Identification of R-peptides in envelope proteins of C-type retroviruses

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Activation of the murine leukaemia virus (MLV) envelope protein (Env) requires proteolytic cleavage of the R-peptide, a 16 amino acid C-terminal part of the cytoplasmic tail (C-tail) of Env. This paper demonstrates the presence of R-peptides in Env proteins of C-type retroviruses of simian, avian and porcine origin. Sequence alignment with the MLV C-tail led to the identification of a conserved hydrophobic protease cleavage motif located in the centre of retroviral Env protein C-tails. Expression of Env proteins, truncated at the predicted cleavage sites, of spleen necrosis virus (SNV), gibbon ape leukaemia virus and porcine endogenous retroviruses resulted in cell–cell fusion as monitored by microscopy and reporter gene fusion assays. Western blot analysis of MLV particles pseudotyped with the SNV Env protein demonstrated proteolytic cleavage of the SNV R-peptide by the MLV protease. Our data suggest that activation of membrane fusion by R-peptide cleavage is a common mode in C-type retroviruses.

Entry of retroviruses into cells occurs through fusion between the cellular and viral membranes, a process that is mediated by the viral envelope glycoprotein (Env). Retroviral Env proteins are trimers of heterodimers that include an extracellular soluble domain (SU), which contacts the virus receptor, and a transmembrane domain (TM), which anchors the protein complex in the viral envelope. SU and TM arise from intracellular proteolytic processing of the Env precursor protein. Different modes of fusion regulation used by enveloped viruses have been identified. The best characterized trigger of fusion activation is low pH. Fusion proteins of the pH-dependent viruses, like influenza virus, which enter the cell via receptor-mediated endocytosis, undergo major conformational changes in the low-pH milieu of the endosomes resulting in the fusion active state (Skehel & Wiley, 2000).

In retroviruses that are fusion active under neutral pH conditions the major trigger seems to be receptor contact. However, receptor binding is not sufficient in some retroviruses. For murine leukaemia virus (MLV) proteolytic processing of the R-peptide has been described as a second mode of fusion activation preceding receptor contact. The R-peptide, as initially detected in MLV, is a fragment of 16 amino acids which is released from the C terminus of the cytoplasmic tail of the TM protein upon particle formation as a result of proteolytic cleavage by the viral protease (Green et al., 1981; Henderson et al., 1984). R-peptide-less recombinant MLV Env proteins generated by insertion of a stop codon at the position of the cleavage site cause the formation of large syncytia when expressed in receptor-positive cells in the absence of all other virus proteins (Ragheb & Anderson, 1994; Rein et al., 1994; Yang & Compans, 1997). Apart from MLV, R-peptide cleavage has been demonstrated for Mason–Pfizer monkey virus, a type D retrovirus, and the lentivirus equine anaemia virus (Brody et al., 1992; Rice et al., 1990). The cleavage sites in the cytoplasmic tails (C-tails) of the Env proteins of these viruses have been mapped based on sequence-specific antibodies. However, no sequence homologies are detectable within the C-tails of these Env proteins and that of C-type retroviruses. Recently, indirect evidence suggested the presence of an R-peptide in the Env protein of another C-type retrovirus, gibbon ape leukaemia virus (GaLV) (Fielding et al., 2000; Christodouloupolous & Cannon, 2001). Here we investigated R-peptide-dependent fusion regulation in C-type retroviruses of simian, porcine and avian origin, in particular, the Env proteins of GaLV, spleen necrosis virus (SNV) and porcine endogenous retrovirus (PERV).

The first aim of this study was the identification of putative R-peptide cleavage sites by aligning their sequences to that of MLV. The alignment was started at the C terminus of the transmembrane domain (TMD) of TM which includes a conserved GPC motif present in all four viruses. All C-tails start with a positively charged amino acid at their N terminus and have a similar length, ranging from 32 to 35 amino acids. It is important to mention that different Env protein sequences with shorter C-tails for SNV and GALV have been published in GenBank. However, these were based on sequencing errors and have been corrected (Engelständter et al., 2000; Ting et al.,...
GaLV wt Xba codon was inserted by PCR using the oligonucleotide primers GaLV (kindly donated by Y. Takeuchi, London, UK) the stop GaLV, SNV and PERV Env protein reading frames. In pALF-«inserted stop codons at the predicted P1

exhibit equivalent functions as the MLV R-peptide. In contrast, the membrane distal part shows less sequence homology. The only conserved motifs are YXXL, an endocytic signal previously identified in several retroviral cytoplasmic domains (Ohno et al., 1997), and the accumulation of negatively charged residues. The central parts are completely hydrophobic and include four (MLV, GaLV, PERV) or six (SNV) amino acid residues. In MLV, the R-peptide cleavage site has been mapped to leucine-646 (P1) and valine-647 (P1′) (arrow in Fig. 1). This LV dipeptide is also present in the GaLV and SNV central parts. Moreover, identical or homologous residues are present in the putative P2 and P2′ positions (second residues N- respectively C-terminally of the scissile bond). We therefore predict R-peptide cleavage to occur between leucine-671 and valine-672 in GaLV and leucine-580 and valine-581 in SNV, respectively. In PERV, as the most likely cleavage site we suggest methionine-643 and valine-644. Then, the P1′ + P2′ residues are identical to those in MLV. Overall, the sequence alignment shows that the membrane proximal and the central part including the R-peptide cleavage site of the MLV C-tail are well conserved among GaLV, SNV and PERV Env protein C-tails. Although the membrane distal parts are less well conserved they might exhibit equivalent functions as the MLV R-peptide.

To test whether the putative R-peptides inhibit fusion, we inserted stop codons at the predicted P1′ positions in the GaLV, SNV and PERV Env protein reading frames. In pALF-GaLV (kindly donated by Y. Takeuchi, London, UK) the stop codon was inserted by PCR using the oligonucleotide primers GaLVvwtXba + (5′ GCTTCTAGAATGATTGCTGCTGGCTCTCC 3′), and GaLV-ARCla − (5′ CCATCGAATTACAGACTTTAACTGTTAGCTCTCC 3′) to generate plasmid pALF-GaLVΔR. For the SNV Env plasmid, pRD134 (Martinez & Dornburg, 1995) was modified by introducing a translational stop at codon 2719 to result in pRD134ΔR, using the QuickChange Site-Directed Mutagenesis Kit (Promega) and oligonucleotide primers SNV-ΔR (5′ CTAGCCTATAGC

CACAGTACAAG 3′) and SNV-ΔR(al-reverse (5′ CTTGTCATGTGGATATAGTGCTAG 3′). From PERV we investigated the Env proteins of the A and B strains, which exhibit distinct tropism and receptor choice (Takeuchi et al., 1998; Czauderna et al., 2000). Accordingly, these Env proteins differ in their SU subunits, but are identical in the TM cytoplasmic domains (Patience et al., 2001). The PERV Env proteins were expressed from plasmids pALF-PERV A and pALF-PERV B, which were kindly provided by Y. Takeuchi (London) and R. Toenjes (Langen), respectively. Stop codons were introduced by site-directed mutagenesis using the oligonucleotides PERV-ΔR (5′ CAGATCTGTTAGGATACTGCAACAG 3′) and PERV-ΔR(al-reverse (5′ CTGTGTCCTAATCATGATCTCCTG 3′).

To investigate the proteolytic processing of the SNV TM protein D17 cells, a dog osteosarcoma cell line permissive for SNV was transfected with plasmid pCG4 encoding the complete genome of SNV (Gelinas & Temin, 1986). Four days after transfection cells were lysed in 50 μM Tris (pH 8.0), 150 μM NaCl, 15 μM MgCl₂, 1% NP40 and 0.1% SDS, and cytoplasmic extract was prepared. Virus particles released from the cells were concentrated by ultracentrifugation (25 000 r.p.m., Beckman SW28 rotor, 2 h) through a 25% sucrose cushion. Proteins from cell extracts and virus particles were then separated on a 12% SDS–polyacrylamide gel and subjected to Western blot analysis using the anti-SNV TM antibody 11A25. Fig. 2(A) shows that TM protein derived from cell lysates migrates at about 20 kDa, while the virus-associated TM protein migrated at a lower molecular mass, about 16 kDa (compare lanes 1 and 2). Next, we expressed the SNV Env protein alone (plasmid pRD134) or in the presence of the MLV Gag/Pol proteins (plasmid pHIT60; Soneoka et al., 1995). Cell extract from pRD134ΔR-transfected cells (lane 4) was used as a molecular mass marker. Interestingly, processing of the TM protein was also detectable here, i.e. in the presence of the MLV protease (compare lanes 5 and 6), whereas it was not detected when expressed alone (lane 3).

From these experiments we conclude that the SNV TM protein is processed upon virus particle formation. Co-migration of the processed TM protein with the SNV EnvΔR protein indicated that the cleavage site must be located at or close to the site predicted in Fig. 1. To test if the processed peptide functions as regulator of Env protein fusogenicity we
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Fig. 2. R-peptide cleavage activates fusion of the SNV Env protein. (A) Proteolytic processing. SNV Env proteins derived from SNV-infected D17 cells (lane 1), released virus particles (lane 2), pRD134-transfected 293T cells (lane 3), pRD134ΔR-transfected 293T cells (lane 4), pRD134/pHIT60-co-transfected 293T cells (lane 5) or particles released from the pRD134/pHIT60-co-transfected cells (lane 6) were detected on 12% SDS–polyacrylamide gels by Western blot (lanes 1, 2) or radio-immunoprecipitation (lanes 3–6) using the anti-TM antibody 11A25. The positions of the processed (TMΔR) and unprocessed (TM) forms of TM are indicated. (B) Syncytia formation in transfected cells. 293T cells were transfected with pRD134 (left panel) or pRD134-ΔR (right panel) and co-cultured with SNV receptor-positive D17 cells. Pictures were taken 48 h after transfection.

expressed the SNV EnvΔR protein in 293T cells and cocultivated these with D17 cells. Fig. 2(B) shows that expression of the truncated protein resulted in the formation of large syncytia, which did not appear in cells expressing the unmodified SNV Env protein. The fusion regulatory function of the membrane distal domain of the SNV Env C-tail was further confirmed in a reporter gene assay (see below), using pT7-luc-transfected D17 cells and pT7-pol/pRD134ΔR-co-transfected 293T cells. Compared to the non-modified Env protein deletion of the putative R-peptide resulted in a sevenfold increase of luciferase activity (not shown).

In contrast to SNV, GaLV and PERV are both able to infect the human cell line 293T (Egliitis et al., 1995; Takeuchi et al., 1998). To investigate the fusion regulatory capacity of their putative R-peptides 293T cells were transfected with plasmids encoding the Env protein variants truncated at the predicted R-peptide cleavage sites, and as a control with the full-length Env proteins. In repeated experiments 293T cells transfected with plasmids pALF-PERV-AΔR formed syncytia at time-points as early as 16 h after transfection. In the following hours these syncytia grew rapidly, containing more than 100 nuclei and finally covering the complete surface of the six-well plate (Fig. 3A, top row). Syncytia induced by the truncated GaLV Env protein appeared a few hours later and were in general smaller (10–50 nuclei) (central row). Cells transfected with pALF-PERV-B also developed syncytia which, however, were significantly smaller (5–10 nuclei) than those observed with the other truncated Env proteins (bottom). No syncytia formation was observed after expression of the wild-type Env proteins (left row). Thus, the Env proteins of all these viruses can be fusion activated by removing the C-terminal amino acids of their cytoplasmic tails.

To quantify the fusion capacity of the GaLV, PERV A and PERV B Env protein variants a modified reporter gene assay was performed, based on T7 RNA-polymerase dependent expression of the firefly luciferase (luc) gene (Nussbaum et al., 1994). Two populations of 293T cells were transfected separately, one with the respective Env expression plasmids and pT7-luc, encoding the luc gene under control of the bacteriophage T7 promoter. The other 293T cell pool was
Fig. 3. For legend see facing page.
transfected with plasmid pCMV-T7pol encoding the T7-RNA polymerase under CMV promoter control. Thus, expression of luciferase in this system requires fusion of both cell populations, and the amount of enzyme generated reflects the cell–cell fusion efficiencies mediated by the expressed Env protein.

After transfection both cell populations were detached, mixed and co-cultivated for 20 h before cell extracts were prepared and assayed for luciferase activity. Fig. 3(B) shows the results obtained after expression of the GaLV and PERV Env protein variants. Although the luciferase levels varied between individual experiments expression of the PERV AΑR protein resulted, reproducibly, in the highest levels of luciferase activity, approaching those of cells co-transfected with pCMV-T7pol and pT7-luc. None of the unmodified Env proteins mediated luciferase activity significantly exceeding that of the negative control (no Env). In contrast, the truncated GaLV and PERV B Env proteins mediated luciferase activities on average eight- or fivefold above the activities obtained after expression of the corresponding wild-type Env proteins. Thus, the quantitative fusion assay was in line with the results obtained by microscopy. The PERV AΑR variant was the most potent mediator of cell–cell fusion in 293T cells followed by the GaLV and PERV-B Env variants.

From the data presented here we conclude that R-peptide cleavage is a common mode of fusion protein activation among C-type retroviruses. This study extends previous publications describing the R-peptide in MLV to viruses of avian, simian and porcine origin. Our sequence analysis indicates that the location and quality of the R-peptide cleavage site is conserved among all C-type retroviruses. Furthermore, within the C-type retroviruses R-peptide cleavage sites can be processed by heterologous proteases, as demonstrated here for the SNV TM protein and the MLV protease. Previously, it was shown that the HIV protease is able to activate the MLV Env protein (Kiernan & Freed, 1998). Thus, activation through R-peptide cleavage must have developed early in the evolution of retroviruses and must therefore be of fundamental relevance. This is in line with the current mechanistic model according to which the R-peptide controls the conformation of the Env protein, owing to being membraneanchored by a lipid modification (Olsen et al., 1999).

GaLV and SNV have both attracted attention as gene transfer systems. SNV is one of the few retroviral systems that allows targeted gene transfer upon modification of its Env protein with single chain antibodies specific for the pre-selected cell type (Jiang et al., 1998; Jiang & Dornburg, 1999; Engelstäder et al., 2000). The fusogenic SNV Env variant may form a useful tool to elucidate the molecular mechanism of SNV targeting which is insufficiently understood (Buchholz et al., 1999). The GaLV Env protein utilizes a receptor that is highly expressed on human haematopoietic stem cells and has therefore been frequently used to pseudo-type MLV and also lentiviral vector particles to achieve efficient gene transfer into haematopoietic cells (Stitz et al., 2000). More recently, a variant of the GaLV Env protein carrying an artificial cytoplasmic tail was shown to fuse, thereby killing tumour cells at high efficiency in vitro and also in vivo (Bateman et al., 2000). Our data, for example the highly efficient formation of syncytia induced by the PERV-AΑR Env protein, provide the basis for optimizing efficacy and safety of this novel class of tumour therapeutic genes. PERVs have recently attracted particular interest as a potential risk after xenotransplantation of swine organs into humans. However, their infectivity for human cells is disputed, especially as their receptor is still unknown (Takeuchi et al., 1998; Fiane et al., 2000; Czauderna et al., 2000). Analysis of syncytia formation in various human cell types upon expression of our fusogenic PERV Env variants offers an attractive approach to predict the biodistribution of PERV in humans and to ascertain the risk of xenotransplantation.

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References


Fig. 3. Comparison of the fusogenic capacities of GaLV, PERV A and PERV B Env proteins. (A) Microscopic examination of transfected cells. 293T cells transfected with pALF-PERV-A (top left), pALF-PERV-AΑR (top right), pALF-GaLV (centre left), pALF-GaLV A (centre right), pALF-PERV-B (bottom left) or pALF-PERV-BAR (bottom right). Pictures were taken 20 h after transfection. (B) Quantitative fusion assays. The reporter gene fusion assay was performed with cells expressing the indicated Env protein variants as described in the text. Luciferase activities were determined 16–20 h after co-cultivation. Relative activities were calculated by normalizing the luciferase activities of the co-cultivated cells to that of pCMV-T7/pT7-luc co-transfected cells, which was set to 100%. Results of three independent experiments as represented by the three columns, respectively, are presented.


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