論 文 の 内 容 の 要 旨

Study on degradation of MCM10 by primate lentivirus accessory proteins Vpr/Vpx
（レンチウイルスアクセサリータンパク質Vpr/VpxによるMCM10の分解制御に関する研究）

常　浩

Background

During long term evolution of viruses driven by natural selection pressure and their antagonism against hosts, not just structural and enzymatic proteins play important roles contributing to infection and pathogenesis, various accessory proteins are also derived for increasing requirements. There is to exception to primate lentiviruses. Viral protein R (Vpr) is one of multifunctional accessory proteins among all primate lentivirus, including human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) and different kinds of simian immunodeficiency viruses (SIVs).

As a small accessory protein, HIV-1 Vpr facilitates viral replication during various stages in host cells. Once encapsidated Vpr released into cytoplasm, it initiates multiple functions which are consistent with well-organized virus replication circle. HIV-1 Vpr promotes accuracy of viral genomic RNA reverse transcription, pre-integration complex (PIC) formation and nuclear localization, transcription regulation of viral and host genes, disorder of splicesome complex processing, dysregulation of cell cycle, selective inhibition of cellular pre-mRNA splicing both in vivo and in vitro and positive and negative regulation of apoptosis, activation of DNA damage response pathways. Of note, Vpr was found to hijack DCAF1 (DDB1)-Cul4-E3 ubiquitin ligase complex to lead numerous cellular targets for ubiquitination and proteasome-dependent degradation.

Minichromosome maintenance protein 10 (MCM10) is newly identified cellular target by HIV-1 Vpr, which is recruited to DCAF1-Cul4-E3 ligase for proteasome-dependent degradation. As a conserved component of eukaryotic replisome, MCM10 contributes to continuous replication process, including initiation of DNA replication, replication fork stability, DNA damage control. The multivalent properties of MCM10 are concordant with cell cycle surveillance and also taken as a common molecular maker in cancer. Previous researches also suggested Vpr hijacked DCAF1-Cul4-E3 ligase for MCM10 degradation to induce G2/M arrest.

Objective

As mentioned above, in terms of sequence homology and functional conservation among various primate lentivirus Vpr, we wonder whether or not such properties are associated with MCM10 proteasome-dependent degradation. Therefore, firstly, we demonstrate whether MCM10 degradation also happens and is correlated with lineages of virus types. Secondly, we examine if there are other roles of Vpr on cellular target regulated by MCM10 degradation. Finally, we clarify this hijack of Vpr on E3 ubiquitin ligase for MCM10 proteolysis are also shared among human relative species such like cell lines derived from monkeys.

Materials and Methods

Phylogenetic analysis and Vpr alleles preparation: 96 full length HIV/SIV Vpr amino acid sequences were obtained from Los Alamos HIV Database and multiple alignments were performed with MUSCLE

algorithm; Plasmids:11 HIV/SIV Vpr and Vpx were synthesized according to nucleotide sequences collected as mentioned above and subcloned into pcDNA 3.1 or pME18neo vectors; specific mutants of HIV-1 Vpr were conducted with standard site-directed mutagenesis kit; Immunofluorescence staining: HeLa cells or HEK293 cells were seeded on cover glasses in a 12-well plate and were transfected with HA-MCM10 either without/with lentiviruses FLAG-Vpr. They were stained with anti-FLAG rabbit or anti-MCM10 rabbit antibody and visualized with confocal fluorescence microscope; Co-immunoprecipitation assay: HEK293T cells were co-transfected with HA-MCM10 together with lentiviruses FLAG-Vprs, harvested and lysed. Total protein was incubated with anti-FLAG M2 affinity gel in binding/wash buffer. The gel was collected by centrifugation, washed and collected for Western Blot analysis; Cell cycle analysis: HeLa cells were seeded in a 6-well plate and transfected with FLAG-HIV-1 Vpr wild type and a panel of mutants mentioned above. After 48h, cells were stained with propidium iodide and performed with standard cell cycle analysis protocol.

Results and discussion

Lentiviruses Vpr alleles preparation and sequences characterization

From HIV Database, 96 full-length Vpr amino acid sequences from divergent HIV/SIV lineages were obtained and global alignments were conducted. To cover most HIV/SIV lineages and minimum selection bias, HIV/SIV Vpr proteins derived from 10 lentivirus strains were chosen. Prototype viruses (Vpr+Vpx-Vpu-) include SIVdeb, SIVsyk, SIVlst, SIVagm and SIVcol Vprs. HIV-1, SIVmus and SIVmon Vprs present as HIV-1 type viruses (Vpr+Vpx-Vpu+). HIV-2 type viruses (Vpr+Vpx+Vpu-) were covered by SIVmac and SIVrcm.

A nuclear magnetic resonance structural analysis revealed that full length Vpr forms three amphipathic alpha helices surrounding a hydrophobic core (α-helix 1, 2 and 3). Interestingly, all lentiviruses Vpr proteins show potential zinc-binding motif (H33, H71, H76 and *78) located in α-helix 2 and 3, which is similar with conserved HIV-2 Vpx zinc-binding motif (HHCC). It was possible that zinc-binding motif was essential for function maintenance of both Vpr and Vpx.

MCM10 down-regulation profiling by primate lentiviruses Vpr/Vpx

To verify how MCM10 expression level changed under various HIV/SIV Vpr/Vpx proteins, we carried out co-transfection with HA-MCM10 and FLAG-Vpr/Vpx in HEK293T cells and monitored MCM10 expression.

The expression of HA-MCM10 was decreased by co-transfection with HIV-1, SIVmus and SIVrcm Vpr proteins while other strains failed to induce similar down-regulation. Through triplicate assays, relative intensity of MCM10 expression was calculated and normalized by internal control Tublin. About 62% of intensity of MCM10 remained in the present of HIV-1 Vpr protein compared with that of only MCM10 control. In addition, MCM10 expression decreased to 40% and 54% in the presence of SIVmus and SIVrcm Vpr proteins, respectively. In addition, endogenous MCM10 expression level was also susceptible by HIV-1, SIVmus and SIVrcm Vpr proteins. However, because of less obvious sequence features, we speculated more than one site or site contributed to the role of MCM10 degradation.

MCM10 degradation by Vpr proteins is proteasome-dependent

To verify whether MCM10 degradation resulted from identical pathways by distinct Vpr proteins, HIV-1, SIVmus and SIVrcm Vpr proteins, MG-132, a reversible proteasome inhibitor was used to monitor the

effects on MCM10 expression. At 43 h of post co-transfection of MCM10 and Vpr proteins, MG-132 or DMSO was treated into 293T and cell samples were harvest for Western Blot analysis after 48 h. For only-MCM10 control, MG-132 had little effect MCM10 expression. In contrast, HIV-1, SIVmus and SIVrcm Vpr proteins reduced MCM10 expression in DMSO-treated cultures but not MG-132-treated cultures. The results were confirmed with another proteasome irreversible inhibitor, lactacystin, which also targets the proteasome 20S. Compared to MG 132, lactacystin treatment led to a greater increase of MCM10 expression.

MCM10 interaction with HIV-1, SIVmus and SIVrcm Vpr

It is important to determine whether the MCM10 degradation by Vpr proteins mediate by the interaction between Vpr proteins and MCM10. Therefore, to demonstrate an interaction between Vpr proteins, including HIV-1, SIVmus and SIVrcm Vpr proteins, which induced MCM10 degradation, and MCM10, we performed co-immunoprecipitation using anti-FLAG beads in HEK293T cells. All the 3 FLAG tagged Vpr proteins were immunoprecipitated with HA-MCM10.

To confirm this interaction, we examined an immuno-fluorescence assay in HeLa cells. HA-MCM10 was found to be concentrated predominantly in the cellular nucleus as form of discrete replication foci. HIV-1, SIVmus and SIVrcm Vpr expression also accumulated mostly in nucleus of HeLa cells. On the other hand, distribution of Vpr proteins was observed to aggregate in dispersed form around the nucleoli. Furthermore, with merge results, the Vpr proteins partially co-localized with the MCM10 foci in the nucleus. The results suggested Vpr proteins form complex with HA-MCM10 in vivo. The interaction showed Vpr proteins interfered the MCM10 function during normal physical environment.

MCM 2-7 interaction domain of MCM10 is susceptible to degradation by Vpr

To investigate the determinant domain of MCM10 susceptible to degradate by Vpr proteins, a panel of truncation mutants of MCM10 was constructed. Recently, amino acids 530-655 of MCM10 was identified to mediate MCM10 interaction with MCM 2-7, which is essential for MCM10 nuclear localization. Here, MCM10 mutants were transiently co-transfected with 3 Vpr proteins, HIV-1, SIVmus and SIVrcm Vpr proteins, respectively. After 48 h, expression of MCM10 mutants was detected with Western Blotting and related intensity was calculated. MCM10 mutant (1-655), losing most parts of CTD but still maintaining MCM2-7 interaction domain, still were susceptible to HIV-1, SIVmus and SIVrcm Vpr. This suggested CTD of MCM10 expression maybe was not affected by Vpr proteins engagement. Collectively, region of 530-655 is a key region induced for MCM10 proteolytic consequence by Vpr.

Moreover, to verify subcellular localization alternation of HA-MCM10 mutants related to their degradation susceptibility, HEK293 cells were transfected with MCM10 mutants and subsequent IFA was performed after 48 h. MCM10 wildtype aggregated in nucleus and formed typical replication foci. In contrast, MCM10 (1-655) mainly distributed in the cytoplasm possibly resulted from lack of unidentified nuclear localization signals (NLSs) in CTD. Besides, a small fraction of MCM10 (1-655) still localized in nucleus but typical foci form disappeared. For mutants MCM10 (1-530), MCM10 (1-427) and MCM10 (1-145), localized predominantly in cytoplasm. This suggested probably cellular distribution of MCM10 might be required for subsequent degradation

MCM10 degradation is specifically associated with HIV-1 Vpr G2/M arrest

We wonder whether other roles of Vpr also were involved positively or negatively in MCM10 degradation. Typical six kind of HIV-1 Vpr mutants were summed up and characterized. Through co-

expression of HIV-1 mutants and HA-MCM10 in HEK293T cell and Western Blot analysis, we found that mutants K27M, C76A and R80A, which all lack of cell cycle blocking, reversed the MCM10 degradation compared to HIV-1 wildtype. However, P35A, W54R and R77Q, playing distinct roles but for G2/M arrest, didn't turn back MCM10 proteasome-dependent degradation. Combining quantitative data of MCM10 degradation profiling and G2/M:G1 ratio rendered by Vpr mutants, high correlation (R2=0.8589) between each function was revealed. Therefore, the correlation data demonstrated that G2/M arrest function of HIV-1 Vpr is specifically correlated with MCM10 degradation. In contrast, R77Q, as an apoptosis induction-deficient mutants, still down-regulated MCM10 expression, which showed MCM10 expression level is not affected by apoptosis function of HIV-1 Vpr. These results showed importance of relationship between MCM10 degradation and cell cycle arrest.

Uniform MCM10 degradation pattern by Vpr proteins in COS-1 cells

Moreover, we wondered whether MCM10 degradation by primate lentiviruses Vpr proteins is also conserved among different species such as monkeys. COS-1 cell, a fibroblast-like cell line, derived from African green monkey was used. Accordingly, MCM10 and Vpr proteins were co-transfected in COS-1 cells for MCM10 degradation profiling. Similarly, HIV-1, SIVmus and SIVrcm Vpr suppressed expression of MCM10 (Figure 8), which is as same as MCM10 degradation profiles in HEK293T cells. In contrast, Vpr proteins from other lineages lost ability of MCM10 degradation. This implicated E3 ligase hijacking by Vpr proteins are functionally conserved among primate species.

Conclusion

This study revealed that distinct MCM10 degradation profiles by primate lentiviruses Vpr/Vpx proteins through E3 ubiquitin proteasome-degradation pathway. Particularly, HIV-1, SVImus and SIVrcm Vpr, curbed MCM10 expression, while Vpr derived from other 8 Vpr/Vpx failed. Interestingly, co-localization and interaction of MCM10 and Vpr proteins also were observed. And MCM10 530-655 region was susceptible to degradation through proteasomal degradation pathway. For HIV-1, G2/M interruption was directly related with MCM10 degradation but other Vpr function defects did not. It's noteworthy that similar human MCM10 degradation profiles by such panel of Vpr proteins were observed in COS-1 cells. This result prompts that capacity of hijacking E3 ligase complex by Vpr proteins in both human and monkey cells is a conversed property. Our unpublished data also showed human MCM10 and Vpr proteins keep the similar co-localization pattern. However, it's still questionable whether such set of DNA damage response proteins exert synergistically a role to react by accessory protein Vpr and it's a proposed direction worth working on.