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Human immunodeficiency virus 1 Vpu protein does not affect the conversion of Influenza A virus hemagglutinin to its low-pH conformation in an acidic *trans*-Golgi compartment

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Summary. – The 81-aa Vpu protein of Human immunodeficiency virus 1 (HIV-1) is a structural analogue of the M2 protein of Influenza A virus (IAV). Expression of Vpu in *Xenopus* oocytes has showed that it can form a voltage-activated ion channel permeable to Na^+ and K^+ ions (Ewart *et al*., 1996). To investigate whether Vpu has a pH-modulating activity comparable to that of M2, Vpu was co-expressed with the pH-sensitive hemagglutinin (HA) from IAV. The results indicated that Vpu was unable to reduce the acidity of the exocytic pathway and reduce the conversion of the pH-sensitive HA to its low-pH conformation during transport to the cell surface. Despite these findings, we did not exclude the possibility that Vpu formed a weak ion channel with almost pore-like characteristics as was recently suggested.

Keywords: Human immunodeficiency virus 1; ion channel; Influenza A virus; M2 protein; Vpu protein

Introduction

Small virus proteins that enable a flux of ions are in some cases either essential for the virus or supportive for the viral life cycle. These proteins are involved in the entry/exit pathway as M2 from IAV (Lamb *et al*., 1985; Sugrue and Hay, 1991), in the formation of vesicles as 2B protein from polio virus (Barco and Carraso, 1995), or in the amplification of viral release as Vpu from HIV-1 (Klimkait *et al*., 1990).

Vpu is a small integral membrane protein of HIV-1 and Simian immunodeficiency virus that is expressed in chimpanzee coordinately with Env protein from bicistronic mRNA (Schwarz *et al*., 1990). It is composed of the uncleaved signal sequence-transmembrane domain (aa 1–27) and cytoplasmic domain (aa 28–81) consisting of two α-helices and two highly conserved casein kinase sites (Schubert *et al*., 1994; Cohen *et al*., 1988; Strebel *et al*., 1988; McCormick-Davis *et al*., 2000; Hout *et al*., 2004). Vpu is not found in the envelope of virus particle, but is incorporated into the membranes of sub-cellular compartments of infected cell (Strebel *et al*., 1989). The protein is responsible for two biological activities that contribute to the pathogenicity of HIV-1 infections in humans (Bour and Strebel, 2003). Vpu enhances the release of newly formed virus particles from the cell (Strebel *et al*., 1988, 1989; Terwilliger *et al*., 1989; Klimkait *et al*., 1990) and targets CD4, the viral receptor to the proteosome for degradation (Schubert *et al*., 1996, Margottin *et al*., 1998; Schubert *et al*., 1998). The transmembrane domain of Vpu is required for the efficient virion release and forms a cation-selective ion channel in the cellular membranes (Ewart *et al*., 1996; Schubert *et al*., 1996b; Romer *et al*., 2004).

The Vpu protein of HIV-1 is a structural analogue of M2 protein of IAV. The M2 protein contains 96 aa forming three structural domains: the aminoterminal extracellular domain (23 aa), transmembrane domain (19 aa), and cytoplasmic domain (54 aa). The native form of Vpu protein is a disulfide-linked homotetramer (Holsinger and Lamb, 1991). The M2 protein forms an ion channel (Pinto *et al*., 1992; Hay, 1992) that plays a role in the uncoating of influenza virions in endosomes

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Abbreviations: araC = cytosine arabinoside C; HA = hemagglutinin; HIV-1 = Human immunodeficiency virus 1, IAV = Influenza A virus; IFA = immunofluorescence assay; TGN = *trans*-Golgi network

(Helenius, 1992) and reduction of the *trans*-Golgi network (TGN) acidity. During virus infection, intralumenal pH of the TGN is kept above the threshold, which triggers the HA to undergo a conformational change to the low pH form (Hay *et al*., 1985; Ciampor *et al*., 1992; Grambas *et al*., 1992).

Both proteins M2 and Vpu cause a similar delay in the progress of viral glycoproteins through biosynthetic membrane systems. M2 delays the transport of viral HA from TGN to the plasma-membrane. This delay in secretion is linked to the M2 ion channel activity (Sakaguchi *et al*., 1996). Vpu inhibits the transport of Env and Gag proteins to the late endosomes leading to viral assembly at the plasma membrane (Van Damme and Guatelli, 2008). Although we do not know, if Vpu is able to dissipate pH gradients, the similarities between Vpu and M2 proteins lead to the hypothesis that Vpu affects the trafficking of Env *via* the ion channel activity of its transmembrane domain.

To gain further insight into the ion channel function of Vpu protein, we have investigated the ability of Vpu protein to modify pH within the TGN by co-expressing Vpu with a pH-sensitive HA from IAV.

Materials and Methods

Cells and virus. CV-1 (ATCC CCL) cells were grown in Eagle's minimal essential medium (MEM) containing 10% calf serum. Recombinant vaccinia virus vTF7-3 that expresses the bacteriophage T7 RNA polymerase gene (kindly provided by Dr. B. Moss) was propagated in HeLa cells (Fuerst *et al*., 1986).

Antibodies. Anti-HA monoclonal antibodies HC2 specific for all pH forms of HA, HC58 specific for the native form of HA, and H9 specific for low-pH form of HA (kindly provided by Dr. A.J. Hay) were previously described (Sugrue *et al*., 1990). Rabbit antisera were produced to the peptide corresponding to the C-terminal sequence SAVDVDDGHFVNIELE of M2 protein conjugated to the keyhole limpet hemocyanin. A rabbit polyclonal antibody HA11 (anti-HA-tagged Vpu) recognizing the influenza virus HA epitope YPYDVPDYAS was obtained from Covance.

Plasmid constructs pVOTE.1-HA, pVOTE.1-M2, and pVOTE.2-Vpu. The plasmids encoding the HA and M2 genes of A/chicken/Germany/34 (H7N1, Rostock strain) were previously described (Betakova *et al*., 2005). The Vpu gene was modified by PCR to contain an *NdeI* restriction endonuclease site at the initiation codon and a sequence encoding the influenza virus HA epitope tag sequence YPYDVPDYAS, followed by a termination signal and a *BamHI* site. The PCR products were cut with appropriate restriction enzymes and inserted into pVOTE.2 (kindly provided by Dr. B.Moss) to generate pVOTE.2-Vpu. All constructs were sequenced to check for unwanted mutations. Plasmid DNA was purified using Plasmid Maxi Kit (Qiagen).

Transfection. Confluent CV-1 cells were infected with 10 PFU/cell of recombinant vaccinia virus vTF7.3 in OPTIMEM containing 40μg/ml of cytosine arabinoside (AraC). After 1 hr of incubation, the infected cells were transfected with plasmids coding the proteins mixed with Lipofectine (Life Technologies).

Immunofluorescence assay (IFA). CV-1 cells were grown on the glass coverslips and transfected with 3 μg of pVOTE.1-HA mixed with 0.5 μg of pVOTE.2-Vpu or pVOTE.1-M2. Four hours after transfection, the cells were overlaid with 1 ml of MEM containing 20% FCS and 40 μg/ml of AraC. After additional 20 hrs, the cells were fixed with 3% paraformaldehyde, permeabilized with 0.05% saponine in PBS and immunolabeled with anti-M2, anti-HA-tagged Vpu or HC2 antibodies diluted in PBS containing 1% bovine serum albumin (BSA). Primary antibodies were visualized using fluorescein- or rhodamine-conjugated secondary antibodies diluted in 1% BSA in PBS. The nuclei were labeled for 10 min with the color Hoechst 33342. The cells were examined using Olympus IX70 microscope and the images were captured using Silicon Graphics Delta vision.

Western blot analysis. The transfected cells were lysed in extraction buffer (1% Triton-X-100, 1 mmol/l EDTA, 20 mmol/l Tris-HCl, pH 7.4) containing proteinase inhibitor Complete Mini (Roche). After 10 mins on ice, the lysates were clarified by microcentrifugation for 1 min and the supernatants were analyzed by electrophoresis on 12.5% polyacrylamide gel. Immunoblotting was done as described by Grambas *et al.* (1992) using rabbit anti-M2 or polyclonal anti-HA-tagged Vpu serum, protein A-horseradish peroxidase conjugate and enhanced chemiluminiscence (ECL) reagent (Amersham).

ELISA. CV-1 cells grown on 96-well plates were transfected with 0.25 μg of pVOTE.1-HA with increasing amount of plasmids encoding the ion channels, as mentioned above. Four hours after transfection, the cells were overlaid with MEM containing 20% FCS, 40 μg/ml AraC, with or without 5 μmol/l rimantadine. Transfected cells were incubated for 20 hrs and fixed with 0.05% glutaraldehyde in PBS and ELISA was carried out on duplicate wells using anti-HA monoclonal antibodies HC2, HC58 or H9 as described by Betakova *et al.* (2005). The change in % of HA recognized by HC58 or H9 antibodies, respectively, was estimated from the ratio of the A_{450}, e.g.: HA [HC58](%) = HA[HC58]/ HA [HC2] (plus M2) x 100 – HA [HC58] /HA [HC2] (no M2) x 100, or HA [H9] (%) = HA [H9]/HA [HC2] (plus M2) x 100 – HA [H9}/ HA [HC2] (no M2) x 100, respectively.

Results

Expression of M2 or Vpu proteins in CV-1 cells

Since we had no suitable antibody against Vpu protein, we prepared the HA-tagged version of this protein. The nucleotides encoding an epitope recognized by anti-HA 11 rabbit serum (anti-HA-tagged Vpu) were inserted into the DNA upstream of the terminal codon of Vpu. The accumulation of co-expressed M2 and Vpu proteins was checked by Western blot analysis with anti-M2 and anti-HA-tagged Vpu antibodies. The amount of protein detected increased substantially with the higher amount of plasmid used for transfection indicating that the cells remained viable (Fig. 1).

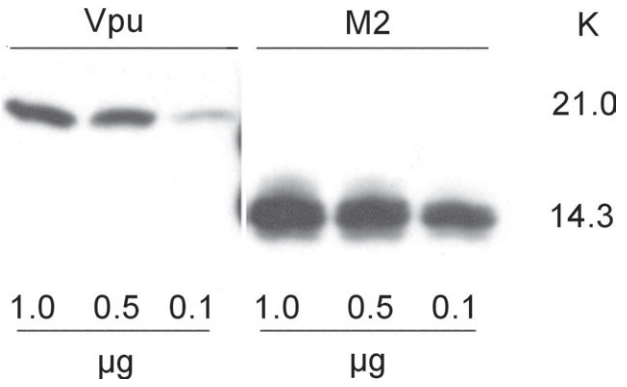

Fig. 1

Expression of M2 and Vpu proteins in CV-1 cells

Western blot analysis of CV-1 cells transfected with 0.1, 0.5, and 1.0 μg of either plasmid expressing Vpu (pVote.2-Vpu) or M2 (pVOTE.1-M2), respectively, and 18 hrs later analyzed for Vpu and M2 using specific anti-M2 or anti-HA-tagged Vpu antibodies. Size markers are indicated on the right.

Co-localization of Vpu and HA proteins co-expressed in CV-1 cells

To determine if Vpu like M2 protein co-localized with the HA, IFA was performed on co-transfected CV-1 cells. The cytoplasm and membranes of CV-1 cells transfected with pVOTE.1-HA were strongly stained with the anti-HA monoclonal antibody HC2 that reacts with all forms of HA (Betakova *et al*., 2006). Anti-M2 and anti-HC2 antibodies were localized at the membranes and cytoplasm of CV-1 cells that co-expressed the HA and M2 proteins (Fig. 2). Vpu protein was detected in the cytoplasm and nucleus, but most of Vpu protein was co-localized in the membranes and cytoplasm with HA like M2 protein (Fig. 2).

Comparison of pH modulating activity of Vpu and M2 proteins

The CV-1 cells in 96-well plates were transfected with 0.25 μg pVOTE.1-HA together with increasing amounts of plasmid DNA encoding M2 or Vpu protein (in duplicate wells), essentially as described previously by Betakova *et al.* (2005). The ability of M2 to elevate TGN pH and protect HA against low pH-induced changes depended on the ratio of expressed HA and M2 proteins and was indicated by the corresponding increases in the proportion of native HA (recognized by HC58 antibody) and decreases in the proportion of low pH HA (recognized by H9 antibody) (Fig. 3). Co-expression of HA with the M2 protein resulted in the 27% increase of native, neutral pH form of HA and

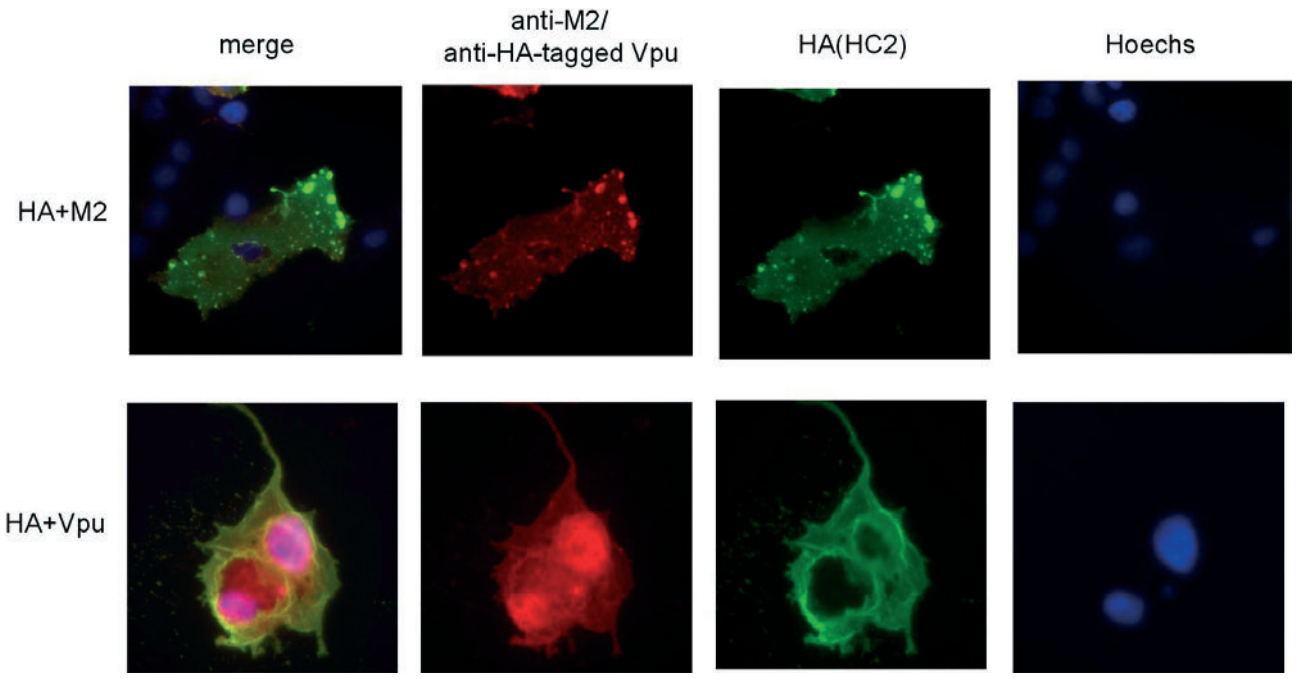

Fig. 2.

Co-localization of M2/Vpu and HA proteins detected by IFA

CV-1 cells were transfected with the plasmid expressing HA and the plasmids expressing either M2 or Vpu. The cells were immunolabeled with antibodies anti-HA and anti-HA-tagged Vpu or anti–HA and anti-M2 antibodies. The nuclei were visualized with Hoechst 33342.

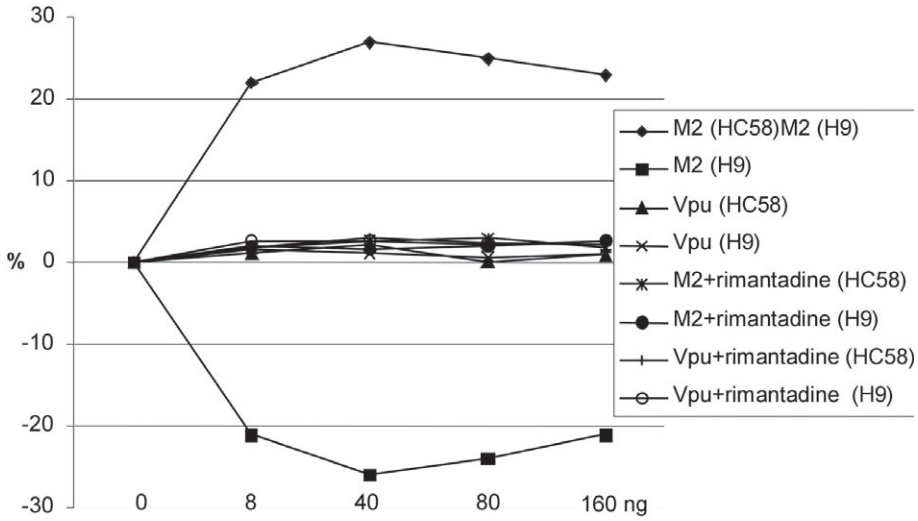

Fig. 3

Comparison of pH – modulating activities of Vpu and M2 proteins

Increased proportions of native HA (positive values) and decreased proportions of low-pH HA (negative values) calculated from ELISA A_{450} values are indicative of pH-modulating activity of M2 or Vpu co-expressed with HA in CV-1 cells (for details see Fig. 2). Abscissa: amount (ng) of transfected plasmids.

the corresponding 26% decrease of low pH form as averaged from six experiments. The changes caused by the M2 protein were specifically inhibited by rimantadine. On the other hand, no significant changes in the relative proportions of native and low pH forms of HA were detected, when co-expressed with the Vpu protein (Fig. 3). Several different amount of HA and Vpu protein as well as the ratios of HA/Vpu were used to enhance the sensitivity of the co-expression assay. However, no changes due to the Vpu expression were detected.

Discussion

The structural similarity of Vpu to M2 has raised the question, whether the Vpu protein might be also a functional analogue of M2 protein and form an ion channel for a long time. The results presented in this paper provide the experimental evidence that Vpu cannot replace M2 protein during transport of HA through the TGN. The avian influenza virus A/chicken/Germany/34 (Rostock strain) H7 HA is cleaved to HA1 and HA2 intracellularly in the TGN (Sugrue *et al*., 1990). To maintain the Rostock HA in its native form during transport through the TGN, a functioning M2 ion channel activity is required, otherwise the HA undergoes its transition to the low-pH form. Previous studies characterized the effects of co-expression of M2 and HA on the transport, cleavage, and stability of HA (Ohuchi *et al*., 1994; Sakaguchi *et al*., 1996; Henkel *et al*., 1999; Betáková and Kollerová, 2006), and the intracellular co-localization of these two proteins (Hughey *et al*., 1992; Betakova and Hay, 2007).

Like M2 protein, Vpu was detected in the TGN and endoplasmic reticulum membranes (Klimkait *et al*., 1990; Willey *et al*., 1992). The second helical structure of Vpu protein regulates the localization of Vpu protein in TGN (Dube *et al*., 2009). Moreover, trafficking of Vpu between TGN and endosomal system is critical for releasing of HIV-1 particles from the infected cells (Dube *et al*., 2009). Our results of indirect IFA indicated that HA co-localized with Vpu protein in the membranes and cytoplasm. Despite of co-localization of Vpu protein with HA, Vpu did not prevent the conformation changes of HA to its low pH form. It is apparent therefore, that the protein has not the ability to modulate pH of TGN.

The Vpu expressed in *Esherichia coli*, purified, and reconstituted in planar lipid bilayer showed the channel activity (Ewart *et al*., 1996). Synthetic peptide corresponding to the putative transmembrane domain also exhibited the channel activity (Shubert *et al*., 1996b; Marrasi *et al*., 1999). These channels were more permeable to the monovalent cations Na^+ and K^+ than to anions Cl^-. Another structural analogue of Vpu is NB protein from the influenza B virus. It was reported that NB might form some kind of channel in the membrane that was permeable to the Na^+ and Cl^- ions (Sunstrom *et al*., 1996; Premkumar *et al*., 2004), but like Vpu protein was unable to modulate pH in the TGN and to protect HA (Betáková and Kollerová, 2006).

On the contrary, the M2 protein forms the H^+-activated, H^+-selective channel, which has low permeability for other physiological ions (Chizmakov *et al.*, 1996; Mould *et al.*, 2000). The transmembrane domain of M2 ion channel protein contains a HXXXW motif, where H and W amino acids are important for the channel function (Mould *et al.*, 2003). BM2 protein of influenza B virus possesses the same HXXXW motif in its transmembrane domain and this pH sensitive ion channel is able to replace functionally M2 and protect co-expressed HA from undesirable conformation changes (Betakova and Hay, 2009).

When A19 was replaced with H in the transmembrane domain of Vpu, HXXXW motif was restored and the resulting Vpu mutant was converted into a rimantadine-sensitive ion channel (Hout *et al.*, 2006a; Park and Opella, 2007). Inhibition of this ion channel by the rimantadine was not specific, because i) too high concentration of rimantadine was needed to inhibit this ion channel (Grambas *et al.*, 1992), and ii) rimantadine does not bind to the H37 in M2 protein (Betakova *et al.*, 2005). However, the fact that this ion channel could be inhibited by a high concentration of the rimantadine let us assume that substitution of A19H made this ion channel pH sensitive like M2 protein. The transmembrane domain of Vpu can be functionally substituted with the transmembrane domain of M2 of IAV. The simian-human immunodeficiency virus with the chimeric Vpu/M2 protein was pathogenic for pig-tailed macaques (Hout *et al.*, 2006b). These data point to the common ancestor of M2 and Vpu proteins. Since glycoproteins of HIV-1 do not require a pH change during uncoating and maturation in the TGN as glycoproteins of influenza viruses, the Vpu evolved into a very weak ion channel.

In conclusion, we have shown that the Vpu protein is not capable of modifying pH within the TGN. However, our finding supported the suggestion of Mehner *et al.* (2008) that Vpu formed a weak ion channel with almost pore-like characteristics. Nevertheless, a further characterization of this ion channel is required.

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