Mutational analysis of the Latency-associated nuclear antigen DNA binding domain of Kaposi’s sarcoma-associated Herpesvirus reveals structural conservation among γ-Herpesvirus origin binding proteins

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The latency-associated nuclear antigen (LANA) of Kaposi’s sarcoma-associated herpesvirus functions as origin-binding protein (OBP) and transcriptional regulator. LANA binds the terminal repeats via the C-terminal DNA binding domain (DBD) to support latent DNA replication. To date, the structure of LANA has not been solved. Sequence alignments among OBPs of γ-herpesviruses revealed that the C-terminus of LANA is structurally related to EBNA1, the OBP of Epstein-Barr virus. Based on secondary structure predictions for LANA_{DBD} and published structures of EBNA1_{DBD}, we used bioinformatics tools to model a putative structure for LANA_{DBD} bound to DNA. To validate the predicted model, 38 mutants targeting the most conserved motifs, namely three α-helices and a conserved proline loop, were constructed and functionally tested. In congruence with EBNA1, residues in both helices 1 and 2 mainly contribute to sequence-specific DNA binding and replication activity, while mutations in helix 3 affected replication activity and multimer formation. Additionally, we isolated several mutants with discordant phenotypes, which may aid further studies into LANA function. In summary, these data suggest that the secondary and tertiary structures of LANA and EBNA1 DBDs are conserved and are critical for (i) sequence-specific DNA binding, (ii) multimer formation, (iii) LANA-dependent transcriptional repression, and (iv) DNA replication.
**INTRODUCTION**

Kaposi’s sarcoma-associated herpesvirus (KSHV/human herpesvirus 8) is a DNA tumor virus associated with KS, primary effusion lymphomas (PEL), and a plasmablastic variety of multicentric Castleman’s disease (MCD) (Cesarman et al., 1995, Chang et al., 1994, Soulier et al., 1995). LANA, encoded by ORF73, interacts with multiple cellular proteins to affect various signal transduction pathways (Gao et al., 1996, Kedes et al., 1996). LANA also functions as origin-binding protein (OBP) by binding to the viral latent origin, which recruits the host cellular replication machinery to ensure replication of viral episomes during S-phase. Additionally, LANA tethers viral genomes to mitotic chromosomes via its N-terminal chromosome binding motif, thereby contributing to episomal maintenance (Ballestas et al., 1999, Ballestas et al, 2001, Barbera et al., 2006, Cotter et al., 1999, Garber et al, 2002, Hu et al., 2002, You et al., 2006).

The C-terminal LANA DNA binding domain (LANA_{DBD} aa 775 to 1003) (Garber et al., 2001) binds cooperatively to LANA binding sites 1 and 2 (LBS1/2) within viral terminal repeats for replication of TR-containing plasmids (Garber et al., 2001, Garber et al., 2002, Hu et al., 2002). LANA predominantly forms dimers and the dimerization domain has been mapped to the LANA_{DBD} (Schwam et al., 2000), which also has partial replication activity (Hu et al., 2002). LANA and EBNA1 are functional homologues with respect to DNA binding and supporting DNA replication by recruitment of cellular origin recognition complex (ORC) proteins. Both proteins form dimers in solution and bind to two sites within their respective origins of replication in a cooperative manner (Lim et al., 2002, Stedman et al., 2004, Schepers et al., 2001, Verma et al., 2006).

Neither the structure of full-length LANA nor its DBD has been determined to date. In contrast, crystal structures of EBNA1 in the presence and absence of DNA (Bochkarev et al., 1996, Bochkarev et al., 1995) and E2, the OBP of Human Papillomavirus (Hegde et al., 1992), have been solved. Although EBNA1 and E2 share very limited primary sequence homology and
are encoded by different classes of DNA tumor viruses, their DBDs revealed a common core domain structure. The core domain consists of a series of interspersed $\beta$-sheets, which form a $\beta$-barrel within the dimer interface, a proline loop, which interacts with cellular proteins, and three $\alpha$-helices, which make direct or indirect contacts to DNA and stabilize higher order multimers (Bochkarev et al., 1996, Bochkarev et al., 1995, Ceccarelli et al., 2000). To get insights into the possible structure of the LANA$_{DBD}$ in the absence of a crystal structure, we performed detailed sequence alignments among LANA$_{DBDs}$ of different rhadinoviruses and performed bioinformatics-based modeling to predict a potential structure. We investigated our model by mutational analysis, and functional testing of mutants targeting residues most conserved between different LANA$_{DBDs}$ and EBNA1$_{DBD}$.

RESULTS

High evolutionary conservation of LANA$_{DBDs}$ in $\gamma$-herpesviruses and bioinformatics-based predicted structure of KSHV LANA$_{DBD}$.

Grundhoff et al. first noted a limited secondary structure homology between the C-termini of LANA and EBNA1 (Grundhoff et al., 2003). Furthermore, sequencing LANA from RFHVMn and MneRV2 revealed that the C-terminal amino acids (aa) of LANAs showed the strongest sequence conservation (Burnside et al., 2006). To further analyze these homologies, we performed aa alignments among LANA$_{DBDs}$ of KSHV, RFHVMn, RRV and EBV EBNA1$_{DBD}$ using bioinformatics programs, the PRALINE, 3D-PSSM and T-COFFEE (Fig. 1 and Table 2). This analysis revealed that KSHV LANA$_{DBD}$ has more than 50% similarity to the DBDs of RFHVMn and RRV. Although EBNA1$_{DBD}$ has less than 16% overall aa sequence identity to LANA$_{DBDs}$ (Table 2), there is significant structural similarity such as the presence of three $\alpha$-helices as previously noted (Grundhoff et al., 2003). In addition, we found a conserved proline-rich loop motif between KSHV LANA$_{DBD}$ ($^{938}$PHPGPDQSP$^{938}$) and EBV EBNA1$_{DBD}$.
(543PGPGPQPGP553) (Fig. 1B) which is important for protein-protein interaction of EBNA1 (Bochkarev et al, 1996). We also note that between KSHV, RFHVMn, and RRV residues within the α-helices are more highly conserved than surrounding residues (Fig. 1A). Based on these observations, we performed bioinformatics modeling to predict KSHV LANA_{DBD} structure, a common approach for related proteins for which crystals cannot easily be obtained (Hantz et al., 2009, Hass et al., 2008, Purta et al., 2005).

KSHV LANA_{DBD} residues 868-960 were modeled with the 3D-Jigsaw modeling tool (Bates et al., 2001) using the EBNA1_{DBD} structure (PDB accession no. 1B3T) as template (Bochkarev et al., 1996). The LANA_{DBD} residues 929-939 did not have defined coordinates after 3D-Jigsaw and were modeled using Modloop (Sali et al., 2003). Despite the relatively low residues homology, the structure for a LANA_{DBD} monomer was very similar to chain A of EBNA1_{DBD}. The Root mean square deviation (RMSD) is 0.85 Å between the EBNA1_{DBD} structure and the predicted LANA_{DBD} model which suggest close similarity.

To predict multimer structures of LANA_{DBD}, M-ZDOCK (Pierce et al., 2005) was run using the LANA_{DBD} homology model to perform a full search of possible homodimeric interfaces. The output models from M-ZDOCK were then filtered based on similarity to the EBNA1_{DBD} dimer interfaces, ability to fit double-stranded DNA and score of the model from ZRANK (Pierce et al., 2007). We next selected two M-ZDOCK models for LANA_{DBD} dimer by these criteria, which were joined to construct a tetramer (Fig. 2A). The RMSD for the LANA_{DBD} dimer versus the two EBNA1_{DBD} chains is 2 Å.

We had previously shown that LANA binds to LBS1/2 within the terminal repeats that are spaced by 21 to 22 nts (Garber et al., 2002) and Wong et al. demonstrated that LANA occupying both sites induces a bend of about 110 degrees (Wong et al., 2005). While the sequence composition between EBNA1 binding sites (AT-rich) and LANA binding sites (GC-rich) are very different, both the spacing and the induced DNA bending are conserved features. Accordingly,
the DNA conformation was initially taken from the structure of EBNA1\textsubscript{DBD} bound DNA (Bochkarev et al., 1996) and fit to the two dimers in the LANA\textsubscript{DBD} tetramer. The linking DNA between the two dimers binding sites was extended from the existing DNA strands. Rosetta program (Havranek et al., 2004) was then used to restore the DNA sequence to the LBS1/2 sequences and repack the LANA side chains accordingly.

The resulting model for the LANA\textsubscript{DBD} tetramer bound to DNA (Fig. 2A) shares the defining $\beta$-barrel core domain structure with both EBNA1\textsubscript{DBD} and E2\textsubscript{DBD}. The tetramer of LANA\textsubscript{DBD} is composed of eight antiparallel $\beta$-strands within their core domains, and flanking domains including helices 1 (Fig. 2A in red) (Bochkarev et al., 1995), which are positioned at the outside of each monomer towards the dimer interfaces. The $\beta$-barrel formation is composed of four $\beta$-strands apart from each monomer, and the $\beta$-strands are crossover connected by the two $\alpha$-helices (within each core domain) (Fig. 2A in blue and green) on the outside of each barrel. Hence, our model incorporated all known data on LANA\textsubscript{DBD}-DNA interaction (Garber et al., 2002, Wong et al., 2005), which suggests similar secondary and quaternary structures for LANA\textsubscript{DBD} and EBNA1\textsubscript{DBD}.

**Mutagenesis of KSHV LANA\textsubscript{DBD} and expression of mutant proteins.** To test the LANA\textsubscript{DBD} model, we performed a detailed mutational analysis by targeting conserved residues in the three $\alpha$-helices and the proline loop. A total of 38 single, double, or triple alanine substitutions were generated by site-directed mutagenesis. Wt and mutant proteins were expressed using the MVA/T7 vaccinia virus expression system in CV-1 cells as previously described (Garber et al., 2001, Garber et al., 2002). Briefly, constructs containing T7 promoter were transfected into MVA/T7 infected cells, the cells were harvested 36 hrs post-transfection and proteins were enriched by affinity purification. Protein expression levels for all mutant proteins were monitored by Western blot (supplementary Fig. S1).
Evaluation of wt and mutant KSHV LANA DBDs for DNA binding by electrophoretic mobility shift assay (EMSA). We previously reported that LANA DBD binds to its high affinity binding site (LBS1) with a $K_d$ of $1.51 \pm 0.16$ nM (Garber et al., 2002). To determine the effect of mutations on DNA binding, equal amounts of wt and mutant LANA DBD proteins were incubated with radio labeled probes containing either LBS1 or LBS1/2 (Fig. 3, A-D). After electrophoresis gels were dried, signals were quantified by phosphoimaging. Shown are representative autoradiographs from three independent experiments.

Most mutants in helix 1 significantly reduced binding affinities to both LBS1 and LBS1/2 (Fig. 3, A-D). Especially, $\epsilon 874 \alpha$ and $\eta 876 \alpha$ reduced DNA binding affinity to less than 20% of wt (Fig. 3A, lanes 5 and 8). Helix 1 ($871 K$ to $882 F$) contains polar residues $871 K$, $873 R$ and $879 Y$ which are highly conserved residues between KSHV, RFHVMn and RRV (Fig. 1A). These residues potentially contact DNA either directly or indirectly by stabilizing the secondary structure of the N-terminal domain of LANA DBD. From the structure of EBNA1 DBD, polar residue $477 K$ within helix 1 and residues $46 K$ to $46 R$ within the N-terminus, have been shown to directly contact DNA (Bochkarev et al., 1996). In congruence with the binding data, the predicted structure (Fig. 2) suggests that the N-terminal residues of helix 1 ($871 K$, $873 R$, and $875 Q$) are located in close approximation to DNA (Fig. 2, B and C). For EBNA1 DBD, residues within helix 2, which was originally termed DNA recognition helix (Bochkarev et al., 1995), also contribute to DNA binding. The recognition helices of all HPV E2 proteins contain several highly conserved residues in a consensus motif $(338 LXXLRY 343)$ which is also conserved in EBNA1 DBD $(517 LYNLR 522)$ (Fujita et al., 2000). Within LANA DBD, $906 PYGLKK 911$ in helix 2 has similar surface charge as EBNA1 DBD helix 2 (Fig. 1). Moreover, in the model, $907 Y$ and $910 KK 911$ like $518 Y$ and $521 RR 522$ of EBNA1 DBD, are predicted to be in close contact to DNA (Fig. 2D). Indeed, all mutants in helix 2,
except T909A showed dramatically reduced DNA binding affinities to both LBS1 and LBS1/2 (Fig. 3, B and D).

For EBNA1DBD, it was shown that the proline loop (545PGPGPQP553) between helix 2 and the β-barrel bundle contributes to DNA binding as well as to protein-protein interaction with cellular transcription factors (Bochkarev et al., 1995, Bochkarev et al., 1996). P932A in the center of the proline loop (930PHPGPDQSP938) of LANA DBD did not reduce DNA binding affinity (Fig. 3B, lane 13) – however, P925A located at the inside of the β-barrel bundle reduced binding affinity by about 50% (Fig. 3B, lane 12).

Helix 3 (950K to 966S) follows the proline loop and continues towards the inside of the β-barrel through an extended strand. In contrast to mutants in helices 1 and 2, helix 3 mutants, except SKK953AAA, 1961A and WE963AA, did not show significant changes in DNA binding affinity (Fig. 3, C and D, and Table 1). SKK953AAA in helix 3 may change folding by interrupting hydrogen bonds with basic residues of helix 1. Thus, these helix 3 residues contribute to stabilize protein-DNA interactions and, in contrast to residues within helices 1 and 2, are not directly involved in DNA binding.

Evaluation of multimerization of KSHV LANA DBD by Co-immune precipitation assays.

Schwam et al. first demonstrated that LANA DBD in solution and in the absence of TR DNA predominantly exists as a homodimer (Schwam et al., 2000). To analyze dimerization of a subset of mutants with reduced DNA binding activities, we performed co-immunoprecipitation assays. Flag-tagged wt or mutant LANA DBDs were tested for their ability to interact with HA-tagged wt LANA DBD. LANA DBD complexes were immunoprecipitated by α-Flag M2 beads and separated on SDS-PAGE. The amount of wt LANA DBD was detected and quantified by Western blot using α-HA-tag antibody. Dimerization activity for each mutant is reported as the percentage of HA and Flag-tagged wt LANA DBD set to 100%.
HIF876AAA and YR879AA in helix 1, which showed drastically reduced DNA binding affinities, did dimerize comparable to wt (Fig. 4A, lanes 5 to 8). G875A and RF881AA only reduced dimerization (Fig. 4B, lanes 5 and 6, and table 1) further suggesting that most helix 1 residues directly contribute to DNA binding but not to dimer formation.

Similarly, except YGL907AAA, which showed a moderate decrease (73%) in dimerization (Fig. 4B, lanes 7 and 8), helix 2 mutants had largely unaltered or increased dimerization activities compared to wt (Fig. 4C, lanes 5 to 8). This result was expected since helix 2 of EBNA1\textsubscript{DBD} and presumably LANA\textsubscript{DBD} functions as a DNA recognition domain. In addition, P925A within β-barrel connected to the proline loop did not effect dimerization (table 1).

Within helix 3 several mutants had reduced dimerization (Fig. 4, D to F). Dimerization for WE963AA and SKK953AAA were reduced by 29% and 64%, respectively (Fig. 4F, lanes 7 and 8, and table 1). Within the EBNA1\textsubscript{DBD} (Bochkarev et al., 1996), the corresponding mutants in helix 3, showed both the loss of dimerization and DNA replication activities (Bochkarev et al., 1996).

Analysis of DNA replication activity of wt and mutant KSHV LANA\textsubscript{DBD}s. Mutagenesis of the LBS1/2 showed that the replication efficiency of TR-containing plasmids is dependent on the LBS1 (Garber et al., 2002). To test the inverse, we chose a subset of mutants with reduced DNA binding or dimerization and performed transient replication assays as previously described (Garber et al., 2002, Hu et al., 2002). Briefly, a plasmid containing four copies of TR was co-transfected with plasmid expressing wt or mutant LANA\textsubscript{DBD} into 293 cells. Replicating DNA was extracted and analyzed by Southern blot after DpnI digestion. As previously described, LANA\textsubscript{DBD} replicates with about 20% efficiency of full-length LANA (compare Fig. 5A, lanes 9 to 11, and B, lanes 7 to 11) (Hu et al., 2002).

All mutants with reduced binding affinity in helix 1 HIF876AAA and YR879AA, helix 2 YGL907AAA, KK910AA and LSQ912AAA and helix 3 SKK953AAA did not replicate to any detectable
levels (Fig. 5A, lanes 12 to 15, and B, lanes 10 and 11). Furthermore, WE963AA in helix 3, which strongly reduced dimerization, was also inactive in replication assays (Fig. 5B, lane 12). In contrast, S966A, which had no phenotype in either binding or dimerization, showed residual replication activity (Fig. 5A, lane 16). These data further confirm that LANA dimerization and high affinity binding to TR are required for replication. Interestingly, while the proline loop mutant P932A bound to DNA and dimerized like wt, it did not support replication (Table 1 and data not shown). For EBNA1\_DBD, it was shown that the proline loop contributes to spatial orientation of helices 1 and 2 and interacts with cellular proteins (Bochkarev et al., 1996, Bochkarev et al., 1995, Ceccarelli et al., 2000).

Transcriptional repressor activity of wt and mutant LANA\_DBDs. We and others have previously shown that TR sequences have enhancer activity which can be repressed by LANA. Furthermore, the LANA\_DBD alone is sufficient for repression (Garber et al., 2001). To test mutants for repressor activity, plasmids encoding wt or mutant LANA\_DBD were co-transfected with pAG/7TR reporter plasmid into 293 cells. Cell lysates were assayed for luciferase activity and normalized as previously described (Renne et al., 2001). The data for mutants in all three helices is shown as percentage repression activity over wt set to 100% (Fig. 6).

Within helix 1, 8 of 11 mutants had only moderately decreased repression activity between 80% and 65% compared to wt. Repression activity of HIF876AAA and F878A was decreased to 41% and to less than 1% of wt, which was concordant with strongly reduced binding activity (Fig. 6A and Table 1). Interestingly, R880A showed only 7% repression activity albeit its DNA binding activity was only reduced to 55% (Fig. 6A and Table 1).

Within helix 2, 4 of 5 tested mutants showed significantly decreased repression activity except for 907A, these were mostly concordant with either loss or strong reduction in DNA binding (Table 1). These data further confirm that residues in helix 2 significantly contribute to
DNA recognition and binding. Interestingly, \( \gamma_{907A} \) was strongly reduced in DNA binding nevertheless displayed 71% repressor activity (Fig. 6B).

In agreement with the DNA binding data, most mutants in helix 3, including the proline loop, had only modest or no effects on transcriptional repression (Fig. 6, B and C). However, \( \text{WE963AA} \) completely abolished repressor activity. Interestingly, while \( \text{WE963AA} \) had only modestly reduced DNA binding (Table 1) but strongly reduced homodimer formation suggests that these residues may interact with helix 1 to stabilize the homodimer or contribute to interactions with cellular proteins conveying transcriptional repression.

In summary, these data show that for most mutants DNA binding and transcriptional repression is similarly affected. However, we observed mutants most notably \( \text{R880A} \) and \( \text{WE963AA} \), which could bind to TR but did not repress transcription and inversely \( \gamma_{907A} \) which poorly binds to TR but still represses.

DISCUSSION

Many mechanistic details on the role of LANA in transcriptional regulation, latent DNA replication, tethering of viral episomes to host chromatin, and interaction with multiple host cellular proteins have been reported (reviewed in Lieberman et al., 2007 and Verma et al., 2007). In contrast, with the exception of a small 23 aa peptide in the N-terminal histone H2A binding domain (Barbera et al., 2006), no structural data on LANA is available. We have expressed LANA\text{DBD} protein using vaccinia virus, baculovirus, and \textit{E. coli} systems but have not yielded concentrations of soluble protein amenable for crystallization. A further complicating factor is that all published DNA binding assays were performed in the presence of BSA whose substitution will be crucial to solve LANA\text{DBD} structure in the presence of its cognate binding site (Ballestas et al., 2001, Cotter et al., 1999, Garber et al., 2002).
In the meantime, we performed bioinformatics modeling based on the observed sequence homology between the DBDs of KSHV, RRV, RFHVMn LANA and the DBD of EBNA1 to predict a structure for KSHV LANA\_DBD. We note that the x-ray structures of the EBNA1\_DBD and E2\_DBD core domains, which show no discernable sequence homology, nearly perfectly superimpose (Bochkarev et al., 1996, reviewed in Grossman et al., 1996, Hegde et al., 1992, Liang et al., 1996). In contrast, the DBDs of LANA and EBNA1 show 14% identity and 53% similarity (table 2) and the highest conservation within motifs that are crucial for the overall core domain structure (Fig. 1B) (Grundhoff et al., 2003). As a result, the predicted model (Fig. 2) indicates high degree of structural relatedness.

To functionally validate this model, we targeted the three $\alpha$-helices and the proline loop which showed highest conservation (Fig. 1B) and for which phenotypes had been described for EBNA1\_DBD. We identified residues in all three $\alpha$-helices which are either crucial for DNA binding (helices 1 and 2) or multimerization (helix 3). Both efficient DNA binding and dimerization are crucial for LANA’s ability to support replication of the TR-containing plasmids. The functional data for all mutants is summarized in Table 1. The key observations are that charged residues within a conserved motif in helix 2 (906PYGLKK911) are crucial for DNA binding (Fig. 2D). Helix 1 of LANA\_DBD also contributes to binding presumably through direct interactions with DNA (Fig. 3, A and D). These data are in congruence with EBNA1\_DBD where both helices 1 and 2 significantly contribute to DNA binding. Interestingly, within the crystal structure, helix 1 of EBNA1\_DBD was located much closer to DNA than helix 2. However, biochemical data by Cruickshank et al. clearly demonstrated that helix 2 is also critical for DNA binding (Cruickshank et al., 2000). To explain the difference between the crystal structure of EBNA1\_DBD bound to DNA and the biochemical data, it was suggested that EBNA1 binds to DNA via a two-step mechanism; sequence-specific binding is initiated by helix 2 followed by interactions of helix 1 residues. The observation that LANA residues from both helices
contributing to binding activity points to a conserved DNA binding mechanism for EBNA1 and the rhadinovirus LANA proteins, which was also suggested for the HPV E2 protein (reviewed in de Prat-Gay et al., 2008, Hegde et al., 1992, Liang et al., 1996).

Most mutations in helices 1 and 2 reduced transcriptional repressor activity as well as reduced DNA binding (Table 1). This data is consistent with the previous observation that high and low affinity of LBS1/2 determine DNA binding and replication (Garber et al., 2002). In contrast, most mutants in helix 3 had only moderate effects on transcriptional suppression; however, WE963AA displayed greatly reduced repression but only moderately reduced DNA binding (Fig. 3C and Table 1), indicating a role in protein-protein interaction that conveys LANA-dependent repression.

These data strongly suggest functional homology between all three α-helices and the proline loop of KSHV LANA_{DBD} and EBNA1_{DBD}. In addition, this analysis yielded at least one mutant in each helix and in the proline loop, which showed discordance in phenotype with regard to DNA binding, homodimer formation, transcriptional repression, or DNA replication. Within helix 1, R880A bound to TR but had nearly no repressor activity. Inversely, Y907A in helix 2 significantly reduced DNA binding but still repressed transcription and WE963AA in helix 3 which had only moderately reduced binding but completely lost repression activity. Finally, proline loop mutant P932A had no defect in either binding or dimerization, but did not support DNA replication. These mutants will be useful for further mechanistic studies on LANA function and some may function as dominant negative proteins, which for LANA have not been described to date.

Previously, two studies have performed mutational analysis of the LANA C-terminal domain. First, Wong et al. introduced a limited set of mutations and analyzed their effect on DNA binding and found that binding to DNA induced 57° or greater for LBS1 and about 110° for occupation on LBS1/2; furthermore mutations preventing bending also greatly affected DNA
binding of LANA (Wong et al., 2005). We observed similar results for mutants (SKK953AAA and
WL963AA) in the helix 3 confirming that changes in DNA bending do contribute to decreased DNA
binding and replication activity (Wong et al., 2005). Additionally, Kelley-Clarke et al. performed
an unbiased mutational analysis across LANA_{DBD} by introducing triple alanine substitutions to
define residues important for binding to TR and attachment to host chromatin (Kelley-Clarke et
al., 2007).

With respect to the importance of helix 2 for DNA recognition our data is in agreement
with both previous studies and adds more details by identifying several residues whose mutation
alone abrogates DNA binding. Especially, 909L, 910K, 911K and 917Q are partly overlapping with the
conserved LXXLRY motif present in the core domains of EBNA1 and many HPV E2 proteins
(Fujita et al., 2000).

With respect to helices 1 and 3 we identified several residues that contribute to DNA binding
but were not previously identified (Kelley-Clarke et al., 2007). Specifically, HIF876AAA, YR879AA
and all corresponding single aa substitutions showed drastically reduced DNA binding (Figs. 3, 4
and Table 1). In congruence with our observation, the corresponding EBNA1_{DBD} residues are also
important for DNA binding and bending either by directly contacting DNA or by stabilizing the
N-terminal domain of DBD (Bochkarev et al., 1996). No significant changes in DNA binding
were observed within helix 3 mutants. However, RL960AA, which was previously shown not to
bind to DNA (Kelley-Clarke et al., 2007), bound to LBS1 or LBS1/2 with wt activity (Fig. 4) and
also formed dimers. Observed differences between both studies may in part be due to differences
in protein expression and purification utilized.

In summary, our data suggest that the LANA_{DBD} has a high degree of structural
conservation with EBNA1_{DBD}, which is critical for sequence-specific DNA binding, multimer
formation, protein-protein interactions required for its DNA replication activity and LANA-
dependent transcriptional repression.
MATERIALS AND METHODS

Amino acid alignments of γ-herpesviruses LANA\textsubscript{DBD} in different primate species and EBNA1. The sequences of KSHV LANA\textsubscript{DBD} (aa 775 to 1003; Gene Bank Accession no. AAK50002), for reference BC-1 KSHV LANA (aa 934 to 1162; Gene Bank Accession no. AAC55944), EBV EBNA1\textsubscript{DBD} (aa 461 to 641; Gene Bank Accession no. NP_039875), RFHV\textsubscript{m} LANA\textsubscript{DBD} (aa 849 to 1071; Gene Bank Accession no. ABH07414) and RRV LANA\textsubscript{DBD} (aa 251 to 448; Gene Bank Accession no. AAF60071) were binary and multiply aligned using the 3D-PSSM (program version 2.6.0; Imperial College of Science, Technology and Medicine [http://www.sbg.bio.ic.ac.uk/servers/3dpssm/index.html], PRALINE program [http://www.ibi.vu.nl/programs] (reviewed in Pirovano et al., 2010) and T-COFFEE (program version 7.71; Comparative bioinformatics group, bioinformatics and genomics program center for genomic regulation) [http://www.tcoffee.org/] (Notredame et al., 2000).

Computational prediction of LANA\textsubscript{DBD} multimer structure. The M-ZDOCK program [http://zlab.bu.edu/m-zdock] was used to predict putative LANA\textsubscript{DBD} dimer and tetramer complexes. The M-ZDOCK, which is a specially developed algorithm for predicting the structure of multimer based on the structure of unbound (or partially bound) monomer (Pierce et al., 2005).

The predicted tetramer of LANA\textsubscript{DBD} bound to LBS1/2 was modeled based on solved structures of EBNA1\textsubscript{DBD} (Bochkarev et al., 1996).

Plasmid constructs. pcDNA 3.1 Flag-LANA\textsubscript{DBD} was previously described (Garber et al., 2001). Fragments containing LBS1 or LBS1/2 used as EMSA probes were produced by \textit{XhoI/XbaI} digestion from pAG31 containing LBS1 and pAG43 containing LBS1/2, respectively, as previously described (Garber et al., 2002).
pPuro/4TR, used for short-term replication assay, was constructed by cloning four TR units from pCRII/4TR (Garber et al., 2002, Hu et al., 2002) into pPur vector (BD bioscience). PGL3/7TR, which contains seven TR units, was constructed from pAG9 (Garber et al., 2001) and used as reporter for LANA-dependent transcriptional repression assays.

Alanine substitution mutagenesis. PCR-based Quickchange site-directed mutagenesis kit (Stratagene) was used to generate alanine substitution mutants in LANA_{DBD} as recommended by the manufacturer. Primers containing the desired alanine substitution were designed using web-based program, Primer X (Bioinformatics.org [http://bioinformatics.org/primers]) (supplementary table S1). All constructs were confirmed by sequencing (Davis Sequencing Co).

Cell lines. CV-1 cells, African green monkey fibroblasts, and 293 cells, human embryonic kidney cells were obtained from American Type Culture Collection. Cell monolayer was maintained in Dulbecco’s modified Eagle’s medium (Mediatech Inc) supplemented with 10% fetal bovine serum (FBS) (Mediatech Inc), 2 mM L-glutamine, penicillin-streptomycin (5 U ml^{-1} and 5 µg ml^{-1}, respectively) at 37 °C under a 5% CO₂ atmosphere.

Expression of wild-type and mutant LANA_{DBD} proteins with the MVA/T7 expression system. Wild-type and mutant LANA_{DBD} proteins were produced by using the modified vaccinia virus T7 (MVA/T7) expression system (Moss et al., 1990). Briefly, highly confluent CV-1 cells in 10 cm plate were infected with MVA/T7 virus as previously described (Garber et al., 2001, Moss et al., 1990) and transfected 3 hrs post-infection using a slightly modified calcium phosphate methods (reviewed in Sambrook et al., 2001). Cells were harvested 36 to 40 hrs post-transfection. His-tagged wt or mutant LANA_{DBD} proteins were purified using Ni^{2+}-TED columns (Active motif Inc.). Protein concentrations were determined by BCA assays (Bio-Rad) and
protein expression levels were determined by western blot analysis using αFlag-tag antibody (Sigma-Aldrich).

**Electrophoretic Mobility Shift Assay (EMSA).** For probe labeling, fragments containing LBS1 or LBS1/2 were labeled using T4 polynucleotide kinase (NEB) in the presence of [γ-32P] ATP (Amersham Biosciences) following the manufacturer’s instructions. EMSA assay were performed as described previously (Garber et al., 2001). Captured Protein-DNA complex signals on phosphor screen were analyzed using a Typhoon 9410 phosphoimager system (Amersham Bioscience).

**Co-immunoprecipitation.** Plasmids expressing wt and mutant Flag-tagged LANA_{DBD} proteins and a plasmid expressing wt HA-tagged LANA_{DBD} were co-transfected to evaluate dimer formation. Co-transfected cells were harvested 36 - 40 hrs post-transfection, lysed in lysis buffer and pre-cleared by centrifugation. Lysates were co-immunoprecipitated with α-Flag M2 beads. LANA_{DBD} complexes were separated on SDS-PAGE and the amount of HA-tagged wt LANA_{DBD} was detected and quantified by Western blot using αHA-tag antibody. Dimerization activity for each mutant was normalized based on the expression level of Flag-tagged wt or mutant LANA_{DBD} proteins.

**Short-term DNA Replication Assays.** 3×10^6 cells 293 cells were co-transfected with 8 µg pPur/4TR plasmid and 2 µg wt or mutant LANA_{DBD} expression plasmids using the TransIT®-293 transfection reagent (Mirus). Transfection efficiency was monitored using pcDNA3/LacZ. Short-term DNA replication assays were performed as previously described (Hu et al., 2002). Captured signals on a phosphor screen were analyzed using a Typhoon 9410 phosphoimager system (Amersham Bioscience).
Luciferase reporter assays. For transcriptional repression assays, 20 ng pGL3/7TR plasmid as a reporter and 380 ng wt or mutant plasmid as an effector were co-transfected into $3 \times 10^5$ 293 cells using the TransIT®-293 transfection reagent (Mirus). To monitor transfection efficiency, pMaxGFP plasmid was co-transfected with those plasmids and transfection efficiency was over 90%. Relative light units (RLUs) were measured 48 hrs post-transfection. Protein concentrations were determined by BCA assay and RLU values were normalized to the protein concentration. This was based on previous observations that LANA modulates a wide range of promoters (Renne et al., 2001). Reporter gene activity values represent the mean of several independent transfections performed in triplicate and the standard deviation from the mean shown as error bars.
Figure legends

**Figure 1.** Sequence alignments of LANA_{DBD}s of γ-herpesviruses and EBNA1_{DBD} reveals structural conservation. (A) Multiple alignment of amino acid sequences among LANA_{DBD}s of KSHV, RFHVMn and RRV using the PRALINE and T-COFFEE programs. Conserved amino acids among OBPs are labeled in bold. (B) Binary aa alignments between EBNA1_{DBD} of EBV and LANA_{DBD}s of KSHV, RFHVMn and RRV using the 3D-PSSM program. Conserved helices between proteins are shown as dark gray boxes. Proline loops are indicated in italic within light gray boxes. Numbers in parentheses are referring to corresponding aa numbers from BC-1 KSHV LANA (Kelley-Clarke et al., 2007).

**Figure 2.** Computational model of the LANA_{DBD} and multimer structure bound to DNA. LANA_{DBD} model with specific DNA binding site by the M-ZDOCK program [http://zlab.bu.edu/m-zdock](http://zlab.bu.edu/m-zdock) based on alignment with the structure of EBNA1_{DBD}. (A) Tetramer by combining two dimers bound to their respective LBS1/2 (DNA helix in light blue and green). β-barrel bundle is made of four β-strands from each monomer at dimer interface; (B) Each monomer is composed of four β-strands and three helices (helix 1 in red, helix 2 in blue and helix 3 in green); (C and D) crucial amino acids for DNA contact or dimerization shown in yellow; (C) 871K and 875Q and 963Y and 964E for helix 1; (D) 907Y, 910K, and 911K for helix 2. Monomer pictures are generated by ViwerLite 4.2 (Accelrys Inc).

**Figure 3.** DNA binding activity of LANA_{DBD} mutants. Purified LANA_{DBD} mutant and wt proteins were incubated with radiolabeled LBS1 or LBS1/2 as previously described (Garber et al., 2001). DNA binding affinity is represented as the percentage of mutants over wt LANA_{DBD} set to 100%. In each assay, all mutants were tested for DNA binding activity in LBS1 (A to C) or
LBS1/2 (D) and arrow indicates specific protein-DNA complexes. EMSA assay for helix 1 mutants (A); helix 2 mutants (B); helix 3 mutants (C); adapted mutants from each helix (D). N, probe alone as a negative control; wt, wt LANA\textsubscript{DBD}.

Figure 4. Co-immunoprecipitation assays (co-IP) with alanine substitution mutants. Dimerization ability of Flag-tagged wt or mutant LANA\textsubscript{DBD}s with HA-tagged wt LANA\textsubscript{DBD} was tested. Dimerization activity for each mutant was normalized based on the expression level of Flag-tagged wt or mutant LANA\textsubscript{DBD} proteins. L; cell lysate, IP; immunoprecipitated samples. Wt (N); only HA-tagged wt as a negative control, Wt (P); Flag-tagged and HA-tagged wt as a positive control.

Figure 5. Analysis DNA replication mediated by alanine substitution mutants using short-term replication assay. LANA\textsubscript{DBD}-expressing constructs were co-transfected with pPuro-4TR into 293 cells. 10 % of Hirt-extracted DNA was digested with \textit{HindIII} as input (A, lanes 1 to 8 and B, lanes 1 to 6) and remaining DNA was double digested by \textit{HindIII} and \textit{DpnI} (A, lanes 9 to 16 and B, lanes 7 to 12). The DNA was detected by Southern blot with radio-labeled 4TR probe. Full length LANA was transfected as a positive control.

Figure 6. Analysis the activity of LANA-dependents transcriptional repression by alanine substitution. Graphical representation of data from luciferase reporter assays. pGL3/7TR luciferase reporter and wt or mutant LANA\textsubscript{DBD} plasmid were co-transfected. RLU values were normalized to total protein concentration as previously described (Renne et al., 2001). The percentage of suppression activity compare to LANA\textsubscript{DBD} wt set to 100%.
Acknowledgements

We thank Dr. Robert Mckenna and Dr. David C. Bloom for helpful advice and critical reading of this manuscript. This work was supported by grants R01 CA88763 and R01 CA119917 from the NIH National Cancer Institute to RR.
References


Figure 1

(A)

(B)
Figure 3

(A) Proline loop

(B) Proline loop

(C) Proline loop

Relative binding affinity (%)

0 20 40 60 80 100

120

Relative binding affinity (%)

0 20 40 60 80 100 120 140 160 180

KDT950AAASKK953AAAVQM956AAA

V956AM958A L961A

Wt Q957A RL960AA R960A WE963AA W963AE964AS966A

NCWt R873AP874AQ875AHIF876AAAYR879AAK871A H876AI877AF878AY879AR880ARF881AAR881AF882A

NC Wt Y907AL909AKK910AA F916AYGL907A

AA

K910AK911ALSQ912AAAQ917AP925AP932A

NCWt SKK953AAAVQM956AAAV956AQ957A L961AKDT950AAA M958ARL960AAR960AWE963AAW963AE964AS966A

NC Wt Y907AL909AKK910AA F916AYGL907A
Figure 4

(A) Wt (N)       Wt (P)       HIF876AAA      YR879AA
          L  IP  L  IP
          100% 127% 73%

(B) Wt (N)       Wt (P)          RF881AA     YGL907AAA
          L  IP  L  IP
          100% 31% 73%

(C) Wt (N)          Wt (P)         KK910AA      LSQ912AAA
          L  IP  L  IP
          100% 210% 248%

(D) Wt (N)          Wt (P)       VQM956AAA
          L  IP  L  IP
          100% 74% 147%

(E) Wt (N)          Wt (P)       RL960AA      WE963AA
          L  IP  L  IP
          100% 88% 29%

(F) Wt (N)          Wt (P)           KDT950AAA            Q957A
          L  IP  L  IP
          100% 194% 29%
Figure 5

(A) 

(B)
Figure 6

(A) Transcriptional repression activity (%)

(B) Transcriptional repression activity (%)

(C) Transcriptional repression activity (%)
Table 1. Summary of LANA<sub>DHD</sub> mutants and their relative activities in DNA binding, dimerization, replication and transcription repression

<table>
<thead>
<tr>
<th>Position</th>
<th>Mutants</th>
<th>&lt;sup&gt;1&lt;/sup&gt;EMSA</th>
<th>&lt;sup&gt;1&lt;/sup&gt;IP</th>
<th>&lt;sup&gt;1&lt;/sup&gt;RA</th>
<th>&lt;sup&gt;1&lt;/sup&gt;Repression Assay</th>
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<td>LBS1/2</td>
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<tr>
<td>K871A</td>
<td>74 (± 4.8)</td>
<td>35 (± 2.2)</td>
<td>74</td>
<td>79 (±8)</td>
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<tr>
<td>R873A</td>
<td>48 (± 2.9)</td>
<td>18 (± 1.6)</td>
<td>135</td>
<td>103 (±12)</td>
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<tr>
<td>P874A</td>
<td>19 (± 1.3)</td>
<td>21 (± 2.1)</td>
<td>98</td>
<td>77 (±13)</td>
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<td>Q875A</td>
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<td>W963A</td>
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<td>S966A</td>
<td>136 (±16)</td>
<td>127 (± 4.6)</td>
<td>188</td>
<td>+/-</td>
<td>82 (±13)</td>
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1. Detection levels by gel shift assay in presence of single or double DNA binding site. Numbers indicate percentages of relative binding affinity compared to wt.
2. Detection levels by immunoprecipitation. Numbers indicate percentages of dimerization activity compared to wt.
3. Replication activity. -, no activity; +/-, reduced activity compared to LANA<sub>DHD</sub>
4. Transcription repression activity. Numbers indicate percentages of transcriptional repression activity compared to wt.
Table 2. Similarity and identity of the C-termini between gamma herpesvirus OBPs

<table>
<thead>
<tr>
<th></th>
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<th>RFHVMn</th>
<th>EBV</th>
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<td>100</td>
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<td>53 (14)</td>
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Numbers indicate percentages of similarity and identity, indicated by parentheses.