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Construction and characterisation of infectious recombinant HIV-1 clones containing CTL epitopes from structural proteins in Nef

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Abstract

In this study the construction is described of HIV-1 molecular clones in which CTL epitopes from RT or Env late proteins were inserted into the Nef early protein. The ectopic epitopes were efficiently processed from the recombinant Nef proteins, were recognized by their cognate CTL in cytolytic assays, and did not perturb virus replication or viral protein expression in vitro. These recombinant viruses will therefore be an important tool in studying the effect of distinct epitope expression kinetics on the efficiency of CTL-mediated suppression of HIV-1 replication.

Keywords: HIV-1; Recombination; CTL; Epitopes

HIV is a lentivirus for which protein expression is strictly regulated during the replication cycle (for a review, see Cullen, 1992). After entry into the target cell, the viral proteins Tat, Rev and Nef are the first to be expressed. The regulatory proteins Tat and Rev are crucial for viral replication, via enhancement of transcription and regulation of HIV-1 mRNA export to the cytoplasm (Cullen, 1992; Jeang et al., 1999; Pollard and Malim, 1998). The accessory genes encoding Vpu, Vif and Vpr are expressed next, together with Env Gag and Pol are the last proteins to become detectable, after which assembly of viral particles begins (Feinberg et al., 1986; Kim et al., 1989).

Infection with HIV leads to a chronic infection and although the virus cannot be cleared, immunity plays a role in suppressing the virus spread. Recent reports have shown that an efficient immune response against the regulatory proteins correlates with the control of primary viremia (Allen et al., 2000) and chronic infection (Geretti et al., 1999) in SIV-infected monkeys, and with a

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better prognosis for HIV-1 infected individuals (van Baalen et al., 1997; Zagury et al., 1998). This is substantiated further by a recent study, which showed no significant differences between the control of viremia in infected individuals and the presence of CTL responses against Tat and Rev, but demonstrated that the breadth of the CTL response against Tat correlates with an efficient control of disease progression (Addo et al., 2001). To test whether the early expression of Tat and Rev contributes to the more effective control of HIV/SIV viremia it was decided to manipulate the expression kinetics of CTL epitopes. Deletion, insertion or mutation in the *tat* and *rev* genes is problematic, because the open reading frames (ORFs) partially overlap with each other and with *env* (Myers et al., 1998). In contrast, the early expressed *nef* gene does not overlap with other ORFs (Myers et al., 1998). Moreover, although Nef-deleted HIV-1 variants display a lower pathogenicity in vivo, they are replication-competent in vitro (Harris, 1996), which makes the *nef* gene a more suitable candidate for genetic modifications.

The construction of full length, infectious HIV-1 molecular clones is described, in which CTL epitopes from the late HIV-1 proteins RT and Env have been inserted in frame into the *nef* ORF. The molecular clone pACH320.2A.2.1 (HIV-$1_{2.1WT}$), which has been described previously (Groenink et al., 1991; Guillon et al., 1995), was subcloned in two halves named pXE2.1 and pEX2.1, respectively (Guillon et al., 1997). DNA was amplified using *Escherichia coli* STBL2 (Gibco-BRL), grown at 30 °C to avoid recombination (Joshi and Jeang, 1993), and purified on cesium chloride gradients using standard methods (Sambrook et al., 1989). We inserted ectopic epitopes in the unique Bpu1102I site (position 67–73 in the *nef* gene), which was located close to a natural CTL epitope of Nef, providing a favourable context for antigen processing (Korber et al., 1999). pEX2.1 subclones were digested with Bpu1102I (MBI Fermentas), dephosphorylated using Calf Intestine Phosphatase (Boehringer–Mannheim), and purified on agarose gels (DNA extraction kit, Boehringer–Mannheim). The nucleotide sequences encoding the $Env_{589-597}$ (ERYLKDQQL) and $RT_{244-252}$ (IVLPEKDSW) CTL epitopes, which are recognised in the context of HLA-B14 and -B57, respectively (Johnson et al., 1992; Klein et al., 1998), were generated by annealing complementary oligonucleotides (Fig. 1A). These sequences were ligated into pEX2.1 to create pEX2.1EN and pEX2.1RN, respectively. Clones were sequenced to confirm the correct presence of the epitopes. To generate the pEX2.1NM constructs, subclones were digested with Xho I (position 101–106 in the *nef* gene) and incubated with Kleenow DNA polymerase (New England Biolabs) in the presence of 30 mM dNTPs. This resulted in a frame shift after amino acid 35 and a truncated Nef protein of 56 amino acids (Fig. 1C). pEX2.1EN, pEX2.1RN and pEX2.1-NM were cloned back into full-length pACH320.2A.2.1 (Fig. 1B) and transfected into 293-T cells for the production of infectious HIV-$1_{2.1EN}$, HIV-$1_{2.1RN}$, HIV-$1_{2.1NM}$ and HIV-$1_{2.1WT}$ stocks, as described previously (Pear et al., 1993).

The replication characteristics of these recombinant viruses were evaluated using TCL2H7 cells, a non-transformed CD4+ T cell line supporting replication of primary HIV isolates and presenting HLA B14- and B57-restricted epitopes (van Baalen et al., 1998). The cells require re-stimulation every 14 days using PHA-L (1 μg/ml) and gamma-irradiated feeder cells, and susceptibility to HIV infection was optimal at day 3 after stimulation (van Baalen et al., 1998). Therefore, virus replication could be monitored for 10 days, without the need for re-stimulation or adding freshly stimulated cells. Cells were incubated with 2-, 20- and 200-fold dilutions of the transfection supernatants for 90 min, and washed subsequently twice to remove unbound virions. Cultures were initiated with 3×10^5 cells, and virus production in the supernatant was measured using a double sandwich p24 ELISA (Cheynet et al., 1993; Guillon et al., 1997). In parallel, 5-fold dilutions of the cells inoculated with the highest virus concentration were cultured in quadruplicate with uninfected TCL2H7 cells for 2 weeks, and the number of positive wells for each of the cell dilutions was used to estimate the initial fraction of infected cells. At the start, cultures infected with HIV-$1_{2.1WT}$, HIV-$1_{2.1EN}$, HIV-$1_{2.1RN}$ or HIV-$1_{2.1NM}$ contained a number of infected cells

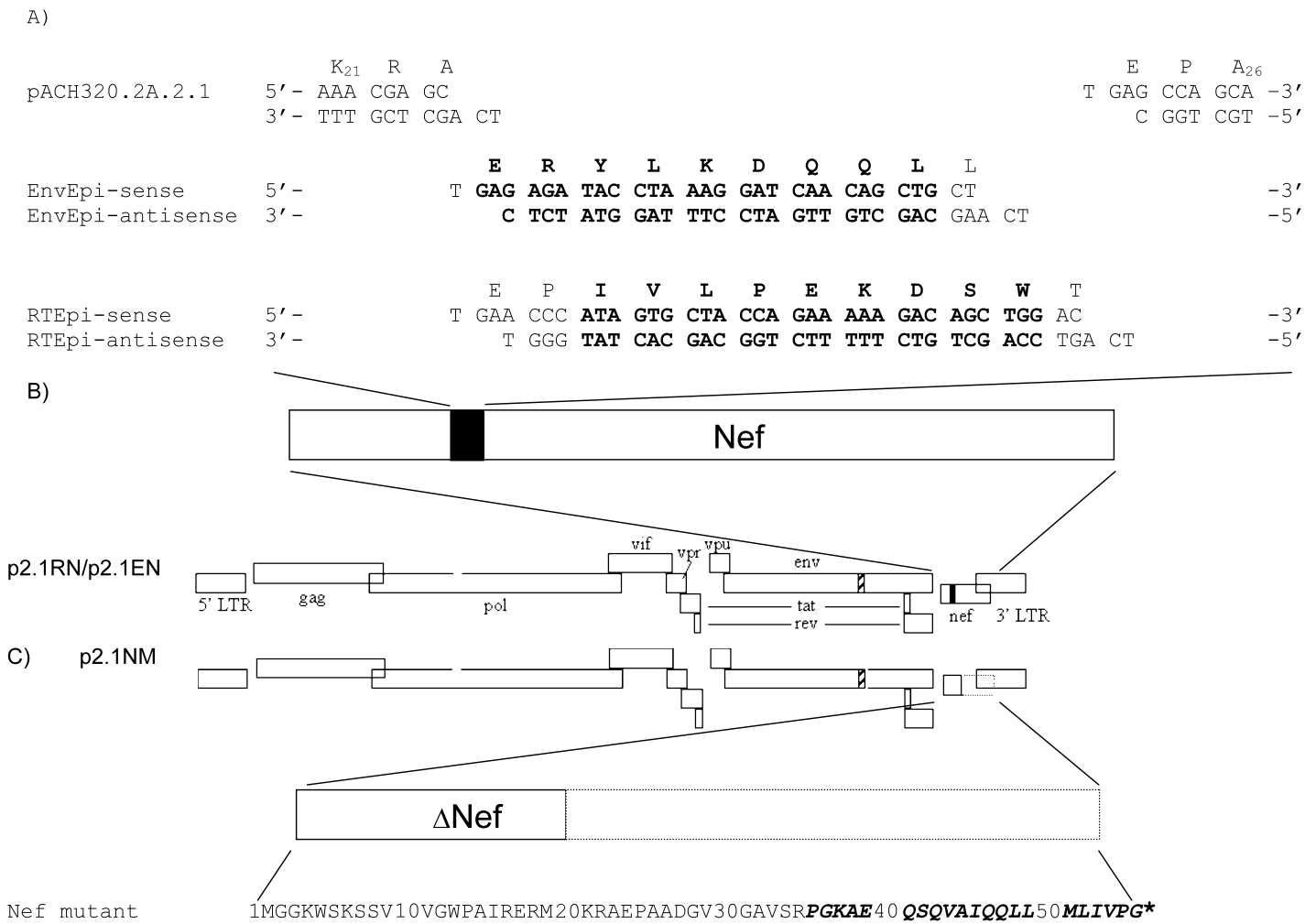

Fig. 1. Generation of the artificial epitopes. (A) Description of the oligonucleotides depicted as annealing product. The DNA strands coding for the epitopes are shown in bold. (B) Recombinant full-length HIV-1 molecular clones generated in this study. The ectopic epitopes are depicted as grey and striped areas at their respective natural position and in the recombinant *nef* gene for the p2.1EN and p2.1RN constructs. (C) Aminoacid sequence of the p2.1NM Nef protein. Residues in italic are the result of the frameshift in the *nef* ORF.

proportional to 418, 187, 17 and 125 ID_{50} per 3×10^5 cells, respectively. As shown in Fig. 2, the insertions into or truncation of Nef did not affect in vitro replication kinetics in TCL2H7 cells. At the peak of virus production, p24 concentrations in the supernatant were similar for all infections, between 500 and 1000 ng/ml. Similar results were observed when viruses were used to infect PHA-stimulated PBMCs (data not shown). The inserts and the truncation were stable, since the *nef* genes of virus isolated at the end of the observation period were identical to the *nef* genes of the input virus (data not shown). The differences in growth kinetics correlated with the differences in the estimated initial fraction of infected cells. Tenfold dilutions of the inoculum resulted in a 1–2-day delay in peak virus production, as was shown previously by Dimitrov et al. (1993).

Infected cells were lysed at the peak of p24 replication to assess Nef and Gag expression from the recombinant viruses by Western blot using Nef-specific monoclonal antibodies EVA3068.1 and EVA3067.5 clone 3A2 (Ovod et al., 1992) or with p24-specific antibody 14D4E11 (Janvier et al., 1990). As shown in Fig. 3, upper panel, Nef expression was detected in HIV-$1_{2.1EN}$ and HIV-$1_{2.1RN}$ infected cells. Nef proteins containing the ectopic epitopes (Fig. 3, lanes 2 and 3) showed a slightly higher apparent molecular weight than the wild-type Nef (Fig. 3, lane 1), but all proteins had a molecular weight around the expected size of 32 kD. Thus, the insertion of Env- or RT-specific epitopes in the *nef* ORF of the full-length ACH320.2A.2.1 molecular clone did not perturb viral replication or Nef expression. Nef could not be detected in cells infected with the Nef-truncated HIV-$1_{2.1NM}$ virus (Fig. 3, lane 4), but Gag proteins were detected by Western blots on the same lysate (Fig. 3, lower panel), indicating that viral proteins were expressed normally, despite the lack of Nef expression of HIV-$1_{2.1NM}$.

To assess whether the ectopic epitopes could be processed and presented from the Nef protein, we constructed recombinant vaccinia viruses (rVV) for the expression of the recombinant Nef proteins. The *nef* genes were amplified from the pEX2.1EN or pEX2.1RN plasmid, with primers 5′Nef-SalI (5′-GTCGACGGGGATGGGTG-GCAAGTGGTCAAA-3′) and 3′Nef-EcoRI (5′-GAATTCTTAGCAGTCCTTGTAGTACTCCG-3′), where restriction sites are underlined. The full-length *nef* ORFs were then cloned into the pTG186.poly plasmid (Kieny et al., 1986) using the SalI and EcoRI restriction sites. Recombinant vaccinia viruses were generated by homologous recombination and TK-selection as described (Kieny et al., 1986). Plaque purified rVV clones containing the correct *nef* gene and showing Nef expression, $rVV_{2.1EN}$ and $rVV_{2.1RN}$, were selected by sequence analyses and Western blots (data not

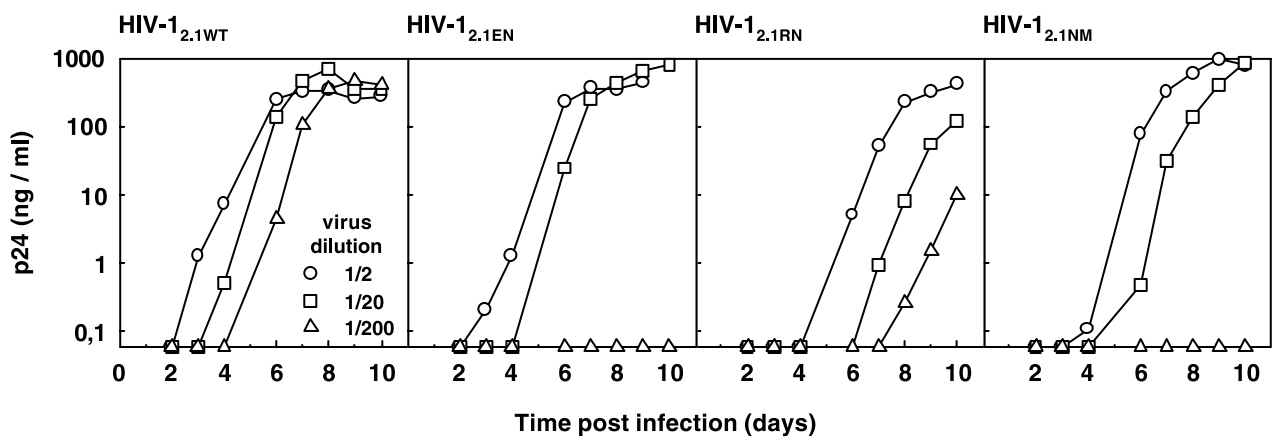

Fig. 2. Kinetics of viral replication in TCL2H7 cells. Levels of p24 were measured in supernatants of cell cultures infected with 2-, 20-, or 200-fold dilutions of HIV-$1_{2.1WT}$, HIV-$1_{2.1EN}$, HIV-$1_{2.1RN}$ or HIV-$1_{2.1NM}$ virus stocks.

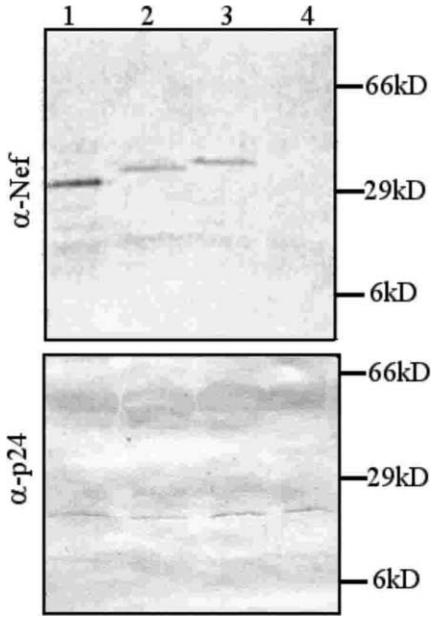

Fig. 3. Nef expression from recombinant HIV-1 viruses in PBMC. Crude cell lysates of HIV-$1_{2.1WT}$, HIV-$1_{2.1EN}$, HIV-$1_{2.1RN}$, HIV-$1_{2.1NM}$-infected PBMC were analysed in Western blot with (upper panel) Nef-specific monoclonal antibodies EVA3068.1 and EVA3067.5 clone 3A2 (Ovod et al., 1992) or (lower panel) with p24-specific antibody 14D4E11 (Janvier et al., 1990). Lane 1, HIV-$1_{2.1WT}$; lane 2, HIV-$1_{2.1EN}$; lane 3, HIV-$1_{2.1RN}$; and lane 4, HIV-$1_{2.1NM}$.

shown). The B-lymphoblastoid cell line BLCL709 (HLA B14, B57) was infected with $rVV_{2.1EN}$ or $rVV_{2.1RN}$ and after o/n incubation cocultured at a 1:10 ratio in a standard 4 h chromium-release assay (van Baalen et al., 1998), using Env-specific CTL clone KMTCC4-1280 ($CD8^+$, HLA-B14-restricted, ERYLDQQL-specific, which is similar to a previously described clone (Johnson et al., 1992)) or RT-specific CTL clone 090TCL1C11 ($CD8^+$, HLA-B57-restricted, IVLPEKDSW-specific (Klein et al., 1998)). As a control, the BLCL709 cells were pulsed with 10 μM of the minimal Env or RT epitope peptide. As shown in Fig. 4, target cells infected by $rVV_{2.1EN}$ or $rVV_{2.1RN}$ were only lysed efficiently by the CTL specific for the inserted epitope. The level of lysis was comparable or higher for rVV-infected cells than for cells pulsed with the minimal epitope peptides (Fig. 4). This showed that the Env and RT-ectopic epitopes were efficiently processed from the recombinant Nef gene and recognised by their specific CTLs.

RT and Env epitopes were inserted in a region of Nef located 4 residues downstream of the C-terminal end of a naturally processed Nef CTL epitope. In line with previous reports which addressed the insertion of CTL epitopes in heterologous proteins (Beekman et al., 2000; Weidt et al., 1995), the antigenic inserts from our constructs were presented efficiently to CTL in a MHC class I restricted manner. One cannot exclude that the heterologous context of the ectopic epitopes might have influenced epitope processing from the EpiNef constructs, either in size of the generated peptides or in the protease pathway involved (Del Val et al., 1991; Niedermann et al., 1999). However, the influence of the site of insertion on epitope processing in our system has to be limited since rVV infected target cells were very efficiently lysed. The insertion of epitopes in Nef did not impair viral replication kinetics, even when the infection was performed at a low MOI, suggesting the recombination did not perturb the natural function of Nef in the replication cycle (Chowers et al., 1994). However, the insertion site

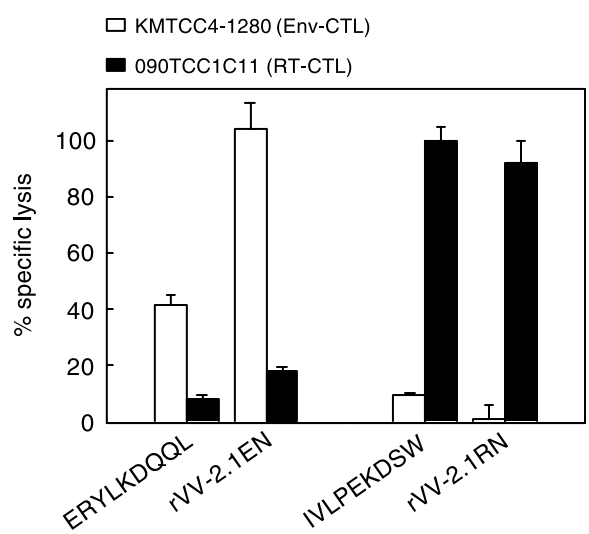

Fig. 4. Recognition of ectopic epitopes by their specific CTL. Percent specific lysis of BLCL709 cells infected with $rVV_{2.1EN}$ or $rVV_{2.1RN}$, or pulsed with peptides corresponding to the minimal epitopes, by Env- or RT-specific CTL. Means of triplicates with standard error are shown.

is located close to a domain involved in the ability of Nef to modulate expression of class I MHC molecules (Collins et al., 1998; Piguet and Trono, 1999), and additional experiments are therefore needed to assess the effect of the insertions in Nef on this mechanism.

In this study, CTL epitopes from the structural HIV-1 proteins Env or RT were inserted in the early expressed Nef ORF of HIV-1. These insertions were not deleterious for HIV-1 replication in vitro, and did not perturb Nef expression. In addition, the inserted CTL epitopes were processed and efficiently recognised by specific CTL despite their ectopic position. Moreover, this system uses a primary HIV-1 isolate and a primary, non-immortalized, CD4-positive T-cell clone to be as close as possible from the in vivo situation. These recombinant viruses have since been used successfully to demonstrate the effect of distinct epitope expression kinetics on the ability of CTL to inhibit HIV replication (van Baalen et al., submitted for publication), and could provide new insights in the mechanisms underlying the control of HIV-1 replication by the immune system in vivo.

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