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RESEARCH ARTICLE

Interaction of Human Immunodeficiency Virus-1 Vpr and Glucocorticoid Receptor using Bimolecular Fluorescence Complementation (BiFC) analysis

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ABSTRACT

Human Immunodeficiency Virus-1 Vpr, a nonstructural protein incorporated into virus particles, possesses several features contributing to virus replication and cytopathic effects. Vpr induced effects are mediated through its interactions with viral and/or host cellular proteins, in particular, the host protein, Glucocorticoid Receptor (GR). Though GR is known to increase Vpr-mediated HIV-1 transactivation, Vpr-GR interaction and its subcellular localization have not been studied in cells. Towards this, we evaluated Vpr interaction with GR in cells using Bimolecular Fluorescence Complementation analysis by generating chimeric Vpr or GR with the N- and C-terminal fragments of Venus protein. Our results showed that interaction between Vpr and GR requires certain Vpr and GR domains. Specifically, leucine residues in the third helical domain of Vpr and N-terminal domain of GR is involved in Vpr-GR interaction. Altering these residues not only interferes with Vpr-GR interaction, but also prevents translocation of this complex into the nucleus. Further, utilizing a mutant Vpr unable to oligomerize, we show that Vpr oligomerization is essential for optimal interaction with GR. In conclusion, by taking advantage of BiFC system, specific residues in Vpr have been found to be associated with binding GR and the subcellular distribution of Vpr-GR complex.

Keywords: HIV-1, Vpr, Glucocorticoid Receptor, BiFC

Introduction

The Human immunodeficiency virus (HIV-1) *vpr* gene encodes a protein of 96 amino acids with a predicted molecular weight of 15 kDa, which is conserved in HIV and SIV (1). Vpr is packaged into assembling virions by binding to the p6 domain of viral $Pr55^{Gag}$ precursor protein. The presence of Vpr in the virus particles enables its participation in the translocation of the preintegration complex (PIC) into the nucleus for successful infection of primary monocytes/ macrophages and other non-dividing cells (2-4). While Vpr is important for viral replication in non-dividing cells (4-7), increased viral replication in T-cell lines as well as activation in latently infected cells have also been reported (8, 9). Vpr transactivates HIV-1 LTR moderately and is suggested to be the basis for increased virus replication compared to Vpr-deleted virus in target cells, specifically, T-cells and macrophages (10-12). It has been suggested that many of the functions of Vpr are likely to be regulated by host cellular factors acting as cofactors as demonstrated for the establishment of infection and replication of HIV-1 (13-15). Analysis of viral sequences using cells/tissues isolated from infected individuals who did not progress to AIDS [i]within a short time (referred as long term non progressors) and nontransmitters (e.g. HIV positive mothers who did not transmit virus to their infants) indicate that nonfunctional Vpr (containing deletions, mutations) results in loss and/or delayed of pathogenesis (16-20). Together, these studies support a role for Vpr in optimal virus replication and disease induction.

HIV-1 virus associated proteins have been shown to interact with host cellular factors and modulate virus replication during infection (21-23). HIV-1 Vpr and host protein interactions are complex and several host cellular proteins are identified as Vpr interacting partners (24). Many of HIV-1 Vpr interacting proteins are linked to its cell cycle and/or apoptotic functions and virus replication (25-27). Recently we and others have shown that Vpr, either in the context of virus particles or as an extracellular protein, transactivates HIV-1 LTR and upregulates viral replication prior to the *de novo* synthesis of Tat in infected cells. Specifically, HIV-1 Vpr-mediated transactivation is shown to occur through steroid receptors and other coactivators (28, 29). Steroid receptors belong to a super family of ligand-dependent transcription factors that are known to interact with proteins containing the signature motif, LxxLL (30, 31). Upon binding to their ligands, steroid receptors translocate into the nucleus and activate/repress transcription depending on the signal (32, 33). HIV-1 Vpr forms a complex with Glucocorticoid Receptor (GR) and p300/CBP coactivators and activates transcription through the Glucocorticoid Response Element (GRE) (34, 35). The Vpr-mediated activator/coactivator function requires interaction of Vpr with its cellular partner, GR. As a coactivator of GR, Vpr interacts with TFIIB and TFIID within the transcription initiation complex (36). More recently, Vpr released from the infected cell is known to cause PARPgamma-GR regulation and disrupt cell cycle control to induce adipose dysfunction and hepatosteatosis (37, 38). Thus, Vpr-GR interaction may result in high virus production through transcriptional regulation and has a role in virus replication in resting PBMCs was reported (39, 40). Together, these studies indicate an important role for Vpr-GR interaction in HIV-1 life cycle.

Direct interaction of Vpr with GR has been demonstrated by using standard immunoprecipitation methods (41, 42). However, the domains of GR involved in binding to Vpr are unknown. Thus, the goals of this manuscript are to identify the regions in GR involved in interacting with HIV-1 Vpr and to further understand whether this interaction results in functional outcome including nuclear translocation of HIV-1 Vpr-GR complex. Using BiFC system, we detected the interaction of two proteins via flow cytometry analyzed subcellular localization of the interaction between Vpr and GR and elucidated the domains of GR required for interaction with Vpr. Further, we showed that oligomerization feature of Vpr is critical for interaction and translocation of Vpr-GR complex.

MATERIALS AND METHODS

Cells

HeLa and HEK293T cells were grown in DMEM supplemented with 10% FCS, 1% glutamine and 1% penicillin-streptomycin.

Plasmids

Human Glucocorticoid receptor alpha (hGR□) and the deletions mutants were generated using GR specific primers and cloned into pcDNA 3.1 (InVitrogen, CA) with flag tag at the N-terminus. All the constructs were sequenced, and the integrity of the mutations/deletions was confirmed. Vpr expression plasmids were generated as described (43). Vpr mutants encoding single amino acid substitutions in the $L^{64}XXLL^{68}$ motif (L64E, L67E, L68E) were generated via mutagenesis. For BiFC assays, sequences encoding the amino (residues 1 to 173, VN) or carboxyl (residues 155 to 238, VC) fragments of Venus fluorescence protein (template

provided by Dr. Ronald Montelero, University of Pittsburgh) were fused to the N terminus of GR full length, GR mutants or HIV-1 Vpr via a six-alanine linker as described (44).

Interaction of Vpr and GR in cells

Interaction of Vpr-GR in cells was evaluated by BiFC methods. HeLa cells were seeded in 6-well plates with glass cover slips and transfected with combinations of Venus-GR and Vpr expression plasmids using Lipofectamine (Life Technologies, NY). Forty hours post-transfection, glass cover slips were removed, cells were washed with PBS and fixed with 2% paraformaldehyde. Transfection efficiency for subsequent experiments was controlled via cotransfection with a β-galactosidase expression vector and β-galactosidase expression was assessed using the β-Glo kit (Promega, WI). Transfection efficiencies were normalized across multiple wells within each experiment. Cells were mounted with Vectashield containing DAPI (Vector Laboratories, CA) and fluorescence was detected using Nikon inverted fluorescence microscope with appropriate filters.

Flow Cytometry

The BiFC interaction of Vpr-GR was analyzed through flow cytometry. HeLa or HEK293T cells were seeded (300,000) on six-well plates and transfected with wild type- or mutant BiFC-Vpr expression plasmids (1µg of each BiFC partner) using Polyjet® reagent (Signagen MD) as per manufacturer's protocols. Thirty-six hours post-transfection, the cells were washed with PBS, fixed with 4% paraformaldehyde for 10 minutes, washed again with PBS, and then resuspended in 2% FCS in PBS for subsequent analysis through flow cytometry. The BDFACSCanto II cytometer and FACSDiva software (BD Biosciences, NJ) were used to analyze the percent positive cells.

Westernblot

Cells (HEK293T) were seeded on 6-well plates and transfected with 1 µg each of Vpr and GR BiFC constructs. Cells were lysed with RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Triton-X-100, 1% NP-40, 0.1% SDS, and SIGMAFAST™ protease inhibitor from Sigma). Samples were electrophoresed by SDS-PAGE under reducing conditions, and transferred to PVDF membrane (Millipore, MA). Membranes were blocked with 5% nonfat dry milk in PBS-T (140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 0.1% Tween-20) at room temperature for an hour, and then incubated with either anti-flag (1:1000), anti-HA (1:5000) or anti-Tubulin (1:1000) for overnight at 4°C followed by incubation with HRP-labeled goat anti-mouse IgG (1:10000) for 90 min. All membranes were visualized using ECL substrate (Thermo Scientific, IL).

RESULTS

Construction and characterization of chimeric HIV-1 Vpr and GR expression plasmids. *In vitro* studies utilizing biochemical and biophysical methods have shown that HIV-1 Vpr and GR interaction is specific (28, 29). However, very little is known about the subcellular localization or the intracellular trafficking of Vpr-GR complexes due to the limitations of the techniques used. For this reason, we performed Vpr-GR interaction using bimolecular fluorescence complementation (BiFC) method as described previously (45, 46). Briefly, Vpr and GR were fused with venus-N and venus-C terminal fragments as fusion proteins with an alanine linker, also containing a hemagluttinin tag for expression analysis, separating the proteins (Fig. 1A). These constructs were characterized for the expression of appropriate chimeric proteins by specific antibodies using transiently transfected 293T cell lysates through immunoblot method (Fig. 1B). Results indicate that both VC/VN-GR and VC/VN-Vpr expressed appropriate chimeric proteins, whereas cells transfected with control vector did not show any band[ii]. Furthermore, cotransfection of VN and VC plasmids expressing Vpr, or GR did not affect the expression of either of the proteins (Fig. 1B). To address the possibility of variable transfection efficiency, these plasmids were also cotransfected with a β-galactosidase expression vector; enzyme activity was quantified and found to be equal across all BiFC-Vpr and BiFC-GR constructs (data not shown).

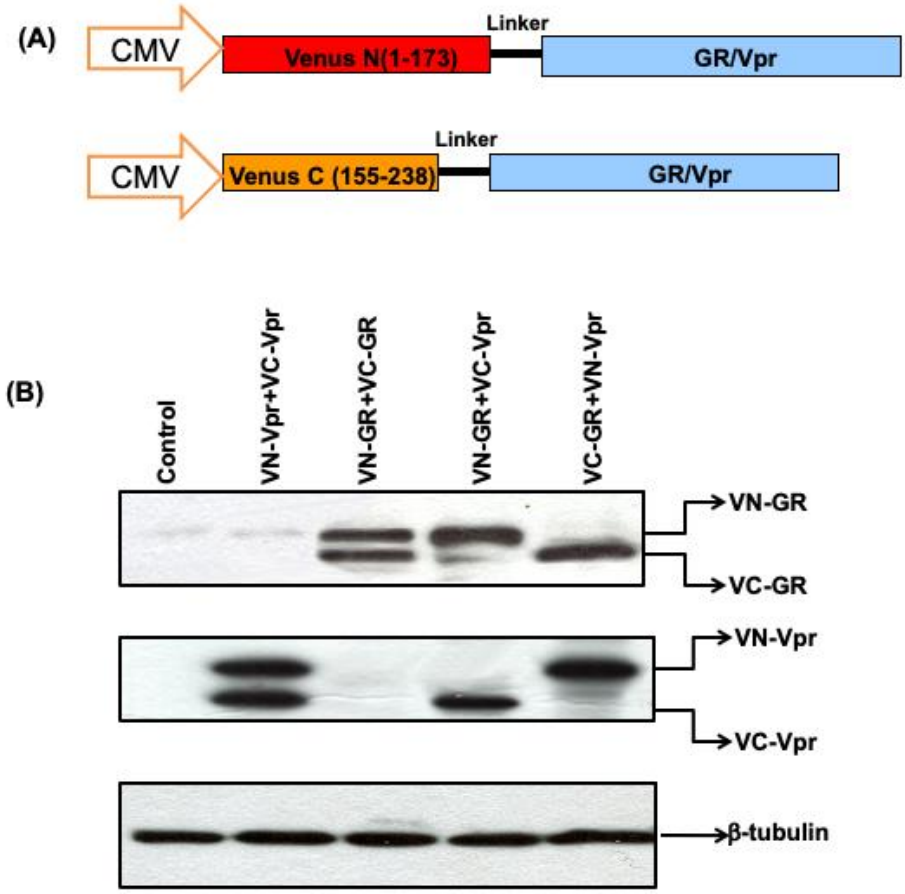


Figure 1

Figure 1. Construction and characterization of Vpr and GR plasmids. (A) Schematic representation of Vpr and GR fused with Venus-N terminal (VN) and Venus-C-terminal (VC) fragments, respectively. **(B)** Expression of chimeric Vpr and GR proteins was assessed in HEK293T cells by transient transfection. HEK293T cells were co-transfected with combinations of Vpr and GR expression plasmids or Vector control plasmid, and assessed by Western blot. Tubulin was used as a loading control. Results represent one out of three independent experiments (n=3).

Interaction and subcellular distribution of HIV-1 Vpr and GR by BiFC: Interaction of Vpr and GR releases GR from hsp70 subunits and results in translocation of Vpr-GR complex into the nucleus (47-49). First, we assessed the physical interaction of Vpr and GR in the presence of other cellular proteins by flow cytometry. Presence of BiFC signal was quantitated in cells transfected with VC and VN combinations of Vpr and GR or with control vector plasmid (Fig. 2A). Results indicate that specific interaction (BiFC positive signal) of Vpr and GR was detected in cells cotransfected with chimeric GR and Vpr plasmid, whereas transfection of a combination of control and chimeric plasmids did not show any BiFC signal as measured by flow cytometry. For instance, 38 and 21% of the cells are BiFC positive in cells transfected with VN-Vpr and VC-GR or VC-Vpr and VN-GR, respectively, whereas VC-GR with control VN or VC plasmid showed baseline signal (~3%). Similar results were observed in HeLa and HEK293T cells (data not shown) suggesting that Vpr-GR interaction is specific and is not cell type dependent.

Next, to determine the sub-cellular localization of Vpr-GR complex, we evaluated the subcellular distribution of BiFC signal resulting from interaction in cells by fluorescence microscopy (Fig. 2B). The results showed cytoplasmic distribution of GR in cells cotransfected with VC-GR and VN-GR similar to cells that are transfected with GR expression plasmid and endogenous GR (50). This suggests that GR in the absence of Vpr forms a homodimer in the cytoplasm as reported previously (32, 51, 52). On the other hand, Vpr showed nuclear localization in cells transfected with VC-Vpr and VN-Vpr plasmids. Interestingly, BiFC signal resulting from Vpr-GR complex was observed in the nucleus. Control plasmid (pcDNA3.1) transfection with either VC-Vpr or VC-GR and vice versa did not show signal indicating specificity. These results indicate that Vpr-GR interaction is specific, independent of VC/VN chimeric molecules and this

interaction results in translocation of Vpr-GR complex into the nucleus.

Figure 2

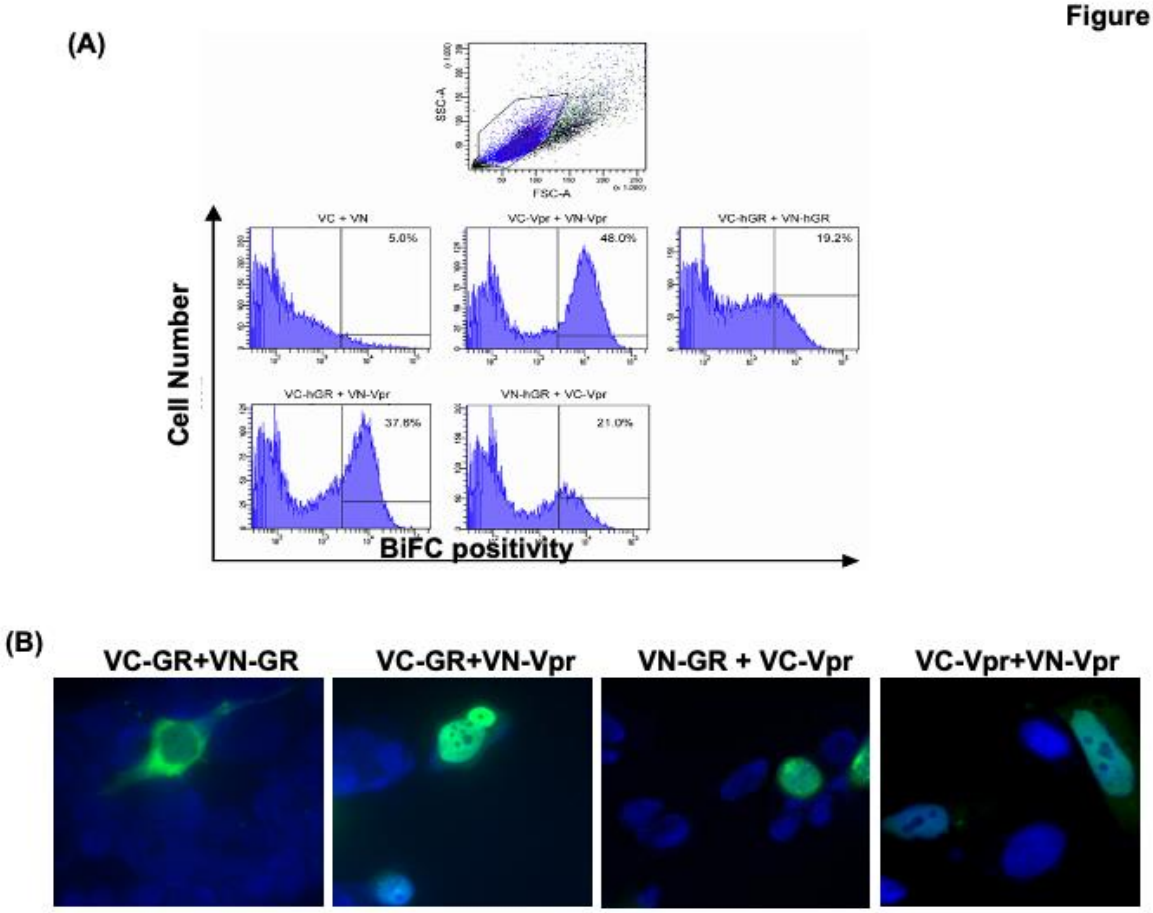


Figure 2. Demonstration of Vpr-GR interaction. (A) Quantitative analysis by flow cytometry of Venus fragment complementation in HeLa cells co-transfected with VC-Vpr and VN-Vpr, VC-Vpr and VN-GR, VN-Vpr and VC-GR, VC-GR, and VN-GR or with control plasmid (VC and VN). Thirty-six hours post-transfection, cells were harvested and analyzed by flow cytometry to determine the percentage of cells positive for BiFC fluorescence. Results represent one of five independent experiments. **(B)** Subcellular localization of the BiFC complex. HeLa cells grown on glass coverslips were cotransfected with VC-Vpr and VN-GR, VN-Vpr and VC-GR, VN-GR, and VN-GR, VN-Vpr and VC-Vpr or with control plasmid pairs. Thirty-six hours post-transfection, cells were fixed, stained with DAPI, and imaged at 60X magnification. Scale bars represent 50 microns. Green, BiFC; Blue, Nucleus. Figure represents one of three independent experiments with similar results (n=3).

Required domain(s) of Vpr for interaction with GR and translocation of GR into nucleus. Previous studies showed that LxxLL domains in helix I and helix III of Vpr play a role in Vpr-GR coactivation and interaction (28, 29). Using BiFC assay, we further assessed the ability of site-specific mutants in helix III to form dimers (Fig. 3A; Table 1). Results indicate that mutants containing substitution of E (glutamic acid) for L exhibited a reduced ability to form dimers. Specifically, mutation at residues 64, 67, and 68 resulted in 9, 22, and 18% BiFC, respectively, compared to 64% BiFC positive cells noted with VC/VN-Vpr^{wt} constructs transfected cells. Additionally, the mean fluorescence intensity (MFI) of the cell population is reduced in BiFC-Vpr mutant pairs, L64E, L67E, and L68E compared to BiFC-Vpr wild type.

Table 1: Mutations in the third helical leucine motif inhibit dimerization of Vpr.

Vpr Mutants			
VC-Vpr	**VN-Vpr**	BiFC Positive Cells (%)	Mean fluorescence intensity (MFI)
Wt	Wt	61.73 ± 4.49	13710.33
L64E	L64E	9.67 ± 4 73	207.67
L67E	L67E	22 ± 6.28	1107.33
L68E	L68E	18.73 ± 6.58	927.67

HEK293T cells were cotransfected with VC-Vpr and VN-Vpr constructs expressing mutations in the LxxLL motif and analyzed by flow cytometry. BiFC percentage and Mean Fluorescence Intensity (MFI) reported as raw values. Results represent the mean of three independent experiments (n=3).

Next, we evaluated the effect of leucine mutants on the interaction with GR by measuring the BiFC signal in cells transfected with VC/VN-Vpr mutants and VN/VC-GR constructs (Table 2). Results indicate that BiFC signal in cells transfected with VC-GR^{wt} and VN-Vpr -L64E, -L67E or -L68E resulted in a reduced number of BIFC positive cells as well as a reduction in overall MFI of the measured cell population compared to VN-Vpr^{wt}. Considering BiFC positive cells as 100% with Vpr^{wt}, the mutant L64E, L67E and L68E exhibited 16, 29, 27% cells positive for BiFC, respectively. Similar results were observed in cells transfected with VN-GR^{wt} and VC-Vpr mutants (Table 2). A reduction of at least 35% BiFC positive cells in Vpr helix III mutants compared to Vpr^{wt} was observed in multiple experiments suggesting that helical domain III plays a major role in Vpr-GR interaction in cells.

Table 2: Mutations in the third helical leucine motif of Vpr inhibit interaction with GR.

Vpr Constructs	**BiFC positive cells (%) compared to Vpr^{wt}**		**Mean fluorescence intensity (MFI)**	
	VC-GR^{wt}	**VN-GR^{wt}**	**VC-GR^{wt}**	**VN-GR^{wt}**
VN-Vpr^{wt}	100	-	18191.33	
VN-Vpr^{L64E}	15.70 ± 8.29	-	1316.67	
VN-Vpr^{L67E}	29.23 ± 10.67	-	5527.33	
VN-Vpr^{L68E}	27.07 ± 12.83	-	6639.67	
VC-Vpr^{wt}	-	100		6757.67
VC-Vpr^{L64E}	-	38.87 ± 18.63		393
VC-Vpr^{L67E}	-	66.33 ± 17.06		831.33
VC-Vpr^{L68E}	-	65.40 ± 17.34		639.33

HEK293T cells transfected with VC-GR and VN-Vpr or VN-GR and VC-Vpr constructs were then analyzed by flow cytometry and normalized to Vpr^{wt} for % positive BiFC levels. Results represent the mean of three independent experiments (n=3).

It is not known whether mutations in the helical domain III prevent Vpr-mediated nuclear translocation of Vpr-GR complex. To address this, cells were cotransfected with VC-GR and VN-Vpr^{wt}, VN-Vpr^{LxxLL} mutants (L64E, L67E and L68E) or VN-GR and VC-Vpr^{wt}, VC-Vpr^{LxxLL} mutants (L64E, L67E and L68E) and BiFC positive cells were assessed by microscopy. Imaging results reveal that signal in

BiFC positive cells exhibited cytoplasmic distribution (Fig. 3B). Further, subcellular distribution analysis of Vpr^{LxxLL} mutants reveals cytoplasmic localization, whereas Vpr^{wt} showed nuclear (mostly) and perinuclear localization. Similar localization patterns were also observed when GR^{wt} and Vpr^{LxxLL} mutants were expressed and stained with appropriate antibodies. To rule out whether a weak signal is due to low level of Vpr mutant expression, we assessed the expression of all the transfected plasmids by immunoblot analysis (Fig. 3C). Results indicate that the expression level of Vpr mutants only slightly differs from Vpr^{wt} and does not explain the highly reduced BiFC signal above, further confirming a role for LxxLL domain in translocation of Vpr-GR complex into the nucleus.

Figure 3

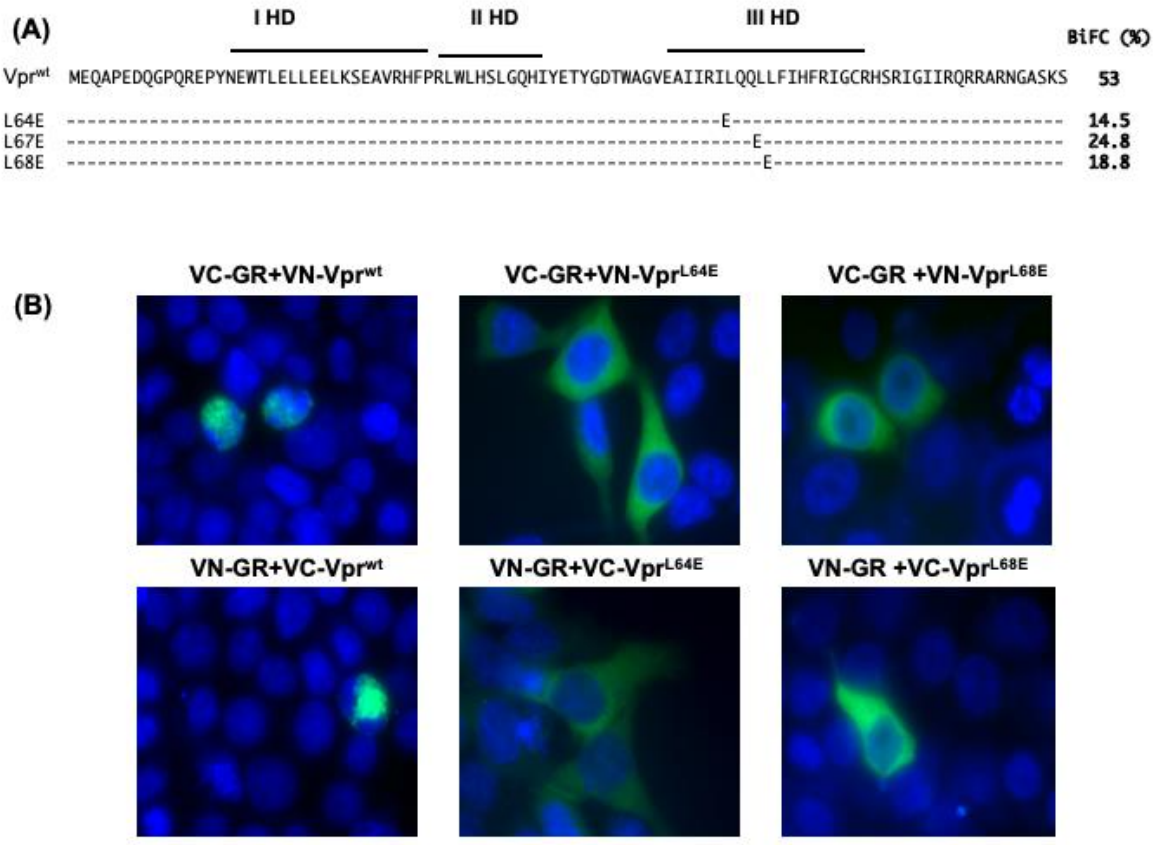


Figure 3C

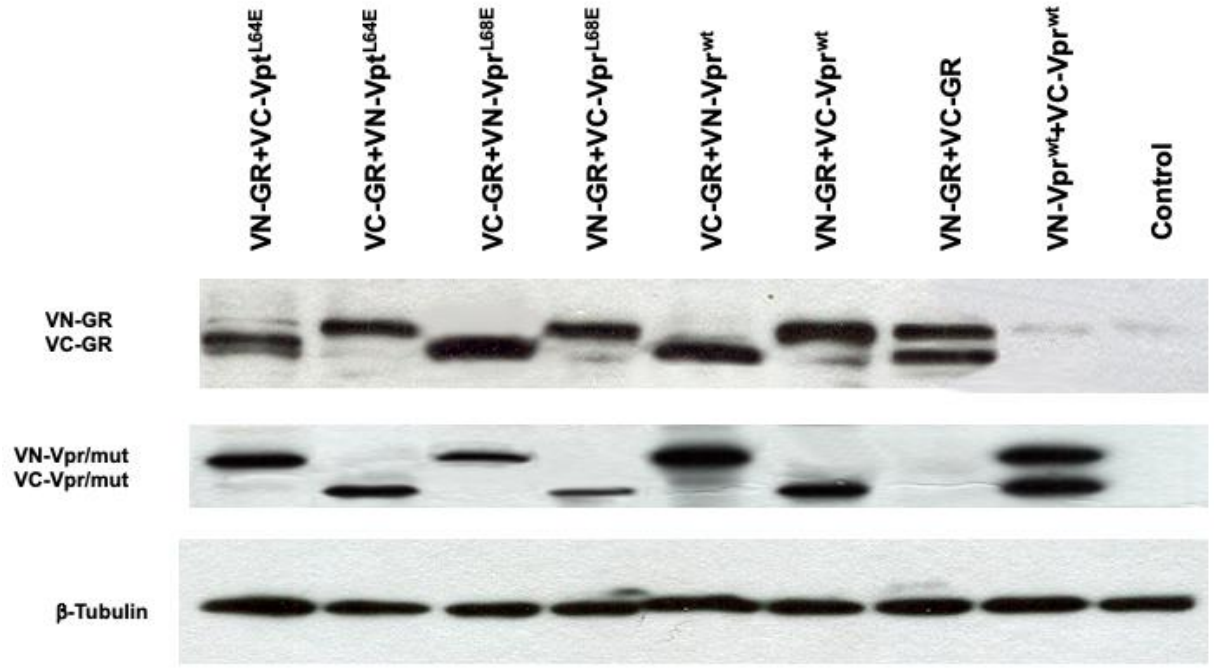


Figure 3. Vpr domains involved in Vpr-GR interaction by BiFC. (A) Schematic representation of HIV-1 Vpr third helical domain (III HD) mutants: Vpr mutants were generated by site directed mutagenesis. Vpr^{wt} represents the *vpr* gene derived from NL43 proviral DNA and the change in residues at a particular position is marked for each mutant. Mutation at position is marked with the substitution residue and the helical domains are marked as I, II and III HD above the amino acid sequence. BiFC positive cells (%) measured in cells cotransfected with VN and VC combination of Vpr constructs is presented. **(B)** Subcellular localization of the BiFC complex with Vpr mutants and GR. HeLa cells grown on glass coverslips were co-transfected with VN-Vpr mutants and VC-GR or VC-Vpr mutants and VN-GR or with control plasmid pairs. Post-transfection, cells were fixed, stained with DAPI, and imaged at 60X magnification. Scale bars represent 50 microns. Green, BiFC; Blue, Nucleus. **(C)** Immunoblot analysis of expression of Vpr mutants. 293T cells were co-transfected with 2 µg of Vpr and GR plasmids. Post-transfection cells were lysed and equal amounts of protein containing total cell lysates were resolved in 12% SDS-PAGE and immunoblotted with HA specific antibody (1:5000 dilution). Vpr mutant constructs representing the residues are marked on top of each lane. The control represents 293T cells transfected with the pcDNA3.1 vector backbone.

Domains of GR required for interaction with Vpr. Although it is well established that GR interacts with Vpr at the LxxLL domains, the domains of GR required for interaction with Vpr are unknown. To identify the GR domains involved in Vpr interaction, several GR deletion mutants were generated as chimeric molecules fused with venus C and venus N terminal fragments (Fig. 4A). Protein expression and stability of GR mutants were assessed by immunobloting with HA antibody (Fig. 4B). Such an analysis indicated that all mutant GR molecules showed an appropriate band corresponding to the calculated molecular weight protein. The additional bands in Fig. 4B indicate the presence of breakdown products which likely occur as a result of cellular processing. The specificity of the antibody was further confirmed by using the lysate of vector plasmid transfected 293T cells, which did not recognize any band (Fig. 4B, first lane). These results indicate that the steady state level of all the GR mutant molecules is comparable. We also assessed the dimerization and localization of GR mutants by cotransfecting VC and VN chimeric GR mutant molecules by BiFC analysis (Table 3; Fig. 4C). The results indicate that chimeric GR mutants 262 (VC-GR^{262} and VN-GR^{262}), and GR- 488 (VC-

GR^{488} and VN-GR^{488}) exhibited dimerization, albeit reduced compared to Venus-GR^{wt}. In particular, VC-GR^{262} and VC-GR^{488} mutants coupled with VN-Vpr^{wt} produced 24% and 31% BiFC signal whereas VC-GR^{wt} produced 44%. A similar trend was observed for the VN-GR mutant and VC-Vpr pairs. Their localization patterns occurred in the cytoplasm similar to venus-GR^{wt}. However, chimeric GR-LBD domain did not show any BiFC signal, suggesting that N-terminal of GR is required for GR dimerization.

Table 3: GR domains involved in GR-Vpr interaction.

GR mutants Used	**BiFC positive cells (%)**		**Mean fluorescence intensity (MFI)**	
	VC-Vpr^{wt}	**VN-Vpr^{wt}**	**VC-Vpr^{wt}**	**VN-Vpr^{wt}**
VN-GR^{wt}	49.6	-	3900	-
VN-GR262	12.9	-	350	-
VN-GR 488	40.7	-	1727	-
VN-GR-LBD	16.7	-	413	-
VC-GR^{wt}	-	47.5	-	9883
VC-GR262	-	47.2	-	7373
VC-GR 488	-	43.6	-	5539
VC-GR-LBD	-	9.8	-	333
Vector control	2		231	

HEK293T cells were cotransfected with VC-GR mutants and VN-Vpr wt and vice versa and Vpr-GR interaction was measured by BiFC signal by flow cytometry. BiFC percentages. Results represent one of four independent experiments (n=4).

Upon expression, GR is localized in the cytoplasm and interaction of GR with Vpr results in nuclear translocation. To identify the domains of GR involved in Vpr interaction and its subsequent translocation, cells were transfected with VC-Vpr^{wt} with VN-GR^{wt} (Fig. 4D). The cells transfected with Vpr and GR^{262} and GR^{488} resulted in BiFC positive cells, whereas Vpr and GR^{LBD} transfected cells did not show BiFC positive cells. Similar results were observed with VN-Vpr^{wt} and VC-GR mutants (data not shown), suggesting that the interaction is specific. Furthermore, we also evaluated the localization of BiFC signal in transfected cells. It was observed that the complex formed by Vpr and GR mutants localized in the cytoplasm, whereas Vpr-GR wild type exhibited nuclear localization. Together these results suggest that GR-N terminal domain is required for Vpr-GR interaction and its subsequent nuclear translocation.

Figure 4

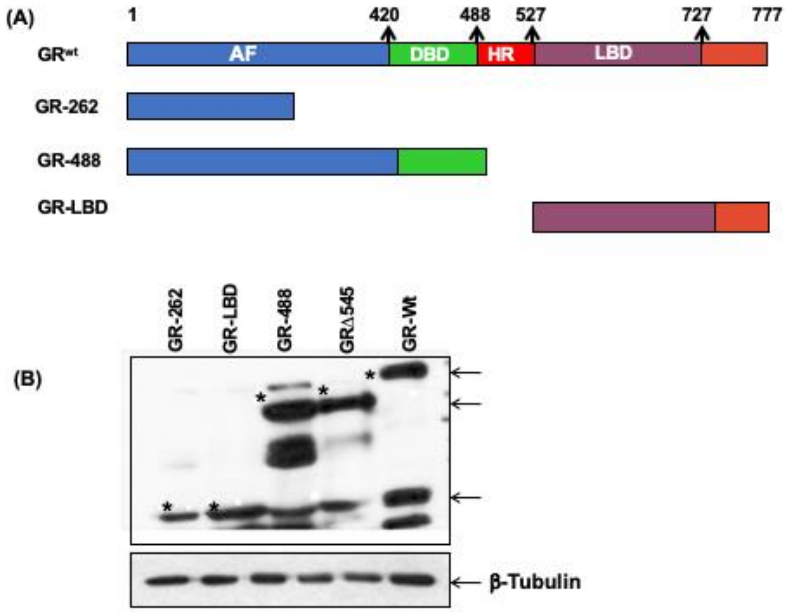
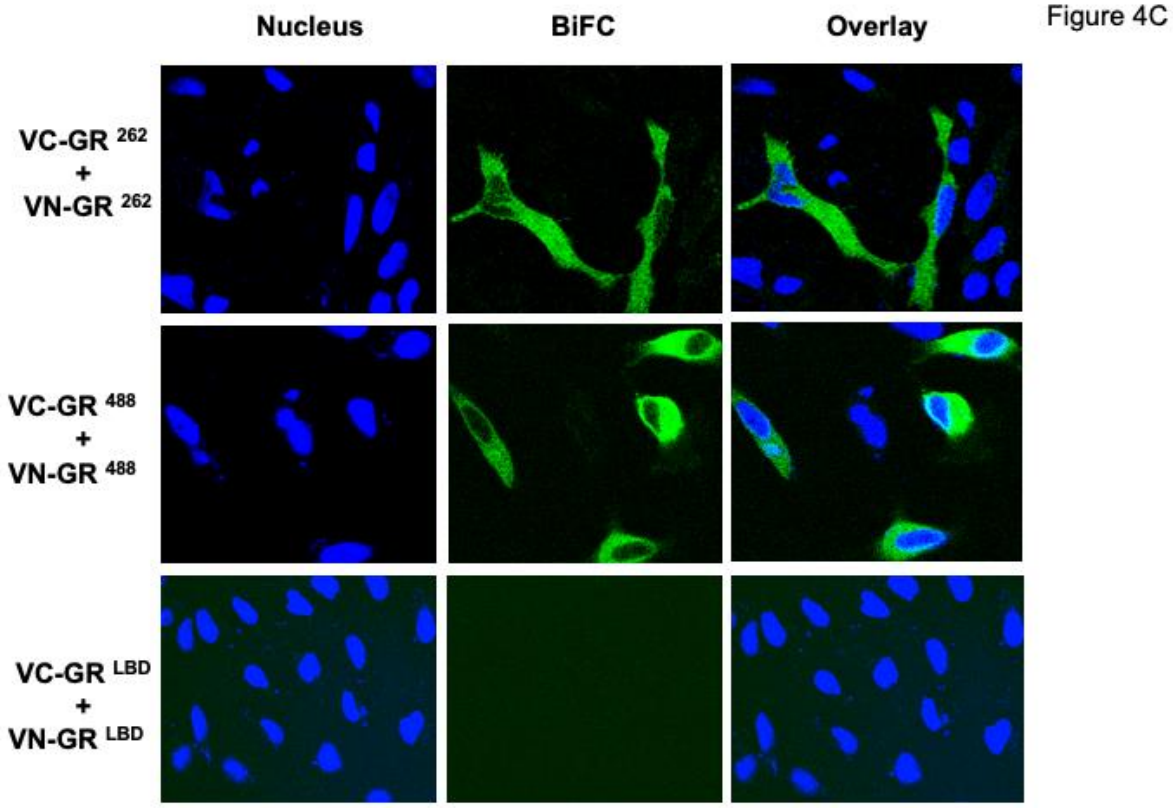
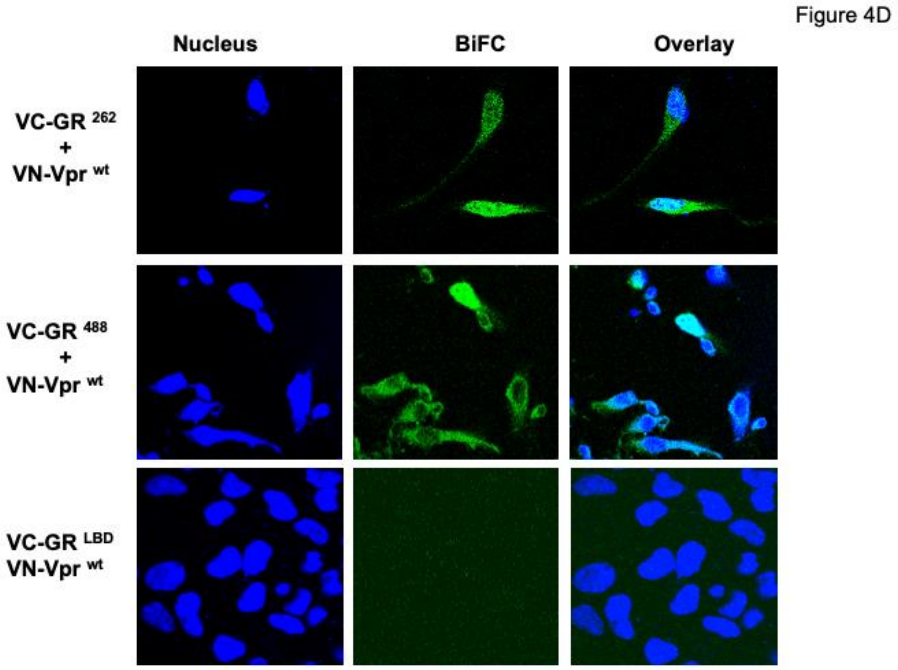


Figure 4C





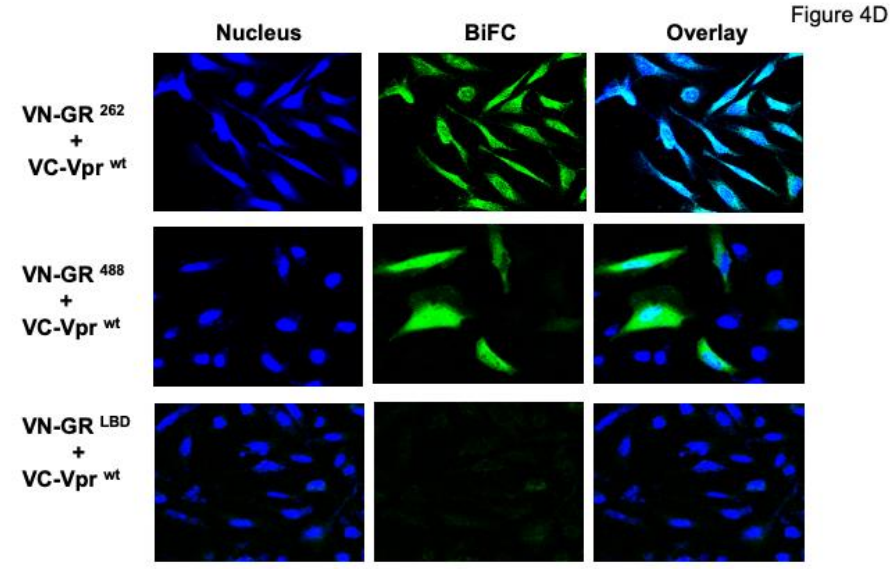


Figure 4. Construction and characterization of GR mutants and their interaction with Vpr by BiFC. (A) Schematic representation of domains in full length GR and GR deletion mutants. Deletion mutants are generated by PCR and fused with VC or VN to generate the chimeric molecules as described in methods. **(B)** Immunoblot analysis of expression of GR mutants: 293T cells were transfected with 3 µg of GR mutant plasmids and 48 hours post-transfection cells were lysed and equal amounts of protein containing total cell lysates were resolved in 8% SDS-PAGE and immunoblotted with HA antibody (1:5000 dilution). Specific bands are marked with * and □-Tubulin was detected as a loading control. **(C)** Subcellular localization of the BiFC complexes. HeLa cells grown on glass coverslips were co-transfected with VN-GR and VC-GR mutants to identify their ability to form dimers and their subcellular distribution. 36 hours post-transfection, cells were fixed, stained with DAPI, and imaged at 60X magnification. It is noted that the cells in the upper panel appear to be undergoing mitosis, which is indicative of their shape. **(D)** Ability of VC-Vpr^{wt} and VN-GR mutants to interact and form BiFC complex. HeLa cells were transfected with combinations of VC-Vpr^{wt} and VN-GR mutants and assessed by the fluorescence microscopy for BiFC positivity. Scale bars represent 50 microns. Cells were stained with DAPI to identify the nucleus. Green, BiFC; Blue, Nucleus.

Role of oligomerization of Vpr in Vpr-GR interaction. It is not clear whether oligomerization of Vpr is essential for the functions mediated by Vpr including binding to viral and cellular proteins. Hence, we asked the question whether oligomerization of Vpr is critical for binding to GR. For this purpose, we selected oligomerization negative Vpr mutant A30L and E48A for generating chimeric protein with the addition of VN or VC fragments of Venus protein. Cotransfection of a combination of VC-Vpr^{A30L} and VN-GR or VN-Vpr^{A30L} and VN-Vpr^{E48A} and VC-GR resulted in the detection of BiFC signaling cells. However, the percent of cells showing the signal was reduced by 50% for HEK 293Ts cotransfected with VC-GR and VN-Vpr constructs and 60% for the cotransfection with the complementary BiFC pair in comparison to the wild type Vpr and GR (Figure 5). Results indicate a reduction in BiFC positive cells as well as lack of nuclear translocation of BiFC complex suggesting that optimal binding to GR and nuclear translocation are facilitated by the oligomerization feature of HIV-1 Vpr.

Figure 5

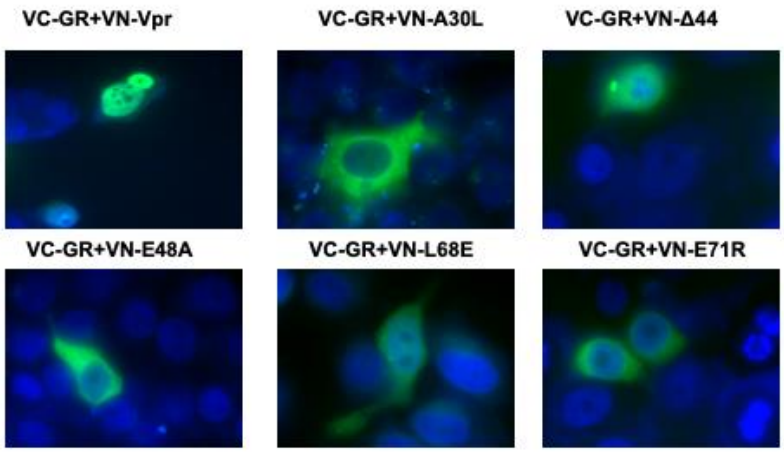


Figure 5. Interaction of oligomerization deficient Vpr mutants with GR is diminished and prevented translocation into the nucleus. pVC-GR^{wt} and pVN-Vpr mutant (VN-A30L, VN-Δ44, VN-E48A, VN-L68E and VN-E71R) constructs were transfected into HEK293T cells and BiFC formation and translocation was assessed by fluorescence microscopy. Figure represents one of three independent experiments (n=3) with similar results.

Discussion

HIV-1 utilizes the components of the host cellular machinery for effective replication and immune evasion (53, 54). Many of these functions are mediated through specific interaction of HIV-1 viral proteins with the proteins of host cells (55, 56). Several laboratories including ours have previously shown that GR is one of the host cellular proteins interacting with Vpr. Importantly, the interaction between Vpr and GR has been beneficial to HIV-1 as it leads to the activation of transcription from HIV-1 and cellular gene promoters. The interaction between these proteins has been demonstrated by pull-down and/or immunoprecipitation assays (28, 29). An obvious limitation with these approaches is the ability to follow the downstream events such as trafficking of the complex following interaction in the cells. This has prompted us to consider strategies for the analysis of the interaction between Vpr and GR and the cellular distribution of this complex in cells. For this purpose, we have utilized BiFC assay in which the candidate proteins (Vpr and GR) are generated as fusion proteins with N- and C-terminal fragments of Venus protein.

HIV-1 Vpr is primarily located in the nucleus and nuclear rim upon expression (57-59), whereas, GR under normal conditions (in the absence of its ligands) is present exclusively in the cytoplasm as a homodimer in combination with heat shock protein 70 (hsp70) (51, 60). HIV-1 Vpr is known to interact with several host cellular proteins and regulate cellular functions. Vpr contains multiple leucine-rich domains (LxxL) in addition to the LxxLL motif in the C-and N-terminal region of the protein. These helical regions are known to interact with its binding partners. HIV-1 Vpr mutational studies have shown that amino acids in third helix and first helix are required for Vpr interaction with GR (28, 29).

Oligomerization is an intrinsic feature of Vpr, which has been demonstrated by utilizing biochemical and live cell approaches (44, 61, 62). Vpr shares this feature along with other HIV-1 encoded proteins, in particular, Vif, Vpu, and Nef (63-65). In an earlier study, we utilized site-specific mutagenesis approaches to analyze the requirement of several residues in Vpr for oligomerization. It was further shown that Vpr lacking oligomerization also failed to be incorporated into the virus particles. Results presented here (figure 5) further suggest that Vpr oligomerization feature is required for translocation of Vpr-GR complex. Formation of BiFC complex with GR and Vpr mutants could be the result of interaction of a single Vpr molecule with GR molecule however, translocation of GR-Vpr complex required Vpr dimeric and/or multimeric forms of Vpr. Functional consequences of Vpr-GR complex require translocation of this complex into the nucleus where transactivation or trans repression of gene expression occurs, thus Vpr oligomerization is an important feature in Vpr-GR mediated effects in host cells.

The results presented here provided evidence in support of Vpr-GR interaction in cells by BiFC analysis, and the importance of the LxxLL motif in the third helical domain of Vpr. Due to our use of BiFC we were able to observe that Vpr-GR interaction results in translocation of Vpr-GR complex into the nucleus in the absence of its natural ligand dexamethasone. Translocation of GR into nucleus is a well-controlled event as GR is held in the cytoplasm by hsp70 and only ligand binding to GR alters the confirmation and frees GR from hsp70, allowing GR translocation (66). Human Glucocorticoid receptor alpha (hGRa) display a modular structure comprised of the amino-terminal A/B region, also called immunogenic or N-terminal domain (NTD), and the C and E regions, which correspond to the DNA- (DBD) and ligand-binding (LBD) domains. These domains are involved in ligand binding, activation, translocation, and activation/repression of gene expression. Using mutants representing various domains of GR, we have identified that GR AF region (1-262 aa) and AF and DBD (1-488) is essential for dimerization, whereas, LBD domain alone is not enough for GR dimerization, suggesting that amino acids 1-488 plays an important role.

Results from BiFC analyses using GR mutants with Vpr^{wt}, indicate that Vpr mimics GR ligands by binding to the N-terminal domain containing the AF and DBD of GR. However, translocation of Vpr-GR occurred with GR mutant 488, whereas the complex remained in the cytoplasm with GR 262, suggesting GR regions spanning aminoacids 262-488 is essential for translocation of Vpr-GR complex into the nucleus. It is not clear whether deletion of this region changes the confirmation and/or exposure of the nuclear location signal (NLS) that requires for translocation. Additional mutants and structural studies are required to identify the role of this domain, which is beyond the scope of this manuscript. Similarly, the inability of GR to form dimers in the absence of the LBD might also alter Vpr-GR interaction. However, additional site-specific mutational studies are required to confirm these predictions.

In our study, we have corroborated previous findings of the interaction between Vpr-GR, and additionally reveal the localization of these interactions. Several Vpr interacting proteins fall in this category further strengthening its role as a regulator of viral and host cellular transcription. As HIV-1 Vpr is associated with the virus particles, Vpr is also available to host cells prior to *de novo* synthesis following infection, implying an immediate early role for this protein in HIV-1 infection (1, 67). Studies have shown that Vpr alters both viral and cellular events by hijacking the glucocorticoid receptor and its normal functional pathway, among other host cellular proteins. Thus, the viral-host cellular complexes may serve as potential targets for combating virus infection and disease progression. Understanding the role of host cellular factors and their interaction with viral proteins will provide additional targets for therapeutic approaches.

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