mutagenesis of charged residues into alanines has been carried out for HHV-1 UL8 and four replication-defective mutants were identified (Muylaert *et al*., 2012). Three of the mutants can be mapped onto the model of HHV-1 (Fig. 2 and Supplementary Fig. S1). Mutants A49 (R640A, D642A and E644A) and A53 (R677A and R678A) displayed a defective interaction with UL52. The interaction between mutant A52 (D671A and E673A) and UL52 was only slightly impaired. In the HHV-1 UL8c model, these positions are close to positions substituted in mutant A53. Our model is in good agreement with these experimental findings, as all the positions affecting interaction with UL52 are on the same side of the model surface.

In addition, a putative protein-binding site is located within the very C-terminus of UL8, extending beyond the modeled structure (Supplementary Fig. S1). The C-terminal region features a short conserved hydrophobic motif (HHV-1 UL8 747-FLF-749) and is apparently disordered, as predicted by PrDOS (Ishida and Kinoshita, 2007) at 5% false positive rate and MetaDisorder3D (Kozlowski and Bujnicki, 2012). A conserved hydrophobic motif within the disordered region is often a signature of protein-binding site. It has been established that HHV-1 UL8 interacts with DNA polymerase, and indirectly the interaction site was mapped within the C-terminal region of UL8 (Marsden *et al*., 1997). These results implicated UL8 residues, important for binding, just upstream of the conserved C-terminal motif. This suggests that the conserved hydrophobic motif is, perhaps, a secondary polymerase-binding site. Alternatively, it may mediate interaction with one of the other multiple binding partners of UL8.

3.3 UL52 and UL5 are related to eukaryotic PrimPol family and Pif1 helicases, respectively

Since UL8 functions as part of the helicase–primase complex, we decided also to look into homologous relationships of the other two components of the complex, UL52 and UL5. HHV-1 UL52 (gi: 9629434) is a 1058-residue long protein with the C-terminal half related to archaeo-eukaryotic primases (Iyer *et al*., 2005). UL52 homologs, identified by sequence searches, fall into several clusters (Supplementary Fig. S2). These include HHV-1 UL52 and other *Herpesviridae* sequences, UL52 homologs from mollusc and cold-blooded animal herpesviruses (*Malacoherpesviridae* and *Alloherpesviridae* families, respectively), A468R-like proteins from *Phycodnaviridae* family and a cluster of viral and eukaryotic homologs. The latter are exemplified by the recently characterized human PrimPol (CCDC111), which has both primase and polymerase activities and plays an important role in replication fork progression through sites of DNA damage (García-Gómez *et al*., 2013; Mourón *et al*., 2013). Human PrimPol is the first example of an eukaryotic protein harboring both primase and polymerase activities. Interestingly, in kinetoplastids this family of proteins has undergone lineage-specific expansion. For example, *Trypanosoma brucei* has four copies of PrimPol homologs. Two of the more distantly related ones to human PrimPol (TbPRI1 and TbPRI2) were shown to be active primases and to have roles essential for cell growth and kinetoplast DNA replication (Bezalel-Buch *et al*., 2013). The other two PrimPol-like proteins act as DNA polymerases with TbPPL1 also having a DNA primase activity (Rudd *et al*., 2013) The PrimPol group includes viral proteins from *Marseilleviridae*, *Mimiviridae*, *Asfarviridae* and *Ostreid herpesvirus 1*. Surprisingly, the latter virus representing *Malacoherpesviridae* has three copies of primases. Two of them (OsHV1_ORF7 and OsHV1_ORF49) clustered with the PrimPol group, whereas the third one (OsHV1_ORF66) was more similar to herpesviral UL52 sequences.

UL5 is a member of the SF1 helicase superfamily having 5′-3′directionality. Using iterative sequence searches with HHV-1 UL5 (gi: 9629385), we readily detected similarity of UL5 to eukaryotic and viral Pif1 helicases as well as to more distantly related homologs of bacterial RecD and T4 Dda helicases. We also identified previously unannotated UL5 homologs in *Malacoherpesviridae* (Supplementary Fig. S3). Clustering of sequence search results revealed that herpesviral UL5 are split into three groups mirroring the UL52 results. Eukaryotic Pif1 homologs belong to a group displaying the closest similarity to UL5. Pif1 is found in nearly all eukaryotes. Most eukaryotes including humans have a single Pif1 family helicase, but *Saccharomyces cerevisiae* has two (ScPif1 and ScRrm3). Interestingly, *T.brucei* has as many as eight Pif1 paralogs, mirroring the expansion of its PrimPol-like proteins. Yeast proteins represent some of the best characterized members of the Pif1 family. ScPif1 affects telomeric, ribosomal and mitochondrial DNA replication, as well as Okazaki fragment maturation (Bochman *et al*., 2010). Recently, ScPif1 was also found participating together with Polδ in recombination-coupled DNA

synthesis (Wilson *et al.*, 2013). ScRrm3 moves with the replication fork during the DNA replication and helps to pass difficult-to-replicate sites (Azvolinsky *et al.*, 2006). ScRrm3 was found to interact with Polε, suggesting that it is a stable component of the replisome (Azvolinsky *et al.*, 2006).

4 DISCUSSION

Our finding that UL8 is a homolog of B-family polymerases and that it has lost the active site explains why no catalytic activity of UL8 could be found. What could be the evolutionary origin of UL8? One possibility is that UL8 originated from the duplication of herpesviral DNA polymerase, UL30. Alternatively, UL8 could be derived from some ancestral form of a B-family polymerase. However, the sequence similarity with B-family polymerases is low, precluding a straightforward answer to this question. The analysis of homologs of UL52 and UL5, the other two subunits of helicase–primase complex, showed that their links to eukaryotic enzymes are even clearer. Thus, all three components of helicase–primase complex have mechanistic and perhaps functional similarities with the corresponding eukaryotic proteins. For example, UL8, an inactive polymerase, may be compared with the C-terminal region of Polε, which corresponds to an inactivated exonuclease-polymerase module serving as a protein-binding platform (Tahirov *et al.*, 2009). UL5 has recently been found to interact with the polymerase UL30 (Weller and Coen, 2012), whereas yeast Pif1 interacts with Polδ (Wilson *et al.*, 2013). UL52–UL5 forms subassembly as part of helicase–primase complex. An interesting question is whether their eukaryotic homologs interact or at least cooperate in certain conditions. Although the direct evidence is lacking, there are some hints that they might. For example, PrimPol and Pif1 both help to bypass difficult-to-replicate sites (Azvolinsky *et al.*, 2006; García-Gómez *et al.*, 2013). The observation of the correlated expansion of the PrimPol-like and Pif1 protein families in trypanosomes is another hint that they might be linked functionally if not physically. At least two pairs of trypanosomal PrimPol and Pif1 homologs participate in the same processes. Thus, TbPRI1 and TbPIF2 function in replication of DNA maxicircles while TbPRI2 and TbPIF1 are involved in replication and segregation of minicircles (Bezalel-Buch *et al.*, 2013). Obviously, differences between the herpesviral and the eukaryotic counterparts are to be expected. However, the consideration of similarities may provide help in advancing the knowledge in both systems.

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