Increase in infectivity of targeted Moloney murine leukemia virus-based gene-delivery vectors through lowering the threshold for fusion

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Many research groups have developed targeted vectors for gene therapy based on Moloney murine leukemia virus (MoMLV). Despite proper binding of the targeted vector to the target molecule, little or no infectivity of human cells expressing the target molecule has been achieved in most studies. One of the reasons for this lack of infectivity may be steric hindrance within the targeted envelope glycoprotein (Env), impeding the conformational changes required for fusion and infection. Here, attempts were made to solve this problem by mutating key residues within Env of two targeted MoMLV-based vectors, MoMLV–E-Sel and MoMLV–FBP. Selection of key residues was based on an Env with reduced threshold for fusion, that of the CD4-independent human immunodeficiency virus type 2 isolate ROD/B. It was shown here that vectors bearing MoMLV–FBP Env with a V512M substitution had higher titres and faster kinetics of entry than vectors bearing parental targeted Env proteins. This could be due to the partial release of steric constraints that result in an Env with a reduced threshold for fusion.

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INTRODUCTION

Retroviral entry begins with a specific interaction between the surface subunit (SU) of the envelope glycoprotein (Env) and the receptor. Binding to the receptor triggers conformational changes in Env, and clustering of receptor molecules and – in human immunodeficiency virus (HIV) at least – co-receptors or co-factors. Formation of the fusion pore and fusion then take place, either at the cell surface or within an endocytic compartment. Mutational and immunological studies have been used to characterize the functional domains within Moloney murine leukemia virus (MoMLV) Env and the interactions between them (Bae *et al.*, 1997; Battini *et al.*, 1995; Denesvre *et al.*, 1995; Lavillette *et al.*, 1998, 2001; Zavorotinskaya & Albritton, 1999a, b; Zhao *et al.*, 1998; Zhu *et al.*, 1998). It has been suggested that transmission of a signal from the receptor-binding domain (RBD), located at the N-terminal end of the surface glycoprotein, SU, through the proline-rich region (PRR) triggers conformational changes in Env leading to the formation of a trimeric coiled coil at the N-terminal region of the transmembrane (TM) subunit (Fass *et al.*, 1996; Lavillette *et al.*, 2001). This major rearrangement of Env is believed to allow the interaction of the fusion peptide with the cellular membrane, in a similar process to the spring-loaded mechanism proposed for influenza virus haemagglutinin (HA) (Carr & Kim, 1993).

MoMLV is widely used as a gene-delivery vehicle for gene therapy. Several groups have inserted targeting domains at the N terminus of MoMLV Env to produce MoMLV-based targeted vectors. Insertion of targeting domains in MoMLV Env dramatically reduces both envelope-dependent syncytium formation and infectivity of murine cells (Cosset *et al.*, 1995b; Marin *et al.*, 1996; Martin *et al.*, 1998; Pizzato *et al.*, 2001; Viejo-Borbolla *et al.*, 2005). This is thought to be partially due to the presence of physical constraints within the chimeric Env that do not permit the conformational changes required for fusion. However, factors other than steric hindrance seem to determine the fate of the vectors. Thus, rerouting of MoMLV-based targeted vectors to a degradative pathway has been shown (Cosset *et al.*, 1995b; Fielding *et al.*, 1998; Zhao *et al.*, 1999). In a similar way, a MoMLV vector targeted to the α folate receptor (αFR; MoMLV–FBP) seems to differ in the route of entry from vectors bearing the MoMLV wild-type (wt) Env (Viejo-Borbolla *et al.*, 2005). Inhibition of endocytic pathways results in higher infectivity levels for this vector, suggesting

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that degradation in lysosomes follows viral internalization (Viejo-Borbolla *et al.*, 2005).

Here, we have shown a novel strategy to improve MoMLV-based targeted vectors by reducing the threshold for fusion of such vectors. Mutations that reduce the threshold necessary to trigger the conformational changes leading to fusion have been described for influenza virus (Daniels *et al.*, 1985), MoMLV (Lavillette *et al.*, 1998) and HIV-2 (Reeves & Schulz, 1997). The HIV-2 CD4-independent ROD/B strain is able to fuse with and infect cells in the absence of either its receptor CD4 or soluble CD4 (sCD4) (Clapham *et al.*, 1992). This is due to the fact that conformational changes in Env induced by sCD4 happen more readily or even spontaneously for these Env proteins (Reeves & Schulz, 1997). The substitutions responsible for this phenotype have been characterized (Reeves & Schulz, 1997). Two of these substitutions, A544T and I546M [A526T and I528M in the work by Reeves & Schulz (1997)] are located within the HIV-2 leucine zipper-like (LZL) domain involved in the formation of the trimeric coiled coil required for fusion and entry (Weissenhorn *et al.*, 1997). We have identified residues in MoMLV Env whose substitutions could have a similar effect to substitutions A544T or I546M found in HIV-2 ROD/B, i.e. that could produce more infectious MoMLV targeted vectors by reducing the threshold for fusion. The following experiments describe the design and introduction of such mutations in the trimeric coiled coil of MoMLV Env and the effect of these substitutions on the entry process of wt MoMLV and targeted MoMLV vectors.

METHODS

Plasmid constructs. MoMLV Env-expressing plasmids FBMOSALF (expressing wt MoMLV Env), MoMov18 (expressing MoMLV Env targeted to the αFR; MoMLV–FBP), E-selectin (expressing MoMLV Env targeted to E-selectin; MoMLV–E-Sel) and FBASALF [expressing wt amphotropic murine leukemia virus 4070A Env], were a gift from Dr Y. Takeuchi (UCL, London, UK) and have been described previously (Pizzato *et al.*, 2001; Viejo-Borbolla *et al.*, 2005). Site-directed mutagenesis was performed with primers V1ALA1 (5′-CTCCAAGCCGCAGCACAGGATGATCTC-3′) and V1ALA2 (5′-GAGATCATCCTGTGCTGCGGCTTGGAG-3′) to substitute V512 to A; V1LEU1 (5′-CTCCAAGCCGCATTACAGGATGATCTC-3′) and V1LEU2 (5′-GAGATCATCCTGTAATGCGGCTTGGAG-3′) to substitute V512 to L; V1ILE1 (5′-CTCCAAGCCGCAATACAGGATGATCTC-3′) and V1ILE2 (5′-GAGATCATCCTGTATTGCGGCTTGGAG-3′) to substitute V512 to I; V1MET1 (5′-CTCCAAGCCGCAATGCAGGATGATCTC-3′) and V1MET2 (5′-GAGATCATCCTGCATTGCGGCTTGGAG-3′) to substitute V512 to M; V2ALA1 (5′-GATCTCAGGGAGGCAGAAAAATCAATC-3′) and V2ALA2 (5′-GATTGATTTTTCTGCCTCCCTGAGATC-3′) to substitute V519 to A; V2LEU1 (5′-GATCTCAGGGAGTTAGAAAAATCAATC-3′) and V2LEU2 (5′-GATTGATTTTTCTAACTCCCTGAGATC-3′) to substitute V519 to L; V2ILE1 (5′-GATCTCAGGGAGATAGAAAAATCAATC-3′) and V2ILE2 (5′-GATTGATTTTTCTATCTCCCTGAGATC-3′) to substitute V519 to I; V2MET1 (5′-GATCTCAGGGAGATGGAAAAATCAATC-3′) and V2MET2 (5′-GATTGATTTTTCCATCTCCCTGAGATC-3′) to substitute V519 to M. MoMLV delH was a gift from Dr F.-L. Cosset (Lyon, France) and has been described previously (Lavillette *et al.*, 2000). Substitutions V512I and V512M were introduced into MoMLV delH through insertion of the *Nsi*I–*Cla*I fragment from MoMLV V512I and MoMLV V512M, respectively. All constructs were sequenced to ensure that only the desired mutations were present.

Western blotting. Western blotting was performed as described previously (Pizzato *et al.*, 2001). Envelope glycoproteins were detected with a goat anti-Rauscher murine leukemia virus (RLV) gp70 polyclonal antibody (diluted 1 : 2000; Quality Biotech), whereas capsid proteins were detected with a goat anti-RLV p30 polyclonal antibody (diluted 1 : 5000; Quality Biotech). As secondary antibody, a horseradish peroxidase-conjugated rabbit anti-goat serum (diluted 1 : 10 0000; Sigma) was used.

Cell lines. All cell lines used in this report were cultured in Dulbecco's modified Eagle's medium containing 10 % heat-inactivated fetal calf serum, 300 μg L-glutamine ml^{-1}, 50 IU penicillin ml^{-1} and 50 μg streptomycin ml^{-1} (Gibco-BRL) (D10 medium). NIH 3T3 (murine embryo fibroblasts; ATCC CRL-1658), XC (muscle epithelial rat cells; ATCC CCL-165), SC-1 (mouse embryo fibroblasts), 208-F (mouse embryo fibroblasts; ECACC 85103116) and NRK (normal rat kidney epithelial cells; ATCC CRL-6509) were the murine cell lines used in this study. The human cell line A431 (human epidermoid vulval carcinoma cells; ATCC CRL-1555) and A431-αFR (A431 cells expressing αFR; Pizzato *et al.*, 2001) were a gift from Dr Massimo Pizzato and Dr Yasuhiro Takeuchi (UCL, London, UK).

TELCeB6 cells (Cosset *et al.*, 1995a) and NIH 3T3 and XC cells expressing soluble RBD (sRBD) were kindly provided by Dr F.-L. Cosset (Lyon, France). TELCeB6 cells were cultured in D10 medium plus 6 μg Blasticidin-S (ICN) ml^{-1}. Virus-producer cells were grown in D10 medium containing 6 μg Blasticidin-S ml^{-1} and 50 μg phleomycin ml^{-1}(Sigma). NIH 3T3 and XC cells expressing sRBD were cultured as described previously (Lavillette *et al.*, 2000). Human 293 cells expressing the murine cationic amino acid transporter type 1 (mCAT-1) receptor (293mCAT-1) were kindly provided by Dr P. Cannon (Los Angeles, USA) and were selected with 0·55 mg G418 ml^{-1} (Sigma).

Syncytium-formation assays. Six-well plates were plated with 3×10^4 293 cells and left to grow in culture medium for 18–24 h. Cells were transfected with 2 μg plasmid DNA expressing non-targeted MoMLV Env or targeted MoMLV–FBP and MoMLV–E-Sel. At 18 h post-transfection, 293 cells were overlaid with 6×10^4 XC cells. After a further 30 h, cells were stained with 1 % methylene blue, 0·025 % carbol fuchsin in methanol. A syncytium was defined as a multinucleated cell with four or more nuclei. Syncytia were counted by light microscopy.

Production of recombinant retroviruses. Recombinant retroviruses pseudotyped with wt or mutant Env were obtained as described previously (Viejo-Borbolla *et al.*, 2005). Viral protein expression (p30 and Env) from TELCeB6 producer cells was assessed as described previously (Viejo-Borbolla *et al.*, 2005). To detect the presence of reverse transcriptase (RT), a commercially available C-type RT kit (Cavidi Biotech) was used. The amount of RT in the virus-producer cell lines was used to normalize the different producer cell lines to the wt MoMLV-producer cell line.

Infection assays. Target cells (6×10^4 or 2×10^5) were plated in 12- or six-well plates, respectively. The following day, cells were incubated for 3–5 h with virus-containing or mock supernatant containing 8 μg Polybrene ml^{-1} (Sigma). The inoculum was then replaced with fresh medium and cells were incubated for 48 h.

Analysis of the kinetics of entry of the recombinant retroviruses was carried out as described previously (Viejo-Borbolla *et al.*, 2005). The effect of recombinant sRBD on virus entry was investigated as described previously (Viejo-Borbolla *et al.*, 2005). Infected cells were

fixed with 0·2 % glutaraldehyde, 2 % formaldehyde in PBS for 10 min at room temperature. Following two washing steps with PBS, cells were incubated with 2·5 mg X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, prepared in DMSO) ml^{-1}, 4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM $MgCl_2$ in PBS for 2–5 h. After the incubation period, cells were washed twice in distilled H_2O and the presence of cells expressing β-galactosidase was detected by light microscopy. Groups or clusters of stained cells were attributed to a single infection event and the titre of each experiment was expressed as β-galactosidase-expressing c.f.u. ml^{-1}. Standard deviation and significant values (Student's *t*-test) were calculated with the Excel program.

Computer modelling. A trimer of HIV-1 gp41 and a trimer of MoMLV TM fusion core were created with the computer program Symgen (provided by Michael Hartshorn, Chemoinformatics, Astex Technology Ltd, Cambridge, UK) by applying a symmetry matrix to the coordinates published in the Protein Data Bank (ID codes: 1MOF for MoMLV; 1AIK for HIV-1; Brookhaven National Laboratory). The computer software Insight II was used to superimpose the MoMLV TM fusion core on that of HIV-1.

RESULTS

Selection of key residues for site-directed mutagenesis in the MoMLV LZL motif

As we have shown previously that two substitutions in the HIV-2 TM protein, A544T and I546M [A526T and I528M in the paper by Reeves & Schulz (1997)] increase the propensity of this Env complex to undergo the conformational changes required for virus–cell fusion, we used computer models to identify the corresponding residues in the MoMLV TM protein.

The crystal structure of a fragment within the LZL domain corresponding to the extended coiled-coil region has been characterized for both HIV-1 and MoMLV Env proteins (Chan *et al.*, 1997; Fass *et al.*, 1996; Weissenhorn *et al.*, 1997). A trimer of HIV-1 gp41 and a trimer of MoMLV TM fusion core were created (Fig. 1a) with the computer program Symgen by applying a symmetry matrix to the coordinates published in the Protein Data Bank. Once the trimers were developed, the computer software Insight II was used to superimpose the MoMLV TM fusion core on that of HIV-1. As shown in Fig. 1(b), HIV-1 I541 corresponds to I546 in HIV-2 and is located at the tip of the trimeric coiled coil (Fig. 1a).

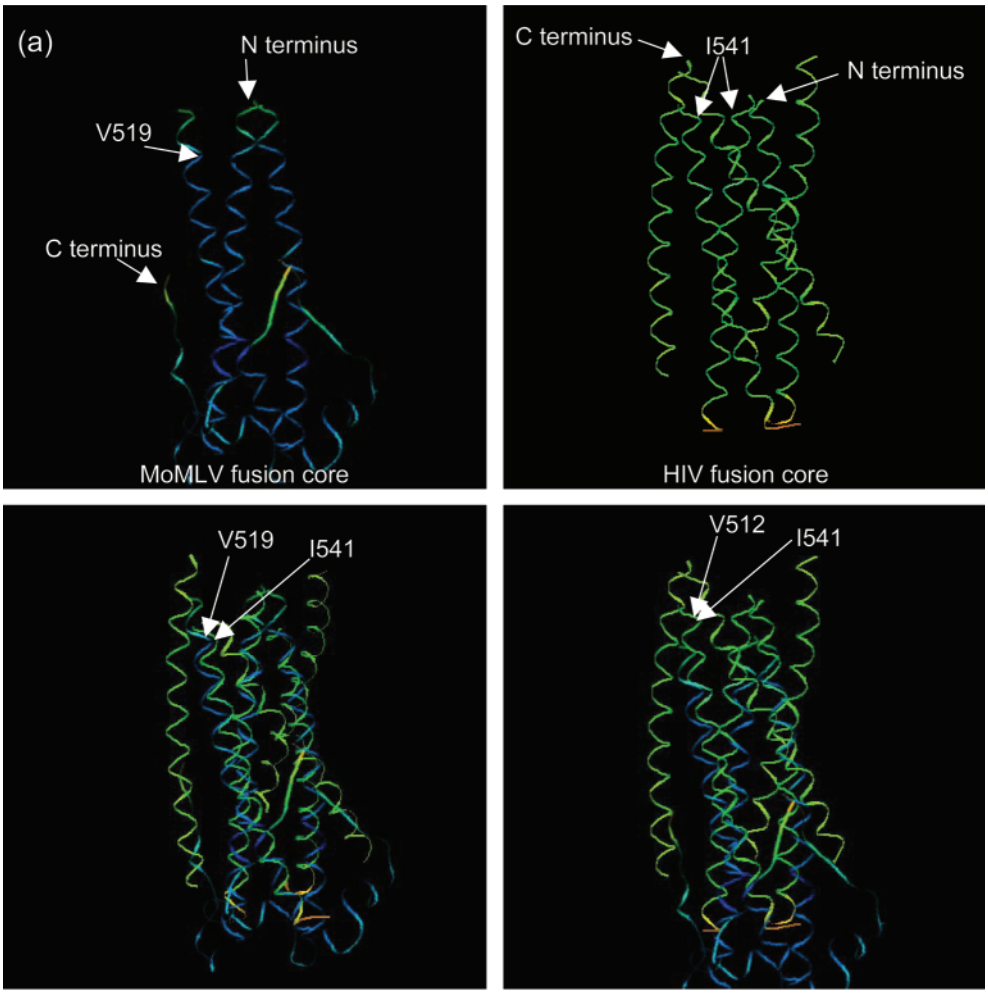
Both rod-shaped structures showed the N-terminal residues at the tip of the trimeric coiled coil, a chain reversal and C-terminal helices (for HIV) or loops (for MoMLV) positioned within the grooves formed by the trimeric coiled coil. I546 is located in position *d* of the trimeric coiled coil. We were interested in MoMLV amino acids located within the N-terminal region of the trimeric coiled coil in position *d* of the heptad repeat. Coiled coils have 3·5 residues per turn with respect to the supercoil axis. Therefore, one of the characteristic features of a coiled-coil structure is that amino acids spaced seven residues apart (two turns of the helix) present their side chains in the same position, on the same face of the helix. Due to this spatial orientation and to the fact that HIV-2 gp41 and MoMLV TM differ in length, we identified two residues in MoMLV, V512 and V519, located 7 aa apart on the helix that could correspond to HIV-2 I546 (Fig. 1). Both residues thus tentatively identified were located in the interface between the three helices composing the trimeric coiled coil, as is I546 in the HIV-2 TM ectodomain. This orientation towards the 'inside' of the helix was in keeping with the hydrophobic character of the two amino acids thus identified, V512 and V519.

Fig. 1(b) shows a direct alignment of the TM protein sequences of MoMLV with those of HIV-1 and -2. Whilst it is possible to tentatively align HIV-1 and -2 sequences in this region, as well as the MoMLV sequence, the similarity between the lentiviral and C-type retroviral groups is too low to permit a reliable alignment. Only the combination of sequence alignments and three-dimensional modelling as shown in Fig. 1(a) could reveal which MoMLV residues corresponded to I546 of HIV-2. We concluded that V512 and V519 were the most likely candidates whose positions in the coiled-coil domain were equivalent to that of I546 of HIV-2. V519 is located within the characterized coiled-coil domain (Fass *et al.*, 1996), whereas V512 is placed within the sequence presumed, but not confirmed, to form part of the coiled-coil domain. Both residues are located on position *d* of the heptad repeat.

Therefore, V512 and V519 were chosen as possible candidates that could play a similar role in MoMLV to that played by I546 in HIV-2 and could perhaps, by lowering the threshold required for MoMLV fusion, release the spatial constraints in targeted MoMLV Env proteins. Both V512 and V519 were mutated to four hydrophobic residues, A, L, I and M, to avoid any major disruption of the trimeric coiled coil, and their effects on MoMLV Env were determined. The substitutions were performed in non-targeted MoMLV and in MoMLV Env proteins targeted to E-selectin (MoMLV–E-Sel) and folate-binding protein (MoMLV–FBP) (Pizzato *et al.*, 2001; Viejo-Borbolla *et al.*, 2005).

Correct expression and incorporation of Env mutants

Substitution of V512 or V519 by A, L, I or M did not have a negative effect on viral-protein expression or incorporation. All mutant Env proteins were expressed properly and incorporated into virions as detected by Western blotting. Thus, all MoMLV virions, whether wt or mutant, showed the SU at 70 kDa (Fig. 2). Similarly, parental and mutant MoMLV–E-Sel Env proteins had the expected size of 70 kDa (not shown). Parental and mutant MoMLV–FBP Env proteins had an apparent molecular mass of 98 kDa (not shown). All vectors showed good expression and incorporation of structural proteins (shown only for MoMLV; Fig. 2). Incorporation of the viral glycoprotein was a little lower for some mutants (V512I, V512M, V519L, V519I and V519M) than for the corresponding wt glycoproteins V512 and V519. This did not, however, correlate with reduced



(b)

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                a  d  a  d  a  d  a  d  a  d  a
MoMLV   QFQQLQAAVQDDLREVEKSISNLEKSLTSLSEVVLQNRRGLDL
        504                                     546
HIV-2     QSRTLLAGIVQQQQQLLDVVKRQQEMLRLTVWGTKNLQARVTA
          538                                   580
HIV-1     QARQLLSGIVQQQNNLLRAIEAQQHLLQLTVWGIKQLQARILA
          533                                   575

                a  d  a  d  a  d  a  d  a  d  a
MoMLV   QFQQLQAAVQDDLREVEKSISNLEKSLTSLSEVVLQNRRGLDL
        504                                     546
HIV-2   ----------▶ QSRTLLAGIVQQQQQLLDVVKRQQEMLRLTVWGTKNLQARVTA
                    538                                 580
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Fig. 1. (a) Computer modelling of MoMLV and HIV trimeric coiled coils. The trimeric coiled coils of MoMLV and HIV are shown at the top. Residue I541 in HIV-1 (which corresponds to I546 in HIV-2 ROD/B) is indicated. Below, the two possible superimpositions of HIV trimeric coiled coil over that of MoMLV are shown. The alignment on the left shows both N termini aligned, with I541 in HIV-1 over V519 in MoMLV. The alignment on the right represents the MoMLV trimeric coiled coil being shifted seven residues downstream of the HIV trimeric coiled coil, where I541 would be over V512. However, V512 is not within the protein fragment whose crystallographic structure has been characterized. (b) Alignment of MoMLV, HIV-1 and HIV-2 sequences corresponding to the LZL domain. The letters *a* and *d* represent the positions of the residues in the heptad repeat of MoMLV. Two possible amino acids in MoMLV, V512 and V519 (underlined), could correspond to I546 in HIV-2. GenBank accession nos: MoMLV, J02255; HIV-1_{HXB2R}, K03455; HIV-2 (ROD), A14064.

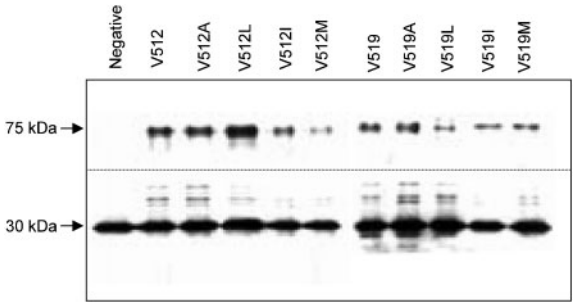

Fig. 2. Western blot showing expression of viral proteins produced by TELCeB6 cells. Correct expression and incorporation into viral particles for parental (V512 and V519) and mutant MoMLV proteins. The dashed line shows where the membrane was cut prior to incubation with the appropriate antibody (see Methods).

infectivity (see Fig. 4, Table 1). Importantly, in spite of the apparently lower incorporation seen with mutant V512M, this mutant turned out to show increased infectivity in some cell lines (see below).

Substitutions in V512 or V519 reduce both cell-to-cell fusion and infectivity of targeted and non-targeted MoMLV Env proteins

To study the effect of the substitutions on the formation of cell-to-cell fusion, syncytium-formation assays were performed as described in Methods. All substitutions in V512 or V519, with the exception of V512M, impaired cell-to-cell fusion (Fig. 3). The differences obtained between the wt and the mutant Env proteins, with the exception of V512M, were significant ($P<0{\cdot}05$).

The infectivity of vectors carrying mutated and parental Env proteins was studied in NIH 3T3 and XC cells (Fig. 4). Vectors bearing Env with substitutions in V512 or V519 were less infectious, with the exception of MoMLV/V512M,

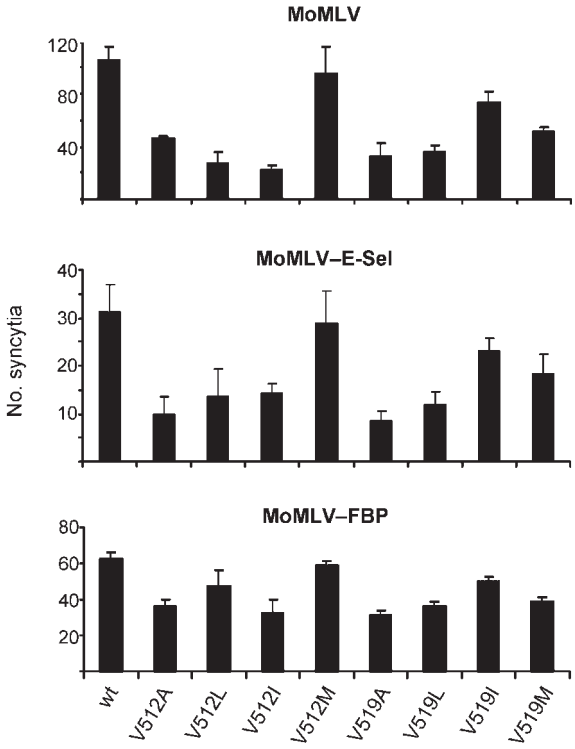

Fig. 3. 293 cells were transfected with Env-expressing plasmids and overlaid with XC cells to assess syncytium formation (fusion from within). Values shown are the means of three wells ± SEM. Targeted Env proteins produced fewer syncytia than wt MoMLV Env. All mutations, with the exception of V512M, resulted in an Env less prone to fusion ($P<0{\cdot}05$).

which showed approximately the same infectivity as wt vectors. Interestingly, MoMLV–FBP/V512M and MoMLV–E-Sel/V519I were more infectious than their respective parental vectors in both NIH 3T3 and XC cells, whilst MoMLV–FBP/V519L and MoMLV–FBP/V519I showed increased infectivity only in XC cells, and MoMLV–E-sel/V519M did so only in NIH 3T3 cells.

Table 1. Infection of NRK, SC-1 and 208-F cell lines

Murine NRK, SC-1 and 208-F cells and human A431 and A431-αFR cells were infected with *lacZ*-containing pseudotyped virus bearing wt or mutant MoMLV–FBP Env proteins. β-Galactosidase-expressing c.f.u. ml^{-1} were counted and normalized to the wt MoMLV RT value as described in Methods. Values shown are the means for three wells ± SEM.

Cell line	Vector		
	MoMLV–FBP	MoMLV–FBP/V512I	MoMLV–FBP/V512M
NRK	30·4 ± 38 (100 %)	0 (0 %)	500 ± 270 (1644·74 %)
SC-1	$2{\cdot}7\times10^4\pm1{\cdot}4\times10^3$ (100 %)	326 ± 65·2 (1·19 %)	$2\times10^5\pm1{\cdot}8\times10^4$ (729·82 %)
208-F	119·33 ± 42·06 (100 %)	0 (0 %)	648 ± 43·2 (543·03 %)
A431	0	0	0
A431-αFR	0	0	0

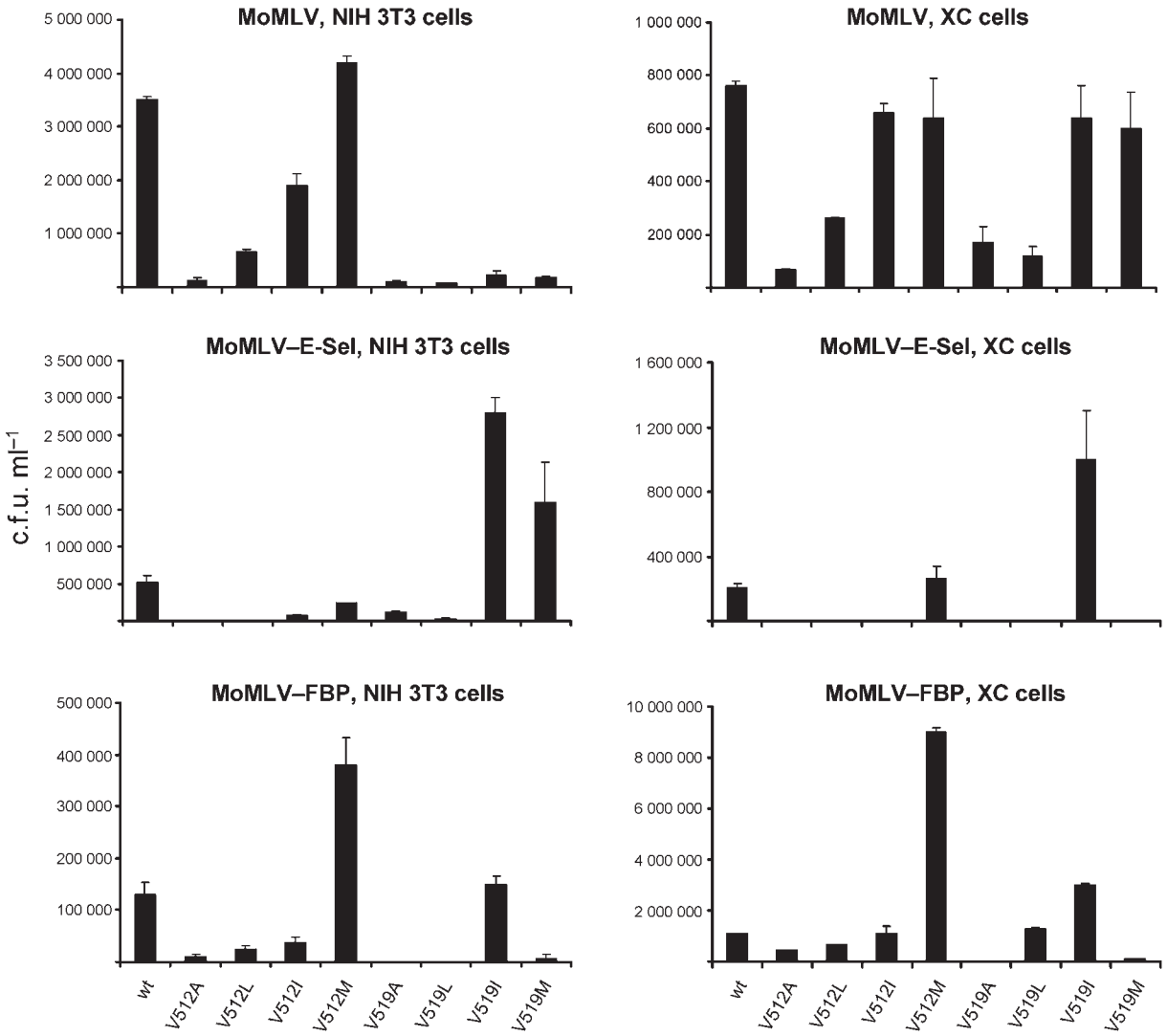

Fig. 4. NIH 3T3 and XC cells were infected with *lacZ*-containing pseudotype virus bearing wt or mutant MoMLV, MoMLV–E-Sel and MoMLV–FBP Env proteins. β-Galactosidase-expressing c.f.u. ml^{-1} were counted and normalized to the wt MoMLV RT value as described in Methods. Values shown are the mean for three wells ± SEM.

Further investigations on the effect of substitutions V512M and V519I on the infectivity of the vectors MoMLV–FBP and MoMLV–E-Sel, respectively, on different cell lines were carried out. As shown in Table 1, MoMLV–FBP/V512M vectors were more infectious than wt MoMLV–FBP in NRK, SC-1 and 208-F cell lines, with titres being five- to 16-fold higher than parental MoMLV–FBP titres. The increase in infectivity due to the V512M substitution was significant ($P<0{\cdot}05$). However, none of the MoMLV–E-Sel/V519 mutants showed higher titres than the parental MoMLV–E-Sel virus in these cell lines (not shown).

As MoMLV–FBP/V512M was more infectious than wt MoMLV–FBP in several murine cell lines, we decided to investigate whether this vector was able to infect human epidermoid carcinoma cells expressing αFR (A431-αFR). We and others have previously attempted to infect A431-αFR cells with MoMLV–FBP without success (Viejo-Borbolla *et al.*, 2005; Pizzato *et al.*, 2001). Neither the V512M nor the V512I substitution changed this phenotype (Table 1).

V512M substitution increases the kinetics of entry of MoMLV–FBP virions

Targeted MoMLV–E-Sel and MoMLV–FBP virions exhibit slower kinetics of entry into SC-1 and XC cells than MoMLV (Viejo-Borbolla *et al.*, 2005). We were interested in investigating whether the increase in infectivity seen with the V512M substitution in MoMLV-FBP virions correlated with an increase in the kinetics of entry. As a control, we also investigated the effect of V512I substitution on the kinetics of entry.

As shown in Fig. 5, wt MoMLV reached 50 % of maximal infectivity levels after 25 min infection on XC cells, whereas MoMLV/V512I required 40–50 min to reach 50 % of the maximum infection level.

Substituting M or I for the wt V512 in MoMLV led to a reduction in the maximum level of infectivity reached, and this reduction was more pronounced for MoMLV/V512I (approx. 50 % reduction) than for MoMLV/V512M (approx. 30 % reduction). However, the kinetics of entry were not strongly affected: 50 % of the maximum levels of infection was reached at approximately 20–30 min for wt MoMLV and MoMLV/V512M, and at approximately 40 min for MoMLV/V512I.

A similar result was obtained when analysing the impact of the V512I and V512M mutations on MoMLV–E-Sel (not shown). All three MoMLV–E-Sel vectors appeared to have slightly delayed infection kinetics compared with wt MoMLV. Again, the V512I mutation reduced the maximum level of infectivity obtained more strongly (approx. 60 % reduction) than the V512M mutation (approx. 30 %) (not shown).

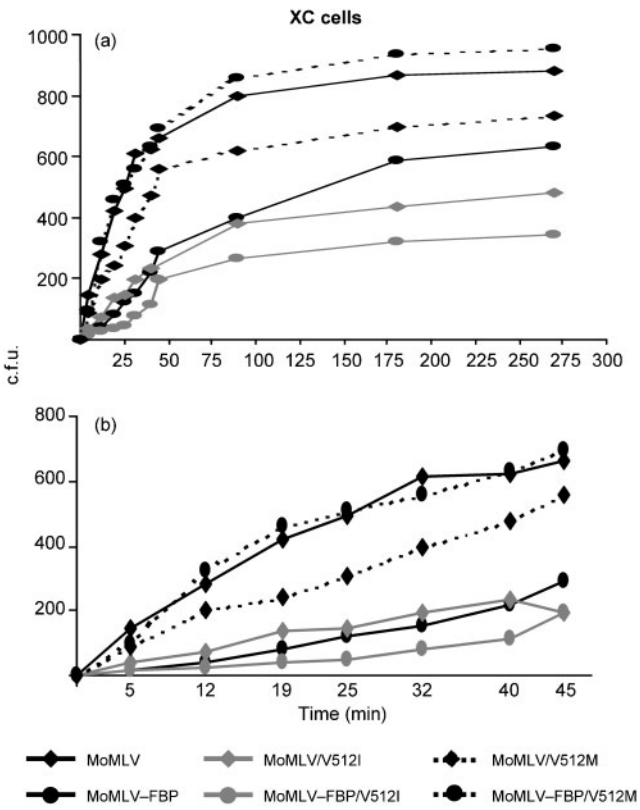

Fig. 5. Kinetics of entry of parental and mutant MoMLV and MoMLV–FBP vectors on XC cells. The *y* axis represents the c.f.u. obtained, whereas the *x* axis shows the different time points at which the non-internalized virions were inactivated with citrate buffer at pH 3. (a) Complete time span of the experiment. (b) First 45 min of the experiment.

MoMLV–FBP/V512M showed a markedly increased infectivity (Fig. 5), which was restored to the levels seen with wt MoMLV. Wt MoMLV and MoMLV–FBP/V512M also had identical infection kinetics, with 50 % of the maximum level reached at approximately 20–30 min, i.e. the V512M substitution in MoMLV–FBP also seemed to restore the more rapid virus entry seen with wt MoMLV compared with the targeted MoMLV–FBP. In contrast to the V512M substitution, V512I had a deleterious effect on MoMLV–FBP, reducing the maximum levels of infectivity by approximately 50 %. Taken together, these results suggest that the V512M substitution improves infectivity of the MoMLV–FBP-targeted Env.

V512M mutation facilitates conformational changes in Env triggered by sRBD

MoMLV–FBP/V512M virions showed higher titres and faster kinetics of entry than MoMLV–FBP parental virions. We were interested in studying whether the V512M mutation increased the propensity of MoMLV Env to induce virus–cell fusion, perhaps by facilitating conformational changes required for the fusion process. To test this hypothesis, we took advantage of an observation by Lavillette *et al.* (2000). This group has shown that addition of the sRBD can restore the infectivity of a fusion-defective Env lacking a His in position 8 of MoMLV (MoMLV delH) and interpreted this to indicate that sRBD could trigger conformational changes in this defective Env. We introduced the V512I and V512M substitutions into the MoMLV delH Env (Fig. 6a) and studied the effect of these substitutions on infectivity following either ecotropic (Moloney) or amphotropic sRBD activation (Mo- and A-sRBD, respectively).

Incorporation of viral proteins into virions was determined by Western blotting. MoMLV delH/V512I and MoMLV delH/V512M Env proteins were expressed correctly and incorporated into virions, at levels similar to those of parental MoMLV delH Env (Fig. 6b).

Virions bearing the MoMLV delH/V512 (parental) Env were not infectious unless a certain amount of sRBD was added to the cells (Fig. 7a–c, e–h). MoMLV delH/V512I virions were not infectious in most cell lines, even when large amounts of Mo-sRBD were added to the cells. Only on rat XC and human 293mCAT-1 (not shown) cells could some infectivity be achieved. This probably reflects our previous experience with this mutant, which had a detrimental effect on virus infectivity in many cells (Table 1 and Figs 4 and 5). That MoMLV delH/V512I was not intrinsically defective was illustrated by its ability to infect XC cells as efficiently as MoMLV delH or MoMLV delH/V512M in the presence of A-sRBD supernatant (Fig. 7d). Comparable levels of gp70 were incorporated into MoMLV delH, MoMLV delH/V512I and MoMLV delH/V512M virions, suggesting that the defect in MoMLV delH/V512I was not due to incorrect processing of Env (Fig. 6b).

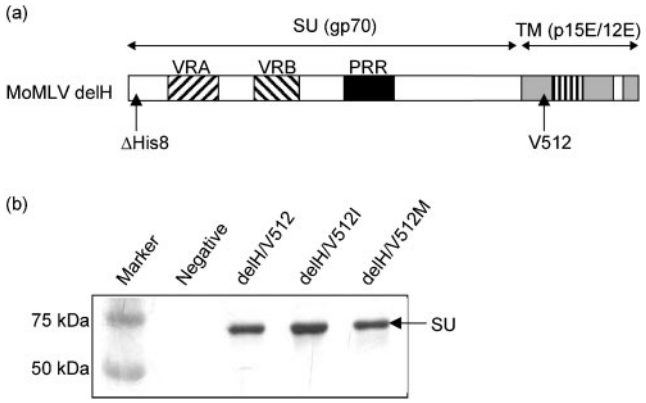

Fig. 6. (a) Schematic representation of delH Env. MoMLV delH was obtained by deleting the H residue at position 8 (Bae *et al.*, 1997; Lavillette *et al.*, 2000). MoMLV delH/V512I and delH/V512M were created by inserting substitutions V512I and V512M into the delH Env as explained in Methods. (b) Western blot showing expression and incorporation into viral particles of parental MoMLV delH and MoMLV delH/V512I and delH/V512M Env proteins. Parental MoMLV delH and mutant MoMLV delH/V512I and delH/V512M were expressed correctly and incorporated into virions. Detection of Env was performed with an anti-RLV gp70 antibody. VRA, Variable region A; VRB, variable region B; PRR, proline-rich region.

Virions bearing MoMLV delH/V512M Env behaved similarly to MoMLV delH virions. They were activated when sRBD was added but, in most of the experiments, the amount of sRBD needed for activation of MoMLV delH/V512M was lower than the amount needed to activate MoMLV delH Env (Fig. 7a, c–f and h). As it is thought that the sRBD triggers conformational changes in Env that lead to fusion (Lavillette *et al.*, 2000, 2001), these data suggest that MoMLV delH/V512M Env is slightly more prone to fusion than the parental MoMLV delH Env. The differences in the infectivity, however, were never high. As shown in Fig. 7(a, b and d), parental MoMLV delH virions had similar maximum infection levels to MoMLV delH/V512M virions, suggesting that the differences in the amount of sRBD needed to trigger the conformational changes in Env were not due to a higher number of infectious particles of MoMLV delH V512M compared with parental MoMLV delH. The level of Env incorporated that was detected in the Western blots also supported this (Fig. 6b).

Taken together, our results suggest that V512M substitution marginally reduces the threshold needed to activate fusion of MoMLV Env.

DISCUSSION

The production of targeted vectors based on MoMLV for *in vivo* gene therapy has encountered several problems. Most of the vectors developed show low or no infectivity of human cells expressing the target molecule (Ager *et al.*, 1996; Benedict *et al.*, 1999; Lorimer & Lavictoire, 2000; Pizzato *et al.*, 2001; Russell *et al.*, 1993; Zhao *et al.*, 1999). Moreover, the targeted vectors have, in general, lower titres in murine cells than the parental vectors (Marin *et al.*, 1996; Martin *et al.*, 1998; Pizzato *et al.*, 2001; Viejo-Borbolla *et al.*, 2005). This is due at least partially to the presence of steric constraints within Env, due to insertion of the targeting domain (Zhao *et al.*, 1999). Such constraints do not allow conformational changes in Env to occur as efficiently as in the parental vector, increasing the threshold for fusion. As an attempt to overcome this problem, we have devised a strategy to reduce the MoMLV Env threshold for fusion. By using computer modelling programs (Insight II and Symgen) and sequence alignments, we determined which residues in MoMLV could play a similar role to A544T and I546M in the CD4-independent HIV-2 isolate ROD/B, i.e. which MoMLV residues would produce a more fusogenic Env. Two residues within the MoMLV TM region, V512 and V519, were chosen as candidates and substituted by A, L, I and M. Mutant Env proteins were expressed correctly and incorporated into viral particles. All the substitutions except V512M were deleterious both in syncytium production and in infectivity of murine cell lines, suggesting that they were not able to produce a more fusogenic Env (Figs 3 and 4 and Table 1). Thus, conservative substitutions in V512 and V519 might negatively affect the conformational changes required for Env to expose the fusion peptide, confirming the importance of this region in the MoMLV infection process.

The presence of an L residue in position *a* and a V residue in position *d* favours the formation of trimeric over dimeric or tetrameric coiled coils. This was the pattern observed in residues L516 (position *a*) and V519 (position *d*) in MoMLV. Thus, disruption of V519 may alter the conformation of the trimeric coiled coil. Previous results (Ramsdale *et al.*, 1996) have shown that the effect of conservative substitutions on some residues (L516A, I523V/I523G, L530A, L533T) in positions *a* or *d* within the heptad repeat results in complete abrogation of both Env-dependent syncytium formation and cell-free infectivity. In our hands, however, conservative substitutions of V512 or V519 did not completely impair either syncytium formation or infectivity by MoMLV.

Non-targeted MoMLV/V512M was nearly as efficient as wt MoMLV both in syncytium-formation assays and in cell-free infectivity assays. A similar phenotype was obtained when mutating V537 to A (Ramsdale *et al.*, 1996). V537 is in position *a* of the heptad repeat, near the base of the characterized MoMLV trimeric coiled coil (Fass *et al.*, 1996). The presence of A or V in position *a* of a trimeric coiled coil occurs with similar frequency, suggesting that V537A substitution may not interfere with the conformation of the coiled coil (Harbury *et al.*, 1994; Lovejoy *et al.*, 1993; Ramsdale *et al.*, 1996).

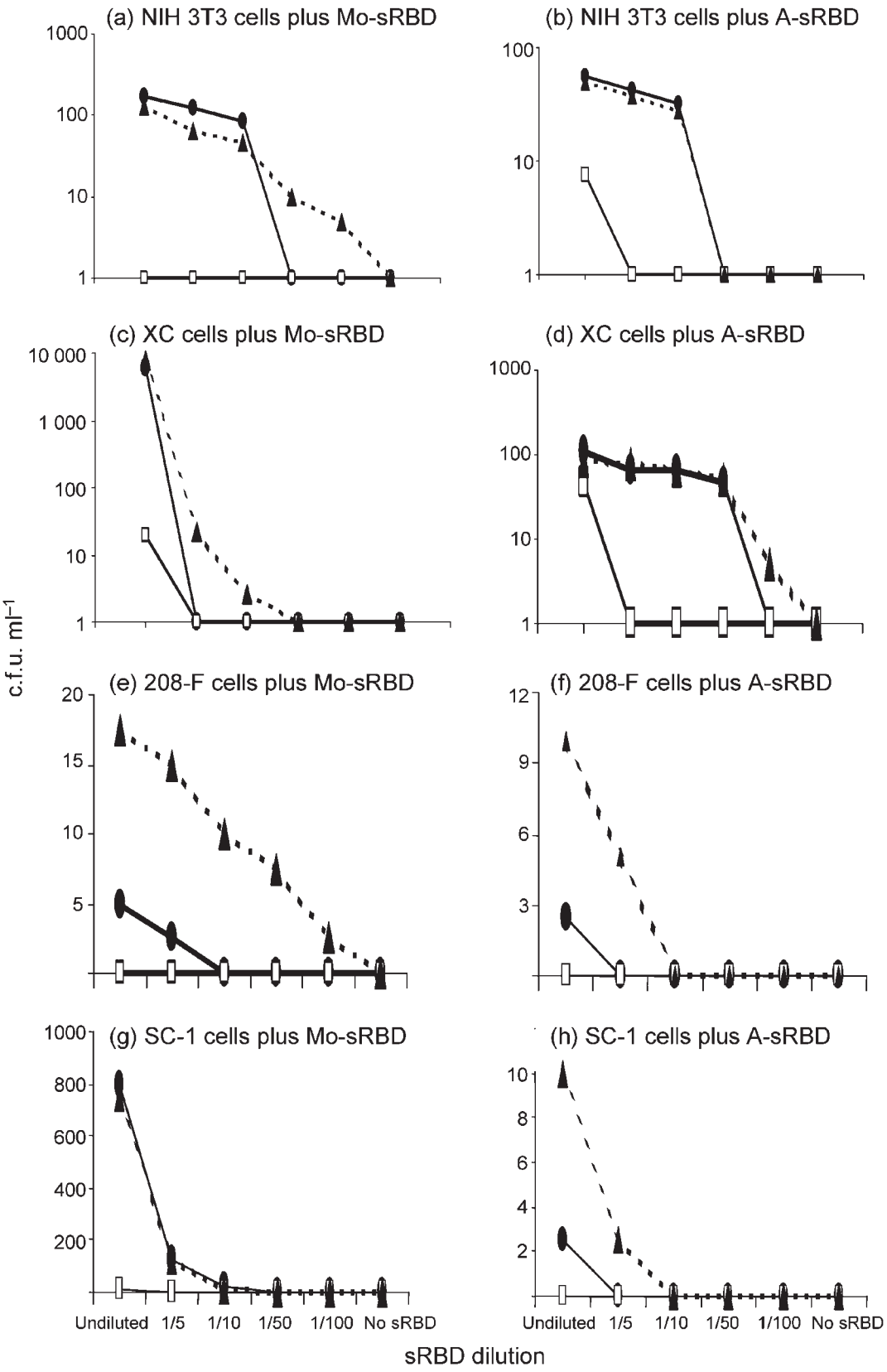

Fig. 7. Titration of the amount of sRBD required to trigger infection of several cell lines by parental and mutant MoMLV delH virions. The *y* axis represents the titre obtained as c.f.u. ml^{-1}. The *x* axis shows the amount of sRBD added to the cells. ●, MoMLV delH; □, MoMLV delH/V512I; ▲, MoMLV delH/V512M.

Interestingly, MoML–FBP/V512M Env was as fusogenic as parental MoMLV–FBP Env, and MoMLV–FBP/V512M vectors had higher infectivity levels, in the range of three- to ninefold, on several murine cell lines compared with MoMLV–FBP vectors (Figs 3 and 4 and Table 1). The magnitude of this effect was greater than that seen with the I546M substitution in HIV-2 alone (Reeves & Schulz, 1997). It is remarkable that, in both HIV-2 and MoMLV Env proteins, substitutions of I to M in position *d* of the heptad repeat increased infectivity. Interestingly, a similar substitution was also described for influenza virus HA (Daniels *et al.*, 1985; see below). Whether this is a coincidence or whether it has a relevant functional explanation requires further experimental work.

Analysis of the kinetics of entry of the targeted vectors showed that the negative effect of the V512I substitution on the wt and targeted Env proteins was more pronounced than in standard infectivity assays. This result suggests that an Env carrying the V512I substitution is defective for a post-binding event. In contrast, the V512M substitution improved the kinetics of entry of MoMLV-FBP vectors, suggesting that it favoured the series of complex steps that take part after binding to the receptor.

Following binding to receptor, clustering of a threshold number of receptors and formation of the fusion pore are crucial steps in the infectious process of retroviruses. Triggering of the fusion signal involves interaction between different domains of Env (Lavillette *et al.*, 2001). The formation of the trimeric coiled coil is important both for the interaction of several Env–receptor complexes and for the formation of the fusion pore. It is possible that the V512I and V512M substitutions may affect the interaction between the different domains within Env.

The improved infectivity and kinetics of entry of MoMLV–FBP/V512M compared with the parental MoMLV–FBP suggested that the V512M substitution reduced the physical constraints, allowing a more efficient reorganization of Env leading to exposure of the fusion peptide. To confirm this hypothesis, the substitutions V512I and V512M were inserted into a binding-competent but fusion-defective Env, MoMLV delH. In this Env, the H8 residue within the PHQV motif has been deleted, affecting the interactions that are presumed to take part between this motif and the disulfide-bonded loop within the C-terminal domain of the SU subunit (Bae *et al.*, 1997; Lavillette *et al.*, 2001). MoMLV delH vectors are defective for the transmission of the fusion signal and can only infect cells if Mo-sRBD or A-sRBD are present during the infection process (Lavillette *et al.*, 2000). It has been proposed that sRBD is activated through mCAT-1 interaction and that activated sRBD interacts *in trans* with the C-terminal SU of the defective Env, allowing MoMLV delH infection (Lavillette *et al.*, 2001). As a threshold of activated RBD is needed to trigger the fusion signal leading to MoMLV delH infection, this allowed us to titrate the amount of sRBD required by MoMLV delH/V512I and MoMLV delH/V512M in comparison with parental MoMLV delH. MoMLV delH/V512M required smaller amounts of sRBD than parental MoMLV delH to infect several murine cell lines (Fig. 7). This was not due to a better expression or incorporation of the MoMLV delH/V512M Env compared with that of the MoMLV delH parental vector (Fig. 6). The difference in the infection levels with respect to parental MoMLV delH was small but reproducible, in keeping with the notion that this substitution reduces the physical constraints only moderately, as does I546M on HIV-2. Further modifications of Env, such as the ones described in the PRR (Lavillette *et al.*, 1998) or V537A (Ramsdale *et al.*, 1996), might further reduce the threshold for fusion of MoMLV and MoMLV targeted vectors. Our result is reminiscent of the I546M substitution in HIV-2 (Reeves & Schulz, 1997) and the HA mutants in influenza virus (Daniels *et al.*, 1985). As mentioned above, V512M is not a very conservative mutation, as M residues are not commonly found in positions *a* or *d* of trimeric coiled coils (Harbury *et al.*, 1994; Lovejoy *et al.*, 1993). V512 is within the LZL sequence that is expected to change its conformation dramatically to produce the rod-shaped structure that would allow interaction of MoMLV fusion peptide with the target cellular membrane. The shapes of the buried side chains in the coiled coils are essential determinants of the coil fold. The sulfur-containing side of the M residue may destabilize the contacts between the helices prior to the formation of the trimeric coiled coil, making the transition to the fusion-active state easier, in a similar way to the HA mutants that fuse at higher-than-normal pH (approx. pH 5·75) (Daniels *et al.*, 1985). An I to M substitution within influenza virus HA results in influenza virus mutants fusing at higher pH than normal, possibly due to destabilization of the normal location of the N-terminal peptide by the longer chain of the M residue (Daniels *et al.*, 1985). Extrapolating the MoMLV delH result to MoMLV–FBP, the effect of V512M on MoMLV–FBP seemed to be that of reducing the effect of the steric hindrance produced by insertion of the single-chain variable fragment targeted to FBP. This reduction in physical constraints might provide the MoMLV–FBP/V512M vectors with a substantial advantage. However, the MoMLV–FBP/V512M vectors were still unable to infect human cells expressing the target molecule (A431-αFR), despite specific binding to the target molecule (not shown). Further modifications in MoMLV–FBP Env may result in vectors able to infect human cells expressing αFR.

Interestingly, despite good expression and incorporation of the MoMLV delH/V512I Env, MoMLV delH/V512I vectors could not infect murine cells, even in the presence of high amounts of sRBD, suggesting that they are defective for a post-binding event. The V512I substitution might affect the receipt of the fusion signal transmitted through the PRR region after binding of activated RBD, a phenomenon suggested by many studies (Barnett & Cunningham, 2001; Barnett *et al.*, 2001; Lavillette *et al.*, 1998, 2000, 2001). This result further supports a functional interaction between

different subdomains within MoMLV Env (RBD and PRR in SU and coiled coil in the TM domain).

In summary, computer modelling based on published crystallographic data was used to select for key residues in the proximity of the trimeric coiled-coil MoMLV Env. The substitution of these amino acids revealed interesting functional characteristics of this region and resulted in a targeted MoMLV with moderately increased infectivity. The use of this strategy combined with other methods could improve the infectivity of targeted MoMLV-based vectors.

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