The proline-rich region of the ecotropic Moloney murine leukaemia virus envelope protein tolerates the insertion of the green fluorescent protein and allows the generation of replication-competent virus

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Sequences encoding the green fluorescent protein (GFP) were inserted into the envelope protein (Env) of ecotropic Moloney murine leukaemia virus, MoMLV. Insertion of these sequences into the proline-rich region (PRR) of Env resulted in a chimeric GFP-Env protein that allowed retrovirus vector transduction of murine cells with titres similar to wild-type Env. However, N-terminal extension with GFP did not result in a functional Env protein. GFP sequences were then inserted into the Env PRR of E-MO virus, a MoMLV that carries epidermal growth factor sequences at the N terminus of its Env protein. The resulting virus, GFP-EMO1, replicates to the same titres as the parental virus. In a chronically infected cell culture, GFP-EMO1 was genetically stable. However, additional insertions of sequences that led to recombination or that may have been incompatible with virus replication were deleted and decreased virus titre. In summary, Env PRR can be used to tag individual virus particles with GFP, which leaves other regions available for modification in studies aimed at altering virus tropism.
only wt(HX) and GFP-Env1 could be detected in vector particles after high-speed centrifugation through a 30 % sucrose cushion (Fig. 1B, lanes 6–8). We and others (Erlwein et al., 2002; Kizhatil et al., 2001; Wu et al., 1998) had shown earlier that the centrifugation step through a sucrose cushion efficiently separates shed surface protein from virions. These results demonstrate that the presence of GFP sequences in the PRR did not interfere with Env function, as no virus-associated GFP-Env2 protein could be detected. However, N-terminal insertion of GFP abrogated Env function and particle association. Kayman et al. (1999) characterized the PRR and reported that deletion or insertion of peptides into the C-terminal part of the variable PRR do not alter the incorporation of chimeric Env into virus particles nor do they alter the fusion activity of the protein. We inserted GFP into that region and did not observe altered Env incorporation (Fig. 1B) or fusion activity with XC cells (ATCC no. CCL165) (data not shown), confirming the data of Kayman et al. (1999). However, N-terminal insertion of GFP abrogated Env function, as no virus-associated GFP-Env2 protein could be detected.

We generated a replication-competent virus expressing the chimeric GFP-Env1 protein. For this, GFP sequences were inserted into the PRR of pE-MO, a plasmid encoding a replication-competent MoMLV with 53 amino acids of the epidermal growth factor (EGF) fused to the N terminus of Env to target virus binding to the EGF receptor (Buchholz et al., 1998). This resulted in GFP-EMO1 (Fig. 2A). When NIH 3T3 cells were transfected, the cells released infectious virus that could be passaged further. We also generated GFP-EMO2, a construct carrying, in addition, the translocation domain (TLD) of the bacterial toxin exotoxin A between the EGF and Env sequences (Fig. 2A). The TLD enables the toxin, after endocytosis by the cell, to escape degradation in the endosomes and to translocate into the cytoplasm (Allured et al., 1986; Hwang et al., 1987). We had found recently that the presence of the TLD sequences in Env allowed the generation of a functionally active Env protein but severely reduced transduction titres (Erlwein et al., 2002). We wanted to investigate whether the replication behaviour of GFP-EMO1 or GFP-EMO2 was affected in comparison to E-MO.

293T cells were transfected with the DNA of the respective plasmids and titres were determined by limiting dilutions on recipient NIH 3T3 cells without the influence of virus replication. Cells were fixed with 1 % formaldehyde in PBS prior to detection of the virus using a goat anti-Env antibody followed by a horseradish peroxidase labelled anti-goat antibody and 3-amino-9-ethylcarbazole (Sigma). Under these conditions, E-MO yielded a titre of 3 \times 10^6 IU ml\(^{-1}\) as did GFP-EMO1. GFP-EMO2 gave titres of 10 IU ml\(^{-1}\). This demonstrates that virus is produced and confirms that the presence of the TLD sequences negatively influences virus titre in NIH 3T3 cells.

NIH 3T3 cells transfected with GFP-EMO1 (NIH 3T3/GFP-EMO1 cells) or GFP-EMO2 (NIH 3T3/GFP-EMO2 cells) were cultured for another 19 days until, in each case, the virus had spread through the whole cell culture, as judged by the expression of green fluorescence (Fig. 2B). Virus growth kinetics were monitored by FACS (Fig. 2B) and titres released from NIH 3T3/GFP-EMO1 remained at 3 \times 10^3 IU ml\(^{-1}\), while the titre for NIH 3T3/GFP-EMO2 increased and also reached about 10^3 IU ml\(^{-1}\) after 16 days (Fig. 2B). As shown by Western blot analysis, viruses released from NIH 3T3/GFP-EMO2 cells contained an Env protein that was slightly smaller than that observed for NIH 3T3/GFP-EMO1 cells (Fig. 3A, lane 4). To investigate whether this was due to rearrangements in the Env protein of GFP-EMO2, we isolated cellular DNA from both chronically infected cell lines and performed PCR analysis. The primers MLV5'ENV (5'-TAACCGGGAGGCCCCCTATCC-3') and BS5

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**Fig. 1.** (A) Schematic representation of chimeric GFP-Env protein constructs. The position of GFP sequences within Env is shown. Numbers refer to the amino acid positions of the mature Env protein. PRR, proline-rich region. Restriction endonuclease sites used are indicated as: H, HindIII; X, XhoI; P, PstI; and S, SgrAI. (B) Western blot analysis of Env proteins in cell lysates (lanes 1–4) and virions (lanes 5–8). A 30 ml sample of supernatant from cells expressing the respective construct was analysed as described (Erlwein et al., 2002). Envelope proteins were detected by incubation with goat antiserum against p70. Neg., untransformed; wt, wild-type; M, molecular mass marker (kDa).
(5’-TCTGAGTGGATCCCAAATGTAAG-3’) bind from position 6663 to 6685 and from position 7578 to 7555 of pE-MO, respectively.

DNA isolated from NIH 3T3/GFP-EMO1 cells gave a PCR product of the expected size of 0.9 kb, like the plasmid DNA used for transfection. However, NIH 3T3/GFP-EMO2 cell DNA yielded several bands smaller than the 1.3 kb fragment from plasmid DNA (Fig. 3B). The amplicons were cloned into pGEM-T (Promega) and sequenced. Two clones for NIH 3T3/GFP-EMO1 were identical to the plasmid DNA, indicating that no recombination events had occurred. However, out of seven clones from NIH 3T3/GFP-EMO2, two groups of deletions emerged (Fig. 3C): one carried deletions from the end of the leader peptide to the first amino acid of Env, resulting in the complete loss of EGF and TLD sequences, while the other group retained the complete leader peptide and some N-terminal amino acids of EGF together with some C-terminal amino acids of the TLD but lacked the intervening sequences. This demonstrates that the TLD sequences were not tolerated and that deletions had occurred during virus replication, creating a virus population similar to wt MoMLV. These viruses were able to outgrow the parental construct, resulting in an increase in virus titre.

Fig. 2. Generation of replication-competent GFP-Env-bearing viruses based on E-MO. (A) Schematic representation of the constructs with GFP sequences inserted between amino acids 264 and 274 of Env. gag-pol indicates the presence of the functional gag and pol open reading frames upstream of env. EGF, epidermal growth factor; TLD, exotoxin A translocation domain; LTR, long terminal repeat. (B) Growth characteristics of the viruses GFP-EMO1 and GFP-EMO2 on transfected NIH 3T3 cells and the respective FACS analysis of chronically infected NIH 3T3 cells at day 16 (right) compared to uninfected cells (left). In addition, UV light photographs of infected cells at day 16 of infection are shown.
the titre for wt(HX) and GFP-Env1 was found to be $5 \times 10^{2}$ UI ml$^{-1}$. In contrast, no β-Gal expression could be detected for wt(HX) or GFP-Env1 in chronically infected NIH 3T3/GFP-EMO1 cells, demonstrating that superinfection was reduced at least 500-fold and that the presence of foreign sequences in the PRR does not affect the specificity of the Env interaction.

Recently, the insertion of GFP sequences between amino acids 6 and 7 of MoMLV Env has been described (Kizhatil et al., 2001). Despite the successful packaging of the recombinant protein into virions, transduction failed due to a fusion defect until an additional double mutation (Q$^{227}$→R, D$^{243}$→Y) was included. In our experiments, GFP-Env2, which has the GFP sequences at the same location (aa 6–7), was also not able to transduce a reporter gene despite good Env protein production in the cell. However, there seemed to be a packaging defect of GFP-Env2 into the virion (Fig. 1B). The reason for the discrepancy to the results described by Kizhatil et al. (2001) is not clear. One explanation could be that GFP-Env2 carries the original GFP (S$^{65}$→T) sequences rather than enhanced GFP, which contains an additional point mutation in the chromophore and preferred human codons that enhance the translational efficiency of the mRNA. Inefficient translation of GFP-Env2 may interfere with proper folding of the protein and hinder incorporation of the protein into the virion.

In contrast, GFP-Env1, having the sequences of enhanced GFP in the PRR, was fully functional without the need of the additional mutations described (Kizhatil et al., 2001). The fact that a replication-competent virus can be generated on the basis of this construct without loss of titre demonstrates that this recombinant protein is not toxic to the cell and that it can be stably expressed. Furthermore, we demonstrated that labelling Env in the PRR does not change the specificity for its receptor, leaving other regions like the N terminus available for further insertions of ligands to target specific receptors. Recently, a retrovirus library based on feline leukaemia virus subtype A has been described that carries random modifications in the Env molecule to target new receptors (Bupp & Roth, 2002). The GFP-bearing virus described by us may prove helpful in screening similar libraries based on MoMLV to visualize the virus entry process and to easily monitor the tissue distribution of MoMLV.

ACKNOWLEDGEMENTS

We thank Catherine Mahoney and Thomas Schulz (Georg-Speyer-Haus, Frankfurt/Main) for critically reading the manuscript. This work was funded by the Deutsche Krebshilfe.

REFERENCES


