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Identification of amino acids within the second alpha helical domain of the human immunodeficiency virus type 1 Vpu that are critical for preventing CD4 cell surface expression

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ABSTRACT

Human immunodeficiency virus type 1 (HIV-1) encodes for a Vpu protein, which interacts with CD4 resulting in its degradation. In this study, we examined the role of the 10 amino acids within the predicted second α-helical domain of the subtype B Vpu cytoplasmic tail in CD4 down-modulation using a VpuEGFP reporter system. Our findings indicate that the invariant leucine at position 63 and, to a lesser extent, the valine at position 68 were required for CD4 down-modulation. Mutation of analogous L63 in Vpu proteins subtypes A2, B(YU-2), C, D, and H also abolished CD4 down-modulation from the cell surface. Co-immunoprecipitation analysis revealed that L63A and V68A mutants were capable of binding CD4 and still retained the ability to interact with h-β-TrCP1. Taken together, these results indicate that amino acid substitutions in the second α-helical domain that retain the predicted structure and binding to h-β-TrCP1 can influence Vpu-mediated CD4 degradation.

Introduction

Viral protein U (Vpu) of human immunodeficiency virus type 1 (HIV-1) is a small transmembrane phosphoprotein that is synthesized off a bicistronic mRNA that also codes for the HIV-1 envelope glycoprotein (Cohen et al., 1988; Strebel et al., 1988; Schwartz et al., 1990). The Vpu protein is an oligomeric protein with a short N-terminal domain, an uncleaved leader sequence that also acts as a transmembrane domain, and a longer cytoplasmic domain (Maldarelli et al., 1993; McCormick-Davis et al., 2000). The cytoplasmic domain contains two predicted α-helical domains that are separated by a highly conserved hinge region that contains two casein kinase II sites. Previous studies showed that one of the important functions of Vpu is to interact with newly synthesized CD4 in the rough endoplasmic reticulum (RER) resulting in its re-translocation across the RER membrane and targeting it for destruction via the proteasome pathway (Fujita et al., 1997; Schubert et al., 1998; Willey et al., 1992). Previous studies in our laboratory have indicated that certain amino acids of Vpu are highly conserved in all strains examined (McCormick-Davis et al., 2000). Highly conserved amino acids reside within the transmembrane domain, the hinge region and the first and second predicted α-helical domains. The most extensively studied of these has been the hinge region. Previous studies showed that phosphorylation of both serine residues at positions 52 and 56, which are highly conserved in group M and O HIV-1 isolates, is essential for CD4 down-regulation (Paul and Jabbar, 1997; Schubert et al., 1994; Singh et al., 2003). Other studies have implicated the amino acid residues within the transmembrane domain as important in CD4 down-regulation (Tiganos et al., 1998; Hout et al., 2005). In a previous study, it was shown that disruption of the predicted α-helical domains resulted in Vpu proteins that were incapable of CD4 degradation (Tiganos et al., 1997). Our own studies indicated that deletion of the carboxyl terminal 13 amino acids did not affect CD4 degradation but that deletion of the carboxyl terminal 23 amino acids, which encompass the second α-helical region, prevented Vpu-mediated CD4 degradation (Pacyniak et al., 2005).

In this study, we used alanine-scanning mutagenesis to identify specific amino acids within the second α-helical domain of the subtype B Vpu that are critical to CD4 degradation. The results identify a critical, highly conserved leucine residue and also a downstream valine that are important for CD4 down-modulation from the cell surface as well as Vpu degradation.

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Results

CD4 surface expression in the presence of the Vpu mutants

The sequence of the mutant Vpu proteins analyzed in this study is shown in Fig. 1. We determined whether any of the mutations would disrupt the predicted α-helical region using three different programs (Predict Protein, PROF, and nnPREDICT). None of the mutations were found to disrupt the α-helical region (data not shown). Previously, we showed that fusion of the Vpu protein to enhanced green fluorescent protein (EGFP) still resulted in the ability to down-modulate cell surface CD4 (Hout et al., 2005; 2006; Singh et al., 2003). We analyzed cell surface CD4 expression in the presence of the 10 Vpu mutant proteins with the unmodified subtype B (VpuEGFP) as a positive control and a Vpu protein with both serine residues of the two casein kinase II sites changed to glycine residues ($Vpu_{S52,56G}$EGFP) as a negative control. HeLa $CD4^+$ cells were transfected with vectors expressing each of the 10 Vpu mutant proteins, EGFP, VpuEGFP, or $Vpu_{S52,56G}$EGFP. At 48 h post-transfection, live cells were immunostained for CD4 and analyzed by flow cytometry to measure the intensity of cell surface CD4 expression.

As shown in Fig. 2A, cells transfected with the vector expressing VpuEGFP prevented CD4 surface expression while $Vpu_{S52,56G}$EGFP did not prevent CD4 expression. Of the 10 Vpu mutants examined, only Vpu_{L63A}EGFP and Vpu_{V68A}EGFP allowed significant CD4 surface expression ($p<0.001$) compared to VpuEGFP (Fig. 2B). We also analyzed a double mutant, $Vpu_{LV63,68AA}$EGFP, containing both amino acid substitutions. There appeared to be an additive effect as surface expression of CD4 was slightly higher than each of the single mutants (Fig. 2B).

Vpu_{L63A}EGFP is expressed in similar compartments as VpuEGFP

As Vpu_{L63A}EGFP was deficient at down-modulation of CD4 from the cell surface, we compared the intracellular expression. Subconfluent monolayers of 293 cells were transiently transfected with either VpuEGFP or Vpu_{L63A}EGFP. At 48 h, cells were fixed and examined by confocal microscopy. As shown in Fig. 3, VpuEGFP or Vpu_{L63A}EGFP were predominantly localized in intracellular compartments, suggesting that the L63A mutation did not drastically alter the intracellular expression of Vpu in cells.

Vpu_{L63A}EGFP and Vpu_{V68A}EGFP mutants still bind to CD4

We next determined if the Vpu mutants Vpu_{L63A}EGFP and Vpu_{V68A}EGFP, which had reduced function down-modulating CD4 expression, were still capable of binding to CD4. 293 cells were co-transfected with plasmids expressing CD4 and wild-type or mutant Vpu proteins. At 48 h lysates were prepared and immunoprecipitations performed using an anti-EGFP antibody. The immunoprecipitates were analyzed by Western blots for the presence of CD4. The results in Fig. 4 indicate that VpuEGFP but not EGFP co-

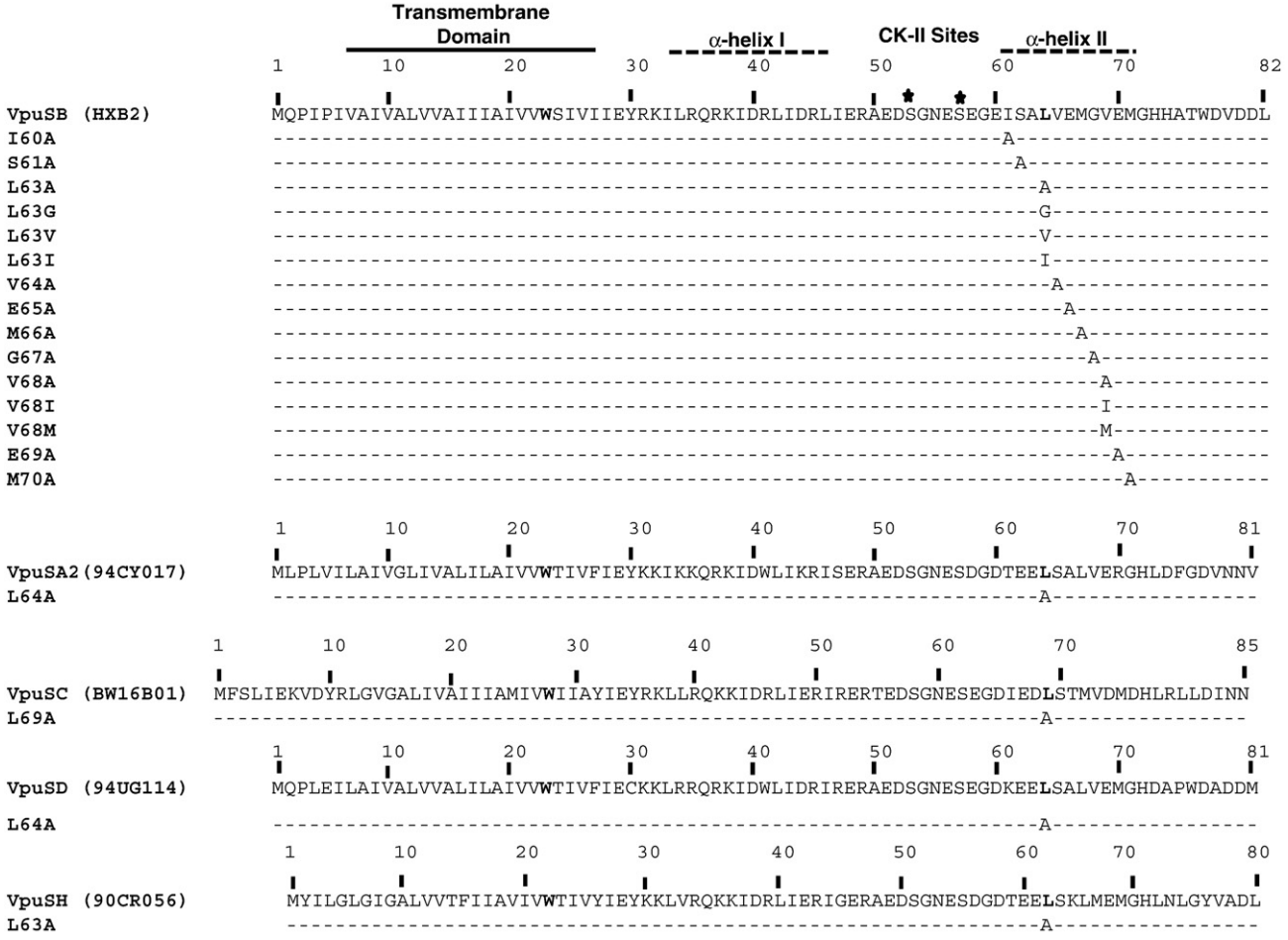

Fig. 1. The sequence of the various mutants analyzed in this study. The transmembrane domains, the two predicted α-helical domains and two phosphoserine residues (indicated by the star). The numbering of amino acid residues used for the HXB2 is based on the original *vpu* sequence of HXB2 in which the initiation codon (ATG) was actually an ACG codon such that residue number 10 is actually 11th residue in the corrected HXB2 open reading frame. The Vpu proteins from other HIV-1 subtypes are numbered starting with methionine residue as 1.