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Definition of an Amino-terminal Domain of the Human T-cell Leukemia Virus Type 1 Envelope Surface Unit That Extends the Fusogenic Range of an Ecotropic Murine Leukemia Virus

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Felix J. Kim‡§, Iban Seiliez‡, Caroline Denesvre¶, Dimitri Lavillette‡**, François-Loïc Cosset‡**, and Marc Sitbon‡‡†† From the ‡Institut de Génétique Moléculaire de Montpellier, IPR24, CNRS-UMR5535 and Université Montpellier II, 1919 Rte de Mende, F-34293 Montpellier Cedex 05, **LVRTG, INSERM U412, Ecole Normale Supérieure de Lyon, 46 allée d’Italie, 69367 Lyon Cedex 07, and ¶Mnsit Cohé de Génétique Moléculaire, 22 Rue Méchain, 75014 Paris, France

Murine leukemia viruses (MuLV) and human T-cell leukemia viruses (HTLV) are phylogenetically highly divergent retroviruses with distinct envelope fusion properties. The MuLV envelope glycoprotein surface unit (SU) comprises a receptor-binding domain followed by a proline-rich region which modulates envelope conformational changes and fusogenicity. In contrast, the receptor-binding domain and SU organization of HTLV are undefined. Here, we describe an HTLV/MuLV envelope chimera in which the receptor-binding domain and proline-rich region of the ecotropic MuLV were replaced with the potentially corresponding domains of the HTLV-1 SU. This chimeric HTLV/MuLV envelope was processed, specifically interfered with HTLV-1 envelope-mediated fusion, and similar to MuLV envelopes, required cleavage of its cytoplasmic tail to exert significant fusogenic properties. Furthermore, the HTLV domain defined here broadened ecotropic MuLV envelope-induced fusion to human and simian cell lines.

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‡‡ Supported by the Institut National de la Santé et de la Recherche Médicale. To whom correspondence should be addressed: Institut de Génétique Moléculaire de Montpellier (IGMM), CNRS-UMR5535, 1919 Rte. de Mende, F-34293 Montpellier Cedex 5, France. Tel.: +33-467-613-640; Fax: +33-467-040-231; E-mail: sitbon@jones.igm.cnrs-mop.fr.

Murine leukemia viruses (MuLV) are simple C-type oncoretroviruses whose genetic organization differ significantly from that of complex retroviruses, such as the human immunodeficiency virus (HIV) and the human T-cell leukemia virus (HTLV), by the lack of accessory and regulatory genes in addition to the gag, pol, and env genes. Each functional retroviral envelope glycoprotein is expressed as a precursor that is cleaved into two associated components, a surface subunit (SU), implicated in receptor recognition, and a transmembrane subunit (TM), which harbors a fusion peptide (1). Current understanding of envelope-mediated fusion suggests that receptor recognition by the SU induces conformational changes that unmask fusion determinants in the TM. MuLV envelopes have weak fusogenic abilities when expressed at the cell surface (fusion “from within”) and stronger fusogenic ability in the context of the viral particle (fusion “from without”). The increased fusogenicity of the MuLV envelopes in virions has been associated with viral protease cleavage of the cytoplasmic TM carboxyl terminus, known as the R-peptide, which occurs late during or after virion assembly (2, 3). Concerning the SU, a common organization in three major domains has been described for all MuLV: (i) the amino terminus comprising two variable regions, VRA and VRB, which define receptor binding specificity (4); (ii) a proline-rich region, which regulates post-receptor binding changes in conformation and fusion ability of the envelope (5, 6); and (iii) the carboxyl terminus, thought to interact with the TM subunit. Also, the three MuLV envelope receptors identified so far are multiple-membrane-spanning proteins (7–11). In contrast to MuLV, HTLV envelope is highly fusogenic in cell-to-cell fusion assays, measuring fusion from within, whereas cell-free virions are reported to be poorly infectious (12–15). Moreover, when expressed at the cell surface, the HTLV envelope induces rapid, rampant syncitia formation (12–15). Furthermore, the SU of HTLV envelope induces conformational changes with a broad range of cell lines, suggesting that the yet unidentified HTLV receptor(s) is a highly conserved and ubiquitous molecule. However, neither the receptor-binding domain nor a particular organization has been reported for the HTLV SU.

Here, we describe conserved determinants in the SU of the two envelopes based on a novel amino acid alignment. Using this alignment, we derived an HTLV/ectropic MuLV envelope chimera that presented a fusogenic range extended to human and simian cell lines while exhibiting the general fusion characteristic of MuLV envelopes.

EXPERIMENTAL PROCEDURES

Construction of the HTLV/MuLV Envelope Chimera—Introduction of a BarGI site into both parental envelope genes, which maintained their wild-type amino acid sequence, was performed by polymerase chain reaction mutagenesis using the following oligonucleotides in which the created restriction sites are indicated in italics and the nucleotide substitutions underlined: AGGTACTAAAATCTtgtacaGG-GAGCT (sense BarGI F-MuLV); AGCTCCGtgtacaGAATTTTAGAACCT (antisense BarGI F-MuLV); GTGACCTtgtacaGAACCTCTA (sense BarGI HTLV-1); TAGGGTTAAGtgtacaAGGGTCA (antisense BarGI HTLV-1). Expression vectors for the HTLV and MuLV envelope have
been described previously (16, 17). The HTLV-1/Friend-MuLV SU chimeric HHproFc reported here was constructed in a pGEM-based plasmid and subsequently cloned into the parental Friend-MuLV envelope expression vector, pCEL/F, at the SpeI and BglII sites. The pCEL/HHproFcLR construct was derived from HHproFc and the FJR envelope. The latter was derived by introducing a stop codon immediately upstream of the first R-peptide codon of the parental Friend-MuLV envelope gene. All mutated regions were sequenced using an ABI Prism sequencer.

**Cell Lines and Fusion Assay**—The following primates and murine cell lines were used in this study: COS (African green monkey kidney cells), HeLa (cervical carcinoma), Dunni (murine, Mus dunni tail fibroblasts), NIH3T3 (murine fibroblasts), and 293 (human fetal kidney cells). Cells used for the fusion assay were stable transfectants of either a β-galactosidase gene (LacZ) under the control of the HIV-1 long terminal repeat (LTR) (CosLTLRlacZ and HeLaCD4LTRlacZ), which has Tat-dependent expression, or cell lines constitutively expressing the Tat protein of HIV-1 (Cos-Tat, Hela-Tat, NIH-Tat, Dunni-Tat) as described previously (16–18).

Envelope-mediated fusion was quantified essentially as described (16, 17) with a few modifications. In this assay, the HIV-1 LTR-driven expression of β-galactosidase is transactivated by the Tat protein upon fusion of envelope-expressing cells with receptor-bearing indicator cells. Envelope genes were transfected into the cell lines described above using polyethyleneimine (25-kDa, water-free; Sigma catalog no. 40,872–7) as described (19). 24 h prior to transfection, 5 × 10^4 cells were seeded per 35-mm well on six-well plates (Nunc) with Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Life Technologies, Inc.), 2 mM L-glutamine, and penicillin-streptomycin. For all cell lines tested, transfections were performed with an amine:nitrogen:phosphate nmol ratio of 10:1, wherein 1 µl of the 10 nmol polyethyleneimine solution, pH 7.0, containing 10 nmol of amine nitrogen, and 1 µg of the envelope gene-containing pCEL expression vectors, were estimated to comprise 3 nmol of phosphate. Between 0.5 and 2.0 µg of envelope-expressing plasmid were transfected, and 24 h post-transfection, 10^5 indicator cells (the Tat expressing cell lines) were co-cultivated with the envelope-presenting cells for 36 to 48 h. Cell-to-cell fusion was measured following fixation with 30% (weight/volume) glutaraldehyde in phosphate-buffered saline (PBS), washed with PBS, and stained by incubation in a 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside solution as described previously (16, 17). Blue syncitia, indicating fusion between the envelope-presenting and Tat-containing indicator cells, were counted regardless of the number of nuclei per syncitia. All data represent the results of at least three independent experiments, with each envelope-to-cell combination performed in duplicate. Statistical analysis was performed using the Student t test. All p values of comparisons considered to be significantly different in this report were p < 0.04.

**Envelope Expression and Maturation**—24 h prior to transfection, 1–5 × 10^4 HeLa, Cos, 293, NIH3T3, and Dunni cells were seeded/10-cm plate of each primary antibody, washed three times with PBS/Tween 20, and probed with the corresponding horseradish peroxidase-conjugated immunoglobulins raised against the species appropriate primary antibody, the immunoblots were washed three times with PBS/Tween 20 and probed with a 1:1000 dilution of goat anti-Rauscher leukemia virus gp69/70 polyclonal antibody (Quality Biotech Inc.) in PBS containing 0.5% polyoxyethyleneorbitanmonalaurate (Tween 20) and 5% milk with a 1:2000 dilution of mouse anti-HTLV-1 gp46/61 monoclonal antibody 1C11 (Epitope). After each respective incubation with the appropriate primary antibody, the immunoblots were washed three times with PBS/Tween 20 and probed with the corresponding horseradish peroxidase-conjugated immunoglobulins raised against the species of each primary antibody, washed three times with PBS/Tween 20, and detected using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech). No differences in expression levels and precursor cleavage were observed between the 24- and 48-h time points or between the different cell lines transfected.

**Envelope Fusion Assay**—24 h prior to transfection, 5 × 10^4 HeLaCD4LTRlacZ and NIH3T3/Tat cells were seeded in separate 35-mm wells. The latter cells, initially selected from NIH3T3 cells to not express the thymidine kinase gene (20), stably expressed the Tat protein upon transfection with a Tat expression vector as described previously (16, 18). Two µg of the HHproFc chimera or the Friend-MuLV envelope gene and 3 µg of the FJR MuLV or the HTLV-1 envelope genes were transfected, as described above, into the

**RESULTS**

**Homologous Determinants between the SU of the HTLV and MuLV Envelopes**—We previously described a conserved SU determinant, comprising the amino acid residues CWLCL, among C- and D-type oncoretroviruses (21). A similar motif, comprising the amino acid residues CIVCI, is located at an equivalent position in the HTLV-1 envelope SU. We used parameters in the Clustal program of the Megalign alignment software package (DNASTar) that favored the alignment of the regions containing the CIVCI and CWLCL sequences without regard to an SU/TM cleavage site alignment. This alignment revealed a striking homology between a RLLNLVQ motif in the Friend-MuLV SU, located immediately downstream of the Friend-MuLV envelope proline-rich region (Fig. 1A), and a KLLTLVQ sequence in the HTLV-1 SU. Furthermore, the latter motif was located at an equivalent distance from the SU/TM cleavage site and immediately downstream of a potential proline-rich region of the HTLV SU. These homologies compelled us to test whether the HTLV SU amino terminus could functionally re-
Expression and cleavage of the HTLV/MuLV envelope chimera. Western blot of HeLa total cell lysates 48 h post-transfection with irrelevant plasmid DNA (Mock), chimeric HHproFc (HH), HHproFcAR (HHΔK), and parental HTLV and MuLV envelope expression vectors. The left panel shows a Western blot probed with the anti-HTLV SU gp46 monoclonal antibody (mAb) 1C11, and the right panel shows a duplicate blot probed with the anti-MuLV SU gp70 polyclonal antibody. Migration of uncleaved envelope precursor (Pr) and cleaved SU are indicated in kilodaltons for both parental HTLV and F-MuLV. Symbol keys are shown next to each identified envelope precursor.

Expression and Maturation of a MuLV Envelope Chimera with an HTLV Amino Terminus—HTLV/MuLV envelope chimeras wherein we replaced the entire MuLV SU with that of HTLV (16, 17) or wherein the exchange border was located between the (K/R)LL(T/N)LVQ and the C(W/I)(L/V)C(L/I) motifs (data not shown) resulted in the translation of envelope precursors that were not efficiently processed through the endoplasmic reticulum. Others have also reported that substitution, deletion, or insertion mutations within various subdomains of the SU of the HTLV envelope led to uncleaved and non-matured envelope precursors (22, 23). Here, we constructed a new HTLV/MuLV envelope chimera (Fig. 1B) in which the exchange border corresponded to the newly introduced leucine and valine amino acid residues 327 and 328 of Friend-MuLV.

Mean number of blue fusion presented as relative or + values: +<10; ++<10–100; +++<100–400; +++<500–9000; ++++<1000. Data shown in Table represent values from three independent experiments performed in duplicate, with all standard errors of the mean between 2 and 30. The parental HTLV envelope induced the formation of significantly larger syncitia than HHproFcAR, although they induce similar numbers of blue fusion after X-gal staining.

Blue foci were counted regardless of syncitia size. Data shown in Table are representative of three independent transfections.

**TABLE I**

Fusion-inducing capacities of F-MuLV, HTLV-1, and HHproFc envelopes, with or without the R-peptide

<table>
<thead>
<tr>
<th>Envelope</th>
<th>Primate cell lines&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Murine cell lines&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HeLa</td>
<td>COS</td>
</tr>
<tr>
<td>Mock</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F-MuLV</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>FAR</td>
<td>–</td>
<td>++</td>
</tr>
<tr>
<td>HHproFc</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>HHproFcAR</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>HTLV-1</td>
<td>++++</td>
<td>+++</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean number of blue fusion presented as relative or + values: –<10; ++<10–100; +++<100–400; +++<500–9000; ++++<1000. Data shown in Table represent values from three independent experiments performed in duplicate, with all standard errors of the mean between 2 and 30. The parental HTLV envelope induced the formation of significantly larger syncitia than HHproFcAR, although they induce similar numbers of blue fusion after X-gal staining.

<sup>b</sup> Blue foci were counted regardless of syncitia size. Data shown in Table are representative of three independent transfections.

**TABLE II**

Specific interference of HTLV-1 envelope-mediated fusion by HHproFc

<table>
<thead>
<tr>
<th>Challenging envelope&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Interfering envelope</th>
<th>No. of blue foci&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>HHproFc</td>
<td>545</td>
</tr>
<tr>
<td>H</td>
<td>HHproFc</td>
<td>52</td>
</tr>
<tr>
<td>F-MuLV</td>
<td>HHproFc</td>
<td>798</td>
</tr>
<tr>
<td>FAR</td>
<td>HHproFc</td>
<td>968</td>
</tr>
<tr>
<td>FAR</td>
<td></td>
<td>913</td>
</tr>
</tbody>
</table>

<sup>c</sup> Challenging HTLV-1 (H) envelope (3 μg) was transfected into NIH3T3/TK/ΔTat cells and cocultivated with HeLa/LTR LacZ cells transfected with interfering envelope (2 μg); challenging FAR envelope (3 μg) was transfected into HeLa/LTR LacZ cells and cocultivated with NIH3T3/TK/Tat cells transfected with interfering envelope (2 μg).

<sup>d</sup> Blue foci were counted regardless of syncitia size. Data shown in Table are representative of three independent transfections.

Upon transfection, the HHproFc chimeric precursor and SU proteins reacted with a monoclonal antibody, 1C11, raised against an HTLV-1 envelope SU synthetic peptide of amino acids 190–209 (24) (Fig. 2, lane 4) located within the potential proline-rich region of the HTLV envelope. Precursor cleavage was observed in both the chimeric and parental envelopes, although cleavage appeared to occur more efficiently in the parental HTLV-1 (Fig. 2, lane 6) and Friend-MuLV (Fig. 2, lane 8) than in the HHproFc chimera (Fig. 2, lane 4). Similar results were obtained for all cell lines tested, including human, simian, mouse, and rat cells (data not shown).

Fusion Properties of the HHproFc Envelope Chimera Extended to Primate Cells—When testing the fusion ability of either the parental ecotropic MuLV or the HHproFc chimeric envelope described above, no detectable cell-to-cell fusion was observed, regardless of the species origin of the target cell (Fig. 3).
3, A, B, E, and F). However, as described previously for amphotropic and ecotropic Moloney MuLV (2, 3), fusion of mouse NIH3T3 cells was detectable only after removal of the envelope inhibitory R-peptide, located at the carboxyl terminus of the TM cytoplasmic domain (Fig. 3D). Therefore, we also tested the fusogenic ability of a HHproFcR construct, corresponding to the HHproFc chimeric envelope lacking the R-peptide. Whereas neither the parental nor the R-peptide-less forms of the ecotropic Friend-MuLV envelope induced fusion with simian and human cells (Table I and Fig. 3, A and C), the HHproFcR envelope was fusogenic toward mouse cell lines as well as human and simian cell lines (Table I and Fig. 3, G and H). It is noteworthy that the parental Friend-MuLV envelope was slightly fusogenic for the mouse Dunni cells in the presence of the R-peptide, whereas deletion of this peptide in HHproFc appeared to be necessary to detect fusion even on this cell line (Table I). Furthermore, despite its extended range of target cells, the HHproFcR envelope remained significantly less syncytial than the parental HTLV-1 envelope (Table I and Fig. 3 [compare panels G and H with I and J]).

**Interference of HTLV-1 Envelope-mediated Fusion by the HHproFc Envelope Chimera**—To assess whether the HTLV/MuLV envelope chimera indeed interacted with the HTLV-1 receptor, we tested the ability of the HHproFc chimera to interfere with HTLV-1 envelope-induced fusion. For this purpose, we tested fusion using NIH3T3(TK<sup>−</sup>)Tat cells because these cells presented smaller fusion foci with the HTLV-1 envelope than any other cells tested (not shown). Using this system, we observed that the HHproFc chimeric envelope specifically inhibited HTLV-1 envelope-mediated fusion by more than 10-fold (Table I).

**DISCUSSION**

Here, we describe homologous motifs between the SU of the Friend-MuLV and HTLV-1, two phylogenetically distant oncoretroviruses. Because the SU is considered to be the most variable region of related retroviral envelopes and because this variability establishes the basis for receptor recognition, our observation may provide important clues concerning the nature of the elusive HTLV envelope receptor(s). Indeed, the MuLV, feline leukemia virus (FeLV), Gibbon ape leukemia virus (GALV), and D-type retrovirus envelope receptors identified thus far belong to a family of multiple-membrane-spanning proteins (7–11, 25, 26), which includes solute transporters (8–10, 26). It is tempting, therefore, to speculate that the HTLV SU receptors may belong to this family as well. Although the HTLV SU receptor remains to be identified, our interference data suggest specific HTLV receptor recognition by the chimeric SU (Table I). Further constructions providing a more precise definition of the receptor-binding domain, proline-rich region, and carboxyl terminus of the HTLV envelope will help in the production of separable domains.

In this report, we replaced the receptor-binding domain and proline-rich region of the MuLV envelope SU with the potentially corresponding domains in the HTLV and postulated that such an HTLV/MuLV SU chimera would broaden the receptor recognition properties of the ecotropic MuLV envelope. Indeed, we observed that HHproFc required R-peptide deletion for fusion with murine and primate cell lines, including human HeLa (Fig. 3) and 293 cells (data not shown), similar to the Friend-MuLV envelope, suggesting that this HTLV/MuLV SU chimera combined the extended host range of HTLV with MuLV envelope fusion characteristics. This envelope represents a novel tool for better understanding the particularly highly fusogenic properties of the HTLV envelope and the search for the HTLV receptor(s).

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