DING Proteins from Phylogenetically Different Species Share High Degrees of Sequence and Structure Homology and Block Transcription of HIV-1 LTR Promoter

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Abstract

Independent research groups reported that DING protein homologues isolated from bacterial, plant and human cells demonstrate the anti-HIV-1 activity. This might indicate that diverse organisms utilize a DING-mediated broad-range protective innate immunity response to pathogen invasion, and that this mechanism is effective also against HIV-1. We performed structural analyses and evaluated the anti-HIV-1 activity for four DING protein homologues isolated from different species. Our data show that bacterial PfluDING, plant p38SJ (pDING), human phosphate binding protein (HPBP) and human extracellular DING from CD4 T cells (X-DING-CD4) share high degrees of structure and sequence homology. According to earlier reports on the anti-HIV-1 activity of pDING and X-DING-CD4, other members of this protein family from bacteria and humans were able to block transcription of HIV-1 and replication of virus in cell based assays. The efficacy studies for DING-mediated HIV-1 LTR and HIV-1 replication blocking activity showed that the LTR transcription inhibitory concentration 50 (IC50) values ranged from 0.052–0.449 ng/ml; and the HIV-1 replication IC50 values ranged from 0.075–0.311 ng/ml. Treatment of cells with DING protein alters the interaction between p65-NF-kB and HIV-1 LTR. Our data suggest that DING proteins may be part of an innate immunity defense against pathogen invasion; the conserved structure and activity makes them appealing candidates for development of a novel therapeutics targeting HIV-1 transcription.

Introduction

DING proteins were recently clustered into a new group of highly conserved proteins found in prokaryotes and eukaryotes throughout the plant and animal kingdoms [1,2,3,4,5,6,7]. Phylogenetically, the DING proteins belong to the superfamily of phosphate-binding proteins (PBP) which comprises also PstS and alkaline phosphatase (AP), both exclusively found in prokaryotes [8]. To date, more than fifty different DING proteins have been reported in all kingdoms of life [9]; however, their complete genomic structure and chromosomal assignment in humans is still unknown.

Structural studies on the human phosphate binding protein (HPBP) and the DING protein from Pseudomonas fluorescens (PfluDING) revealed interesting details about their configuration [10,11]. Similarly to the PstS, the DING proteins are formed by two globular domains linked together by a flexible hinge allowing a “Venus flytrap” movement [12]. However, the structural differences on four external loops and presence of two disulfide bridges in DINGs stand them apart from other PBPs and suggest an autonomous class of proteins [12]. The phosphate molecule in the DING protein resides in the vicinity of the binding cleft formed by the two globular domains. The binding of this phosphate ion occurs between four conserved residues in each globular domain, that form a complex network of 12 hydrogen bonds [11]. It has been shown that presence of an aspartic acid (D62) in this position is required for the activity of DING proteins [13]. The structural studies on the human phosphate binding protein (HPBP) and the DING protein from Pseudomonas fluorescens (PfluDING) revealed interesting details about their configuration [10,11]. Similarly to the PstS, the DING proteins are formed by two globular domains linked together by a flexible hinge allowing a “Venus flytrap” movement [12]. However, the structural differences on four external loops and presence of two disulfide bridges in DINGs stand them apart from other PBPs and suggest an autonomous class of proteins [12]. The phosphate molecule in the DING protein resides in the vicinity of the binding cleft formed by the two globular domains. The binding of this phosphate ion occurs between four conserved residues in each globular domain, that form a complex network of 12 hydrogen bonds [11]. It has been shown that presence of an aspartic acid (D62) in this phosphate-binding pocket is important for the distinction between the phosphate and other closely-related ions such as sulfate or arsenate [13,14].

The biology of eukaryotic DING proteins as a group remains to be defined, but most DING proteins isolated from eukaryotes were related to a broad range of disorders and biological processes [1,2,6,7,15,16,17]. For example, the synovial stimulatory protein...
(SSP) has the capacity to induce proliferation of the peripheral blood T cells in patients with rheumatoid arthritis [6]. Another DING protein, the steroidogenesis-Inducing protein (SIP) has mitogenic activity toward the ovarian epithelium and might be involved in the etiology of ovarian cancer [10]. In contrast, a recent report by Hendricks et al indicated that dysfunction of the newly-characterized bovine DING homologue, the gonadotropin surge-Inhibiting/attenuating factor (GnSIF/AF) could lead to polycystic ovarian syndrome [15]. The crystal adhesion inhibitor (CAI), found in monkey and in human renal epithelial cells, has been shown to inhibit the growth of kidney stones [16]. Finally, several members of the DING family inhibit HIV-1 replication through blockage of the LTR transcriptional activity [4,7,19,20]. This comprises HPBP from human plasma [19], X-DING-CD4 from CD4+ T cells [7,21] and p27SJ [and its full-length form, p38SJ] from the medicinal plant Hypericum perforatum [4,20]. It has been shown that primary target for X-DING-CD4 in blocking HIV-1 activity was NF-kB [22,23] whereas p38SJ, called hereafter pDING, exhibits the ability to interact with C/EBPβ and Tat, and dephosphorylates the CTD domain of the RNA polymerase II. All these interactions can participate in anti-HIV-1 activity of DING proteins [4,20,24]. Subsequent studies showed the ability of pDING and X-DING-CD4 in regulating various cellular genes including MCP-1 and IL-8 by either C/EBPβ- and/or NF-kB-dependent pathways [25,26]. In addition to the in vitro studies, the clinical evaluations indicated that HIV-infected patients have an increased concentration of DING proteins [27], and the increased expression of the X-DING-CD4 mRNA correlates with the cellular restriction of HIV-1 replication in human macrophages [21].

The NF-kB system is involved in the immediate signaling mechanisms of the innate immune responses against infecting pathogens, including HIV-1 [28]. Activation of NF-kB-dependent transcription of cytokines and other mediators of inflammation exerts protective anti-inflammatory function. Transcription of several viruses such as Herpes Simplex Virus (HSV), Simian Virus 40 (SV40) and HIV-1 depends on NF-kB signaling [29]. The anti-NF-kB activity of DING proteins is of particular interest in the design of novel anti-viral therapies.

The conserved nature and anti-HIV-1 properties of these DING homologues indicate their function as innate immunity effectors molecules and highly recommend consideration of DING proteins as a potential antiretroviral. With this in mind, we performed structural analyses and anti-HIV-1 activity studies for four DING homologues isolated from cells of Pseudomonas fluorescens (PfluDING), Hypericum perforatum (pDING) and Homo sapiens (HPBP and X-DING-CD4). To date, HPBP represent the only DING homologue that blocks Tat, and dephosphorylates the CTD domain of the RNA polymerase II. All these interactions can participate in anti-HIV-1 activity of DING proteins [4,20,24]. Further studies showed the ability of pDING and X-DING-CD4 in regulating various cellular genes including MCP-1 and IL-8 by either C/EBPβ- and/or NF-kB-dependent pathways [25,26]. In addition to the in vitro studies, the clinical evaluations indicated that HIV-infected patients have an increased concentration of DING proteins [27], and the increased expression of the X-DING-CD4 mRNA correlates with the cellular restriction of HIV-1 replication in human macrophages [21].

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Materials and Methods

The studies reported here using human peripheral blood lymphocytes were granted exempt status by the St. Luke’s-Roosevelt Institutional Review Board under qualifications listed in section 45.101 (b) [4].

Cell cultures and reagents

Human astrocytoma U87 MG and MAGI-CCR-5 [32] cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The 1G5 cells stably expressing a luciferase gene driven by the HIV-1 long terminal promoter (LTR) [33] were obtained through the NIH AIDS Research and Reagent Program Division of AIDS, NIAID. The U87 MG and MAGI-CCR-5 cell lines were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Inc.), antibiotics (100 U/ml penicillin and 10 mg/ml streptomycin) and glutamine, while the 1G5 cells were cultured in RPMI 1640 (Sigma) supplemented with 5% FBS, antibiotics and glutamate. Peripheral blood lymphocytes (PBls) were obtained by elutriation from the whole blood of healthy, HIV-1 negative volunteers. Before the experiment, cells were stimulated for 2 days in Dulbecco’s Modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), phytohemagglutinin (PHA, 5 μg/ml; Sigma), interleukin 2 (10 U/ml; R&D Systems), antibiotics and glutamate. Subsequently, cells were cultured without PHA. All cell cultures were incubated at 37°C in a 7% CO2, 95% air-humidified incubator.

DING proteins

The detailed information referring to the isolation and purification of X-DING-CD4, HPBP and pDING was previously reported [4,7,10,34]. PfluDING protein was used in the form of a site-specific mutation (S29G), altering one of the phosphate-binding residues, and resulting in a protein purified by the same method as the wild-type protein [34]. For evaluations of the biological activity, all four DING homologues were dialyzed against 10 mM Tris-HCl, pH 8.0 using benzoylated cellulose tubing with a MW cut-off of 1.2 kDa (Sigma). Subsequently the dialyzed material was concentrated by diallylization and stored at 4°C.

Tertiary structure prediction

Sequences of DING proteins (NCBI Accession Number: AAW57408, ADT62916, YP_002872202.1 and P85173.1 for pDING, X-DING-CD4, PhluDING and HPBP) have been aligned using ClustalW 2.1 [35]. The structure of X-DING-CD4 and pDING was related to sequences of HPBP and PhluDING as a template. Structure alignment was performed by MODELLER 9.11 software [36]. Heteroatoms of pdb files have been removed except the phosphate molecule on the PhluDING structure. The 3D structures were validated using RAMPAGE software [37]. Pymol was used to represent DING protein structures [38].

SDS PAGE

A total of 50 ng of each DING protein sample was resolved by SDS-PAGE and transferred to supported nitrocellulose membranes as described previously [4]. Proteins were visualized by Coomassie brilliant blue or by western blot using rabbit polyclonal antibody to pDING with the enhanced chemiluminescence detection system (Amersham Pharmacia). Antibody specific for pDING (anti-p27SJ rabbit polyclonal antibody) was obtained from Lampire Biological Laboratories, Inc. Pipersville, PA.

ChIP assay

The U87 MG cells were transfected using the FuGENE 6 transfection reagent (Roche). Briefly, 1×10⁶ cells were cultured overnight in 100 mm plates. Subsequently, cells were transfected with 1 μg of pGL3-Luc LTR plasmid in the presence of 200 ng/ml of each DING protein, respectively. The pGL3-Luc LTR
plasmid used for the chromatin immunoprecipitation (ChIP) assay was generated from the HIV-1 LTR (−374/+138 bp) DNA fragment cloned into the pGL3-basic vector (Promega), as we described before [4]. At 48 hours post-transfection, proteins were cross-linked with 1% formaldehyde for 10 min at 37°C; then cells were washed twice in ice-cold PBS supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin and 1 μg/ml pepstatin A, and pelleted for 4 min at 2000 rpm at 4°C. The cell pellets were lysed in 200 μl of lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Nonidet P-40 and protease inhibitors as above) for 10 min on ice. Subsequently, cell lysates were sonicated to shear the genomic DNA to fragments between 200 and 1000 bp, cleared by centrifugation for 10 min at 13,000 rpm at 4°C; and the supernatant was diluted 10-fold in ChIP dilution buffer (0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl). To remove the nonspecific background, all protein extracts were pre-cleared with 80 μl of salmon sperm DNA/protein A agarose-50% slurry for 30 min at 4°C. For the IP reaction, 250 μg of each protein sample was incubated overnight at 4°C with 2 μl of anti-p65 NF-kB (F-6) antibody (Santa Cruz Biotechnology) or 2 μl of control normal mouse serum (Pierce Chemical). The IP complexes were precipitated by addition of 60 μl of salmon sperm DNA/protein A agarose slurry for 1 hour at 4°C and collected by centrifugation at 1000 rpm at 4°C. Following washing for 3–5 min at room temperature (RT) with 1 ml of low salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), high salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), LiCl immune complex wash buffer (0.25 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1), and 1X TE buffer, the IP complexes were eluted for 15 min with the elution buffer (50 mM Tris-HCl, pH 8.1, 150 mM NaCl). The DNA by protein assay (Bio-Rad). The HIV-LTR inhibition values were established from the formula: [(LUC1-LUC0)x100/Z]-100, where LUC1 is the value obtained from PMA-treated cells treated with a specific dilution of DING protein, and LUC0 is the basal value obtained from uninduced, untreated cells; Z is the absolute luciferase induction by PMA calculated as Z = LUCmax-LUC0, where LUCmax is the value of 100% luciferase expression in PMA-induced, untreated cells.

**MAGI assay**

The MAGI assay [32] was performed with modifications as described before [7]. Briefly, MAGI-CCR-5 cells were seeded 24 hours prior to assay in a 96-well plate (Costar Scientific) at 6.2×10^3 cells well in DMEM supplemented with 10% fetal bovine serum, antibiotics and glutamate (all from Sigma). Subsequently, cells were exposed to DING protein or control C3 Peptide P16 treatments. Twenty four hours later cells were infected with 0.1 pg/cell NL4-3 HIV-1 isolate [40]. Replication of virus was evaluated 48 hours later after fixation of cells with 1% formaldehyde and 0.2% glutaraldehyde in PBS. The expression of β-galactosidase was visualized by 50 min exposure to X-GAL (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), 0.4 mg/ml 2 mM MgCl2, 4 mM potassium ferricyanide at 37°C. After enumeration of the infected (blue) cells, all cells were lysed and subjected to the protein assay (Bio-Rad) to establish total protein sample concentration, and all data were normalized by total protein concentration. The values for inhibition of HIV-1 replication were calculated based on the formula [(R1-R0)/R0]100, where R1 is the value obtained from uninfected, untreated cells; R0 is the absolute value obtained from the uninfected, untreated cells; Z is the absolute HIV-1 replication value calculated as Z = Rmax-R0, where Rmax is the value representing 100% inhibition of HIV-1 replication in the untreated cells.

The IC50 values were calculated from titration curves using two adjoining dilutions for each DING protein that showed inhibition close to 50% inhibition of LTR (RSA) or HIV-1 replication (MAGI).

**Evaluation of DING protein-mediated antiviral activity in primary human cells**

3×10^9 PBLS/well in a 24-well plate (Costar Scientific) were cultured in 1 ml RPMI medium supplemented with antibiotics, glutamate and 1 μg/ml of each DING protein, respectively. Six hours after the initial exposure to the DING treatments, the culture medium was supplemented with 3%/Vol FBS. One day after treatment, cells were infected with 0.01 pg/cell NL4-3 HIV-1 isolate [40] and cultured as described above, except that the concentration of FBS was adjusted to 5%/Vol. The experimental control consisted of HIV-1-infected but untreated PBLS. Replication of virus was evaluated at five and seven days after infection by Elisa assay of the intracellular HIV-1 p24 core antigen (Perkin Elmer). The viability of cells was assessed by the dye exclusion method [41] at 1, 2, 3, 5 and 7 days after DING treatments.

**Results**

The structure of DING proteins

As shown in Figure 1A, the four DING proteins migrated with similar mobility of about 39 kDa. All of them are recognized by antibody generated to pDING (Fig. 1B), and that seems to be due to the high sequence identity shared by the four DING homologues (Fig. 2).
The only existing crystal structures of DING proteins were derived from the HPBP [10] and the bacterial PfluDING [11]. We used this information for prediction of the X-DING-CD4 and pDING structures (Fig. 3). Our analysis showed that all tested proteins share the same topology with closely-similar structures. The protein backbone is perfectly superimposed between the four DING proteins, particularly in the zone implicated in phosphate binding (Fig. 3, sphere). The structural difference was noted only in the length of protuberant loops, which generated a visible disparity in some regions between the four DING proteins.

Testing the anti-HIV-1 therapeutic utility of DING protein variants

Independent studies demonstrated that phylogenetically-distinctive DING proteins might have similar HIV-1 blocking activity [4,7,19]. To better understand the specific anti-viral characteristics

Figure 1. Testing the mobility of DING protein variants in SDS-PAGE. (A) Coomassie brilliant blue staining of SDS PAGE gel. The first and last lanes correspond to the molecular weight marker (respectively Rainbow and ProSieve). Lanes 1–4 correspond to pDING, HPBP, X-DING-CD4, and PfluDING. (B) Western Blot of DING proteins. The first lane corresponds to molecular weight marker (ProSieve), bands lower than 39 kDa in lane 4 indicate degradation products of PfluDING.

doi:10.1371/journal.pone.0069623.g001

Figure 2. The amino acid alignment for pDING, PfluDING, X-DING-CD4 and HPBP proteins. Accession numbers for each protein are indicated between bars, identical residues are highlighted with black. The sequence alignment was done using Bioedit software.

doi:10.1371/journal.pone.0069623.g002
of these DING proteins, we compared their antiviral potential in a uniform experimental setting. We utilized the rapid suppression assay (RSA) [30] which tests directly the inhibition of HIV-1 transcription, the MAGI assay which tests the inhibition of HIV-1 replication in a single cycle infection [32], and evaluated the DING-mediated inhibition of HIV-1 infection in human PBLs.

As shown in Fig. 4A, HPBP, pDING, PfluDING and X-DING-CD4 blocked transcription of HIV-1 LTR by 75% at concentrations ranging from 0.1–1 μg/ml. The HIV-1 LTR IC_{50} for human X-DING-CD4 was 52 ng/ml and 449 ng/ml, respectively (Fig. 4A and Table 1). The IC_{50} values for plant pDING and bacterial PfluDING were 160 ng/ml and 254 ng/ml, in that order (Fig. 4A and Table 1). Treatment of cells with control C3 Peptide P16 had only a minor effect on HIV-1 LTR activity, blocking its expression by 4–9%.

Similarly to the evaluations of DING-mediated inhibition of HIV-1 LTR transcription, the MAGI assay showed that replication of HIV-1 was also blocked by all tested DING protein homologues (Fig. 4B). This data suggested that successful replication of HIV-1 was also blocked by all tested DING protein homologues (Fig. 4B). This data suggested that successful replication of HIV-1 was also blocked by all tested DING protein homologues.

The DING proteins form an intriguing family of biologically active factors contributing to protective [7,13,16,27,42,43] or possibly adverse [1,6] cellular functions. In this work we selected four distinct members of the DING family to ascertain their structural and functional properties with specific interest in their anti-HIV-1 activity.

The structural studies indicated that four tested DING proteins have highly-conserved protein backbones and differ in the length of the protuberant loops. The perfect superimposition observed in the phosphate-binding cleft confirmed that these proteins have been specifically designed to bind a phosphate ion.
The differences in the length of protuberant loops could be connected to the various physiological functions attributed to DING proteins and may be related to their specific protein/protein interactions [PON1, NF-κB, Tat or C/EBPβ] [4,20,22,25,26,27,44,45]. Nevertheless, the native function of DING proteins, and particularly pDING, is yet to be determined, but plant DING proteins associate with germin-like proteins (GLPs) [46] known to have multiple functions including pathogen elicitation [47].

As a dose response assay has been performed only for HPBP in a previous study [19], it was worthwhile to determine the IC_{50} values for all tested DING proteins in a single experiment. Our result confirmed those previously obtained for HPBP; the value of 150 ng/ml obtained from this experiment for the inhibition of HIV-1 replication was close to that published by Cherrier et al (190 ng/ml) [19]. However, while IC_{50} values obtained for X-DING-CD4, pDING and PfuDING corresponded closely for the RSA and MAGI assays, this was not the case for the HPBP. We think that it may be due to the instability of this protein (E.C. personal communication), since the value obtained with the MAGI assay was significantly low and close to that obtained by Cherrier et al [19]. This might also explain the results obtained in primary human cells. Results from this study also permitted the determination of an order of efficiency of DING proteins for HIV-1 inhibition. In fact, all DING proteins do not inhibit HIV-1 similarly. We showed that X-DING-CD4 was the most potent inhibitor of HIV-1 transcription and replication, followed by HPBP and pDING approximately at the same level and finally by PfuDING. This difference of HIV-1 inhibition efficiency is likely to be related to a specific part of the DING sequence (or structure) that needs to be determined. It also might be related to specific posttranslational modifications, absent in bacterial DING. We found both methylated and un-methylated (E68) forms of X-

![Figure 4](image_url)
DING-CD4 protein during its purification from cell culture supernatants [7].

Results from NF-κB binding assays indicated that the four tested DING proteins are able to block the formation of the p65-NF-κB/HIV-1 LTR complex. The NF-κB family is composed of five proteins (p50, p52, p65, RelB and c-Rel) that form various complexes of transcription factors involved in inflammation, cell proliferation and immunity [48]. The activation of NF-κB may occur in response to different stimuli, including bacterial and viral infections, and is triggered via different pathways [48,49]. Out of 15 known NF-κB homo- and heterodimers formed in cells, the p65/p50 NF-κB is most abundant [50], rapidly activated [51] and most importantly, used by HIV-1 during LTR-transactivation [52]. Densitometry analysis showed no significant differences in the rate of inhibition between the four DING proteins that ranged from 61.5 to 62.9%. This result indicates that all tested DING proteins inhibit NF-κB binding to LTR similarly when used at concentrations exceeding the IC_{50} value (200 ng/ml). The discrepancy between the IC values obtained from the efficacy evaluations (RSA and MAGI) and those obtained from ChIP assay could be explained by use of distinct experimental tools. For example, the RSA measures DING-mediated inhibition of the whole LTR promoter, while the ChIP assay is performed upon the LTR probe encompassing only the NF-κB binding sequences. We believe that minor structural differences between DING proteins could impose unique physiological functions upon individual DING variants, such as targeting several events in transcription from the HIV-1 promoter, as reported for pDING [4,20,44]. Nonetheless, the inhibition of NF-κB/LTR binding seems to be a common trait for all tested DING variants.

We indicated before that the eukaryotic genes encoding DING proteins might originate from evolutionary conservation [33]. The notable preservation of their structure and function could have its origin in the selective pressure necessary to maintain successful clearance of invading pathogens. Maintenance of these genes throughout the process of evolution was likely related to the retention and development of functions essential to survive the pathogen invasion. These studies provide direct proof for this hypothesis. DING homologues from distinctly diverse organisms retain a structural and functional resemblance. The bacterial, plant and human DING proteins blocked transcription of the HIV-1 LTR promoter in cell-based assays suggesting that these proteins might permeate the cell membrane or interact with a cell-surface receptor in similar ways. The mechanism of X-DING-CD4-cell membrane interactions and downstream effects of these interactions is currently under investigation.

We believe that the LTR-blocking activity is, in part, a consequence of a broad-spectrum DING-mediated mechanism to block pathogen-induced activation of NF-κB-dependent promoters [22,25,26]. This function becomes a strategic advantage in the event of HIV disease. Nevertheless, the presence of such proteins in some opportunistic pathogens (like Pseudomonas) may interfere with this mechanism of innate immunity. In fact it has been shown that X-DING-CD4 is able to block LPS-mediated induction of NF-κB-dependent IL-8 transcription, and thus interfere with the inflammatory process [25]. That means that it is not to be excluded that bacteria may use their own DING proteins in order to block the host’s inflammatory mechanism upon invasion.

**Figure 5. Human, plant and bacterial DING proteins block replication of HIV-1 in human PBLs.** Replication of HIV-1 was assessed by measurements of HIV-1 p24 core antigen (bars, right axis) and the viability of cells was established by the dye exclusion method (lines, left axis). The results are representative of at least three separate measurements.

doi:10.1371/journal.pone.0069623.g005
Previous study showed the ability of pDING to interfere with other critical events involved in HIV-1 replication that include association of C/EBP\(b\) with the HIV-1 genome, nuclear localization of HIV-1 Tat, and phosphorylation of C-terminal polymerase by pTEF [20]. Based on extensive sequence homology, it is anticipated that the other members of the DING family can also exert activities similar to pDING on the other events that may impact LTR transcription and replication. In earlier studies, we showed that some HIV-1-infected individuals might control virus replication through the induction of X-DING-CD4 gene activity [21], and new studies indicate that expression of X-DING-CD4 mRNA is significantly higher in peripheral blood mononuclear cells (PBMCs) from elite HIV-1 controllers than in AIDS patients or uninfected controls [54]. This alone indicates that X-DING-CD4, and possibly HPBP, function as molecules of the human innate immunity system, while their counterpart, the pDING, may have a similar function in plants. The fact of pDING isolation from plant callus tissue [4] indicates co-localization of this DING protein within the injured tissue caused by pathogenic invasion.

In summary, we conclude that DING proteins form a distinctive group of highly conserved biomolecules with highly redundant properties, some of which are directed to protective anti-microbial function across the species. Four members of this family block HIV-1 transcription and their role in cellular innate immunity responses needs further investigation. DING proteins are also an attractive target for drug development, in particular because none of the existing components of antiretroviral therapy targets HIV-1 transcription.

**Acknowledgments**

We appreciate the past and present members of the Department of Neuroscience/Center for Neurovirology and the basic Science Core I of the NIMH-funded Comprehensive NeuroAIDS Center (CNAC) for preparation of highly purified cultures of primary human brain cultures and HIV-1 viral stocks as a service to the NeuroAIDS community. The CNAC offers its unique expertise to study the pathogenesis of HIV neurological diseases from both the basic science and clinical perspectives.

We thank to Galina Bentsman for technical support in performing the p24 Elisa.

**Author Contributions**

Conceived and designed the experiments: KK SA EC KS MS. Performed the experiments: RS ND DG AD AS. Analyzed the data: KK EC MS. Contributed reagents/materials/analysis tools: KK EC KS MS. Wrote the paper: MS. Edited the manuscript: KS.

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**Figure 6. Human, plant and bacterial DING proteins block the recruitment of p65-NF-\(\kappa\)B to HIV-1 LTR.** (A) The p65 NF-\(\kappa\)B occupancy on HIV-1 LTR was tested in U87MG cells exposed to four DING proteins. The 300 bp HIV-1 LTR fragment was amplified with a pair of primers used for detection of NF-\(\kappa\)B binding. (B) the optical density of PCR amplicons was tested by the Adobe Photoshop software and ImageJ analysis Program. The (input) represents total amount of HIV-1 LTR DNA before the immunoprecipitation; (IP:p65 NF-\(\kappa\)B) – represents the amount of p65 NF-\(\kappa\)B/LTR complex in cells treated or not (Mock) with each DING protein. (IP: mouse serum) – indicates immunoprecipitation with a non-specific serum. Data were analyzed using Excel software. The results are representative of at least two separate experiments.

doi:10.1371/journal.pone.0069623.g006
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