Receptor co-operation in retrovirus entry: recruitment of an auxiliary entry mechanism after retargeted binding

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We have constructed Moloney murine leukemia virus (MoMLV)-derived envelope glycoproteins (AMO) displaying an amino-terminal Ram-1-binding domain in which a variety of different amino acid spacers have been inserted between the displayed domain and the MoMLV surface (SU) subunit. Titres of retroviruses generated with these chimeric envelopes were enhanced on cells expressing both Ram-1 and Rec-1 receptors compared with the titres on cells expressing only one or other receptor type. The absolute viral titres and the degree of titre enhancement due to receptor co-operativity were highly variable between the different chimeric envelopes and were determined primarily by the properties of the interdomain spacer. An extreme example of receptor co-operativity was encountered when testing Ram-1-targeted AMOPRO envelopes with specific proline-rich interdomain spacers. AMOPRO viruses could not enter cells expressing only Rec-1 or only Ram-1 but could efficiently infect cells co-expressing both receptors. The data are consistent with a model for receptor co-operativity in which binding to the targeted (Ram-1) receptor triggers conformational rearrangements of the envelope that lead to complete unmasking of the hidden Rec-1-binding domain, thereby facilitating its interaction with the viral (Rec-1) receptor which leads to optimal fusion triggering.

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Introduction

Retroviral infection is initiated by the attachment of the virion to specific cell surface molecules, the viral receptors. This attachment is mediated by the surface (SU) subunit of the retroviral envelope glycoprotein trimer and involves a receptor-binding domain located in the retroviral SU (Weiss, 1993). For murine leukemia viruses (MLVs), the receptor-binding domain has been located in the first half of the SU (Heard and Danos, 1991; MacKrell *et al.*, 1996), and two hypervariable subregions, VRA and VRB, have been shown to contribute to receptor binding (Battini

et al., 1992; Ott and Rein, 1992; Morgan *et al.*, 1993). The fusion machinery of the MLV envelope is most probably located in the transmembrane (TM) subunit (Jones and Risser, 1993) and also involves the C-terminal half of the SU (Pinter *et al.*, 1986; Nussbaum *et al.*, 1993). This latter C-terminal protein domain is separated from the amino-terminal receptor-binding domain by a long polyproline-rich region thought to be a hinge between the two functional domains of the SU (Kabat, 1989).

Both SU and TM are thought to undergo major conformational rearrangements following receptor binding, allowing the second retroviral envelope glycoprotein subunit, the TM protein, to become active and to trigger the fusion between the viral and the cytoplasmic lipid bilayers. The 18 final residues of the TM cytoplasmic tail have been shown to modulate the fusogenicity of the viral particles (Ragheb and Anderson, 1994; Rein *et al.*, 1994). Some studies suggest the involvement in infection of a co-receptor that would be available in limited quantities in target cells (Wang *et al.*, 1991; Siess *et al.*, 1996). Whether a co-receptor or an auxiliary entry mechanism exists or not, it is highly remarkable that all primary receptors identified so far for the type C mammalian retroviruses belong to a family of multi-TM proteins that have transport functions (Weiss and Tailor, 1995). Given the similar overall structures of type C mammalian retrovirus envelope glycoproteins, this may reflect the natural selection and evolution of this group of retroviruses to adapt and to use, for viral fusion and/or entry, specific determinants or functions shared by the transporters. Thus there is the possibility that only few cell surface molecule types might be used for viral penetration.

Host-range modifications of retroviruses is an approach currently carried out in our laboratory to investigate retrovirus entry (Valsesia-Wittmann *et al.*, 1994; Cosset *et al.*, 1995a). Ligands for several cell surface molecules have now been displayed as N-terminal extensions on MLV envelopes (Cosset and Russell, 1996). Although such chimeric envelopes generally allow a retargeted binding, infection via the targeted cell surface molecule has been found to be poor (Cosset *et al.*, 1995a; Somia *et al.*, 1995; Marin *et al.*, 1996; Valsesia-Wittmann *et al.*, 1996) if not non-existent (Cosset *et al.*, 1995a; Ager *et al.*, 1996; Schnierle *et al.*, 1996). It is assumed that most cell surface molecules cannot act as retroviral receptors and that the intrinsic fusogenicity of this type of chimeric envelope is weak (Cosset and Russell, 1996).

So far, strategies to retarget retrovirus entry via specific cell surface molecules expressed on human cells have relied on host-range extensions, i.e. by N-terminal addition of new binding domains on Moloney murine leukemia virus (MoMLV) ecotropic envelopes. It now seems clear that these approaches suffer two major drawbacks. (i) Due to the ectopic location of the new binding determinants

Fig. 1. Schematic diagrams of envelope chimeras. The plasmid construct coding for chimeric envelope glycoproteins in which the Ram-1-binding domain was fused to MoMLV SU is displayed. The general format is shown diagrammatically and the amino acid sequence of the interdomain spacer between the displayed domain and the SU protein is shown in detail. Vertical arrows, protein cleavage sites; SU, surface protein; TM, transmembrane protein; SP, env signal peptide; T, transmembrane domain; Ram-1 ligand, MLV-A receptor-binding domain. Black boxes, MoMLV-derived env sequences; gray boxes, MLV-A-derived env sequences; white boxes, other MLV-derived sequences. All env genes were expressed using the same promoter (LTR) and polyadenylation sequence (polyA) from a sub-genomic mRNA using retroviral splice donor (SD) and acceptor (SA) sites with an identical 190 nucleotide intron containing the end of the *pol* gene (ΔPOL). The position of some restriction sites is indicated.

in these chimeric envelopes, the fusion trigger cannot be transmitted optimally to the viral fusion domain. (ii) Due to the absence of ecotropic receptors on the surface of the targeted (human) cells and thus to the lack of retroviral receptor-mediated fusion triggering, the fusogenicity of such retroviruses is not exploited fully. We report here a strategy to overcome these two problems and to optimize the fusogenicity of N-terminally extended envelopes, thus facilitating the entry of retargeted retroviruses by recruiting, as a second step in the entry process, a fusion trigger mediated by the interaction with the retroviral receptor.

Results

We have previously reported a set of MoMLV-derived envelopes retargeted to the Ram-1 phosphate transporter, generated by N-terminal fusions with a Ram-1-binding domain provided by the first 208 amino acids from the 4070A SU (Valsesia-Wittmann *et al.*, 1996). The amino acid sequences of interdomain spacers separating the Ram-1- and the Rec-1-binding domains in some of these constructs are shown in Figure 1. We have also reported (Valsesia-Wittmann *et al.*, 1996) that retroviruses generated with the different chimeric envelopes could bind TE671 human cells as efficiently as viruses with wild-type 4070A envelopes, and most of them, with the notable exception of AMOPRO, could infect TE671 cells. However, viruses generated with all chimeras could efficiently infect NIH 3T3 cells bearing both Ram-1 and Rec-1 receptors (Table I). It was assumed, therefore, that infection of AMOPRO viruses occurred in mouse cells via Rec-1 and that the lack of infectivity in human cells was due to

both the absence of Rec-1 molecules and the inability of Ram-1 to function as retrovirus receptor for this chimeric envelope.

Ram-1 and Rec-1 co-operate for virus entry

Receptor interference assays were performed on NIH 3T3 cells chronically infected by either MoMLV (which blocks Rec-1) or 4070A-MLV (which blocks Ram-1) and compared with normal NIH 3T3 cells (Table I).

Viruses with AMO envelopes could infect the two former 3T3-derived target cell types, with an efficiency similar to or slightly higher than that of parental 3T3 cells (Table I), suggesting that AMO viruses could use either of the two receptors. Compared with AMO and relative to infection of normal 3T3 cells, viral particles coated with the other Ram-1-targeted envelopes were not able to use Rec-1 or Ram-1 efficiently when either of the receptors was expressed alone. For example, when 100 infectious particles (as determined on 3T3 cells) coated with AMOFx envelopes were used for infection on 3T3 interferent cells, only four viral particles could enter via Rec-1 when Ram-1 was blocked, and only two viral particles could enter via Ram-1 when Rec-1 was blocked. These data indicated a loss of 94% in virus infectivity compared with when both receptors were available, suggesting that Ram-1 and Rec-1 could co-operate to optimize virus entry. Similar results were obtained with viruses carrying AMO1 and AMO1Fx chimeric envelopes (Table I).

This phenomenon of receptor co-operation was considerably enhanced in the case of viruses carrying AMOPRO envelopes. Viruses with AMOPRO could not infect Rec-1-blocked 3T3 cells at all and could hardly infect Ram-1-blocked 3T3 cells. When both Ram-1 and Rec-1

Table I. Titrations of Ram-1-targeting envelopes on 3T3 interferent cells

env ^a	3T3 ^b Ram-1 + Rec-1 ^c	3T3 _A ^b Rec-1 ^c	3T3 _E ^b Ram-1 ^c
MO	92 000 000	46 000 000	40
A	12 000 000	240	8 000 000
AMO	240 (100)	320 (266.7)	240 (150)
AMOFx	440 000 (100)	8000 (3.6)	6000 (2)
AMO1	1 920 000 (100)	78 000 (8.1)	62 000 (4.8)
AMO1Fx	1 620 000 (100)	60 000 (7.4)	74 000 (6.8)
AMOPRO	60 000 (100)	4 (0.013)	<1 (<0.0025)
AMOΔPRO3	280 000 (100)	40 (0.029)	64 000 (34.3)

^aEnvelopes depicted in Figure 1 and expressed on lacZ virions.

^bTitres as lacZ i.u./ml. Abbreviations for cell lines: 3T3_A, MLV-A-infected 3T3 cells; 3T3_E, MoMLV-infected 3T3 cells. To correct the slight variations in retrovirus infectivity between the different target cells lines, the percentage infectivity (brackets) relative to infection of 3T3 cells is calculated according to the formulae: (3T3_A titre/3T3 titre)/(46/92)×100 for titres via Rec-1 and (3T3_E titre/3T3 titre)/(8/12)×100 for titres via Ram-1.

^cReceptors available on indicated target cells.

Fig. 2. β -turn probability profile of the PRO spacer. The y axis values represent the probability $P(\text{turn}) \times 10^{-4}$ (Chou and Fasman, 1978) at each residue of the PRO spacer (x axis). The predicted β -turns in the Δ PRO spacers are underlined. The β -turn analysis was performed with the help of the PC/Gene (release 6.26, IntelliGenetics, Belgium) suite of programs.

were co-expressed on the cells, infection could proceed efficiently and reached titres of up to 6×10^4 lacZ i.u./ml (Table I).

Interdomain spacer structure influences receptor co-operation

The data in Table I suggested that specific interdomain spacers could regulate receptor co-operation. To analyze this phenomenon further, we made the following changes in the experimental procedures: (i) rather than blocking one of the two receptors by superinfection interference, we used for infection assays cells that naturally expressed only Ram-1 (TE671 human cells) or retroviral receptor-negative cells (CHO cells) which were engineered to express only Rec-1 (Cerd9 cells) or both Rec-1 and Ram-1 (Cear13 cells); (ii) we constructed a set of AMO-derived chimeric envelopes carrying interdomain spacers with different lengths and/or amino acid compositions (Figure 1); (iii) in an attempt to dissect the particular features of the AMOPRO envelopes in which the PRO interdomain

spacer consists of the regular repetition of 11 predicted β -turns (Figure 2), three Ram-1-targeted envelopes carrying truncated proline-rich amino acid spacers were constructed (Δ PRO2, Δ PRO3 and Δ PRO4) containing either the first 9 amino acids, the first 13 amino acids or the first 19 amino acids of the PRO spacer, respectively (Figures 1 and 2). The length of these three spacers was chosen firstly to encompass the two (Δ PRO2), three (Δ PRO3) or four (Δ PRO4) first predicted β -turns of the PRO spacer (Figure 2) and secondly to allow the direct comparison of AMO Δ PRO2, AMO Δ PRO3 and AMO Δ PRO4 envelopes with the AMO1Fx (10 amino acid long spacer), the AMOG1x (15 amino acid long spacer) and the AMOG2x (20 amino acid long spacer) envelopes that contain short interdomain spacers but with different amino acid compositions (Figure 1).

Envelopes, including the control envelopes from ecotropic (MO) and amphotropic (A) MLV, were expressed in TELCeB6 cells (Cosset *et al.*, 1995b) which provide MLV gag-pol core particles and an nlslacZ retroviral vector.

Fig. 3. Viral incorporation of chimeric envelope glycoproteins. Immunoblot of pellets of viral particles produced from TELCeB6 cells transfected with the envelopes shown in Figure 1. The blot was stained with an SU antiserum. The immunoblot was cut at 46 kDa, and the lower part was stained with a p30 antiserum to detect the p30-CA capsid protein (CA). The position of MoMLV env surface (SU) proteins is shown.

Immunoblots were performed to analyze envelope expression in transfected cells by using antibodies against MLV SU. For all chimeric envelopes, both a precursor and a processed SU product were detected at ratios similar to wild-type envelopes (not shown), suggesting that the mutants were expressed and processed correctly. Cell surface expression of mutant envelopes was examined by FACS analysis of producer cells, using antibodies against the SU. All transfected cells were stained with the anti-SU antibodies (not shown).

To demonstrate incorporation of the chimeric envelope glycoproteins into retroviral particles, supernatants from the various TELCeB6-transfected cell lines were ultracentrifuged to pellet viral particles. Pellets were then analyzed on immunoblots for their content of gag (p30-CA) and envelope proteins (Figure 3). To compare the efficiency of viral incorporation between the different envelopes, equivalent amounts of viral particles were loaded on gels, as judged by immunostaining for viral core proteins using anti-capsid protein antibodies. Viral SU could be detected for all mutants with only a slightly weaker env:gag ratio compared with wild-type envelope for most of the chimeric envelopes. Only in the case of the mutants AMOG3Fx and AMOΔPRO4 was significantly less envelope found in the viral pellet (4 and 7% respectively, compared with wild-type ecotropic envelopes MO). As expected, no SU was found in pellets when either of the envelopes were transfected in TELac2 cells, which do not express gag and pol proteins (Takeuchi

et al., 1994), thus demonstrating that the SU found in the pellets of env-transfected TELCeB6 cells was associated with gag-pol viral particles.

Infection assays were carried out to determine virus infectivity via Ram-1 (TE671 cells), Rec-1 (Cerd9 cells) or via both Ram-1 and Rec-1 (Cear13 cells). The results of a typical experiment are shown in Figure 4. Judging from infectious titres, the chimeric envelopes could be separated into four groups.

The first group consisted of the AMO envelopes that, when expressed on the virus surface, could infect cells via Ram-1 or via Rec-1. However, titration of AMO viruses on cells co-expressing both receptors was usually lower compared with titres obtained in cells expressing either receptor alone. The second group of chimeric envelopes comprised mutants AMOFx, AMO1, AMO1Fx, AMOG1Fx, AMOG2, AMOG2Fx and AMOG3Fx. Compared with AMO, the efficiency of infection of viruses of this second group was up to 50-fold higher on TE671 cells and up to 1000-fold higher on Cear13. This was probably due to a more relaxed envelope structure which may favor envelope conformational rearrangements after receptor interaction. In support of this hypothesis, viruses of this second group could infect cells expressing Rec-1 alone more efficiently than viruses with AMO envelopes, with increases in infection reaching up to 200-fold.

Viruses belonging to this second group could enter cells when one of the two receptors was present alone, yet infection titres increased when both receptors were present. This is in contrast to results obtained with AMO viruses (where infection titres decreased upon receptor co-expression) and consistent with the results in Table I. For example, viruses with AMOG1Fx envelopes had a titre of 4.5×10^4 lacZ i.u./ml on cells expressing Ram-1 alone and a titre of 8.7×10^4 lacZ i.u./ml on cells expressing Rec-1 alone, with the titre on cells expressing both receptors being 4×10^5 lacZ i.u./ml. These results suggested that an efficient infection required both receptors and supported the hypothesis of a weak cooperation between the two cell surface molecules.

The absolute requirement for co-expression of the two receptors was noticed in the case of viruses carrying AMOPRO envelopes forming group 4. Such viruses could not infect cells that expressed either Ram-1 or Rec-1 alone (Figure 4). The co-expression of both Ram-1 and Rec-1 allowed efficient infection, though 10–20 times less efficiently than infection with viruses bearing envelopes of the second group, like AMO1Fx or AMOG1Fx. In an attempt to dissect the interdomain spacer inserted in AMOPRO envelopes, the 9, 13 or 19 first amino acids of the PRO spacer were used to construct AMOΔPRO2, AMOΔPRO3 or AMOΔPRO4 chimeric envelopes, respectively (Figures 1 and 2). Viruses generated with this latter type of envelope, i.e. group 3, now referred to as 'AMOΔPRO envelopes', had a phenotype intermediate between viruses of the second group and viruses carrying AMOPRO envelopes (Figure 4). Similarly to viruses of the second group, virions with AMOΔPRO envelopes could infect cells expressing Ram-1 alone efficiently, and infection was ~10 times more efficient when both receptors were co-expressed on the same target cell. This discrepancy did not simply imply that 90% of AMOΔPRO-carrying virions were entering directly through Rec-1 on cells expressing

Fig. 4. Infection by virions expressing targeting envelopes. Virions generated with the Ram-1-targeted envelopes depicted in Figure 1 were used to infect TE671 human (gray bars), CEAR13 (striped bars) or CERD9 (black bars) cells. In the same experiment, titres of viruses with ecotropic (MO) envelopes were 2×10^7 lacZ i.u./ml on CEAR13 cells and titres of viruses coated with wild-type amphotropic (A) envelopes were 1×10^7 lacZ i.u./ml on CEAR13 cells.

both receptors. Indeed, infection of cells that only expressed Rec-1 was very poor on Ram-1-blocked 3T3 cells (Table I), if not undetectable on CERD9 cells (Figure 4).

Masking of post-binding envelope functions

Taken together, these results point to the existence of important interactions between the fused domains of these chimeric envelope glycoproteins, and suggest that the amino acid spacer inserted downstream of the N-terminal binding domain could exert a control on the process of viral entry after retargeted binding. This control seemed to be spacer type-specific and to be related to the amino acid composition of the spacer. A first possibility was that particular interdomain spacers could induce the N-terminal displayed domain to mask the Rec-1-binding sites of the SU domain to which it was fused, thereby interfering with virus attachment to the CERD9 cells. Alternatively, it could be that attachment to Rec-1 might proceed normally and that the N-terminal binding domain might sterically hinder conformational rearrangements or domains of the trimeric SU-TM complex involved in fusion.

To discriminate between the two possibilities, SU binding assays were performed on cells expressing either Ram-1 alone (TE671 human cells) or Rec-1 alone (CERD9 cells) with virions belonging to the different phenotypic groups: AMO (group 1), AMOG1Fx (group 2), AMOΔPRO3 (group 3) and AMOPRO (group 4). Cells were incubated with virus supernatants, and binding of viral

envelopes to the target cell surface was analyzed by FACS using 83A25 monoclonal antibodies raised against the MLV SU. As expected, no binding to TE671 cells was detected for viruses carrying MO ecotropic envelopes, whereas all chimeric envelopes with the Ram-1-binding domain could bind TE671 cells at a similar efficiency compared with binding of amphotropic A envelopes (Figure 5A). Conversely, no binding to CERD9 cells was detected for viruses carrying amphotropic A envelopes, whereas all chimeric envelopes could bind CERD9 cells at a similar efficiency compared with the binding of ecotropic MO envelopes (Figure 5B).

However, it remained a possibility that the chimeric envelopes are unable to interact with Rec-1 receptors whilst they are trapped in trimeric complexes on the viral particles but that they can bind to Rec-1 as soluble monomers after they have been shed from the viral particles, and thus they could be detected in SU binding assays. Indeed, previous reports from our laboratory have shown that <1% of chimeric SU is associated with virions (Cosset *et al.*, 1995a). Therefore, binding assays were performed on the same cell types by using anti-TM antibodies to detect SU-mediated binding of viral particles. Using this assay, virions with envelopes from all different groups were shown to bind TE671 cells with no significant differences in their binding efficiencies, as compared with each other (data not shown). Virions with chimeric envelopes belonging to the first three groups were found

Fig. 5. Envelope and virion binding assays. TE671 (A) or Cerd9 (B and C) cells were used as target. The background of fluorescence was provided by incubating the cells with DMEM only (white histograms). Binding assays (black histograms) were performed with some of the envelopes shown in Figure 1. The env glycoprotein content of the different samples was normalized by immunoblot on viral supernatant (A and B) or viral pellet (C).

to bind Cerd9 cells, though with a much lower efficiency compared with binding of retroviruses generated with ecotropic MO envelopes (Figure 5C). Interestingly, viral particles generated with AMOPRO envelopes could not bind these cells, suggesting that the Rec-1-binding domain was not accessible (Figure 5C).

Discussion

MoMLV-based chimeric envelopes displaying a Ram-1 amino-terminal binding domain were generated. Our data demonstrate that it is possible to engineer chimeric retroviral envelopes that allow infection of mammalian cells via two distinct receptors: Ram-1 and Rec-1. Our studies show that the interdomain spacer inserted between the two binding domains plays a critical role in the regulation of the efficiency of infection. Short amino acid spacers (between 4 and 15 amino acids) had to be used to optimize virus entry through either of the two receptors, although the simultaneous presence of both receptors was found to enhance the infectivity of the retroviruses. Interestingly, retroviruses generated with AMOPRO chimeric envelopes displaying a 59 amino acid long proline-rich spacer (PRO) could not infect cells that expressed only one or other of the two receptors, although infection was shown to proceed efficiently upon receptor co-expression. Receptor binding assays showed that virions coated with AMOPRO envelopes could interact with Ram-1, yet they were not able to bind cells that expressed only Rec-1. Moreover, a minimal core motif (Δ PRO2, Δ PRO3 or Δ PRO4),

encompassing the first 9, 13 or 19 amino acids of the PRO spacer, displayed some features of the parental peptide.

Receptor co-operation

Previous studies from our laboratory performed with different chimeric MLV envelopes have shown that the retroviral receptor-binding domain located underneath the amino-terminally displayed domains is accessible and can interact with the cognate retroviral receptor and lead to infection (Cosset *et al.*, 1995a; Ager *et al.*, 1996; Marin *et al.*, 1996; Nilson *et al.*, 1996).

In the case of the Ram-1-targeted envelopes described in this report, retroviruses generated with AMO chimeric envelopes carrying no spacer (Figure 1) could bind Cerd9 cells expressing Rec-1 alone efficiently, although infection was poor. Interestingly the co-expression of both receptors resulted in a significant decrease in the efficiency of infection, and these viruses could infect more efficiently cells where either Ram-1 or Rec-1 was expressed alone. It is possible that the closeness of the binding domains of the two receptors prevents optimal envelope conformational rearrangements and impairs envelope fusogenicity when such virions are simultaneously bound to the two receptors.

Compared with this latter AMO envelope, the strong enhancement of infectivity for retroviruses generated with envelopes belonging to the second group (AMOFx, AMO1, AMO1Fx, AMOG1Fx, AMOG2, AMOG2Fx and AMOG3Fx) might therefore be explained by the increased size of the spacer between the two different binding

domains. An optimized interdomain spacing may indeed diminish the steric hindrance caused by the displayed Ram-1-binding domain and may favor conformational rearrangements that follow binding to Ram-1 and/or Rec-1. It is noteworthy that the relaxing of the interdomain spacing favors infection whether it occurs via either of the two receptors alone or via both of them. The decrease in infectivity for viruses coated with other envelopes from the same group, AMOG2, AMOG2Fx and AMOG3Fx, that have longer spacers may be caused by a lower density of these chimeric envelopes on the virions (Figure 3). Alternatively, interdomain spacers longer than 15 amino acids may also be unable to transmit a 'fusion trigger' optimally to the SU-TM trimer and to induce sufficient conformational changes required for viral fusion to occur.

The observation that viruses coated with AMOPRO envelopes cannot infect cells expressing Ram-1 alone might also be explained by the important size of the PRO interdomain spacer which may prevent full envelope conformational rearrangements. In contrast to AMOPRO-, AMO Δ PRO2-, AMO Δ PRO3- and AMO Δ PRO4-carrying viruses are able to infect cells expressing Ram-1 only. This might be explained by the short size of the Δ PRO2, Δ PRO3 and Δ PRO4 interdomain spacers which, like the short spacers of the second group (1Fx, G1x and G2x), should be able to transmit the fusion trigger efficiently, independently of the presence of the Rec-1 receptor.

Our data suggest that the inability of AMOPRO viruses to infect Cerd9 cells is caused by masking of the Rec-1-binding domain in the envelope trimer (Figure 5C). Although retroviruses generated with AMO Δ PRO envelopes display the same properties as viruses coated with AMOPRO envelopes regarding their inability to infect Rec-1-expressing cells, the Rec-1 receptor-binding domain seems partially accessible in AMO Δ PRO3-carrying virions (Figure 5C). It is tempting, therefore, to speculate that the inability of viruses with AMO Δ PRO envelopes to achieve post-binding events after Rec-1 receptor interaction on Cerd9 cells is caused by the masking of envelope functions involved in fusion, such as, for example, TM and/or C-terminal SU regions. It is likely that the interdomain spacers inserted in envelopes belonging to groups 3 and 4 share similar properties that can lead to steric hindrance of different envelope domains or post-binding functions. For example, a particular feature shared by these interdomain spacers may be capable of inducing a backward tilt of the N-terminally displayed domain which may inhibit either the Rec-1-binding domain (for AMOPRO) or post-binding rearrangements (for envelopes of group 3). A striking property of both types of interdomain spacer is that inhibition of envelope fusogenicity can be reversed upon binding to Ram-1. Interaction with Ram-1 may modify the positioning of the interdomain spacers and this may lead to unveiling of either the Rec-1-binding domain or the fusion functions. Thus binding to Ram-1 of the AMO Δ PRO-carrying retroviruses would unmask the fusion functions of the envelope and would allow infection to proceed via Rec-1. On the other hand, binding of AMOPRO-coated virions to Ram-1 would unveil the Rec-1-binding domain and would allow Rec-1-mediated infection.

Our results may have interesting parallels with the mechanism of human immunodeficiency virus (HIV) entry

in which primary virus attachment to CD4 leads to a conformational rearrangement in gp120-SU and to secondary virus attachment to recently characterized HIV co-receptors (Feng *et al.*, 1996; Premack and Schall, 1996). C-type retroviral vectors with engineered SU glycoproteins could therefore be developed as model systems to investigate the entry mechanisms of naturally occurring viruses, such as HIV.

Proline-rich spacers

Taken together, these data suggest a dynamic role for the PRO spacer and particularly for its first 9–19 amino acids, the Δ PRO spacers (Δ PRO2, Δ PRO3 and Δ PRO4). In the constructs described in this report, the PRO interdomain spacer was derived from 4070A-MLV (nucleotides 751–927) (Ott *et al.*, 1990).

Most type C mammalian retrovirus envelopes share similar sized proline-rich regions located in the middle of their SUs. These regions provide a hinge separating the two functional domains of the SU (Kabat, 1989): the N-terminal receptor-binding domain (Battini *et al.*, 1992, 1995) and the C-terminal domain implicated in post-binding entry events (Pinter *et al.*, 1986; Nussbaum *et al.*, 1993). Such proline-rich sequences are thought not to fold as stable secondary structures but rather as a random coil conformation (Kabat, 1989). However, as noticed by others for MoMLV (Gray and Roth, 1993), the regular arrangement of proline residues (GPRV/IPIGNPI/L) conserved among the beginnings of proline-rich sequences for all MLV strains argues for the importance of this region. Indeed, several reports suggest that the proline-rich domain is not merely a flexible linker but rather a functional domain. First, in studies performed with chimeric SU glycoproteins, although not directly involved in receptor binding, the proline-rich regions of some MLV strains were found to influence receptor recognition (Ott and Rein, 1992; Battini *et al.*, 1995). Secondly, the MoMLV proline-rich region was found to be important for stabilizing SU-TM interaction (Gray and Roth, 1993). Thirdly, several point mutations in the MoMLV proline-rich region were found to affect virus fusogenicity, perhaps by altering glycosylation (Andersen, 1994).

The proline-rich region of 4070A-MLV is not compatible with either α -helix or β -sheet secondary structures by Chou–Fasman analysis (Chou and Fasman, 1978), but rather is predicted to form a regular arrangement of 11 β -turns induced by the majority of the proline residues (Figure 2). This highly ordered structure is consistent with the polyproline β -turn helix form of secondary structure associated with the proline-rich repeat sequences from an array of diverse proteins (Matsushima *et al.*, 1990). Interestingly, both feline leukemia virus A (FeLV-A) (Fontenot *et al.*, 1994) and 4070A-MLV (Figure 2) proline-rich sequences display the regular arrangement of 10–11 polyproline β -turns. A recent report using synthetic peptides derived from the FeLV-A proline-rich region has shown that this region most likely exists as a polyproline β -turn helix, a particularly highly ordered and stable structure, and can self-assemble into complex ordered multimers (Fontenot *et al.*, 1994). Together with our computer predictions, this latter study strongly supports the proposal that both the proline-rich region of 4070A-MLV and the Δ PRO peptides, which contain the first two

to four β -turns of the 4070A-MLV proline-rich region (Figure 2), may fold as polyproline β -turn helices. As shown for the dynamic β -spirals of bovine elastin (Urry, 1988) and as suggested for the FeLV-A proline-rich domain (Fontenot *et al.*, 1994), such a repetitive turn motif in MLV proline-rich regions may display some very unusual physical properties such as: (i) self-assembly to form oligomeric quaternary structures; (ii) increasing structure order with increasing temperature; and (iii) development of elastomeric forces coincident with molecular ordering (Urry, 1988).

Consistently with preliminary results of our laboratory (S.Valsesia-Wittman and F.-L.Cosset, data not published), it seems likely that these properties of the polyproline β -turn helices can account for the behavior of retroviruses coated with AMOPRO and AMO Δ PRO envelopes. On the one hand, the Ram-1 N-terminal binding domain fused to either PRO or Δ PRO interdomain spacers may form a weak trimeric cap on the MoMLV envelope glycoprotein. Such a cap may compromise the accessibility of downstream envelope functional domains, as for example the Rec-1-binding domain which seems completely masked in the envelopes carrying the PRO peptide which displays the strongest predicted oligomeric structure. This may explain why such retroviruses are unable to interact with Rec-1 (in the absence of Ram-1) and why the Rec-1-binding domain was found accessible for soluble (monomeric; Figure 5B) but not for virion-associated (trimeric; Figure 5C) AMOPRO envelopes. On the other hand, the binding of the AMOPRO and AMO Δ PRO viruses to Ram-1 may stretch the β -turn helices of the PRO and Δ PRO interdomain spacers. On return, the extended β -turn helices would tend to recover from deformation and re-fold in the relaxed (non-stretched) state. Such a libration may either disrupt the oligomeric cap, thus causing unmasking of the proximal Rec-1-binding domain, or induce further changes in the conformation of the envelope, thus resulting in a better reactivity during the fusion process.

It is noteworthy that the polyproline-rich regions of the wild-type MLV envelope glycoproteins are also likely to fold as β -turn helices. Based on the particular properties of these helices, our data may provide some insights into the mechanism of fusion triggering after the attachment of MLVs to their receptors. Similarly to chimeric envelopes displaying the Ram-1-binding domain fused to PRO or Δ PRO interdomain spacers, it is possible that the function of the polyproline-rich regions in wild-type MLV envelopes is to provide a trimeric cap which would lock downstream fusion domains until the viruses have interacted with the receptors. This may explain how the fusion peptide located at the amino-terminus of the TM subunit is kept buried inside the envelope glycoprotein trimer before virus attachment and how retrovirus binding to its receptor can activate the fusion domains and trigger the initiation of fusion events via an elastic process. This involvement of the polyproline-rich region in the activation of MLV fusion is now supported by recent evidence from our laboratory (D.Lavillette and F.-L.Cosset, manuscript in preparation).

Targeting by host-range restriction

Our data suggest that the infectivity of retroviruses displaying an N-terminal targeting moiety can be reversibly

inhibited by fusing poly- β -turn peptides between the displayed binding domain and the MoMLV SU envelope glycoprotein. Infectivity via the MoMLV receptor can be restored upon interaction of the new binding domain with its cognate cell surface molecule.

These results suggest a novel approach to retarget virus entry in a two-step targeting manner where the retroviral moiety (SU + TM) of an N-terminally extended chimeric envelope serves as an auxiliary mechanism facilitating retrovirus entry that is conditionally recruited after the binding to a specific molecular target. So far, most of the strategies explored to retarget retrovirus entry have been to engineer new binding domains onto the SU glycoproteins of ecotropic MLVs in the hope that virus tropism will be extended to human cells bearing the targeted cell surface molecule. It is now becoming clear that most cell surface molecules cannot be used by retroviruses to penetrate the cells to which they have bound (Cosset and Russell, 1996). It is expected that a two-step targeting strategy may overcome this main limitation. New binding domains fused to proline-rich sequences displayed on 4070A-MLV envelope glycoproteins may therefore be an attractive tool in this respect and currently are being studied in our laboratory.

Materials and methods

Cell lines

The TELCeB6 cell line (Cosset *et al.*, 1995b) was derived from the TELac2 line (Takeuchi *et al.*, 1994) after transfection and clonal selection of cells containing a plasmid expressing MoMLV gag and pol proteins. TELCeB6 cells produce non-infectious viral core particles, carrying an nlslacZ reporter retroviral vector.

TE671 (ATCC CRL8805) were grown in Dulbecco's modified Eagle's medium (DMEM; Life-Technologies) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL). Cerd9 and Cear13 (Kozak *et al.*, 1995) were grown in DMEM (Life-Technologies) supplemented with 10% FBS and with proline (Life-Technologies). NIH 3T3 and NIH 3T3-derived cell lines were grown in DMEM (Life-Technologies) supplemented with 10% newborn bovine serum (Life-Technologies).

Plasmids, transfection and virus production

Plasmids encoding the ecotropic (FBMOSALF), amphotropic (FBAS-ALF) and AMO chimeric (FBAMOSALF) envelopes have been described elsewhere (Cosset *et al.*, 1995a). Expression plasmids for AMOFx, AMO1, AMO1Fx, AMO Δ PRO3 and AMOPRO chimeric envelopes were described elsewhere (Valsesia-Wittmann *et al.*, 1996).

To generate expression plasmids for the AMO Δ PRO2 and AMO Δ PRO4 envelope glycoproteins, an upper oligonucleotide (805FC: 5'-TCC AAT TCC TCC CAA GGG GC), located just upstream of the *Xho*I site of the 4070A *env* gene (at nucleotide 594) (Ott *et al.*, 1990), was used in combination with oligonucleotides providing an *Eag*I site [AMO Δ PRO(-H+P-A): 5'-TAT GAG CGG CCG GGT TGG GCC CTA TGG GGA C and AMO Δ PRO(+H+S-A): 5'-TAT GTG CGG AGG AAG GGA GTC TTT GGT C] to generate by PCR a 210 and a 247 bp fragment encompassing the Δ PRO2 and Δ PRO4 interdomain spacers, respectively, on the 4070A *env* gene used as template. These PCR-amplified fragments were then digested with *Xho*I and *Eag*I and cloned into *Xho*I-*Not*I-digested FBAMOSALF (Cosset *et al.*, 1995a), a plasmid expressing the AMO envelope.

To construct chimeric envelopes displaying the Ram-1-binding domain fused to the G1Fx, G2, G2Fx and G3Fx interdomain spacers (Figure 1), PCR fragments encoding the spacers were generated by using the same 3' oligonucleotide (envseq7: 5'-GCC AGA ACG GGG TTT GGC C) located downstream of the *Bam*HI site in the MoMLV *env* gene (at position 5638) (Shinnick *et al.*, 1981) and specific 5' oligonucleotides encompassing a *Not*I restriction site for all four spacer types: for the G1Fx spacer, primer NL1FXMo1bak (5'-GCA AAT CTG CGG CCG CAG GTG GAG GCG GTT CAATCGAGG GAA GGG CTT CGC C); for the G2 spacer, primer NL2Mo1bak (5'-GCA AAT CTG CGG CCG

CAG GTG GAG GCG GTT CAG GCG GAG GTG GCT CTG CTT CGC CCG GCT CCA GTC C); for the G2F_x spacer, primer NL2FXMolbak (5'-GCA AAT CTG CGG CCG CAG GTG GAG GCG GTT CAG GCG GAG GTG GCT CTA TCG AGG GAA GGG CTT CGC C; and for the G3F_x spacer, primer NL3Molbak (5'-GCA AAT CTG CGG CCG CAG GTG GAG GCG GTT CAG GCG GAG GTG GCT CTG GCG GTG GCG GAT CGG CTT CGC CCG GCT CCA GTC C). Plasmid FBMOSALF was used as template to generate the PCR fragments encoding the G2 spacer, whereas a plasmid encoding the AMOIF_x chimeric envelope glycoprotein was used to derive PCR fragments encoding the G1F_x, G2F_x and G3F_x spacers. All PCR fragments were digested with *NotI* and *BamHI* and then subcloned into the AMO1 *env* gene from which the original *NotI*–*BamHI* had been removed.

All envelope constructs were expressed as *BglIII*–*ClaI* fragments (corresponding to positions 5408 and 7676 in MoMLV) (Shinnick *et al.*, 1981), cloned between *BamHI* and *ClaI* sites of the FBMOSALF expression vector, carrying a phleomycin resistance gene (Cosset *et al.*, 1995a).

Envelope expression plasmids were transfected by calcium phosphate precipitation into TELCeB6 cells as previously described (Cosset *et al.*, 1995a). Transfected cells were selected with phleomycin (50 µg/ml) and phleomycin-resistant colonies were pooled. Virus-containing supernatants were collected after an overnight production from freshly confluent *env*-transfected TELCeB6 cells in regular medium.

Immunoblots

Virus producer cells were lysed in a 20 mM Tris–HCl buffer (pH 7.5) containing 1% Triton X-100, 0.05% SDS, 5 mg/ml sodium deoxycholate, 150 mM NaCl and 1 mM phenylmethylsulfonyl fluoride (PMSF). Lysates were incubated for 10 min at 4°C and centrifuged for 10 min at 10 000 *g* to pellet the nuclei. Supernatants were then frozen at –70°C until further analysis. Virus samples were obtained by ultracentrifugation of viral supernatants (10 ml) in a SW41 Beckman Rotor (30 000 r.p.m., 1 h, 4°C). Pellets were suspended in 100 µl of phosphate-buffered saline (PBS), and frozen at –70°C. Samples (30 µg for cell lysates, or 10 µl for purified viruses) were mixed 5:1 (v/v) in a 375 mM Tris–HCl (pH 6.8) buffer containing 6% SDS, 30% β-mercaptoethanol, 10% glycerol and 0.06% bromophenol blue, boiled for 3 min, then run on 10% SDS–acrylamide gels. After protein transfer onto nitrocellulose filters, immunostaining was performed in TBS (Tris base saline, pH 7.4) with 5% milk powder and 0.1% Tween. Antibodies (Quality Biotech Inc., USA) were goat antisera raised against either Rausher leukemia virus (RLV) gp70-SU protein or RLV p30-CA protein, and were diluted 1/1000 and 1/10 000, respectively. Blots were developed using horseradish peroxidase-conjugated rabbit anti-goat Ig (immunoglobulins) antibodies (DAKO, UK) and an enhanced chemiluminescence kit (Amersham Life Science).

Binding assays

Target cells were washed in PBS and detached by a 10 min incubation at 37°C with versene 0.02% in PBS. Cells were washed in PBA (PBS with 2% fetal calf serum and 0.1% sodium azide). Then 5×10⁵ cells were incubated with viruses for 45 min at 37°C. Cells were then washed with PBA and incubated with 83A25 (Evans *et al.*, 1990) or 9E8 monoclonal antibodies (Lostrom *et al.*, 1979) for 45 min at 4°C. Cells were washed twice with PBA and incubated with anti-rat Ig FITC-conjugated antibodies (DAKO, UK). Five minutes before the two final washes in PBA, cells were counterstained with 20 µg/ml propidium iodide. Fluorescence of living cells was analyzed with a fluorescent-activated cell sorter (FACSCalibur, Beckton Dickinson).

Infection assays

Target cells were seeded in 24 multi-well plates at a density of 5×10⁴ cells per well. Viral supernatant dilutions containing 5 µg/ml polybrene were added and cells were incubated for 3–5 h at 37°C. Viral supernatant was then removed and cells were incubated in regular medium for 48 h. X-Gal staining and viral titre determination were performed as previously described (Cosset *et al.*, 1995a) as lacZ i.u./ml.

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