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Critical Role of Leucine-Valine Change in Distinct Low pH Requirements for Membrane Fusion between Two Related Retrovirus Envelopes

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\textbf{Background:} The mechanism of viral membrane fusion is still poorly understood.

\textbf{Results:} We show that a leucine-valine change in the JSRV and ENTV Env is responsible for their distinct low pH requirements for fusion.

\textbf{Conclusion:} The Leu-Val change likely stabilizes an intermediate induced by receptor binding.

\textbf{Significance:} This work represents a unique example whereby a simple Leu-Val change has critical impact on virus entry.
Abstract

Many viruses use a pH-dependent pathway for fusion with host cell membrane, the mechanism of which is still poorly understood. Here we report that a subtle leucine (Leu)-valine (Val) change at position 501 in the envelope glycoproteins (Envs) of two related retroviruses, jaagsiekte sheep retrovirus (JSRV) and enzootic nasal tumor virus (ENTV), is responsible for their distinct low pH requirements for membrane fusion and infection. The Leu and Val residues are predicted to reside within the C-terminal heptad repeat (HR2) region of JSRV and ENTV Envs, particularly proximal to the hairpin turn of the putative six-helix bundle (6HB). Substitution of the JSRV Leu with a Val blocked the Env-mediated membrane fusion at pH 5.0, whereas replacement of the ENTV Val with a Leu rendered the ENTV Env capable of fusing at pH 5.0. A Leu-Val change has no apparent effect on the stability of native Env but appears to stabilize an intermediate induced by receptor binding. These results are consistent with the existence of at least two metastable conformations of these viral glycoproteins, the native prefusion conformation and a receptor-induced metastable intermediate. Collectively, this work represents an interesting perhaps unique example whereby a simple Leu-Val change has critical impact on pH-dependent virus fusion and entry.

Enveloped viruses must fuse with the target cellular membrane to initiate infection, a process that is mediated by the viral fusion proteins present on the virus surface (1). Although highly divergent in primary sequence, viral fusion proteins share remarkable similarities in the high resolution structure, allowing them to be grouped into three classes (2). Among these, class I viral fusion proteins, as exemplified by the influenza virus hemagglutinin (HA), HIV-1 envelope (Env), and paramyxovirus F proteins, have been extensively studied and provided significant insights into our current understanding of membrane fusion and viral infection (2–5). Class I viral fusion proteins are typically synthesized as a precursor, and then cleaved by cellular proteases into two subunits; this process confers the viral fusion proteins to be metastable, with the membrane-distal subunit being responsible for recognizing cellular receptors and the membrane-anchored subunit directly mediating the virus-cell membrane fusion (6). The membrane-anchored transmembrane subunit comprises a fusion peptide at or near its N terminus, two central heptad repeats (HRs) known as HR1 and HR2 (or HRA and HRB), a membrane-spanning domain (MSD), and a cytoplasmic tail (CT). HR1 and HR2 are the key elements that are directly involved in the formation of a stable post-fusion conformation, i.e., the six-helix bundle (6HB) (6).

The Env proteins of retroviruses belong to the class I viral fusion protein family, consisting of a surface (SU) subunit and a transmembrane (TM) subunit. SU is responsible for binding to the specific cellular receptors or coreceptors, whereas TM is directly involved in fusion between virus and cell membrane (7). Upon receptor binding, a conformational change occurs in the SU subunit of Env, leading to large-scale conformational rearrangements of TM that drive membrane fusion (3). Although most retroviruses are currently believed to fuse directly at the plasma membrane (8), several retroviruses have been found to require a low pH (8–13), low pH-dependent protease activities (14), or receptor priming plus low pH(15, 16) for fusion activation. The underlying mechanisms for these pH-dependent fusion activation pathways are currently not well defined.

Jaagsiekte sheep retrovirus (JSRV) and enzootic nasal tumor virus (ENTV) are two highly related simple retroviruses that cause lung and nasal tumors in sheep and goats (17). Unlike most oncogenic retroviruses, the native Env proteins of both JSRV and ENTV...
induce oncogenic transformation in addition to mediating virus entry into host cells (18–23). Interestingly, despite significant sequence homology in the Env (~90% identity in the full-length) proteins and that JSRV and ENTV share the same cellular receptor, hyaluronidase 2 (Hyal2) for viral entry (19, 24), the fusogenicity of ENTV Env is much lower than that of JSRV Env, and ENTV Env also requires an unusually low pH (pH < 4.5) for fusion activation compared with that of JSRV (pH < 6.3) (25). We recently determined that the SU subunit of ENTV Env is primarily responsible for its low fusogenicity (25), yet found interestingly that this is not due to the receptor binding per se but lies in its intrinsic insensitivity to the receptor-mediated triggering (26). Here, we find, surprisingly, that a single leucine (Leu)-valine (Val) change in the HR2 region of JSRV and ENTV TM subunits is critical for the differential low pH requirements of these Envs for fusion activation and virus entry. We present evidence that the Leu-Val change alters the stability of receptor-mediated intermediate, likely explaining, at least in part, the distinct low pH thresholds of fusion between JSRV and ENTV Envs.

Experimental Procedures

Reagents and Cell Lines

The fluorescent dye 5-(and 6)-((4-chloromethyl)benzoyl)aminotetramethylrhodamine (CMTMR) and Lipofectamine 2000 were purchased from Invitrogen. The anti-FLAG monoclonal antibody and the secondary anti-mouse IgG coupled to phycoerythrin, fluorescein isothiocyanate (FITC), or horseradish peroxidase (HRP) were purchased from Sigma. Leupeptin was purchased from Calbiochem. The peptides corresponding to the N-terminal heptad repeat (HR1) (DKKIEDRLSALYDVVRVLGEQVQSIKNFRMKIQC) and C-terminal heptad repeat (HR2) with Leu-501 (FNTNLSLDLQLHNEILDIES) or Val-501 (FNTNVSLDLQLHNEILDIES) were synthesized by Alpha Diagnostic International (San Antonio, TX).

HTX (a subclone of HT1080), 293T/GFP (expressing GFP), 293/LH2SN (overexpressing human Hyal2), HTX/LH2SN (overexpressing human Hyal2), NIH 3T3/LH2SN (expressing human Hyal2), and 293/GP-LAPSN (a packaging cell line expressing MLV Gag-Pol and alkaline phosphatase) cells have been previously described (27, 28). All cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, Utah) and maintained at in a 10% CO2/air atmosphere at 100% relative humidity.

Env Constructs

The parental JSRV Env derived from the JSRV7 strain (JS7) (29) and the parental ENTV Env derived from an ENTV isolate of sheep (also known as ENTV-1) (30) were tagged with a FLAG sequence at both N and C termini, and both were cloned into the pCI-Neo expression vector (25, 27). The chimeric Envs between JSRV and ENTV were generated by overlapping PCR of the different regions of the TM subunit. The point mutations in the JSRV and ENTV ectodomains were generated similarly by overlapping PCR, with the specific mutation sites being included in the designed primers. All PCR products were cloned into
the pCI-Neo-based, parental JSRV or ENTV Env backbones, with resulting constructs containing a FLAG tag at both the N and C termini. All mutants were confirmed by DNA sequencing.

**Syncytia Induction and Cell-Cell Fusion Assays**

The syncytia induction assay was performed as previously described (16, 25, 27). Briefly, 293/LH2SN or 293 cells were seeded onto 6-well plates and transfected with 2 μg of plasmid DNA encoding Env plus 0.5 μg of pCMV-GFP-encoding plasmid (kind gift by François-Loïc Cosset, Lyon, France) using the calcium phosphate method. Unless otherwise indicated (such as in the case of peptide inhibition experiments), at 24 h post-transfection, cells were treated with PBS, 10mM HEPES, and 10mM MES (Sigma) at the indicated pH for 1 or 5 min at 37°C. Cells were then incubated at 37°C in complete growth medium for 1 h before being photographed using a fluorescence microscope (Carl Zeiss, Goettingen, Germany). For peptide inhibition assay, a soluble form of Hyal2 (sHyal2) was added to the transected 293 cells in the presence or absence of HR1 or HR2 peptides for 30 min prior to a low pH pulse (16).

The cell-cell fusion assay was also performed as previously described (25, 27), with some minor modifications. Briefly, 293T/GFP cells were transfected with 2 μg of DNA encoding individual Envs using Lipofectamine 2000 (Invitrogen). At 24 h post-transfection, cells were detached using PBS plus 5 mM EDTA, and co-cultured at a 2:1 ratio with target HTX/LH2SN cells that were pre-labeled with CMTMR by following the manufacturer’s instructions. After 1–2 h co-culture at 37°C, cells were treated for 1 or 5 min with different pH buffers, depending on the Envs and specific experiments, and allowed to recover in normal growth medium for 1 h. Where applicable, HR1 or HR2 peptides were added during the 1-h recovery period. Cells were trypsinized and analyzed by flow cytometry using a FACSCalibur (BD Bioscience). The surface expression of Env in the effector 293T/GFP cells was determined by anti-FLAG staining and analyzed by flow cytometry.

**Retroviral Pseudotypes and Infection**

The green fluorescent protein (GFP)-encoding retroviral pseudotypes bearing JSRV/ENTV Env or Ebola GP (a kind of gift of Gary Kobinger, National Microbiology Laboratory, Winnipeg, Canada) proteins were produced by co-transfection of 293T cells with plasmids encoding the individual Envs or Ebola GP, a packaging plasmid encoding Moloney MLV Gag-Pol (pCMV-gag-pol-MLV), and a transfer vector encoding GFP (pCMV-GFP-MLV) (the two latter plasmids were kind gifts of François-Loïc Cosset, Lyon, France). Alternatively, Moloney MLV pseudotypes encoding alkaline phosphatase (AP) were produced by transfection of the 293/GP-LAPSN packaging cell line (28) with Env or Ebola GP-encoding plasmids. The Env surface expression was determined by anti-FLAG staining of the cells as described previously (25, 27). Viral supernatants were harvested 48–72 h post-transfection, and cell debris was removed by centrifugation at 3,200 μg for 5 min. For infection, HTX or HTX/LH2SN cells were seeded onto 12-well plates, and infected with appropriate amounts of GFP- or AP-encoding Env pseudovirions in the presence of Polybrene (5 μg/ml). If applicable, cells were pre-treated with leupeptin or ammonium chloride (NH₄Cl) for 1 h, and infected with viruses in the presence of the drugs for 4–6 h; uninternalized
viruses were inactivated by treating cells with citrate buffer (pH 3.15), and viral titers were determined by flow cytometry (for GFP virus) or AP staining 48–72 h after infection.

**Low pH Rescue of BafA1-Mediated Arrest of Pseudoviral Infection and Measurement of the Stability of Receptor-Induced Intermediate**

We used a previously described method (31) to examine the effect of HR2 peptide on pseudoviral infection as well as determine the stability of receptor-induced intermediate. Briefly, NIH 3T3 cells expressing human Hyal2 (NIH 3T3/LH2SN) were pretreated with 20 nM bafilomycin A1 (BafA1) for 1 h at 37°C, and spinoculated with Env pseudovirions expressing AP by centrifugation at 1,680 × g for 2 h at 4°C. Unbound viruses were washed 3 times with cold PBS, and the virus-cell complex was switched to 37°C; at different time points, cells were incubated with a pH 4.5 buffer for 5 min, followed by continuous incubation at 37°C in the presence of 20 nM BafA1 for 6 h. Where applicable, HR2 peptides harboring Leu or Val were added at the time of temperature switch. Note that BafA1 was constantly present during the 6-h infection except during the 5-min pH 4.5 treatment period. Viral titers were determined by staining the AP positive cells 72 h post-infection. The stability of receptor-induced intermediate was assessed by comparing the ability of low pH to rescue the BafA1-mediated arrest of JSRV or JSRV L501V pseudoviral infection at different time points.

**Metabolic Labeling**

The metabolic labeling assay was performed as previously described (27). Briefly, 293T cells were transfected with Env-encoding plasmids using the calcium phosphate method; 18–24 h post-transfection, cells were starved in cysteine/methionine-free medium for 30 min at 37°C, and pulsed with 62.5 μCi of [35S]cysteine/methionine for 1 h at 37°C. The radiolabeled proteins were initially chased for 3 h, followed by an additional chase in the presence of the indicated amounts of sHyal2 (16). Env proteins in supernatants and cell lysates were immunoprecipitated using anti-FLAG beads (Sigma), resolved on SDS-PAGE, and visualized using a PhosphorImager and quantified using Quantity One.

**Statistical Analysis**

Student’s t tests were used for all statistical analyses, and typically data from three to six independent experiments were used for the analysis.

**Results**

**The Ectodomain of TM Determines Distinct Low pH Requirements of JSRV and ENTV Envs for Fusion**

We recently showed that the SU subunit of ENTV Env is responsible for its intrinsic low fusogenicity, whereas the TM subunit dictates the differential low pH requirements for membrane fusion between JSRV (pH < 6.3) and ENTV Env (pH < 4.5) (25). To identify which domain(s) of the TM are responsible for the differential low pH requirements for fusion between JSRV and ENTV Envs, we created a series of reciprocal chimeras in the CT, MSD, and ectodomain of the TM (fig. 1A), respectively, and characterized their fusion
properties. All the Env chimeras were processed properly into SU and TM, and had approximately equivalent levels of expression on the cell surface and in the cells, as measured by flow cytometry and Western blot (data not shown).

**Figure 1.** The ectodomain of JSRV and ENTV TM is responsible for their differential low pH requirements for fusion. (A), Schematic diagrams of JSRV and ENTV Envs and their chimeras. Gray areas indicate the regions where the Env sequences between JSRV and ENTV were swapped. M, MSD. The nomenclatures of the chimeras are based on the order of Env sequences, JSRV or ENTV, that are present in the four-boxed areas, i.e., SU, the ectodomain of TM, MSD, and CT. (B), Syncytium induction. 293/LH2SN cells overexpressing human Hyal2 were transfected with an Env-expressing plasmid along with a plasmid encoding GFP. 24 h after transfection, cells were treated with pH 5.0 buffer for 1 min, and pictures were taken 1 h after the treatment. Note the morphological differences between cells expressing EJEE or ENTV, and between cells expressing JEJJ or JSRV. (C), Cell-cell fusion. 293T/GFP cells were transfected with plasmid DNA encoding individual Envs; 24 h post-transfection, cells were co-cultured with the CMTMR-labeled HTX/LH2SN cells overexpressing Hyal2 for 1 h. Co-cultured cells were then treated with pH 5.0 buffer for 1 min, and cell-cell fusion was analyzed by flow cytometry 1 h after the treatment. The percentages of double positive (GFP and CMTMR) cells for each construct were plotted at different pH conditions. No Env, 293T/GFP cells not transfected with an Env-encoding plasmid were used as a negative control. Note that a ~2–3% background level at pH 7.0 (dashed line) was normally seen due to cell doublets (because of Env-receptor interaction). Comparisons were made between JSRV Env and chimeras containing JSRV SU (i.e., JEJJ, JJEJ, and JJJE), or
between ENTV and chimeras containing ENTV SU (i.e., EJEE, EEJE, and EEEJ). Averages plus S.D. of at least 3 independent assays are shown. * indicates \( p < 0.05 \), ** indicates \( p < 0.01 \).

We first examined the syncytia induction of these Env chimeras in 293/LH2SN cells overexpressing the JSRV/ENTV receptor, Hyal2. We found that distinct from the parental ENTV Env, replacement of the ectodomain of ENTV TM with that of JSRV (termed as EJEE) resulted in a small but reproducible syncytia formation following a typical pH 5.0 treatment for 1 min (fig. 1B); a prolonged treatment for 5 min at pH 5.0 further increased the size and numbers of syncytia (data not shown). In accordance, replacement of the ectodomain of JSRV TM with that of ENTV (termed as JEJJ) completely abolished the syncytia induction of JSRV Env at pH 5.0 following a 1-min treatment (fig. 1B). No apparent effect was observed for other Env chimeras (fig. 1B). We next performed quantitative cell-cell fusion assays; again, EJEE fusion activity increased from 2 to 3% of background level to 5–6% relative to the parental ENTV Env (\( p < 0.01 \) at pH 5.0; \( p < 0.05 \) for pH 4.5), and JEJJ showed a reduced cell-cell fusion compared with the wild type JSRV Env (from 4.5–10 to 2.5–4%) at all three acidic conditions tested (\( p < 0.05 \) or 0.01) (fig. 1C). Although decreased fusion activities were also noticed for JJEJ (\( p < 0.05 \)) and JJJE (\( p < 0.01 \)) at pH 4.5, no increased fusion was observed for their reciprocal chimeras, EEJE and EEEJ (fig. 1C). Taken together, these data revealed that the ectodomains of ENTV and JSRV Envs, rather than the more divergent MSD and CT regions (<50% identity, fig. 2A), are responsible for their differential low pH requirements for fusion.

**A Leu-Val Difference in Ectodomain of Env Is Critical for Distinct Low pH Requirements of Fusion between JSRV and ENTV**

There are 6 amino acid residues in the ectodomain of Env that are different between ENTV and JSRV (fig. 2A). To identify which of these residues are critical for their differential low pH requirements for fusion, we made reciprocal point mutations at each of the six positions, and determined their fusion activities. The surface expressions of three JSRV Env mutants, F483Y, L501V, and D530N, were comparable with that of wild type JSRV Env, whereas the other three mutants, K489E, K548Q, and T549S exhibited reduced expression by ~50% relative to the parental JSRV Env (fig. 2B). Of note, ENTV Env and all its point mutations, particularly E489K, exhibited relatively higher levels of surface expression as compared with those of JSRV (fig. 2B). These results indicate that the expression of JSRV and ENTV Env can be modulated by amino acid changes in the ectodomains, reflecting possible differences in the local Env structures between JSRV and ENTV.
Figure 2. Sequence alignment of the JSRV and ENTV TM and surface expression of Env mutants.

(A), Sequence alignment. JSRV-7 and ENTV-1 are two representative strains of JSRV and ENTV isolated from sheep. Alignment was performed using Clustal W (asterisk, invariant; colon, highly similar; dot, similar). The positions of the two heptad repeats (HR1 and HR2) are indicated. Six reciprocal mutation sites in the ectodomain of TM between JSRV and ENTV Env are shown in bold. (B), Env surface expression. 293/GP-LAPSN cells were transfected with plasmid DNA encoding Envs, and the surface expression of SU was assessed by staining cells using anti-FLAG antibody and anti-mouse IgG coupled to FITC and analyzed by flow cytometry. The relative surface expression of each mutant was calculated using their geometric means of FITC fluorescence relative to the parental JSRV Env (set to 100%, dashed line). Averages and S.D. of at least three independent experiments are shown. * indicates $p < 0.05$, ** indicates $p < 0.01$.

The membrane fusion activities of these point mutations were examined. Because of the relative lower fusion activity of ENTV Env as compared with that of JSRV, a longer incubation was required to observe robust cell-cell fusion for ENTV Env (5 min) than that of JSRV Env (1 min) (25). The JSRV L501V mutant consistently exhibited a reduced fusion activity compared with that of parental JSRV Env ($p < 0.01$) (fig. 3, A and B). Conversely, the reciprocal ENTV V501L mutant showed an increased fusion, albeit not as great as that of JSRV L501V relative to the parental ENTV Env at both pH 5.0 and 4.0 ($p < 0.05$) (fig. 3,
A and B). Reduced cell-cell fusion activities were also observed for several JSRV and ENTV mutants, likely due to their reduced surface expressions (fig. 2B) and/or the nature of their specific amino acid changes (see next and “Discussion”). Effects of the Leu-Val change on membrane fusion of JSRV and ENTV Envs were also examined in syncytia induction assays, with similar patterns obtained (data not shown). Taken together, these data indicate that the Leu-Val change in the ectodomain of TM is critical for the differential low pH requirements of JSRV and ENTV Envs.

Figure 3. A Leu-Val change in the ectodomain of TM is critical for the distinct low pH requirements of membrane fusion between JSRV and ENTV Envs. Cell-cell fusion was performed as described in the legend to figure 1C, except different durations of pH treatment were applied. (A), representative dot plots of cell-cell fusion are shown, with the percentages of fused cells being indicated in the upper-right quadrant. (B and C), Cell-cell fusion activities of JSRV and ENTV Env point mutants. Averages and S.D. representatives of 3 independent assays are shown. * indicates $p < 0.05$, ** indicates $p < 0.01$. Dashed lines indicate the background levels of fusion (at pH 7.0). No Env, effector 293T/GFP cells were not transfected with an Env-encoding plasmid. (D and E), Relative cell-cell fusion activities of Env mutants at different pH conditions. In both figures, the maximal fusion activities of the parental JSRV or ENTV Envs at pH 4.0 were set as 100%. Averages and S.D. of 3 independent experiments are shown.

Reciprocal Substitution between Leu and Val in Env Chimeras Rescues, Albeit Not Completely, Differential Low pH Requirement for Fusion between JSRV and ENTV

The incomplete switch of fusion phenotypes between JSRV L501V, ENTV V501L, and their parental Envs observed above could be explained by sequences other than the single Leu-
Val difference in the ectodomains of the TMs, or in other regions of the Envs (fig. 2A). We therefore created reciprocal L501V and V501L mutants in the backbones of the chimeric EJEE and JEJJ Envs (fig. 1A), respectively, and asked if the altered fusion phenotypes of these chimeras, particularly their differential low pH requirements, could be reversed. We found, indeed, that the JEJJ V501L mutant readily induced larger numbers of syncytia at pH 5.0 following the typical 1-min treatment, similar to the JSRV Env wild type, and the EJEE L501V mutant exhibited virtually no syncytia following the same pH 5.0 treatment, resembling the wild type ENTV Env (data not shown).

We next employed flow cytometry-based cell-cell fusion assays and compared the membrane fusion activities of these constructs with their wild types at different pH values. Although JSRV Env induced robust membrane fusion at a wide range of pH, as we had shown previously (< pH 6.0) (25), JEJJ only induced low levels of fusion at the very low pH values (pH < 4.5) (fig. 3D). Importantly, JEJJ V501L restored, albeit not completely, the fusion activity of JEJJ at both pH 5.0 and 4.5 (fig. 3D). In comparison, EJEE exhibited robust fusion activity at pH 6.0 and lower, yet the EJEE L501V mutant displayed a much reduced fusion activity, a pattern similar to the ENTV wild type (fig. 3E). Collectively, these data demonstrated that reciprocal substitutions between Val and Leu at position 501 in the Env chimeras switch, albeit not completely, the differential low pH requirements for fusion between ENTV and JSRV. These results suggest that additional amino acids besides the Leu and Val residues in the ectodomain and possibly other regions of Env participate in modulating the differential low pH requirements for fusion between JSRV and ENTV Env.

The Leu-Val Change in Ectodomain of TM Distinguishes Sensitivity of JSRV and ENTV Env Pseudovirions to Lysosomal Protease Inhibitor, Leupeptin
We previously demonstrated that, despite similar pH-dependent entry processes by JSRV and ENTV, the ENTV Env-mediated pseudovirion infection is substantially enhanced by some lysosomal protease inhibitors, such as leupeptin, as compared with that of JSRV (25). We proposed that the enhanced infection of ENTV Env pseudovirions is likely due to a block of viral particle degradation in the late endosome or lysosome where ENTV likely fuse (as a result of its extremely low pH requirement) (25). Here we asked if the Val residue in the HR2 region of ENTV TM might be associated with the enhanced entry of ENTV by leupeptin and whether or not a substitution of the Val with Leu could reverse this phenotype.

We infected the target HTX/LH2SN cells overexpressing the Hyal2 receptor (which permits infection of both JSRV and ENTV) with GFP-encoding Env pseudovirions at relatively low but comparable multiplicity of infections in the presence or absence of leupeptin, and determined the viral infection using flow cytometry. We observed that, different from the parental ENTV Env pseudovirions, the infection of ENTV V501L pseudovirions was not increased by the presence of 25, 50, or 100 μM leupeptin (p < 0.05 for 25 and 50 μM) (fig. 4, A and B). In contrast, the JSRV L501V mutant became sensitive to leupeptin treatment and exhibited an enhanced infection profile relative to the wild type JSRV (p < 0.01 for 50 μM and p < 0.05 for 100 μM), similar to that of ENTV wild type (fig. 4, A and B). As a positive control, the infection of Ebola GP pseudovirions, whose entry is known to be dependent on the low pH-activated host protease activities (32–34), was markedly blocked by the
leupeptin treatment in a dose-dependent manner ($p < 0.01$) (fig. 4, A and B). We also treated HTX/LH2SN cells with 15 and 30 mM NH$_4$Cl, a reversible lysosomotropic agent, and noted that, whereas JSRV infection was potently inhibited by NH$_4$Cl in a dose-dependent manner, the infection of the JSRV L501V mutant was rather enhanced by 30mM NH$_4$Cl similar to that of ENTV Env (25) (data not shown). Collectively, these results show that Val and Leu residues at position 501 of the Envs modulate differential endosomal entry of ENTV and JSRV into cells, consistent with their distinct low pH requirements for membrane fusion.

Figure 4. A Leu-Val change in the ectodomain of TM distinguishes the sensitivity of JSRV and ENTV Env pseudovirions to lysosomal protease inhibitor, leupeptin. HTX/LH2SN cells were pretreated with leupeptin of the indicated concentrations for 1 h and infected with Env pseudovirions encoding GFP (multiplicity of infection between 0.01 and 0.10) for 4–6 h in the presence of the drug. The percentages of GFP positive cells (infection %) were measured 48 h post-infection by flow cytometry. (A), representative infection profiles examined by flow cytometry. The percentages of infected cells for each construct were shown in the lower-right corner of the box. (B), summarized data for the effects of leupeptin treatment on pseudovirion infection. Relative infections were calculated by setting the percentages of infection without leupeptin as 100% (dashed lines). Comparisons were made between ENTV and ENTV V501L, or between JSRV and JSRV L501V at the indicated concentrations of leupeptin. For Ebola virus, the comparisons were made
between the absence and presence of different concentrations of leupeptin. Averages and S.D. of at least three independent experiments are shown. * indicates \( p < 0.05 \), ** indicates \( p < 0.01 \).

**Hydrophobic Residues with Bulky Side Chain at Position 501 of Env Are Critical for Membrane Fusion and Infection of JSRV and ENTV Envs**

The observation that a simple Leu to Val change, and vice versa, can dramatically affect the fusion and infection of ENTV and JSRV prompted us to further examine the amino acid requirement of position 501 for fusion and cell entry. To this end, we substituted the JSRV Leu and ENTV Val residues with several amino acids with different charges, polarities, or other properties, and examined their effects on membrane fusion and pseudovirion infection. All these mutant Envs were processed into SU and TM and expressed in the transfected cells, despite that L501A, L501R, V501G, and V501Q exhibited a decrease in the SU surface expression (by 20–40%) as determined by flow cytometry (fig. 5A and data not shown).

Replacement of the Leu of JSRV with Ala (L501A), Glu (L501E), Gln (L501Q), or Arg (L501R) dramatically decreased the titers of Env pseudovirions in HTX cells overexpressing human Hyal2 (HTX/LH2SN), with the Arg (L501R) and Gln (L501Q) mutants almost completely abolishing viral infection \( (p < 0.01 \) for all mutants) (fig. 5B). Similar patterns were also observed in HTX cells (data not shown). The decreased viral titers correlated with membrane fusion activities, with all these mutants exhibiting either much reduced or no detectable levels of fusion at all acidic conditions \( (p < 0.01 \) (fig. 5D). Interestingly, we found that, replacing the Val of ENTV with Gly (V501G) or Gln (V501Q) resulted in almost no observable fusion and infection titer in HTX/LH2SN cells \( (p < 0.01 \), mutating the Val of ENTV to Ala (V501A) had no apparent effect on viral titer and fusion, and substituting the Val with Ile (V501I) even enhanced viral infection and membrane fusion similar to that of ENTV V501L \( (p < 0.05 \) (fig. 5, C and E). Although discrepancy exists for Ala substitutions between JSRV (L501A) and ENTV (V501A), these data strongly support the notion that position 501 within the HR2 regions of JSRV and ENTV Envs is sensitive to amino acid substitutions, and that hydrophobic residues with bulky side chains at this position are critical for membrane fusion and viral infection.
Figure 5. Hydrophobic residues with a bulky side chain at position 501 are critical for JSRV and ENTV Env-mediated fusion and infection.

(A–C), The 293/GP-LAPSN packaging cell line was transfected with a plasmid DNA encoding Env, and virions were harvested 48 and 72 h post-transfection. (A), The surface expression of Env in the virion-producer cells was measured by flow cytometry using anti-FLAG antibody and anti-mouse IgG coupled to FITC. The relative surface expression of each mutant was calculated by comparing its geometric means of FITC fluorescence with that of parental JSRV Env (set as 100%, dashed line). (B and C), Viral titer was meas-
ured by infection of HTX/LH2SN cells in the presence of Polybrene. AP-positive foci (focus-forming unit, FFU) were counted 72 h post-infection. Comparisons were made between JSRV and its Leu-501 mutants, or between ENTV and its Val-501 mutants. Averages ± S.D. of at least 3 infections are shown. (D and E), membrane fusion activities of JSRV Leu and ENTV Val mutants. Cell-cell fusion assays were performed as described in the legend to figure 6C except different pH conditions and durations of treatment were applied. Comparisons were made between JSRV and its Leu-501 mutants or between ENTV and its Val-501 mutants at the respective pH conditions. Averages ± S.D. of at least 3 infections are presented.

In all cases, * indicates \( p < 0.05 \), ** indicates \( p < 0.01 \).

**Synthetic HR2 Peptides Harboring Leu or Val Inhibit Membrane Fusion and Pseudoviral Infection**

To understand the possible mechanisms by which the Leu and Val residues modulate membrane fusion of JSRV and ENTV Env, we synthesized peptides homologous to the predicted HR1 and HR2 sequences of JSRV and ENTV Envs (fig. 2A), and evaluated their effects on membrane fusion and infection. The HR1 peptide, which is common to both JSRV and ENTV Env sequences, showed no effect on syncytia formation and cell-cell fusion, even at the 100 \( \mu g/ml \) concentration (fig. 6A). In contrast, the HR2 peptide containing the JSRV Leu inhibited JSRV Env-mediated syncytia formation in 293 cells induced by sHyal2 and low pH (16) (fig. 6A), as well as cell-cell fusion in 293/LH2SN cells albeit with less efficiency (~50%, \( p < 0.05 \) or 0.01) (see below explanations). Interestingly, we found that the HR2 peptide containing Val inhibited the membrane fusion activities of Env as effectively as did the HR2 peptide containing Leu at all the acidic pH conditions tested (fig. 6, A and B, data not shown). Similar results were also obtained for ENTV (data not shown).
Figure 6. Effects of HR1 and HR2 peptides on syncytia formation, cell-cell fusion, and pseudoviral infection. 

(A), JSRV Env-induced syncytia formation in 293 cells is inhibited by HR2 but not HR1 peptide. 293 cells expressing JSRV Env were incubated with 5 μg of sHyal2 in the absence or presence of the indicated amounts of HR1 or HR2 peptides for 30 min, followed by treatment with a pH 5.0 solution for 1 min. Cells were fed with normal growth media containing the peptides for 1 h, and photographed. The effect of HR2-Val peptide on JSRV Env-mediated fusion was similar to that of HR2-Leu peptide and was therefore not pictured. Arrows indicate the fused cells when fusion was not obvious, note the size differences between 10 and 30 μg/ml of HR2-Leu. Cells treated with a comparable concentration of dimethyl sulfoxide (DMSO) or cells not expressing Env served as controls. 

(B), Cell-cell fusion was performed for JSRV Env as described in the legend to figure 1C, except that the indicated concentrations of HR1 or HR2 peptides were added to the co-cultured cells for 30 min and included during the 1-h recovery period. Averages plus S.D. of 3 independent experiments are shown. 

(C), effects of HR2 peptides on pseudoviral infection. NIH 3T3/LH2SN cells expressing human Hyal2 were pretreated with 20 nM BafA1 for 1 h, and spinoculated with JSRV or JSRV L501V pseudovirions encoding AP for 2 h at 4°C. 30 μg of HR2 peptides harboring Leu or Val were added concurrently at the temperature switch to 37°C and incubated for 1 h before the virus-cell-HR2 complex was treated by a pH 4.5 buffer for 5 min, followed by continued incubation with BafA1 for 6 h. Viral titer was determined by AP staining 72 h postinfection. The typical titers of JSRV and the JSRV L501V mutant in untreated NIH 3T3/LH2SN cells were ~5 × 10^4 and 1 × 10^4 FFU/ml, respectively. BafA1 reduced the infection rates by ~90% and low pH rescued the effect of BafA1 by roughly 60%, see figure 7B and text for details. The effects of HR2 peptides on JSRV and L501V infection were calculated by comparing their titers in the absence (cells were treated with comparable concentrations of DMSO, set to 100%) and presence of peptides. Averages plus S.D. of 4 independent experiments are shown. 

In all cases, * indicates p < 0.05, ** indicates p < 0.01.
We next examined the effects of these peptides on pseudoviral infection of JSRV and JSRV L501V mutant in HTX cells; however, we failed to observe any apparent effect on viral titer (data not shown), likely due to the poor penetration of these peptides into the endosomal compartments as is the case for most pH-dependent viruses. We then tested these peptides in NIH 3T3 cells expressing human Hyal2 (NIH 3T3/LH2SN) using a different strategy: we used BafA1 to block the endosomal entry of the virus and artificially forced viral entry through the cell surface by applying an extracellular low pH buffer (pH 4.5). NIH 3T3/LH2SN cells were chosen because they can sustain a pH 4.5–5.0 treatment for 5 min or longer (31), allowing significant rescue to occur. In this case, HR2 peptides harboring Leu or Val were added at the time of temperature switch and were present during the 6-h infection period. We observed that, indeed, both Leu and Val containing HR2 peptides were able to block pseudoviral infection of JSRV Env or L501V by ~50% (p < 0.05 or 0.01) (fig. 6C), roughly comparable with their effects on cell-cell fusion in figure 6B, albeit not as great as the inhibition seen in 293 cells when only a portion of Env was induced by sHyal2 and low pH (fig. 6A). The difference may be due to experimental conditions, that is, in the traditional cell-cell fusion assay (fig. 6B) and in the infection assays (fig. 6C) the very high levels of cell surface expression of Env and membrane-bound receptor Hyal2 infer that fusion involves a very large number of Env trimers, perhaps so many that the peptide is unable to compete for more than half the molecules of receptor-induced intermediates and block completion of membrane fusion. In contrast, in the experiment shown in figure 6A, 5 μg/ml of soluble receptor (sHyal2) was applied to trigger Env in the absence or presence of peptide prior to low pH exposure, conditions that may induce only a portion of Env trimers enabling the peptide to more successfully compete for receptor-induced intermediates.

The Leu to Val Change Does Not Affect SU-TM Association of Native JSRV Env but Stabilizes Receptor-Induced Intermediate

Our previous studies demonstrate that the membrane fusion activation of JSRV Env (16), and likely ENTV Env as well (25, 26), requires receptor-mediated priming, leading to formation of an intermediate that is further triggered by a low pH (known as a two-step triggering process). Here we tested the possibility that the intermediate of ENTV Env or the JSRV L501V mutant may be relatively more stable than that of ENTV V501L or JSRV Env and therefore requires a lower pH threshold for fusion. Alternatively, the native Env of JSRV L501V or ENTV may be more stable than that of JSRV or ENTV V501L, thus contributing to their differential low pH thresholds for fusion. We first performed metabolic labeling and examined the SU-TM association of Env in the presence or absence of a soluble form of the viral receptor, sHyal2. Although ENTV Env exhibited relatively higher basal levels of SU shedding in the absence of sHyal2, sHyal2 did not apparently increase the ENTV SU shedding (fig. 7A), a result that was in line with our recent reports (26). Importantly, we found no apparent difference in SU shedding between JSRV Env and its L501V mutant, nor between ENTV Env and its V501L mutant, in the absence of sHyal2 treatment (fig. 7A), suggesting that the change between JSRV Leu and ENTV Val at position 501 does not affect the Env stability at the native metastable state.
Figure 7. The Leu-Val change does not affect the SU-TM association of native JSRV/ENTV Env but influences the stability of their receptor-induced intermediate. (A), 293T cells transfected with plasmids encoding individual Env were metabolically labeled for 1 h and chased for 3 h before the addition of the indicated amounts of sHyal2. Cells were chaced for an additional 3 h before being lysed. Cell lysates and culture media were harvested and immunoprecipitated using anti-FLAG beads. Samples were resolved by SDS-PAGE and analyzed by autoradiography. A representative experiment from five independent experiments performed is shown for Env proteins and subunits in the cell lysates (upper panel) and SU that were shed into the culture medium (lower panel). The relative intensity of each SU band shedded was calculated by setting the signal of JSRV in the absence of sHyal2 as 1.00. ‡ indicates a nonspecific band consistently pulled down by anti-FLAG from 293T cells. (B), examination of the stability of Hyal2-induced intermediates of JSRV and the L501V mutant in the context of pseudoviral infection. The experimental procedures were the same as described in the legend to figure 6C except that a low pH 4.5 buffer was applied at the indicated times following the temperature switch from 4 to 37°C. Values shown are the mean ± S.D. from five independent infection experiments. Comparisons were made between JSRV and JSRV L501V at each time point, and p values for 90–210 min were between 0.06965 and 0.1559 (see text). Mock, the infection rates of JSRV and L501V in the absence of BafA1 and low pH pulse were set to 100%.
We next examined the stability of receptor-induced intermediates of JSRV and its L501V mutant. Because ENTV SU is not sensitive to sHyal2-mediated triggering (26), ENTV Env and its V501L mutant were not tested. The rationale is that if the intermediate of the JSRV L501V mutant is more stable than that of JSRV Env, it should persist longer following receptor-mediated triggering and remain responsive to a low pH stimulus for a longer period of time. To test this hypothesis, we pretreated NIH 3T3/LH2SN cells expressing human Hyal2 with BafA1 to neutralize their low pH compartments prior and spinoculated JSRV or L501V pseudovirions at 4°C for 2 h; at different time points following a temperature switch to 37°C, the virus-cell complex was treated with a pH 4.5 buffer for 5 min, and infection was continued for a total of 6 h at 37°C in the constant presence of BafA1. Consistent with our previous results (31), low pH rescued the BafA1-mediated arrest of JSRV pseudoviral infection by ~60% at 30 and 60 min following receptor-induced triggering (fig. 7B); however, the efficiency of rescue decreased over time, with only ~20% at 4 h post-infection. In contrast, low pH rescue of the JSRV L501V pseudoviral infection persisted at levels of ~60% throughout the 4-h study period, with only slight reductions over time (fig. 7B). These results suggest that the intermediate of JSRV L501V is relatively more stable than that of JSRV Env, especially at time points between 90 and 210 min following the initiation of infection ($p < 0.06965–0.1559$, $n = 5$), and explain, at least in part, the lower pH requirement of the L501V mutant for membrane fusion.

Discussion

The Env proteins of JSRV and ENTV share significant sequence homology (30) and both bind to the same cellular receptor, Hyal2, for virus entry (19, 24, 35, 36). However, ENTV Env exhibits a much lower fusogenicity and requires an extraordinarily acidic pH for membrane fusion compared with that of JSRV (25). Our recent data indicate that the low fusogenicity of ENTV Env is not because of receptor binding per se, but lies in its intrinsic insensitivity to receptor-mediated triggering for membrane fusion (26). Here, we show that the distinct low pH requirements for fusion and infection between JSRV and ENTV are associated with a Leu-Val change within the HR2 region of the Envs.

Importantly, the Leu and Val residues are absolutely conserved among all the JSRV and ENTV sequences (also known as ENTV-1 in sheep) published to date, including the 10 ENTV strains recently isolated from North America (37). Also of note, all the endogenous sheep retrovirus (enJSRV) Envs reported so far possess a Val residue (38, 39), whereas the two full-length ENTV sequences isolated from goats (also known as ENTV-2, GenBankTM accession numbers AY197548 and HM104174) (40) harbor an Ile residue at position 501. Although the low pH requirement for fusion of ENTV isolated from goats is currently unknown, the enJSRV5F16 (GenBank accession number AF136224) Env, which is one of the representative strains of enJSRVs (38), has been determined to require a very acidic pH (pH < 4.5) for membrane fusion similar to that of ENTV in sheep.5 Hence, evolutionarily the Leu, Val, and Ile residues are conserved among these different groups of sheep retrovirus Envs at position 501, and they appear to be functionally linked to the differential low
pH requirements for membrane fusion and cell entry. Our current work represents an interesting and perhaps unique example where a Leu-Val difference in closely related viruses can have important influence on the pH-dependent membrane fusion and cell entry.

How might the conservative Leu to Val substitution cause such striking changes in the fusion capacity of Env and the pH required to trigger membrane fusion? One possible explanation for its mechanism of action is that the L501V change stabilizes the native pre-fusion structure of Env. However, we found here that SU shedding of JSRV L501V and ENTV V501L mutants did not change in the absence of sHyal2; each phenocopied their respective wild type parent Env (fig. 7A), strongly arguing that the Leu-Val change does not affect the stability of native JSRV and ENTV Env. Another possibility is that the L501V change stabilizes the receptor-induced intermediate and therefore necessitates a lower pH for triggering the final conformational rearrangements required for 6HB formation and membrane fusion. This idea is based on our previous finding that both receptor binding and low pH are required for triggering fusion and that the triggers have a requisite order, i.e., receptor binding followed by low pH exposure (16). Increasing the stability of the receptor-induced intermediate would in effect raise the energy barrier between the metastable intermediate state and the final 6HB conformation. In support of this hypothesis, we found here that the sHyal2-induced intermediate of the JSRV L501V mutant was stable for at least 4 h following sHyal2 triggering (fig. 7B). In contrast, the wild type JSRV Env lost substantially its sensitivity to low pH-mediated rescue of viral infection (fig. 7B). It is unlikely that these differences were due to differences in the internalization rates of the wild type and its L501V mutant because these Env have identical SU subunits that should bind identically to the Hyal2 receptor and be internalized at comparable rates. We also applied HR2 peptides harboring Leu or Val at different time periods following the sHyal2-mediated triggering (0–2 h), but observed similar efficiencies of blocking JSRV and JSRV L501V pseudoviral infection (~50%, data not shown). We reason that this was likely due to their less differences in the kinetics of intermediates at the earlier time points (fig. 7B) as well as the less potency of these peptides in blocking membrane fusion (fig. 6B). Although additional experiments are needed to further determine the biochemical properties of the Hyal2-induced intermediate, these initial data support the notion that the Leu-Val change influences the stability of the receptor-induced intermediate and likely contributes to the differential low pH requirements between JSRV and L501V mutant.

We cannot exclude the possibility that the L501V change may affect the pH-sensing mechanism, although it is unlikely that the Leu and Val residues at position 501 would directly sense the low pH environment of endosomes for membrane fusion. Pioneering work on influenza virus have suggested that residues in the fusion peptide and interface between the membrane-distal and anchored domains are important for modulating the pH threshold for viral fusion activation (41). More recent studies on influenza and other viruses suggest that ionic residues present in the viral fusion proteins can affect the stability of 6HB and therefore pH-dependent fusion (42–44). In particular, histidine (His) residues have been shown to be crucial for fusion activation of a number of pH-dependent viruses covering all three classes of viral fusion proteins (45–51). Given that the pH threshold of JSRV for membrane fusion is pH 6.3 (27), which is very close to the side chain pKₐ of His (52), and that the ectodomain of TM dictates the pH threshold as shown in this study (fig.
1), it is likely that His residues residing in the TM serve as pH sensors mediating low pH triggering of JSRV Env. Examination of JSRV and ENTV Envs reveals five conserved His residues in the ectodomain of TM, three of which reside within or between the HR1 and HR2 regions (fig. 2A). Although the side chain pKₐ of His is 6.5 when measured as a pure L-amino acid in aqueous solution, in the context of a protein its pKₐ can vary widely (52). The best demonstrated correlation is that His residues exposed on the surface and buried within the core structure typically have pH 6.5 values for their pKₐ, whereas partially buried His show lower pKₐ down to very acidic values in a reported range of 5.4 to 3.5 (53). It is thus possible that the smaller more compact Val side chain induces a change in structure that results in partial burial of the side chain of nearby critical pH-sensing His residues, lowering their side chain pKₐ to pH ~4.5. Along this line, we noticed that several JSRV and ENTV point mutants exhibit much reduced fusion activities relative to the parental Envs (fig. 3, B and C), especially ENTV E489K, which is one amino acid apart from the residue His-491 (fig. 2A). It is possible that Glu-489 and His-491 engage in a cation-π-electron interaction that regulates the pH threshold for fusion activation. Work is in progress to identify pH sensors of ENTV and JSRV Envs responsible for their distinct low pH requirements for membrane fusion activation and determine their relationship to a L501V change.

A recent study of HIV-1 gp41 indicates that residues proximal to the hairpin turns of the 6HB are critical for membrane fusion, proposing that these residues are likely the first to be involved in the nucleation process between HR1 and HR2 and therefore crucial for driving the 6HB formation (54). Evidently, position 501 of JSRV and ENTV Env resides at the beginning of their putative hairpin turns, occupying the “d” position in the first 3–4 heptad repeat of HR2 (fig. 2); this could provide an additional explanation of why a small change between Leu and Val can have a dramatic effect on membrane fusion. In support of this hypothesis, we found that position 501 of JSRV and ENTV Env is sensitive to amino acid substitutions, with hydrophobic residues possessing a bulky side chain generally promoting membrane fusion and viral infection compared with other residues (fig. 5). Ultimately, a comparison of the three-dimensional structures of JSRV and ENTV Envs, in both prefusion and postfusion conformations, will be required to answer some of the remaining critical questions, including how the conformational coupling occurs between SU and TM during the unconventional two-step fusion activation process.

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Notes

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4. Abbreviations: Env, envelope protein; JSRV, jaagsiekte sheep retrovirus; ENTV, enzootic nasal tumor virus; SU, surface subunit; TM, transmembrane subunit; MSD, membrane-spanning domain; CT, cytoplasmic tail; Hyal2, hyaluronidase 2; MLV, murine leukemia virus; HIV, human immunodeficiency virus; HR1, heptad repeat 1; HR2, heptad repeat 2; 6HB, 6-helix bundle; CMTMR, 5-((4-chloromethyl)benzoyl)amino)tetramethylrhodamine; GFP, green fluorescent protein; HTX, a subclone of HT1080; 293/LH2SN, a 293 cell clone overexpressing human Hyal2; HTX/LH2SN, a HTX cell clone overexpressing human Hyal2; 293/GP-LAPSN, a 293 packaging cell line expressing MLV Gag-Pol and alkaline phosphatase; AP, alkaline phosphatase; BafA1, bafilomycin A1.


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