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HIV-1 auxiliary regulatory protein Vpr promotes ubiquitination and turnover of Vpr mutants containing the L64P mutation

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Abstract The auxiliary regulatory protein Vpr of HIV-1 possesses several biological activities which are believed to facilitate HIV-1 replication and pathogenesis. In this report, experimental evidence suggests a novel biological activity of Vpr: facilitation of the turnover of Vpr mutants bearing the L64P mutation. This novel activity of Vpr was shared by Vpr molecules from different subtypes of HIV-1. Co-expression of the wild type Vpr with the VprW54A/L64P mutant resulted in normal synthesis of the mutant mRNA but enhanced ubiquitination and turnover of the mutant protein. These results suggest that Vpr may interact with the ubiquitin/proteasome pathway to regulate the stability of viral or cellular proteins.

Key words: Vpr; Turnover; Ubiquitination; Proteasome; Apoptosis; Subtype

1. Introduction

HIV-1 Vpr is a 96-amino acid, 14-kDa auxiliary regulatory protein synthesized in the late phase of the HIV-1 life cycle and packaged into the virion through interaction with Gag [1–3]. Potential functions of Vpr during the HIV-1 life cycle include facilitation of the nuclear transport of the HIV-1 preintegration complex [4], transcriptional activation [5–9], and cell cycle arrest [10–12]. Vpr has also been shown to induce apoptosis in different cell types and transgenic mice [13–20]. In groups of so-called long-term non-progressors, mutations in Vpr are found to reduce or abolish the pro-apoptotic potential of Vpr [17,21], suggesting that the pro-apoptotic activity of Vpr may contribute to the pathogenesis of AIDS. Currently, it remains unclear which of the Vpr activities is most important for Vpr function during HIV-1 replication in vivo, and whether there is a common biochemical mechanism underlying some of the Vpr activities.

The function of Vpr appears to be most critical for HIV-1 replication in macrophages [22]. This is proposed to be due to the requirement for the active nuclear transport function of Vpr since macrophages do not have nuclear division [4]. As nuclear transport function has also been assigned to the HIV-1 integrase [23] and the MA component of Gag [24], it remains possible that Vpr has an as yet undefined unique mechanism of function for HIV-1 replication under in vivo conditions. Under most in vitro conditions the existence of the Vpr gene enhances HIV-1 replication only modestly and therefore the mechanism of Vpr function during the HIV-1 life cycle has been difficult to discern. We recently reported that a single L64P mutation of Vpr causes a profound enhancement of the pro-apoptotic potential of Vpr [25]. In this study, co-expression of the wild type Vpr with L64P-containing Vpr mutants is shown to severely reduce the protein level of the Vpr mutants. This effect is correlated with an enhanced ubiquitination and a faster rate of turnover of the Vpr mutants in the presence of the wild type Vpr, and not due to effects on the mRNA level. This study suggests a novel biological activity of Vpr which may facilitate the identification of a unique functional mechanism for Vpr.

2. Materials and methods

2.1. Cell culture, transfection, and Western blots

293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin/streptomycin, and transfected with Lipofectamine 2000 (Invitrogen) under conditions previously described [25]. For analysis of Vpr dimerization in vivo, 293 cells transfected for 2 days were cross-linked in vivo by incubation with 5 mM of DSS (Pierce) for 30 min before lysis of the cells for Western blot. HeLa cells were transfected with the Polyfect reagent (Qiagen) under recommended conditions. Briefly, HeLa cells were plated the day before transfection at 1.5×10^5 cells/well density. The medium was replaced with fresh medium before transfection. DNA precipitation was performed with 0.8 μg of DNA and 6 μl of the Polyfect reagent in a total volume of 50 μl at room temperature for 5–10 min. The DNA precipitates were added to cells and the cells incubated in a CO_2 incubator for 2 days before lysis for Western blot analysis. Conditions for Vpr Western blots and analysis of apoptotic DNA ladders were described before [25].

2.2. Analysis of ubiquitination

293 cells were co-transfected with 0.5 μg of the pCMV-$(HA\text{-}Ub)_8$ plasmid [26], and 0.75 μg each of pFSZ3-F-W54A/L64P and pFSZ3-Vpr. Two days after transfection, cells were treated with 50 μM of the proteasome inhibitor MG132 (Sigma) for 2 h, and then lysed with the immunoprecipitation buffer containing 50 mM Tris–HCl (pH 7.5), 1% Triton X-100, 0.5 M NaCl, 0.5 mM dithiothreitol (DTT), and the complete protease inhibitor cocktail (Roche). Cell lysates were immunoprecipitated with the Flag M2 antibody (Sigma), and the immunoprecipitates were examined by Western blot with the Flag antibody or the HA antibody [27].

2.3. Subcellular fractionation

Transfected 293 cells were harvested and incubated with a hypotonic buffer (20 mM HEPES, pH 7.5, 10 mM KCl, 2 mM $MgCl_2$, 0.5 mM DTT and 0.2 mM phenylmethylsulfonyl fluoride) for 5 min on ice. After passing through a 27G needle 10 times, cells were centrifuged for 3 min at $600\times g$ to separate the cytoplasmic fraction from the nuclear pellet. The nuclear pellet was washed once with the hypotonic buffer containing 20% glycerol. For Western blots, the cytoplas-

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mic fraction and the nuclear pellet were directly mixed with an sodium dodecyl sulfate (SDS) sample loading buffer. For radiolabeled cells, the nuclear pellet was extracted with the immunoprecipitation buffer for 30 min at 4°C. Both the cytoplasmic and the nuclear fractions were immunoprecipitated with the Flag antibody and analyzed.

2.4. DNA cloning

Expression constructs for Vpr, VprL64P, VprL22A, VprE29A, VprW54A and VprI61A/L64P were described before [25]. New double point mutants of Vpr used for this study, including L22A/L64P, E29A/L64P and W54A/L64P, were constructed by replacing the *Bam*HI/*Eco*RI restriction fragment of pFSZ3-VprL64P [25] with the corresponding fragment from the expression constructs for VprL22A, VprE29A, and VprW54A, respectively. For expression of N-terminally Flag-tagged Vpr proteins, the wild type or mutant Vpr genes were polymerase chain reaction (PCR)-amplified with primer 1 ACATGCGGATCCATGGCAGACTACAAGGACGACGACGACAAGGGGATGGAACAAGCCCCAGAAGACC and primer 2 ACATCACTCGAGCTAGGATTTACTGGCTCCATTTCTTGTTCTCC. The PCR DNA was digested with *Bam*HI/*Sal*I and cloned into the *Bam*HI/*Sal*I sites of the pFSZ3 vector [25].

2.5. Reverse transcription (RT) PCR

Experimental conditions were modified from previous studies [28]. Briefly, 293 cells in a six-well plate were co-transfected with 2 μg each of pFSZ3-F-Vpr and pFSZ3-W54A, or pFSZ3-F-Vpr and pFSZ3-W54A/L64P [25]. mRNA was purified from the transfected cells using the mRNA mini-prep kit (Amersham/Pharmacia), and precipitated with ethanol. The purified mRNA (0.25 μg) was used for RT reactions in the presence or absence of the reverse transcriptase under previous conditions [28] using primer 3 (GCTTCATTCTTAAGCTCCTC). The RT products were serially diluted and used for PCR reactions with primer 3 and primer 4 (GACTAGCGGAGGCTAGAAGGAG). Sequences for both primer 3 and primer 4 were derived from the HIV-1 genomic clone 89.6 (GenBank accession number U39362). PCR reactions were performed for 21 cycles with 2 min annealing at 55°C and 2 min extension at 68°C. PCR products were subjected to polyacrylamide gel (6%) electrophoresis (PAGE) followed by staining with Vistra Green (Amersham), and quantified by fluorescence-image analysis (Storm 840, Molecular Dynamics).

2.6. Pulse-chase protein labeling with [^{35}S]methionine

293 cells in 12-well plates were transfected with pFSZ3-F-W54A/L64P and pFSZ3-W54A/L64P, or pFSZ3-F-W54A/L64P and pFSZ3-Vpr. Forty hours later, transfected cells were pre-incubated with methionine-free medium (Invitrogen) for 30 min, and labeled for 30 min with 0.3 ml of methionine-free medium containing 0.25 mCi [^{35}S]methionine/ml of Redivue Protein Labeling Mix (Amersham) and 10% fetal bovine serum. Labeled cells were washed and then incubated with regular medium. At various time points of the incubation, cells were lysed with 0.4 ml of the immunoprecipitation buffer used for ubiquitination analysis. The lysates were immunoprecipitated with the Flag antibody and analyzed by SDS–PAGE followed by phosphor-image analysis (Storm 840, Molecular Dynamics). For inhibition studies, transfected cells were labeled as described above and then treated with 50 μM of MG132 for additional 45 min before lysis of the cells for analysis of Vpr expression. For subcellular fractionation studies, cells treated as described above were washed and incubated with the regular medium for additional 40 min to allow nuclear migration of the newly labeled proteins. Cytoplasmic and nuclear fractions were prepared as described above.

3. Results

3.1. The wild type Vpr inhibits VprL64P-induced apoptosis

Based on nuclear magnetic resonance (NMR) studies [29], Vpr has three helical domains and two flexible terminal domains (Fig. 1A). During our previous studies [25], we introduced point mutations into the various structural domains of Vpr to facilitate analysis of Vpr functions. After DNA transfection into 293 cells, small molecular weight cellular DNA, which is present only in apoptotic cells, was isolated and analyzed for the existence of a DNA ladder of 200-bp

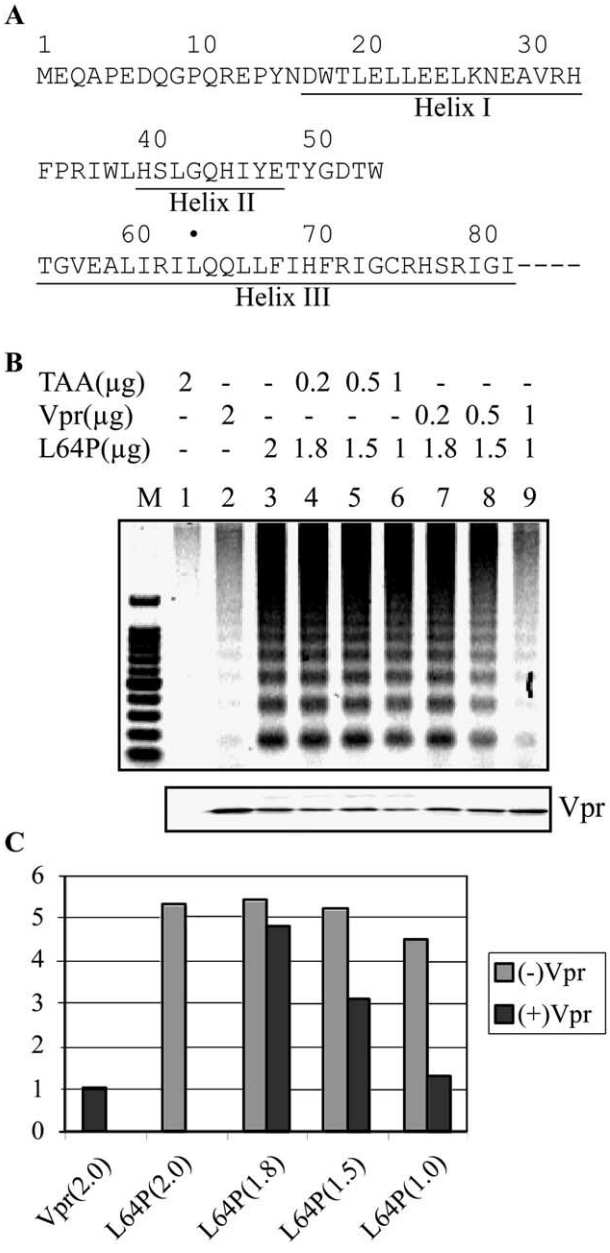

Fig. 1. Inhibition of VprL64P-induced apoptosis by the wild type Vpr. A: Amino acid sequence of the Vpr N-terminal region, with the three helical regions underlined [29]. Leu64 is indicated by a dot. B: Inhibition of VprL64P-induced apoptosis by the wild type Vpr. VprL64P was expressed either alone or together with the wild type Vpr by transfection of 293 cells. The amount of the plasmids used was as indicated on top of the figure. Two days after transfection, small molecular weight cellular DNA was prepared and analyzed. TAA: transfection with the pFSZ3-TAA plasmid which carries a premature codon at Vpr amino acid #5 [25]. Lower panel: Western blot with the Vpr polyclonal antibody [25]. C: Quantification of the DNA ladders in B. Value for lane 1 was deducted from other lanes as the background. Net value for lane 2 (the wild type Vpr) was normalized to 1. Numbers in parentheses indicate the amount of DNA (μg) used for transfection.

increments, which is the defining feature of apoptosis. One of the mutants, VprL64P (Fig. 1B, lane 3), has a dramatically enhanced pro-apoptotic activity compared to the wild type Vpr (lane 2). We subsequently examined the effect of

the wild type Vpr on the pro-apoptotic activity of VprL64P by co-expression of both proteins. Surprisingly, the wild type Vpr inhibited apoptosis induction by VprL64P in a dose-dependent manner (Fig. 1B, compare lanes 7–9 with lanes 4–6). With equal amounts of DNAs used for expression of the wild type Vpr and VprL64P, apoptosis induced by VprL64P was almost completely abolished (compare lane 9 with lane 6). Western blot suggested that the total level of the wild type Vpr and VprL64P was higher when VprL64P-induced apoptosis was inhibited (Fig. 1B, lane 9, lower panel).

3.2. *The VprL64P protein level is reduced in the presence of the wild type Vpr*

In the above Western blot analysis, the wild type Vpr and VprL64P could not be distinguished. Thus, we engineered a Flag epitope tag to the N-terminus of the wild type Vpr and VprL64P, so that the Flag-tagged Vpr proteins and their untagged counterparts can be distinguished during Western blot analysis. The Vpr with double mutations, I61A/L64P, which has approximately 20% of the pro-apoptotic activity of VprL64P [25], was also modified to have a Flag tag as a control. To examine the pro-apoptotic activities of the Flag-tagged Vpr proteins, total cellular DNA, which includes DNA from both apoptotic and non-apoptotic cells, was isolated after DNA transfection, and analyzed for the existence of a DNA ladder. As shown in Fig. 2A, F-VprL64P (lane 5) had a reduced apoptotic potential compared to the untagged counterpart VprL64P (lane 3), but remained much more pro-apoptotic than F-Vpr (lane 4). F-I61A/L64P (Fig. 2A, lane 6) had a pro-apoptotic activity similar to that of F-Vpr (lane 4). Western blot with the Flag antibody showed that all Flag-tagged Vpr proteins were expressed as expected (Fig. 2A, bottom panel).

To examine whether Vpr can affect the level of the VprL64P mutant protein, the wild type Vpr was co-expressed with F-Vpr, F-VprL64P, and F-I61A/L64P, and cell lysates were examined by Western blot with the rabbit polyclonal antibodies to Vpr (Fig. 2B). Co-expression of the wild type Vpr with F-Vpr resulted in F-Vpr being expressed at a slightly lower level than Vpr (Fig. 2B, lane 1), possibly due to competition during protein synthesis. In contrast, co-expression of Vpr with F-VprL64P (lane 2) and F-I61A/L64P (lane 3) rendered the Flag-tagged Vpr mutant proteins to be produced at an extremely low level. To examine if VprL64P also affected the levels of the Flag-tagged Vpr proteins, VprL64P was co-expressed with these proteins and cell lysates examined the same way (Fig. 2B, lanes 4–6). As shown, in the presence of F-Vpr, VprL64P protein was almost abolished (Fig. 2B, lane 4). However, VprL64P was co-expressed well together with F-VprL64P (Fig. 2B, lane 5) and F-I61A/L64P (lane 6), although both Flag-tagged proteins were expressed at a slightly lower level than VprL64P. These results suggest that the wild type Vpr is capable of reducing the protein level of L64P-containing Vpr mutants.

To examine if Vpr-induced decrease in the level of the L64P-bearing mutants can occur in different cell types, we used HeLa cells to express the Flag-tagged VprW54A/L64P or VprL64P mutant either alone or together with the wild type Vpr. As shown in Fig. 2C, expression of the mutant proteins was extremely poor, rendering the effect of the wild type Vpr difficult to discern.

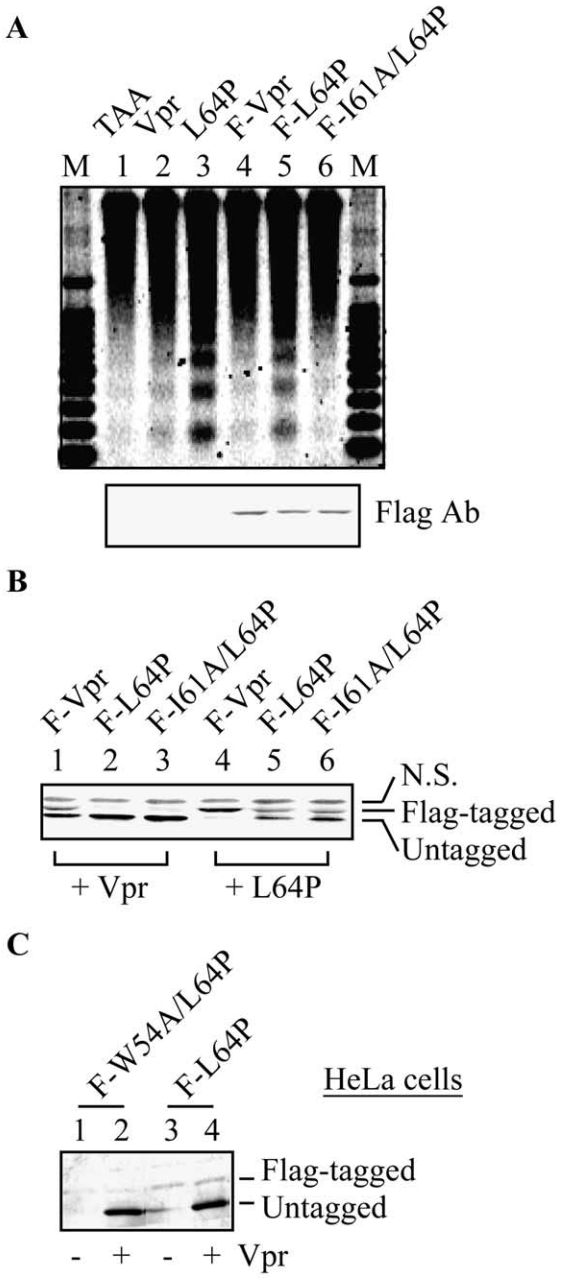

Fig. 2. Protein level of L64P-containing Vpr mutants is reduced in the presence of the wild type Vpr. A: Expression and pro-apoptotic activities of N-terminally Flag-tagged Vpr and mutants. All proteins were expressed in 293 cells by transfection. Two days after transfection, total cellular DNA, including DNA from both apoptotic and normal cells, was prepared and analyzed. Lower panel: Western blot with the Flag monoclonal antibody. B: Protein levels of the VprL64P and I61A/L64P mutants are reduced in the presence of the wild type Vpr. Expression constructs for the Flag-tagged Vpr and untagged counterparts were co-transfected in combinations as indicated. Flag-tagged proteins are labeled on top of the figure and untagged proteins are labeled on the bottom of the figure. Two days after transfection, cells were lysed and examined by Western blot analysis with the Vpr polyclonal antibody. N.S.: non-specific band. C: L64P-bearing mutants of Vpr are expressed poorly in HeLa cells. F-W54P/L64P and F-L64P mutants were expressed in HeLa cells either alone or together with the wild type Vpr. Cell lysates were examined by Western blot as in B.

3.3. Ability to reduce the protein level of Vpr mutants is shared by Vpr molecules from different subtypes of HIV-1 and is independent of apoptosis

The observation that protein level of the F-I61A/L64P mutant was reduced by the wild type Vpr (Fig. 2B, lane 3) suggested that the observed effect was independent of apoptosis. To examine this point further, we constructed additional Vpr mutants carrying another single point mutation in addition to L64P. As shown in Fig. 3A, both the L22A/L64P (lane 4) and the E29A/L64P (lane 5) mutants were as efficient as VprL64P (lane 3) in induction of apoptosis. However, the pro-apoptotic activity of the W54A/L64P mutant was abolished (lane 6). These mutants were then co-expressed with the Flag-tagged wild type Vpr, F-Vpr, and examined for their pro-apoptotic activity by DNA analysis. Vpr proteins were examined by Western blot with the Vpr anti-serum. As shown in Fig. 3B, the pro-apoptotic activities of the three highly apoptotic Vpr mutants were effectively inhibited by F-Vpr (lanes 3–5). Western blot (Fig. 3B, lower panel) showed that inhibition of apoptosis was correlated with a severe reduction in the protein levels of the Vpr mutants. Interestingly, the W54A/L64P mutant was negative in apoptotic activity, and yet its protein level was also reduced by F-Vpr (Fig. 3B, lane 6). Thus, it seemed that Vpr-induced reduction in the protein levels of L64P-containing mutants is independent of the pro-apoptotic activity of the mutants.

We previously reported that Vpr molecules from different HIV-1 subtypes have different pro-apoptotic potentials possibly due to sequence variations [25]. The Vpr molecule used for most experiments in this study is from a subtype B HIV-1 isolate [25]. Importantly, a subtype D Vpr (rD) has no detectable pro-apoptotic activity but acquires the same level of pro-apoptotic potential as VprL64P after substitution of Leu64 with a Pro residue [25]. In contrast, a subtype A/G hybrid Vpr (rAG) has a low level of pro-apoptotic activity that cannot be enhanced by the L64P mutation [25]. To examine if the Vpr molecules of different subtypes of HIV-1 can all reduce the protein level of L64P-containing mutants, the Flag-tagged Vpr double mutant, F-W54A/L64P, was co-expressed with its untagged counterpart W54A/L64P (Fig. 3C, lane 2), Vpr (lane 3), rD (lane 4), or rAG (lane 5). The results showed that the Vpr molecules from the three different HIV-1 subtypes all reduced the protein level of F-W54A/L64P despite their significant sequence variations [25].

3.4. Vpr-induced reduction in the protein level of L64P-containing mutants is not due to changes in mRNA level

Next, we examined if Vpr-mediated protein inhibition was exerted at the mRNA level. Since the vector and promoter used for expression of both the wild type Vpr and all of the

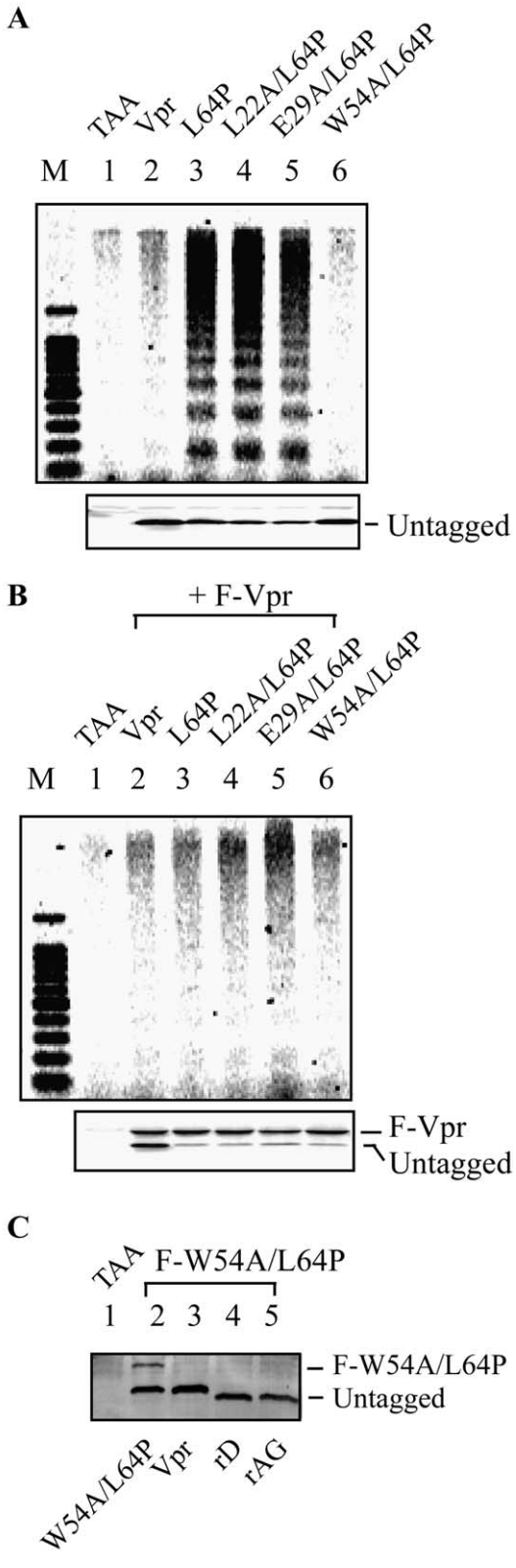

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Fig. 3. Ability to reduce the protein levels of L64P-containing Vpr mutants is shared by Vpr molecules from different subtypes of HIV-1 and is independent of apoptosis. A: Apoptosis induction by L64P-containing Vpr mutants. Expression constructs were transfected alone. Untagged proteins are indicated on top of the figure. Other conditions were the same as in Fig. 1B. B: Inhibition of L64P-containing Vpr mutants by the wild type Vpr. Flag-tagged wild type Vpr was co-expressed with untagged wild type Vpr or its mutants (as indicated on top of the figure). Other conditions were the same as in A. C: Protein level of F-W54A/L64P is reduced in the presence of Vpr molecules from different HIV-1 subtypes. Western blot conditions were the same as in A. Expression and sequence properties of the different Vpr molecules have been described previously [25]. Untagged Vpr proteins are indicated on bottom of the figure. rD and rAG are Vpr molecules from a subtype D and a subtype A/G recombinant HIV-1, respectively.

Vpr mutants were the same, preferential effects of the wild type Vpr on the mRNA level of the L64P-containing mutants were considered unlikely. To confirm this, F-Vpr and W54A/L64P were co-expressed by DNA co-transfection, and the relative mRNA levels of F-Vpr and W54A/L64P were analyzed by RT-PCR. As a control, F-Vpr was also co-expressed with the VprW54A mutant, which was not affected by co-expression of the wild type Vpr. Two RT-PCR primers were designed to simultaneously amplify both the F-Vpr mRNA and the W54A/L64P mRNA (Fig. 4A), resulting in two DNA fragments separable on polyacrylamide gels. The RT-PCR product from F-Vpr mRNA was longer due to the insertion of the Flag epitope-coding sequence (Fig. 4A). During the PCR step, the template (RT reaction product) was serially diluted so that some of the PCR reactions would not progress to saturation.

Cell samples used for RT-PCR analysis were first examined for Vpr expression by Western blot. As shown in Fig. 4B, control analysis of VprW54A showed that VprW54A was not significantly inhibited by co-expression of F-Vpr (Fig. 4B, compare lane 1 with lane 3). In contrast, W54A/L64P expression was undetectable in the presence of F-Vpr (Fig. 4B, lane 4), and normal in the absence of F-Vpr (lane 2). Cell samples corresponding to lanes 3 and 4 of Fig. 4B were subjected to RT-PCR analysis. This analysis revealed that the mRNA level of W54A/L64P (Fig. 4C, lower band in lanes 6–8) was similar to the mRNA levels for F-Vpr (upper band in lanes 2–4 and 6–8) and VprW54A (lower band in lanes 2–4). When the RT reaction was carried out in the absence of the reverse transcriptase enzyme (Fig. 4C, lanes 1 and 5), the PCR reaction did not yield any product, suggesting that the observed PCR signals were all from the specific mRNA molecules. Thus, inhibition of the W54A/L64P protein by F-Vpr was not due to a potential suppression of the mRNA for the W54A/L64P mutant protein.

3.5. *Turnover of the VprW54A/L64P mutant protein is enhanced by the wild type Vpr*

To examine if Vpr-mediated inhibition of L64P-containing mutants was correlated with a more marked turnover of the mutants, F-W54A/L64P was co-expressed with its untagged counterpart W54A/L64P (Fig. 4E, lanes 1–4, upper panel), or the wild type Vpr (lower panel). Transfected cells were labeled with [^{35}S]methionine, and then washed and incubated with regular medium for different lengths of time before lysis for examination of the F-W54A/L64P protein. All cell lysates

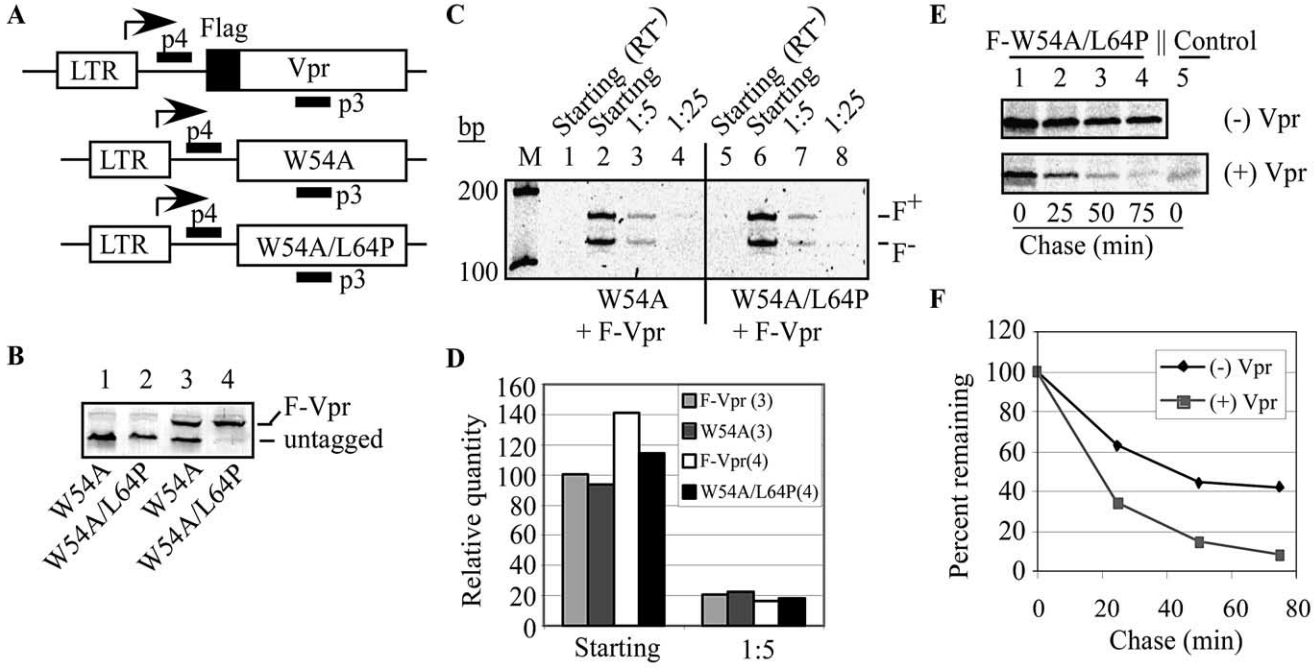

Fig. 4. Vpr-induced reduction in the protein level of the W54A/L64P mutant is correlated with a faster rate of turnover and not with changes in the mRNA level. A: Illustration of Vpr expression constructs used for co-transfection. Arrows indicate transcriptional start sites. Bars indicate positions of the primers (p3 and p4) for RT-PCR. Primer sequences are listed in Section 2. B: Inhibition of VprW54A/L64P, but not VprW54A, by the Flag-tagged wild type Vpr. VprW54A and the W54A/L64P mutant (as indicated on the bottom of the figure) were either expressed alone (lanes 1 and 2), or co-expressed with F-Vpr (lanes 3 and 4). Western blot was performed with the Vpr polyclonal antibody. C: RT-PCR analysis of cell samples corresponding to lanes 3 and 4 of B. RT-PCR products were examined as described in Section 2. 'Starting': starting dilution of the RT reaction product used for the subsequent PCR reactions. 'RT$^-$': RT enzyme was omitted during RT reaction. F$^+$ and F$^-$: RT-PCR DNA product from F-Vpr mRNA and the untagged Vpr mutants (as indicated on the bottom of the figure), respectively. M: 100-bp ladder marker (Promega). D: Quantification of data in C. Methods were the same as in Fig. 1C. Numbers in parentheses correspond to the lane number of B. E: Turnover rate of the F-W54A/L64P mutant protein is enhanced by the wild type Vpr. F-W54A/L64P was co-expressed with its untagged counterpart (lanes 1–4, upper panel) or with the wild type Vpr (lanes 1–4, lower panel). Cells were subjected to pulse-chase labeling as described in Section 2, and F-W54A/L64P examined by immunoprecipitation, SDS–PAGE, and phosphor-image analysis. Lane 5: control transfection with the Vpr-null construct pFSZ3-TAA. The control lane 5 has a non-specific band at a slightly lower position than F-W54A/L64P. F: Quantification of F-W54A/L64P. For both sets of samples, the signal intensity at the beginning of the chase was normalized to 100.

were immunoprecipitated with the Flag antibody and the immunoprecipitates were examined by SDS–PAGE, followed by phosphor-image analysis. As shown in Fig. 4E,F, the rate of F-W54A/L64P turnover was much faster in the presence of the wild type Vpr than in its absence. Thus, Vpr-induced reduction in the protein level of L64P-containing mutants appears to be correlated with a faster turnover rate of the mutant proteins.

3.6. *The wild type Vpr facilitates ubiquitination of the VprW54A/L64P mutant protein*

Degradation of many cellular proteins is mediated by the ubiquitin/proteasome pathway [27]. To examine if Vpr-promoted turnover of the W54A/L64P mutant protein was through the proteasome pathway, we expressed F-W54A/L64P either alone or together with the wild type Vpr. Two days after transfection, cells were labeled with [^{35}S]methionine and then treated with the proteasome inhibitor MG132. The level of the F-W54A/L64P mutant protein was examined by radio-immunoprecipitation. As shown in Fig. 5A, treatment of cells with MG132 increased the level of F-W54A/L64P in the absence and in the presence of the wild type Vpr. However, the extent of MG132-induced increase in F-W54A/L64P was most striking in the presence of the wild type Vpr (Fig. 5A, lanes 4 and 5).

To examine if the observed effect of MG132 was correlated with enhanced ubiquitination of the VprW54A/L64P mutant, we co-expressed the F-W54A/L64P mutant protein with the wild type Vpr as well as with HA-tagged ubiquitin. The transfected cells were treated with MG132 and analyzed for F-W54A/L64P and HA-ubiquitin by immunoprecipitation with the Flag antibody followed by Western blots. As shown in Fig. 5B, expression of F-W54A/L64P alone generated a low level of poly-ubiquitinated F-W54A/L64P (lane 2, upper panel). In contrast, co-expression of F-W54A/L64P with the wild type Vpr generated a much higher level of poly-ubiquitinated F-W54A/L64P (Fig. 5B, lane 3, upper panel), despite the fact that F-W54A/L64P was detected at a much lower level in the presence of the wild type Vpr than in its absence (Fig. 5B, lower panel). These results suggest that the wild type Vpr enhances the ubiquitination of F-W54A/L64P and its subsequent degradation through the proteasome pathway.

We previously demonstrated that Vpr purified from bacteria exists as an oligomer [30]. One hypothesis for Vpr-promoted ubiquitination/degradation of the VprW54A/L64P mutant is that the wild type Vpr forms a complex with the mutant protein due to their tendency to form oligomers. To examine if Vpr in mammalian cells also exists as an oligomer, transfected 293 cells were cross-linked with DSS in vivo, and Vpr was detected by Western blot. As shown in Fig. 5C, DSS treatment induced a higher molecular weight form of the wild type Vpr, which was consistent with a Vpr dimer (lane 3). The Flag-tagged Vpr (Fig. 5C, lane 5) was also cross-linked into an apparent dimer of a higher molecular weight than the untagged Vpr dimer (lane 3). Interestingly, when Vpr and F-Vpr were co-expressed, an apparent Vpr/F-Vpr heterodimer was detected (Fig. 5C, lane 4). These results showed that the wild type Vpr forms a dimer in vivo. Similar analysis of the VprW54A/L64P mutant also revealed a dimer of the mutant protein (data not shown). However, co-expression of the wild type Vpr with the VprW54A/L64P mutant failed to reveal a heterodimer of these proteins (see Section 4).

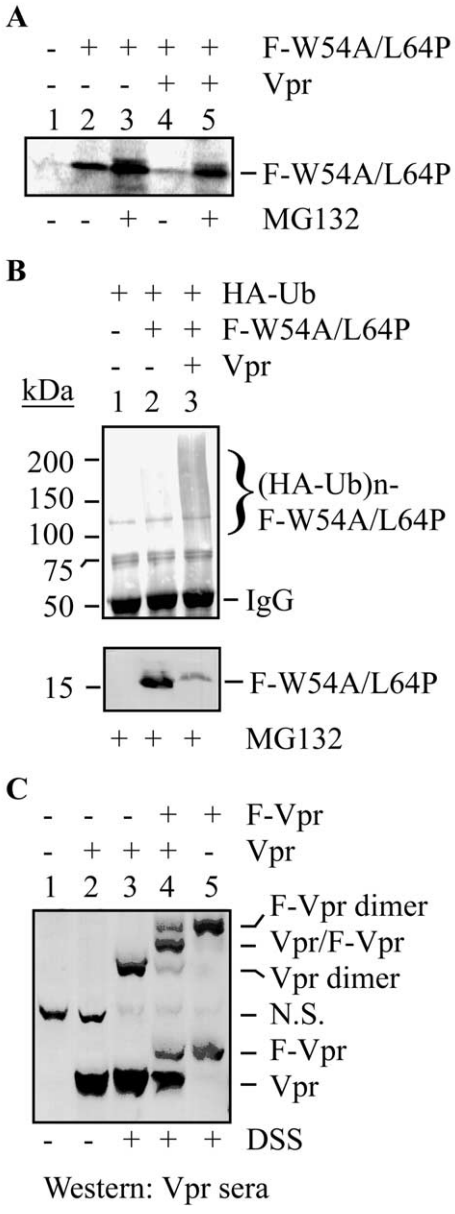

Fig. 5. Vpr forms a dimer in vivo and promotes ubiquitination of the VprW54A/L64P mutant protein. A: Proteasome inhibitor MG132 enhances the protein level of F-W54A/L64P in the presence and absence of the wild type Vpr. F-W54A/L64P was expressed either alone (lanes 2 and 3) or in the presence of the wild type Vpr (lanes 4 and 5). Cells were labeled with [^{35}S]methionine, and either treated or not treated with MG132 as indicated. Cell lysates were immunoprecipitated with the Flag antibody and analyzed as described in Fig. 4E. B: The wild type Vpr enhances ubiquitination of F-W54A/L64P. HA-tagged ubiquitin (HA-Ub) was co-expressed with F-W54A/L64P (lane 2) or F-W54A/L64P together with the wild type Vpr (lane 3). Cells were treated with MG132 and cell lysates immunoprecipitated with the Flag antibody. The immunoprecipitates were examined by Western blot with the HA antibody (upper panel) or the Flag antibody (lower panel). Positions of the poly-ubiquitinated F-W54A/L64P are indicated. C: Vpr forms a dimer in vivo. 293 cells expressing Vpr (lane 3), F-Vpr (lane 5) or both proteins together (lane 4) were cross-linked with DSS and examined by Western blot with the Vpr antibodies. 'Vpr/F-Vpr' indicates the heterodimer between Vpr and F-Vpr. N.S.: non-specific band.

3.7. Inhibition of proteasome-mediated degradation strongly enhances the cytoplasmic fraction of the VprW54A/L64P mutant

Since protein ubiquitination can occur both in the cytoplasm and in the nucleus, we first examined if the L64P mutation affects the subcellular distribution of Vpr. 293 cells expressing either the wild type Vpr or the VprL64P mutant were fractionated into cytoplasmic and nuclear fractions and Vpr expression examined by Western blot. By this analysis, the wild type Vpr (Fig. 6A, lanes 1 and 2) and the VprL64P mutant (lanes 3 and 4) were shown to have a similar pattern of distribution with a higher amount of the Vpr proteins found in the nuclear fraction. Analysis of the nuclear protein, poly-ADP-ribosyl polymerase, and the cytoplasmic protein actin revealed their predominant nuclear and cytoplasmic localization, respectively.

To examine the potential effect of proteasome inhibitor MG132 on the subcellular localization of Flag-tagged VprW54A/L64P mutant in the presence and absence of the wild type Vpr, transfected cells were metabolically labeled with [^{35}S]methionine, treated with MG132, and then incubated in regular medium for 40 min to allow newly synthesized proteins to enter the nucleus. Cells were fractionated and

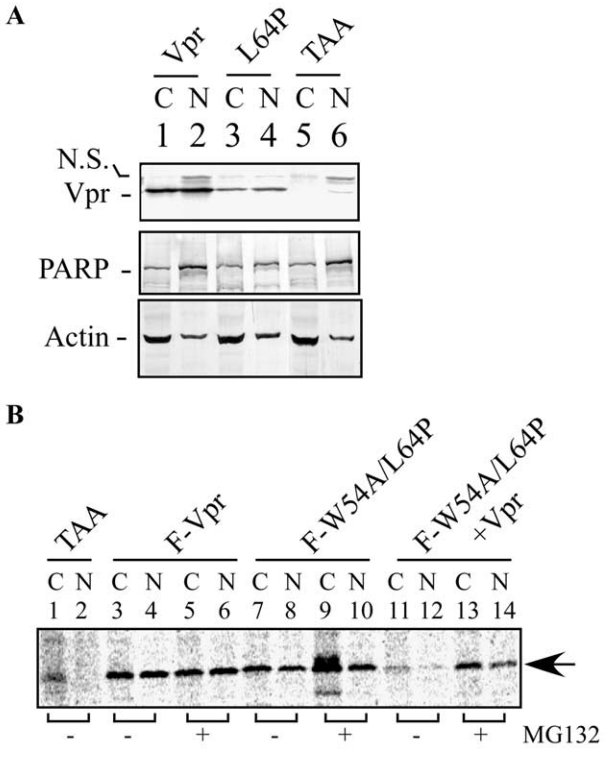

Fig. 6. MG132 treatment enhances the cytoplasmic fraction of the VprW54A/L64P mutant in the presence and absence of the wild type Vpr. A: Subcellular distribution of Vpr and VprL64P. Cytoplasmic (C) and nuclear (N) fractions of 293 cells transfected with the indicated expression constructs were examined by Western blot with antibodies to Vpr (top panel), poly-ADP-ribosyl polymerase (PARP, middle panel), and actin (bottom panel). N.S.: non-specific band. B: MG132 treatment enhances the cytoplasmic fraction of the VprW54A/L64P mutant. Cytoplasmic and nuclear fractions of radiolabeled cells were examined by immunoprecipitation followed by phosphor-image analysis. Treatment with MG132, as indicated on the bottom of the panel, is described in Section 2. The expressed Vpr proteins were labeled on the top of the figure. Arrow indicates the position of the Flag-tagged Vpr proteins.

presence of F-W54A/L64P in the cytoplasmic and nuclear fractions examined by radio-immunoprecipitation. As shown in Fig. 6B, in the absence of MG132, F-W54A/L64P assumed a similar nuclear/cytoplasmic distribution pattern (lanes 7 and 8) as the wild type F-Vpr (lanes 3 and 4). MG132 treatment did not affect either the distribution or the expression level of F-Vpr (Fig. 6B, lanes 5 and 6). In contrast, MG132 treatment significantly increased the amount of F-W54A/L64P in the cytoplasmic fraction both in the absence (Fig. 6B, lane 9) and in the presence (lane 13) of the wild type Vpr. Thus, ubiquitination/degradation of the VprW54A/L64P mutant appears to be predominantly in the cytoplasm.

4. Discussion

Vpr remains to be one of the major puzzles of HIV-1 replication and pathogenesis. While it is non-essential for viral replication in vitro, the Vpr gene is highly conserved among various subtypes of HIV-1. In addition, the many interesting biological activities of Vpr, such as pro-apoptosis and cell cycle arrest, render it possible for Vpr to exert significant effects during HIV-1 replication and pathogenesis in vivo. Despite this, the biochemical mechanism of Vpr function remains unclear. While it is logical to propose that Vpr has multiple functions with different biochemical mechanisms, it is also critical to consider the hypothesis that Vpr has a major function with a specific biochemical mechanism that facilitates HIV-1 replication and pathogenesis in vivo.

The novel observation in this article is that the wild type Vpr promotes ubiquitination/degradation of the L64P-bearing Vpr mutants. This effect is specific and uni-directional: the mutant protein does not affect the stability of the wild type Vpr, suggesting a dominant activity of the wild type Vpr. It is interesting to note that the likelihood of the wild type Vpr to co-exist with the mutant Vpr in vivo is low. Most importantly, however, our unique experimental system has allowed the detection of this novel activity of Vpr and the generation of experimental results that strongly suggest an interaction between the wild type Vpr and the cellular ubiquitination pathway.

Beyond its traditional role in protein degradation, protein ubiquitination has recently been shown to be an important player in a variety of cellular processes, including transcription, endocytosis, cell cycle, and apoptosis [31]. This important advance is made possible by the biochemical and structural characterization of the major factors involved in ubiquitination, including E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase) [32,33]. 'Adapter' molecules in conjunction with the E3 enzyme are responsible for recruiting specific protein substrates for regulated ubiquitination [34]. Protein substrates may be also post-translationally modified, such as phosphorylated, to provide a signal for recognition by the ubiquitination enzyme complex.

Since the wild type Vpr promotes the ubiquitination of the VprW54A/L64P mutant, it may be hypothesized that Vpr simultaneously interacts with the Vpr mutant protein and a cellular factor involved in protein ubiquitination. Similarly, the wild type Vpr may form a ternary complex with the Vpr mutant protein and an unknown cellular factor, enhancing the post-translational modification of the mutant Vpr protein. The post-translationally modified mutant Vpr protein may

be more susceptible to ubiquitination and degradation through the proteasome pathway. Biochemical studies of Vpr suggest that Vpr is capable of forming oligomers in vitro [30] and dimers in vivo (Fig. 5C). By a similar analysis, the VprW54A/L64P mutant also formed a dimer in vivo, and yet a heterodimer between the wild type Vpr and the VprW54A/L64P mutant could not be detected (data not shown). The failure to observe a complex between the wild type Vpr and the mutant Vpr may be due to several reasons. First, the protein level of the mutant Vpr is extremely low in the presence of the wild type Vpr, rendering the analysis of the potential complex difficult. Second, the complex could be unstable. This would be consistent with the observation that only the mutant protein and not the wild type protein is ubiquitinated and degraded.

This model of Vpr function also suggests the possibility that a cellular factor may be destabilized in the presence of Vpr by direct interaction with Vpr. This cellular factor may be inhibitory to HIV-1 replication under physiological conditions but may be difficult to identify due to its degradation enhanced by Vpr. This potential mode of Vpr function is similar to the mechanism of function of HIV-1 auxiliary regulatory proteins Vif and Vpu. Vif has been shown to interact with and promotes the degradation of the cellular cytidine deaminase, CEM15/APOBEC3G which is expressed only in non-permissive cell lines and inactivates the HIV-1 genome by introducing multiple mutations during the process of reverse transcription [35,36]. Vif-mediated degradation of CEM15/APOBEC3G has also been shown recently to be through the ubiquitin/proteasome pathway [37–42]. Vpu has been shown to interact simultaneously with CD4 and β-TrCP [43]. β-TrCP is responsible for the recruitment of the Vpu–CD4 complex to the E3 ubiquitin ligase complex for ubiquitination and degradation [43].

Pro mutations are expected to disrupt protein helical structures. To gain a better view of the L64P-induced thermodynamic changes in Vpr helix III, free-energy calculation of VprL64P mutant was carried out using Swiss-pdbViewer and the recent Vpr structural data acquired by NMR studies [29]. L64P mutation would increase the free energy of Vpr structure from (−)3853 kJ/mol to (+)2297 kJ/mol. Free-energy penalties result mostly from the existence of un-bonded side chains of Leu60, Ile61, and Pro64. Thus, helix III would be disrupted from residue Leu60 to residue L64P. It has recently been shown that Vpr interacts with cyclophilin A (cypA) and this interaction is critical for the functional expression of Vpr [44]. CypA specifically targets the Pro35 residue of Vpr and exerts its effects during the translation of Vpr. As suggested, structural modifications of Vpr by cypA are critical for the biological activity of Vpr. It is possible that cypA-induced modifications of Vpr mutants bearing the L64P mutation also affect the interaction between these Vpr molecules and the cellular protein ubiquitination pathway.

Despite the structural changes induced by the L64P mutation, the subcellular distribution of the L64P-bearing Vpr mutants appears to be similar to that of the wild type Vpr (Fig. 6). This is consistent with the observation that Vpr has two independent domains for nuclear transport [45–47]. The VprL64P and W54A/L64P mutants were also incorporated normally into HIV-1 virion-like particles (data not shown). By radiolabeling of cells followed by fractionation, the subcellular distribution of the VprW54A/L64P mutant appeared to be similar in the absence and presence of the wild type Vpr (Fig. 6B). However, while MG132 treatment did not affect the expression or localization of the wild type Vpr, it significantly enhanced the cytoplasmic fraction of the VprW54A/L64P mutant (Fig. 6B). Since MG132 inhibits proteasome degradation and not ubiquitination, these results suggest the interesting possibility that the VprW54A/L64P mutant is ubiquitinated in the cytoplasm and ubiquitination retards its nuclear migration. Since the wild type Vpr promotes ubiquitination and degradation of the VprW54A/L64P mutant, it seems possible that the wild type Vpr may target a ubiquitination/proteasome pathway in the cytoplasm.

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