Mutational analysis of the R peptide cleavage site of Moloney murine leukaemia virus envelope protein

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Moloney murine leukaemia virus (MoMLV) enters host cells by membrane fusion between the viral envelope and the host cell membrane. The cytoplasmic tail (R peptide) of the MoMLV envelope protein (Env) is cleaved by the viral protease during virion maturation. R peptide-truncated Env induces syncytia in susceptible cells but R peptide-containing Env does not, indicating that the R peptide inhibits membrane fusion. To examine the function of amino acid residues at the R peptide cleavage site in virus entry, mutant Env expression plasmids containing amino acid substitutions at these cleavage site residues were constructed. Some of these mutants induced syncytia in NIH 3T3 cells, even though they expressed the R peptide, indicating the importance of these residues for membrane fusion inhibition by the R peptide. Some mutants in which R peptide cleavage was detected had comparable transduction efficiency to wild-type Env, but mutants in which R peptide cleavage was not detected had lower transduction efficiency than wild-type Env. This result strongly supports that R peptide cleavage is required for virus entry.

To understand the role of the R peptide cleavage site of MoMLV Env protein in syncytium formation, incorporation into virus particles, R peptide cleavage by the viral protease and entry into host cells, plasmids encoding mutant Env proteins containing amino acid substitutions at the R peptide cleavage site were constructed by PCR-mediated mutagenesis (Cheng et al., 1994; Higuchi et al., 1988; Kubo et al., 1994). The leucine residue at the N-terminal side of the R peptide cleavage site (position 616) was changed to arginine (L\textsuperscript{616R}), alanine (L\textsuperscript{616A}), valine (L\textsuperscript{616V}) and isoleucine (L\textsuperscript{616I}). The valine residue at the C-terminal side of the R peptide cleavage site (position 617) was changed to translation termination (R\textsuperscript{−}), arginine (V\textsuperscript{617R}), alanine (V\textsuperscript{617A}), leucine (V\textsuperscript{617L}) and isoleucine (V\textsuperscript{617I}).
The number of syncytia per microscopic field was scored as +++++ (60 syncytia), + + + (10–60 syncytia), + (1–10 syncytia) and − (0 syncytia).

†The titre of wild-type Env was deemed 100 %. Titres of the mutants were scored relative to that of wild-type Env. Thus, +++ (90–100 %), ++ (60–90 %), + (10–60 %) and − (<10 %).

To determine the transduction titres of MoMLV vectors expressing mutant Env proteins, TELCeB6 cells transfected with these mutant Env expression plasmids were inoculated to NIH 3T3 cells in the presence of polybrene (4 μg ml⁻¹). Cells were then stained with X-Gal. The transduction titre of a retrovirus vector carrying the wild-type Env protein was 1–8 × 10⁴ c.f.u. ml⁻¹. The transduction titre of a MoMLV vector carrying the R− Env protein was about 1/10 times lower than that of the wild-type Env protein (Fig. 2A). Titres of the L⁶¹⁶V-, L⁶¹⁶I-, L⁶¹⁶R-, V⁶¹⁷I- and V⁶¹⁷L-transfected cells. This result indicates that the residuals at the R peptide cleavage site are important for the inhibition of membrane fusion by the R peptide.

Reduced levels of the mature SU protein were detected in virion preparations from the R− and L⁶¹⁶R- and V⁶¹⁷R-transfected cells compared to those from cells transfected with the wild-type Env expression plasmid (Fig. 1A). Equal amounts of the mature CA protein, however, were detected in all virion preparations, indicating that the preparations contain equal amounts of virion. The precursor Env and Gag proteins were not detected in the virion samples, confirming the virion preparation and minimal contamination of cells. These results indicate that the R peptide truncation and the amino acid substitutions of the residues at positions 616 and 617 by arginine (L⁶¹⁶R and V⁶¹⁷R) impair the incorporation of Env protein into virions.

Expression plasmids encoding mutant Env proteins C-terminally tagged with the influenza virus haemagglutinin (HA) epitope were constructed to detect the cleaved R peptide. These HA-tagged mutant Env expression plasmids were transfected to TELCeB6 cells and their transduction titres were measured in NIH 3T3 cells. As shown in Fig. 2(B), transduction titres by MoMLV vectors expressing the L⁶¹⁶A-HA, V⁶¹⁷A-HA and V⁶¹⁷L-HA Env proteins were

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*The amount of Env protein expression was detected by Western immunoblotting using anti-SU antiserum. Similar amounts of Env proteins were detected in transfected TELCeB6 cells as determined by X-Gal staining of NIH 3T3 cells transfected with the wild-type Env expression plasmid (Fig. 1A). Equal amounts of the mature CA protein, however, were detected in all virion preparations, indicating that the preparations contain equal amounts of virion. The precursor Env and Gag proteins were not detected in the virion samples, confirming the virion preparation and minimal contamination of cells. These results indicate that the R peptide truncation and the amino acid substitutions of the residues at positions 616 and 617 by arginine (L⁶¹⁶R and V⁶¹⁷R) impair the incorporation of Env protein into virions.

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To examine the fusogenicity of these mutant Env proteins, 293T cells were transfected with these mutant Env expression plasmids using Trans IT LT1 Polyamine reagent (Mirus). NIH 3T3 cells were then added. The R−, L⁶¹⁶R, L⁶¹⁶A, V⁶¹⁷R, V⁶¹⁷A and V⁶¹⁷L mutants induced syncytia (Table 1). However, syncytia were not detected in wild-type, L⁶¹⁶V-, L⁶¹⁶I- and V⁶¹⁷I-transfected cells. This result indicates that the residues at the R peptide cleavage site are important for the inhibition of membrane fusion by the R peptide.

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comparable to that of a vector expressing the wild-type Env protein C-terminally tagged with the HA epitope (wild-type-HA). Transduction titres of vectors expressing the other mutant Env proteins tagged with the HA epitope, L616R-HA, L616V-HA, L616I-HA, V617R-HA and V617I-HA, were less than 25 % of that of the vector expressing wild-type-HA. These results obtained from the HA-tagged Env proteins was consistent with those from the non-tagged Env proteins (Fig. 2A), indicating that the C-terminal tagging of the Env protein by the HA epitope had little effect on Env protein function. Furthermore, fusogenicity of these HA-tagged mutant Env proteins was similar to that of the untagged mutant Env proteins (data not shown).

To analyse the R peptide cleavage of the HA-tagged mutant Env proteins, TELCeB6 cells were transfected with the HA-tagged mutant Env expression plasmids. Cell lysates were subjected to Tris/Tricine-PAGE and Western blotting using the anti-HA antibody (B).

Fig. 1. Western blot analysis of viral proteins. TELCeB6 cells were transfected with the non-tagged mutant Env expression plasmids. Virus particles were pelleted by ultracentrifugation of the culture supernatant (A). Western blotting using the anti-SU (upper panel) and the anti-CA (lower panel) antiserum was then performed. TELCeB6 cells were transfected with the HA-tagged mutant Env expression plasmids. Cell lysates prepared from the transfected cells were subjected to Tris/Tricine-PAGE and Western blotting using the anti-HA antibody (B).

Fig. 2. Transduction titres of MoMLV vectors expressing mutant Env protein. TELCeB6 cells were transfected with the non-tagged mutant Env expression plasmids (A). NIH 3T3 (upper panel) and XC cells (lower panel) were inoculated with culture supernatants of the transfected cells. Relative values to transduction titre by wild-type Env protein are indicated. The titre of the wild-type MoMLV vector was 1–8 x 10⁴ c.f.u. ml⁻¹. Transduction titres in NIH 3T3 cells by the HA-tagged mutant Env proteins (wild-type-HA) are indicated in (B). Relative values to transduction titre by the wild-type Env protein tagged with HA (wild-type-HA) are indicated. The titre by wild-type-HA was 9 x 10⁵ to 5 x 10⁶ c.f.u. ml⁻¹.
same mutant does not. Four independent L616R expression plasmids constructed by independent PCR, however, all induced syncytia in our study. Nucleotide sequences of the L616R expression plasmids were determined and no unexpected mutations were detected. Rein et al. (1994) used CHO cells as donor cells expressing Env protein, while 293T cells were used in our study. When CHO, mink lung and HeLa cells were used as donor cells, the L616R Env protein also induced syncytia by mixed culture with NIH 3T3 cells (data not shown). The amino acid substitution of hydrophobic leucine by basic arginine in the L616R mutant Env should induce a dramatic change in the three-dimensional structure around the mutated site and suppress R peptide function to inhibit membrane fusion. The L616R, L616A, V617R, V617A and V617L mutant Env proteins induced syncytia in NIH 3T3 cells (Table 1). It has been reported that mutations of the leucine residue at position 618 make the Env protein fusogenic (Yang & Compans, 1997). These results suggested that the amino acid residues at positions 616, 617 and 618 are important for the inhibition of syncytium formation by the R peptide.

To detect the R peptide cleavage of the mutant Env proteins, plasmids encoding mutant Env proteins C-terminally tagged with the HA epitope were constructed. Epitope tagging did not affect transduction efficiency (Fig. 2B) and fusogenicity of the Env proteins. It has been reported that linker insertions around the C-terminal region of the R peptide of MoMLV Env protein do not affect their surface expression and transduction efficiency (Rothenberg et al., 2001). This finding is consistent with our result. Therefore, it is unlikely that the C-terminal HA tagging of the Env protein affects the R peptide cleavage. R peptide cleavage of the L616R, L616V, L616I, V617R and V617I mutant Env proteins was not detected (Fig. 1B). It has been reported using synthetic peptides as protease substrates that amino acid sequences recognized by MoMLV protease are not so specific but that hydrophobic amino acids are involved (Boross et al., 1999; Menendez-Arias et al., 1993, 1994). Therefore, it was interesting that R peptide cleavage of the L616V, L616I and V617I Env proteins was not detected, as these amino acids are also hydrophobic. Granowitz et al. (1996) have reported that R peptide cleavage of L616I is defective, as was seen in our study. The defect in the R peptide cleavage of the L616R and V617R mutant Env proteins could be due to the amino acid substitution of the hydrophobic leucine and valine residues by basic arginine or the impaired incorporation of the L616R and V617R Env proteins into virions, as R peptide cleavage occurs after incorporation of Env protein into virions (Green et al., 1981; Henderson et al., 1984).

These results are summarized in Table 1. MoMLV vectors carrying the L616A, V617A and V617L mutant Env proteins in which R peptide cleavage occurred showed comparable transduction titres to the wild-type MoMLV vector. Vectors expressing the L616V, L616I and V617I Env proteins in which the R peptide cleavage was not detected showed much lower transduction titres than the wild-type vector. This result strongly supports that R peptide cleavage is necessary for efficient transduction by the Env protein.

R peptide cleavage of the L616V, L616I and V617I Env proteins was not detected (Fig. 1B). These Env proteins introduced syncytia in XC cells but not in NIH 3T3 cells, even though they have the R peptide (Jones & Risser, 1993; Kubo et al., 2002). This suggests that the vectors with these mutant Env proteins specifically transduce XC cells, but, like NIH 3T3 cells, they did not (Fig. 2A). This result suggests that the syncytium formation in XC cells by the R+ Env protein is not associated with the membrane fusion required for virus entry into cells.

The results reported here indicate that the amino acid residues at the R peptide cleavage site are important for inhibition of membrane fusion by the R peptide as well as for R peptide cleavage by the viral protease. These results also strongly support the previous finding that the R peptide cleavage of the Env protein is required for virus entry into host cells.

ACKNOWLEDGEMENTS

We thank F.-L. Cosset for TELCeB6 cells, A. Rein for an anti-TM antiserum and A. Ishimoto, H. Sato and N. Yamamoto for discussion. We also thank R. Fujita, M. Katane, E. Takao and N. Sasaki for assistance and A. Koshiyama for secretarial support. This work was supported by the Gene Science Research grant of RIKEN to H. Amanuma. Y. Kubo was a special research fellow of RIKEN.

REFERENCES

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