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Small molecule and peptide-mediated inhibition of Epstein-Barr virus nuclear antigen 1 dimerization

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ABSTRACT

Latent Epstein-Barr virus (EBV) infection is associated with human B cell lymphomas and certain carcinomas. EBV episome persistence, replication, and gene expression are dependent on EBV-encoded nuclear antigen 1 (EBNA1)'s DNA binding domain (DBD)/dimerization domain (DD)-mediated sequence-specific DNA binding activity. Homodimerization of EBNA1 is essential for EBNA1 DNA binding and transactivation. In this study, we characterized a novel small molecule EBNA1 inhibitor EiK1, screened from the previous high throughput screening (HTS). The EiK1 compound specifically inhibited the EBNA1-dependent, OriP-enhanced transcription, but not EBNA1-independent transcription. A Surface Plasmon Resonance Biacore assay revealed that EiK1 associates with EBNA1 amino acid 459–607 DBD/DD. Consistent with the SPR data, *in vitro* gel shift assays showed that EiK1 suppressed the activity of EBNA1 binding to the cognate familial repeats (FR) sequence, but not control RBP-Jκ binding to the Jκ site. Subsequently, a cross-linker-mediated *in vitro* multimerization assay and EBNA1 homodimerization-dependent yeast two-hybrid assay showed that EiK1 significantly inhibited EBNA1 dimerization. In an attempt to identify more highly specific peptide inhibitors, small peptides encompassing the EBNA1 DBD/DD were screened for inhibition of EBNA1 DBD-mediated DNA binding function. The small peptide P85, covering EBNA1 a.a. 560–574, significantly blocked EBNA1 DNA binding activity *in vitro*, prevented dimerization *in vitro* and *in vivo*, associated with EBNA1 *in vitro*, and repressed EBNA1-dependent transcription *in vivo*. Collectively, this study describes two novel inhibitors of EBNA1 dimerization. This study demonstrates that EBNA1 homodimerization can be effectively targeted by a small molecule or peptide.

1. Introduction

It has been well documented that EBNA1 is essential for EBV episome persistence in dividing cells and that it enhances episome transcription [1,2]. In latently EBV-infected cancer cells, the EBV viral genomes replicate and persist as extra-chromosomal episomes by virtue of EBNA1 dimerization-dependent DNA binding activities. EBNA1 has 3 domains that are essential for EBV genome persistence and transcription: the arginine-glycine-rich domain 1 (RG1, amino acids (a.a.) 33–89), RG2 (a.a. 328–386), and the dimerization domain (DD)/cognate DNA binding domain (DBD, a.a. 459–607) [3,4]. Both RG1 and RG2 are necessary and sufficient for efficient association of EBNA1 with host chromosomes. RG1 (also called as a Linking Region 1, LR1) can further be subdivided into the AT-rich binding AT-Hook 1 (ATH1, a.a. 40–65) and unique region 1 (UR1, a.a. 66–89). The UR1 mediates DD-independent, Zinc-dependent-dimerization and contributes to EBNA1-dependent transactivation, as well [5]. The EBNA1 DD/DBD is essential for EBV genome persistence and transcription [3,4]. The DBD is almost functionally inseparable from the DD. The DBD/DD homodimer binds to the viral origin of plasmid replication (OriP), consisting of a series of 20 copies of familial repeats (FR) and 4 copies of dyad symmetry (DS) [2]. FR functions as an enhancer for EBV gene expression and a partitioning element for EBV genome segregation, whereas DS acts as an origin of replication upon EBNA1 binding. EBNA1 dimerization is required for EBNA1-dependent DNA binding, transactivation, and replication. These experimental lines of evidence confirm that EBNA1 is essential for EBV episome persistence in dividing cells. Thus, a selective EBNA1 inhibitor could terminate latent EBV infection and abort EBV-dependent growth of nonmalignant and malignant cells.

In our previous report, in which we undertook a cell-based EBNA1 high throughput screen (HTS) in search of EBNA1-selective small molecule inhibitors, we characterized the inhibitory

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mechanism of Roscovitine on EBNA1 function. Roscovitine selectively inhibited cyclin-dependent kinase (CDK)-directed phosphorylation of EBNA1 serine residue 393, EBNA1 nuclear localization, and EBNA1-dependent OriP episome maintenance or persistent infection [6].

In this study, we characterized the inhibitory mechanisms of 2 EBNA1 dimerization inhibitors: a small molecule (EiK1) and an EBNA1 peptide encompassing a.a. 560–566. Both the EiK1 compound and the peptide exhibited the ability to bind the EBNA1 DD, inhibited EBNA1-dependent DNA binding, and interfered with EBNA1 homodimerization, leading to inhibition of EBNA1-dependent, but not EBNA1-independent, reporter transcription. Given that EBNA1 a.a. 459–607 DD forms a strong dimer in high salt conditions and the dimerization interface formed by the β-sheet 3 strands (a.a. 560–566) was buried within the hydrophobic interior of the globular EBNA1 structure [3],[4,7], destabilization of EBNA1 dimer has been believed to be extremely difficult. Contrary to this low expectation, the result in this study proves that targeting of the EBNA1 DD can be experimentally achievable by using a specific sequence peptide or small molecule.

2. Materials and methods

2.1. Materials

The EBNA1-dependent, EBV OriP-enhanced, EBV Cp promoter-driven reporters—p**O**riPCp-**F**L (firefly luciferase) (OF), and p**O**riPCp-**S**EAP (secreted alkaline phosphatase) (OS)—and the EBNA1-independent reporter, pSV40p-RL (Renilla luciferase) (Promega, Madison, WI) were previously described [6]. Compounds EiK1 (Hit2lead, ID 6190924), H31 (Hit2lead, ID 5551021), and others used in the structure and activity relationships (SAR) study were purchased from a vendor as described. Peptides were kindly provided by Dr. Fred Wang of the Channing Laboratory at Harvard Medical School. Peptides fused to HIV TAT were synthesized (Sigma–Genosys, The Woodlands, TX).

EBV-negative BL BJAB cells, BJAB-derivative expressing FLAG-EBNA1 (BJ-FE1), BJAB-FE1-derivative with stably integrated SV40p-RL (BJRL-FE1), BJRL-FE1-derivative harboring episomal OriPCp-FL-SV40p-Puro (OF) (BJRL-FE1-OF), and BJ-FE1-derivatives harboring episomal OriPCp-SEAP-SV40p-Puro (OS) (BJ-FE1-OS) were previously described [6] [8].

2.2. Surface Plasmon resonance Biacore

Each Biacore CM5 sensor chip had two flow cells. EBNA1 a.a. 387–641 or a.a. 459–607 was immobilized on a sensor chip through primary amine groups. Compounds were flowed at a rate of 30 μL/min for 180 s to allow an association, followed by 180 s for dissociation over immobilized protein in TBS-P/2% DMSO running buffer (25 mM Tris–HCl, 137 mM NaCl, 3 mM KCl, 0.005% P20 surfactant, 2% DMSO, pH 7.4). EiK1 compounds were tested for binding in a 1:2 dilution from 20 μM–625 nM. Peptides were tested for binding in a 1:10 dilution from 1 μM to 10 nM. Normalization of the data involved transforming the *y*-axis so that the theoretical maximum amount of binding for a 1:1 interaction with the protein surface corresponded to a sensor response of 100 relative units (RU).

2.3. Membrane-permeable EBNA1 peptide effect on transcription

EBNA1 wild-type sequence peptide a.a. 559–567 (V_{560}CYFMVFL$_{566}$Q), a mutated peptide (V_{560}AAAAAAA$_{566}$Q), or a control Bovine Papilloma virus (BPV), E2 $_{373}$QAQILITFG$_{381}$ was fused at the N-terminus to a membrane-permeable peptide of HIV TAT sequence (YGRKKRRQRRR) at the C-terminus. The peptide N-terminus was tagged with fluorescein isothiocyanate for delivery imaging. The BJAB-derivative BJRL-FE1-OF cells were treated with peptides at the indicated concentration (20 μM) for 4 days. FL, RL, and SEAP activities were determined as described above.

2.4. In vitro Dissuccinimidyl suberate (DSS) cross-linker-mediated dimerization assay

Plasmids for expression of EBNA1 DBDs of a.a. 379–641, 387–641, 459–641, and 459–607 in *Escherichia coli*, all with a 6 × His tag at the amino terminus of EBNA1, were also previously described [6]. Each 5 μg of 6 × histidine-tagged EBNA1 dimer (a.a. 459–607 and a.a. 387–641 DBD/DD) was incubated without (−) or with (+) 1 mM DSS cross-linker at room temperature in the presence of various compounds for 2 h to allow a preexisting dimer to be covalently cross-linked. After incubation, 5× SDS sample buffer was added to each sample, which were separated on an SDS denaturing gel, transferred to nitrocellulose membrane, and blotted with a mouse anti-His antibody.

2.5. In vivo yeast two hybrid (Y2H)-mediated dimerization assay

To further substantiate and reconfirm the inhibitory roles in dimer destabilization, the EBNA1 a.a. 387–641 and EBV SM a.a. 1–479 ORFeome were transferred from entry clones to both DBD and AD vectors by Gateway recombinational cloning [9] (Invitrogen, Carlsbad, CA). The mated yeast 1C09 clone # 5 cells containing EBNA1 a.a. 387–641-AD and EBNA1 a.a. 387–641-DBD were used. SM–SM interaction was used as a negative control [9].

2.6. In vitro EBNA1 electrophoretic mobility super-shift assay (EMSA) and screen for EBNA1 inhibitory peptides

To further identify peptides that are critical for inhibition of dimerization-mediated DNA binding, 15-mer peptides overlapping by five residues from a.a. 459–641 were tested for inhibition of EBNA1 binding to DNA *in vitro* by using an histitine tagged EBNA1 EMSA as previously described [10].

3. Results

3.1. Cell-based EBNA1 HTS identified EiK1 as an EBNA1 inhibitor

From the previous HTS screen, EiK1 (denoted as K1 in Figs. 1 and 2) was identified as an EBNA1-selective inhibitor and reproducibly inhibited an EBNA1-dependent, EBV OriP-enhanced, EBV Cp promoter-driven reporter in a dose-dependent manner at the indicated concentrations, at which no discernible toxicity was observed (Fig. 2D). Structurally flat EiK1 nearly met the Lipinski 5 rule: LogD 1.75, Charge -2, TPSA 95.94, HBA/D 5/2, Refractivity 132.16 (Fig. 1A), suggestive of good therapeutic potential, solubility, and flexibility with six rotational bonds. It had quite a flat and flexible structure with 6 rotational bonds.

3.2. EiK1 suppressed EBNA1-mediated sequence-specific DNA binding to OriP in vitro

Using purified EBNA1 DBD/DD from *E. coli*, we performed an *in vitro* EBNA1 antibody-induced EBNA1 EMSA assay to see whether EiK1 can inhibit EBNA1-dependent sequence-specific binding to the FR of the EBV OriP enhancer element *in vitro*. The protein-probe complexes were clearly EBNA1 bound to FR because the protein-bound FR probe was super-shifted by the EBNA1 monoclonal. Whereas DMSO or a control chemical Puromycin (PURO) did not affect binding, the EiK1 clearly inhibited the EBNA1 a.a. 379–

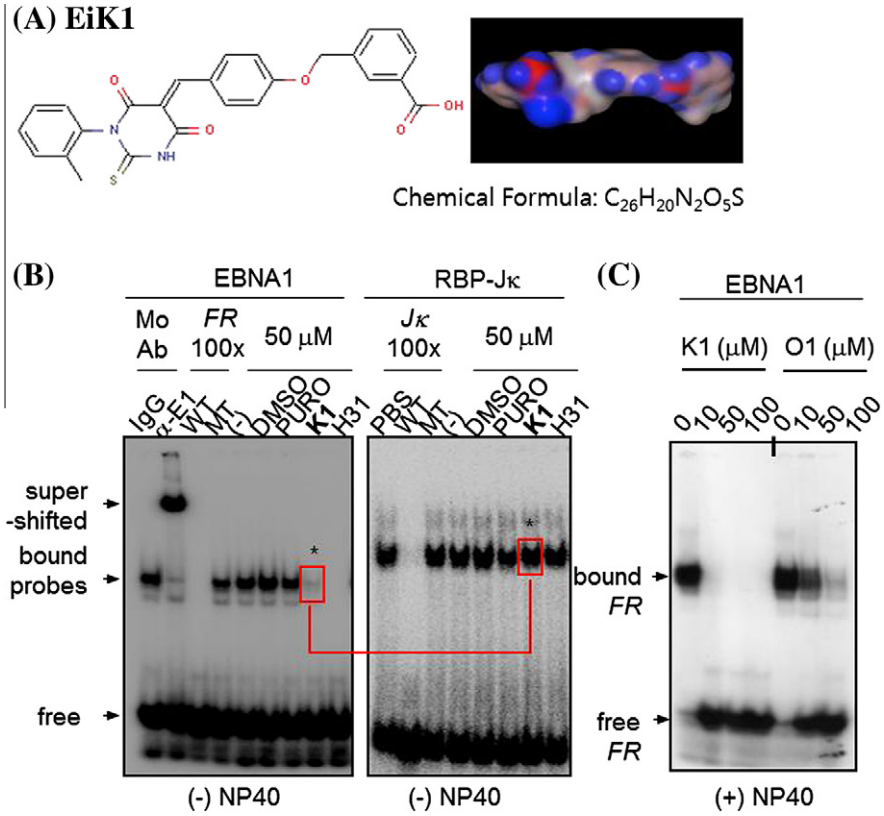

Fig. 1. Chemical and 3D structure of the EBNA1 inhibitor EiK1 and its inhibitory activity on EBNA1-mediated sequence-specific DNA binding to the cognate *FR* sequence in the OriP element *in vitro*. (A) Chemical and 3D structure of EiK1 (B) Left: EiK1 inhibition of EBNA1 a.a. 379–641 DNA binding to FR *in vitro*. Right: EiK1 did not affect RBP-Jκ-dependent binding to the Jκ site *in vitro*. (C) EBNA1 a.a. 379–641 DNA binding assay in the presence of 0.005% NP-40 detergent in modified gel shift buffer. Note that NP-40 detergent was used to solubilize aggregates of hydrophobic compounds, eliminating nonspecific inhibitory action of associated with promiscuous inhibitors.

641-mediated DNA binding to the cognate FR element *in vitro*, but not RBP-Jκ DNA binding activity (Fig. 1B). A positive control H31, a congener of the published H20 [6], exhibited similar results in the inhibition of EBNA1-specific DNA binding (Manuscript in preparation).

To rule out the possibility of EiK1 acting as a promiscuous and nonspecific inhibitor [11], dose-dependent EBNA1 EMSAs were performed with a modified gel shift condition, in which the detergent NP-40 was included to solubilize any potential hydrophobic aggregates of EiK1 (Fig. 1C). The results shown in Fig. 1C are essentially the same as those in Fig. 1B, in which the detergent was excluded from the DNA binding reaction. EBNA1-mediated DNA binding activity was hampered by EiK1 in a dose-dependent manner, but to a much lesser extent by the EiK1-like O1, indicating that EiK1 is a soluble, selective EBNA1 inhibitor that is not a promiscuous chemical inhibitor (Fig. 1C).

3.3. EiK1 inhibited EBNA1 homodimerization in vitro and in vivo

Since dimerization of EBNA1 is required for DNA binding, we further investigated whether EiK1 can affect EBNA1 homodimerization *in vitro*. Consistent with known reports [3,4,7], native EBNA1 a.a. 459–607 DD/DBD was predominantly a dimer in high salt elution buffer conditions, as calculated from retention time by size exclusion gel filtration chromatography (data not shown). The presumably native EBNA1 dimer was incubated with (+) 1 mM DSS cross-linker at room temperature for 2 h to facilitate formation of covalently cross-linked dimers. DSS covalently cross-linked monomers into dimers (Supplementary Fig. 1). The preexisting dimeric EBNA1 molecules dissociated into monomers under denaturing conditions in the absence of DSS cross-linker (Supplementary Fig. S1, DSS (−), and Fig. 2A, lane 1). Upon addition of DSS cross-linker, however, those EBNA1 dimers became covalently linked to form a resultant dimer (~20%) and multimer (~1%), and were resistant to SDS denaturants (Fig. 2A). While DMSO, H31 (unpublished data), and negative control compound Puromycin (PU) did not affect dimerization, EiK1 (K1) and K1-like EiL1 (L1) significantly interfered with cross-linker-mediated covalent EBNA1 dimer or multimer formation *in vitro* (Fig. 2A) (see Supplementary Table for the limited structure and relationship study (SAR) on the EiK1).

To further substantiate the above *in vitro* results of the inhibitory role of EiK1 on dimer destabilization, a Y2H *in vivo* assay was employed, in which EBNA1 DD protein–protein interaction-dependent cell growth and reporter assays can be used to monitor the extent of homodimerization [9]. The mated yeast 1C09 clone # 5 cells containing EBNA1 a.a. 387–641-AD and EBNA1 a.a. 387–641-DBD were treated with compounds for 1–2 days in a selective media (SC-Leu, -Trp, -His + 20 mM 3-amintotriazole (AT)) for their ability to grow in an interaction-dependent manner, and in yeast extract, peptone, dextrose (YPD) media to assay for compound toxicity and subjected to a Cell-Titer Glo assay and β-galactosidase reporter. The EiK1 significantly inhibited EBNA1 DD-mediated, homodimerization-dependent cell growth by ~60%, while it did not affect SM dimerization (Fig. 2B, left). These data were consistent with the results of the dimerization-dependent β-galactosidase reporter (Fig. 2B, right).

3.4. EiK1 associated with DBD dimer of EBNA1 a.a. 459–607 and inhibited an EBNA1-dependent reporter, but not an EBNA1-independent reporter

We performed a SPR Biacore Assay to explore whether the selected EiK1 compound can bind directly to the preexisting EBNA1

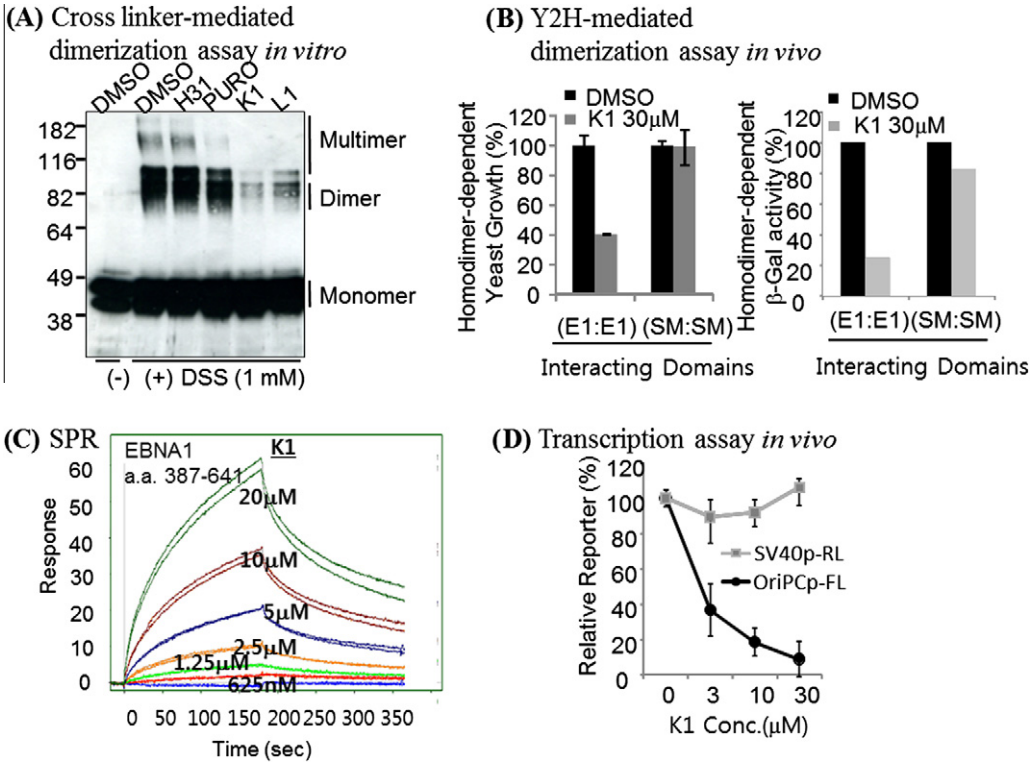

Fig. 2. EiK1 inhibited EBNA1 homodimerization, associated with the EBNA1 dimer, and suppressed the EBNA1-dependent reporter, but not the EBNA1-independent reporter. (A) DSS cross-linker-mediated EBNA1 DD dimerization in the presence of 200 μM indicated compounds. The preexisting dimeric EBNA1 dissociated into monomers in denaturing conditions in the absence of DSS (lane 1). Upon addition of DSS, EBNA1 dimers became covalently linked to form resultant dimers and multimers (lane 2). While DMSO, H31 (unpublished data), and Puromycin (PU) did not affect, EiK1 (K1) and K1-like EiL1 (L1) interfered with EBNA1 dimer and multimer formation. (B) The EiK1 (K1) significantly inhibited EBNA1 DD homodimerization while not affecting SM dimerization both in homodimerization-dependent cell growth (left) and β-galactosidase reporter transcription (right) in Y2H assays. (C) EiK1 (K1) association with the preexisting EBNA1 dimer by SPR Biacore assay. (D) EiK1 dose-dependent inhibition of EBNA1-dependent reporter activity (OriPCp-FL), but not EBNA1-independent reporter activity (SV40p-RL).

DBD/DD dimers predominant in purified solution as discussed above and previously known [3]. The SPR study exhibited promising binding curves in the 20 μM range. Compound EiK1 exhibited good association and dissociation profiles to the preexisting dimeric EBNA1. However, the association did not reach equilibrium for these compounds, and incomplete dissociation between sample injections produced less than ideal-looking curves. Modeling of the association kinetics suggested that association did not follow the 1:1 Langmuir binding model, making it harder or impractical to calculate a K_D value (Fig. 2C).

The inhibitory role of EiK1 was further validated by determining the effect of EiK1 on EBNA1-dependent reporter gene transcription in cells. The EiK1 inhibited EBNA1-dependent, OriP-enhanced, EBV Cp promoter-driven reporter (OriPCp-FL) activity in a dose-dependent manner, but not EBNA1-independent SV40p-RL (Fig. 2D).

3.5. In vitro screen of EBNA1 peptides for EBNA1 DNA binding activity identified a hydrophobic peptide as a homodimerization inhibitor

As described, EBNA1 dimerization is required for DNA binding of EBNA1. EBNA1 dimerization involves intermolecular, symmetric associations between 2 β3 a.a. 559–567 strands and 2 β4 a.a. 592–600 strands, as well as asymmetric intramolecular interactions of the β4 strand with a.a. coil 601–607, and the β2 (a.a. 531–539) strand with coil a.a. 531–5394 [3,7], as deletion of the 557–564 β3 strand abrogated dimerization [12]. Therefore, we have undertaken a rational biochemical screening of EBNA1 peptides to identify minimal peptides that may affect homodimerization-mediated DNA binding. A total of 64 EBNA1 peptides of 15 a.a. in length and overlapping by five residues from a.a. 387–641 were tested for inhibition of EBNA1 binding to DNA *in vitro* (Supplementary Table 2).

3.6. The screened EBNA1 DNA binding inhibitory P85 peptide associated with EBNA1 and interfered with EBNA1 homodimerization

Seven peptides abolished EBNA1 DNA binding activity: P70 (a.a. 500–514), P75 (a.a. 520–534), P83 (a.a. 552–566), P84 (a.a. 566–587), P85 (a.a. 560–574), P91 (a.a. 584–598), and P92 (a.a. 588–602), all of which consist of a beta strand in intra- or intermolecular dimeric interfaces. Among them, 3 consecutive peptides, P83, P84, and P85, which share the markedly hydrophobic a.a. $_{560}$CYFMVFL$_{566}$, almost completely inhibited EBNA1 binding to DNA at 1.2 μM, and even at 0.6 μM in the case of P85 (Fig. 3A). By contrast, the neighboring peptides P86 and P87 did not exhibit any inhibitory activity.

The inhibitory activity on DNA binding is probably through destabilization of dimers, otherwise stabilized by intermolecular interactions [3,7]. As expected and interestingly, P85 interfered with DSS cross-linker-mediated EBNA1 homodimerization *in vitro*, whereas the control peptide P41 that exhibits similar hydrophobicity to P85 did not affect homodimerization (Fig. 3B). In addition, P85 strongly associated with, but apparently did not dissociate from, the EBNA1 dimerization/DBD domains in duplicate SPR Biacore assays (Fig. 3C). These data indicate that EBNA1 P85 (a.a. 560–574) irreversibly interacted with EBNA1 DD, suggesting that the P85 (a.a. 560–574) hydrophobic peptide may nucleate disruptive interactions with EBNA1.

3.7. Membrane-permeable P85 inhibited EBNA1-dependent transcription but not EBNA1-independent transcription

To characterize the peptide's effects in cells, EBNA1 WT (E1WT) sequence peptide 559–567 (V_{560}CYFMVFL$_{566}$Q), a mutated peptide

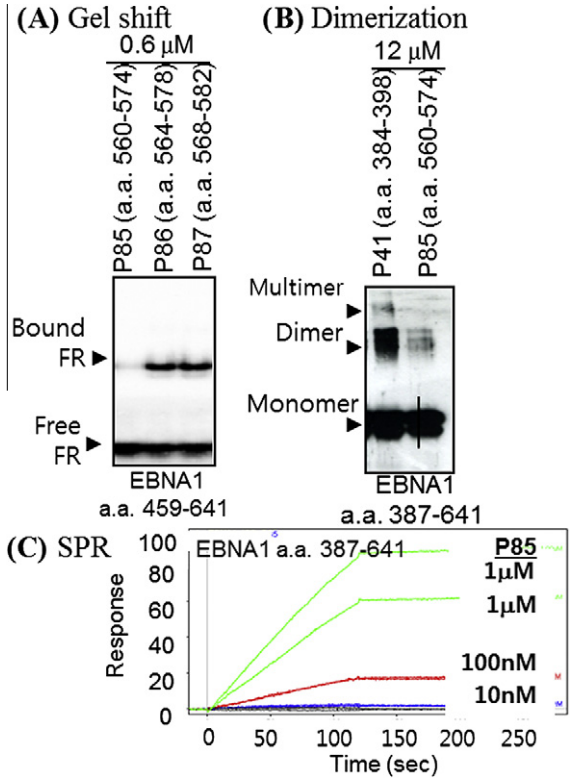

Fig. 3. EBNA1 P85 peptide associated with EBNA1, inhibited EBNA1 DNA binding and homodimerization. (A) The screened hydrophobic EBNA1 peptide P85 encoding a.a. 560–574 abolished EBNA1-dependent sequence-specific DNA binding to the cognate FR sequence in the OriP element, while P86 and P87 did not. (B) P85 interfered with DSS cross linker-mediated EBNA1 a.a. 387–641 homodimerization *in vitro*, whereas control peptide P41 did not. (C) P85 strongly associated with, but apparently did not dissociate from, the EBNA1 DD/DBD dimer in duplicate SPR Biacore assays.

(E1MT, $V_{560}AAAAAAA_{566}Q$), or a control Bovine Papilloma virus E2 (BPVE2, $_{373}QAQILITFG_{381}$) was fused at the N-terminus to a membrane-permeable peptide of the HIV TAT (YGRKKRRQRRR) at the C-terminus [13]. BJAB-derivative cells expressing EBNA1, OriPCp-FL, and SV40p-RL were treated with 0.02% acetic acid solvent or peptides for 4 days, and subjected to reporter assays. Compared to those not treated with E1WT peptide, the E1WT peptide treatment significantly inhibited the EBNA1-dependent, OriP-enhanced, EBV Cp promoter-driven FL activity, but not EBNA1–independent SV40pRL activity. As expected, mutated E1MT and control BPVE2 peptides did not affect either reporter (Fig. 4A).

To confirm that the effect of the peptides was not attributable to the FL reporter used in the assay, but to the effector protein EBNA1 and enhancer element OriP, BJAB-derivative cells expressing EBNA1 and OriPCp-SEAP were treated with E1WT-TAT or BPVE2-TAT peptides. The E1WT-TAT peptide inhibited EBNA1-dependent, OriP-enhanced SEAP transcription in a dose-dependent manner whereas control BPVE2-TAT peptide did not. These results indicate that E1 peptide a.a. 560–566 binds to EBNA1, and represses EBNA1-dependent DNA binding and transcription activity, which is most likely mediated by interfering with EBNA1 homodimerization.

4. Discussion

In this study, we characterized a previously screened small molecule (EiK1) in EBNA1 cell-based HTS assay, and novel EBNA1 peptide (a a. 560–566) as selective inhibitors of EBNA1 homodimerization, leading to defects in EBNA DNA binding and transactivation activity. The HTS against 40550 compounds in our previous study that measured EBNA1 interaction with OriP element-dependent transactivation activity in cells yielded several inhibitory compounds including EiK1 [6]. Since the primary read-out in the HTS and secondary assay was the EBNA1-dependent transcription [6], and the EBNA1 DD is critical for the transactivation, DNA binding, and replication functions, we focused this study on the compound effect on the DD-mediated dimerization and transcription [9,14].

It is known that some hydrophobic compound often promiscuously interferes with a variety of biochemical reactions by self-aggregating in detergent-free conditions, but not in compound-solubilizing, detergent-containing conditions [11]. In this regard, we have ruled out the possibility that the compound EiK1 is a potentially promiscuous inhibitor by adopting the gel shift conditions

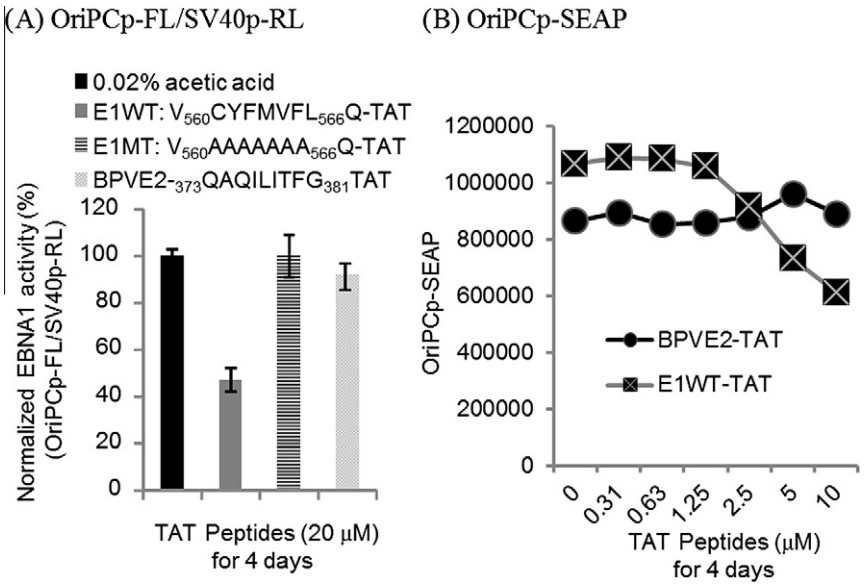

Fig. 4. Membrane-permeable P85 inhibited EBNA1-dependent transcription but not EBNA1-independent transcription. (A) E1WT-TAT only inhibited EBNA1-dependent, but not –independent transcription. (B) E1WT-TAT, but not control BPVE2-TAT, interfered with EBNA1- and OriP-dependent transcription of SEAP reporter in cells in a dose-dependent manner.

under compound-solubilizing NP-40 conditions (Fig. 2C). The results clearly demonstrate that the inhibitory function of EiK1 was not attributable to any promiscuous activity by the compound, as EiK1 in the presence of detergent was still as effective as in the absence of it for inhibition of EBNA1 DNA binding (Fig. 2C), indicating that EiK1 was not a promiscuous compound.

DSS cross-linker covalently links two proteins in direct contact or in close proximity. With greater than 10–20% efficiency, DSS was able to link preexisting predominant EBNA1 dimers or minor multimer, rendering those cross-linked dimers resistant to dissociation to monomers upon addition of reducing agent and SDS denaturant in this study. Most importantly, EiK1 and the dimeric interface peptide decreased the fraction of dimers and multimers more so than the untreated control did.

Because EBNA1 a.a. 459–607 DD forms a strong dimer in high salt conditions and the dimerization interface formed by the β-sheet three strands (a.a. 560–566) was buried within the hydrophobic interior of the globular EBNA1 structure [3,4,7], it would be hard for a compound or peptide to dissociate the dimer or target the dimeric interface. Contrary to this low expectation, the result in this study proves that targeting of the EBNA1 DD can be achievable by using a specific sequence peptide or small molecule. Conceivably, the EiK1 and peptide 85 in this study may break the preexisting native dimers into monomers, interfere with *de novo* formation of or dynamic interchange to dimers from monomers in a dynamically regulated equilibrium. Though the underlying inhibitory mechanism has yet to be resolved, the effect of P85 on DNA binding may be to destabilize preformed dimers, otherwise stabilized by intermolecular interactions [3,7]. This assumption is supported by the data showing P85's irreversible association with EBNA1 DD in the SPR Biacore assay, and its interference with DSS-mediated cross-linking (Fig. 3). Consistent with the contribution of endogenous a.a. 560–574 to dimer formation, Y561 forms an intermolecular stabilizing hydrogen bond to Y561 in another monomer, and M563 forms hydrophobic interaction with M563 (Supplementary Fig. S2A and B) [3,4,7,15,16]. Mutation of tyrosine residue 561 to alanine (Y561A) was tested for the effect on EBNA1 DNA binding *in vitro*. As expected and consistent with the results of the peptide screening, Y561A significantly abrogated DNA binding activity and OriP enhancer–mediated transactivation, probably due to its inability to form dimers (Supplementary Fig. S2C and D).

In addition, the experimental data from homodimerization-dependent cell growth and reporter transcription assay in Y2H study suggest that the peptide that falls into the dimeric interface appears to interfere with *de novo* dimer formation, or that it interferes with high order structures during or after protein translation rather than breaking already formed dimers into monomers.

Collectively, these data *in vitro* and *in vivo* again indicates that dissociation or destabilization of the EBNA1 homodimer *in vivo* was experimentally achievable. Therefore, the novel dimerization inhibitors in this study might be useful probes for dissecting EBNA1 functions *in vitro* and *in vivo*, and for controlling EBV-associated disorders. Further elaboration of EiK1 and P85 to derive better compounds is required in order to achieve considerable therapeutic efficacy.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.06.095.

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