Journal of General Virology (2001), **82**, 2735–2745. Printed in Great Britain

Genetic and functional analysis of the human immunodeficiency virus (HIV) type 1-inhibiting F12-HIV*nef* allele

Paola D'Aloja,[1,2] Anna Claudia Santarcangelo,[1] Stefan Arold,[3] Andreas Baur[2] and Maurizio Federico[1]

[1] Laboratory of Virology, Istituto Superiore di Sanità, Viale Regina Elena, 299, 00161 Rome, Italy

[2] University of Erlangen, Department of Dermatology, Erlangen, Germany

[3] Department of Biochemistry and Molecular Biology, University College London, London, UK

The primary human immunodeficiency virus type 1 (HIV-1) Nef mutant F12-HIVNef is characterized by three rare amino acid substitutions, G^{140}E, V^{153}L and E^{177}G. It was reported previously that the expression of F12-HIVNef in the context of the highly productive NL4-3 HIV-1 strain blocks virus replication at the level of virus assembly and/or release by a mechanism depending on the presence of the CD4 intracytoplasmic tail. Here, it is reported that NL4-3 HIV-1 strains expressing F12-HIV*nef* alleles that were back-mutated in each amino acid substitution readily replicated in CD4$^+$ cells. Attempting to correlate possible functional alterations with antiviral effects, both F12-HIVNef and its back mutants were tested in terms of well-characterized markers of Nef expression. Both F12-HIVNef and its G^{177}E back mutant did not down-regulate CD4 as the consequence of a greatly reduced rate of CD4 internalization. On the other hand, F12-HIVNef as well as the E^{140}G and L^{153}V back mutants failed to activate the p62 Nef-associated kinase (p62NAK). Thus, only F12-HIVNef was defective in both accelerated rates of CD4 internalization and p62NAK activation, whereas at least one Nef function was restored in all of the back mutants. Infection of cells expressing Nef-resistant CD4 molecules with HIV-1 strains encoding F12-HIVNef back mutants showed that both the lack of accelerated CD4 endocytosis and an, as yet, still unidentified function are required for the F12-HIVNef inhibitory phenotype. These results provide a detailed functional analysis of the F12-HIV*nef* allele and support the idea that both CD4 accelerated internalization and p62NAK activation are part of the essential steps in the virus replication cycle.

Introduction

The human immunodeficiency virus/simian immunodeficiency virus (HIV/SIV) Nef protein is a regulatory protein with a molecular mass of between 27 and 34 kDa and which is expressed in large amounts in infected cells and arises early in the virus replication cycle. The observation that most cell types will replicate *nef*-deleted HIV posed difficulties in delineating a clear role of Nef in the virus replication cycle. Nef seems to act at two levels: it increases the efficiency of proviral synthesis, as demonstrated in quiescent peripheral blood lymphocytes (Miller *et al.*, 1994; Spina *et al.*, 1994), and plays a role in virus particle release, although this role is still characterized poorly (Fackler *et al.*, 2000). On the other hand, increasing evidence demonstrates that Nef is a multifunctional protein that interacts with different cellular protein partners. In particular, Nef down-regulates CD4 by both accelerating its internalization rate and inducing recruitment of CD4-containing clathrin-coated pits for routing to lysosomes (Aiken *et al.*, 1994; Greenberg *et al.*, 1997; Piguet *et al.*, 1998, 1999). Through distinct genetic determinants, Nef down-regulates major histocompatibility complex (MHC) class I molecules, mainly by misrouting them to degradative endosomal pathways (Greenberg *et al.*, 1998; Le Gall *et al.*, 1998; Mangasarian *et al.*, 1999; Schwartz *et al.*, 1996). Furthermore, Nef alters cellular signalling pathways, e.g. by engaging the ζ chain of the T cell receptor (Xu *et al.*, 1999). In addition, a peculiar proline-rich motif allows Nef to interact with several tyrosine as well as serine/threonine kinases (reviewed by Saksela, 1997). All of

Author for correspondence: Maurizio Federico.
Fax +39 06 49903002. e-mail federico@iss.it

0001-7748 © 2001 SGM

these functions have been correlated with effects that could indirectly favour the dissemination of the virus within the host. In fact, a diminished CD4 exposition could enhance the efficiency of infectious HIV release (Lama *et al*., 1999; Ross *et al*., 1999). Decreased amounts of exposed MHC class I molecules should lead to an impaired immunological response, mainly in terms of both anti-HIV cytotoxic T lymphocyte activity (Collins *et al*., 1998) and natural killer activity (Cohen *et al*., 1999). Similarly, alterations in T cell receptor signalling could induce anergy in the infected host (Bell *et al*., 1998; Xu *et al*., 1999).

F12-HIVNef is a primary Nef mutant characterized by three rare amino acid substitutions, $G^{140}E$, $V^{153}L$ and $E^{177}G$ (Carlini *et al*., 1992). We reported recently that the *in cis* expression of F12-HIVNef transforms the highly productive NL4-3 HIV-1 into a replication-defective strain (Olivetta *et al*., 2000). We were interested in defining Nef functions involved in the mechanism of the F12-HIVNef antiviral effect. We have already established that (i) the expression of F12-HIVNef fails to down-regulate CD4 (D'Aloja *et al*., 1998), (ii) the block of virus replication occurs at the level of virus assembly and/or release (D'Aloja *et al*., 1998; Olivetta *et al*., 2000), and (iii) both CD4 and HIV-1 gp41 Env intracytoplasmic tails are essential for this antiviral effect (Olivetta *et al*., 2000). Here, we report that the lack of activation of the p62 Nef-associated kinase (p62NAK), a serine/threonine kinase with a molecular mass of 62 kDa, which is typically phosphorylated upon wild-type (wt) Nef expression (Sawai *et al*., 1994), correlates with the F12-HIVNef antiviral phenotype. In addition, we demonstrate that the concomitant lack of both CD4 accelerated endocytosis and an, as yet, still unidentified function are required for the inhibitory effect of F12-HIVNef.

Methods

Cell cultures and transfections. C8166 and CEMss cells were grown in RPMI 1640 (Life Technologies) supplemented with 10% heat-inactivated foetal calf serum (FCS). 293, 293T, 293/CD4 (Olivetta *et al*., 2000), HeLaCD4 cells and derivatives thereof were cultivated in Dulbecco's modified minimal essential medium supplemented with 10% heat-inactivated FCS. 293 cell populations expressing the human CD4 molecules mutated in the dileucine motif of the intracytoplasmic tail ($LL^{413}AA$) were recovered as described previously for 293/CD4 cells (Olivetta *et al*., 2000). Stably transfected cell lines were maintained in the presence of 0·5 mg/ml of G418 antibiotic (70% activity) (Gibco). Transfections and co-transfections were performed using the calcium–phosphate method (Wigler *et al*., 1979). Pools of HeLaCD4 cells stably expressing F12-HIVNef back mutants were obtained, as described previously (D'Aloja *et al*., 1998), by transfecting vectors based on pcDNA3 (Invitrogen) that express Nef. Nef-expressing cell clones were selected from G418-resistant cell populations.

HIV-1 infections and protein detection. Supernatants from transiently transfected 293 cells were the source of NL4-3 strain HIV-1 and derivatives thereof. Infections were performed by adsorbing the virus inoculum onto either cell monolayers (for adherent cells) or cell pellets (for non-adherent cells) for 1 h at 37 °C with occasional shaking. Cells were then washed extensively and re-fed. Detection of virus infection in supernatants was performed by a reverse transcriptase (RT) assay (Rossi *et al*., 1987). RT activity was measured as c.p.m./ml and normalized for 10^6 cells after background subtraction. Virus titrations were carried out either by scoring the number of syncytia in C8166 cells at 5 days after challenge (Federico *et al*., 1993) or by evaluating the number of blue cells at 2 days after the infection of HeLaCD4-LTR-βgal cells (Bryant *et al*., 1991). To determine Nef expression from chimeric viruses expressing the F12-HIVNef back mutants, Western blot analyses on 293 cells transiently transfected with the different HIV-1 molecular clones were performed by means of the enhanced chemiluminescence method (Amersham), as described previously (D'Aloja *et al*., 1998). The stability of CD8Nef chimeric proteins was assessed either by Western blot or by anti-CD8 immunoprecipitation of lysates from ^{35}S-labelled cells (D'Aloja *et al*., 1998) followed by 12% PAGE.

Mutagenesis, cloning and subcloning. To introduce site-specific mutations into the F12-HIV*nef* allele, the overlapping PCR technique was carried out as described previously (Taddeo *et al*., 1996). A first amplification step using F12-HIV*nef* as the template served to generate two half fragments of *nef*. Internal primer couples carried the back mutations. The external primers contained *Hin*dIII (5′) and *Eco*RI (3′) restriction enzyme sites. Full-length *nef* fragments were generated by second-round PCR using only the external primers. The PCR mixture contained 20 mM Tris–HCl (pH 8·8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0·2 mM dNTP, 0·6 µg of each primer, 0·5 µg of DNA template and 2 U of Vent DNA polymerase (New England Biolabs) in a final volume of 50 µl. Reaction mixtures were subjected to 15 cycles of 95 °C for 1 min, 58 °C for 1 min and 72 °C for 2 min. Finally, full-length *nef* fragments were digested with *Hin*dIII/*Eco*RI and inserted into the homologous sites of the pcDNA3 vector.

Construction of the pNL4-3 molecular clone expressing F12-HIVNef was described previously (Olivetta *et al*., 2000). pNL4-3 clones expressing each F12-HIVNef back mutant were obtained in a similar way. Briefly, *nef* mutants were amplified by PCR using the pcDNA3*nef* constructs as a template and a pair of oligoprimers carrying the *Mlu*I (5′ end) and *Cla*I (3′ end) restriction sites, which overlapped the *nef* initiation and stop codons, respectively. Amplified *nef* genes were then inserted into a derivative of the pNL4-3 plasmid, where the *Mlu*I (whose restriction site was present in a linker inserted between the *env* stop and *nef* start codons) and *Cla*I (downstream to the *nef* stop codon) sites had been created previously. The infectious molecular clone expressing the NL4-3 HIV-1 genome defective in *nef* expression has been described previously (Chowers *et al*., 1994).

CD8Nef-expressing vectors were obtained by inserting in-frame *nef* mutants obtained by PCR using oligoprimers carrying *Bam*HI (forward) and *Sal*I (reverse) restriction sites into the homologous unique sites of the pEF-BOS-CD8T vector (Lu *et al*., 1996, 1998). The vector expressing the green fluorescence protein (GFP) has been described previously (Palm *et al*., 1997). The pCMX/CD4$LL^{413}AA$ construct expressing a CD4 molecule defective in the spontaneous internalization process was a generous gift from C. Aiken.

All of the sequences obtained by PCR amplification were rigorously checked by the dideoxy chain-termination method using the Sequenase II kit (US Biochemicals).

Analysis of the steady-state levels, internalization and recycling of CD4. Steady-state levels of CD4 were assayed by membrane immunolabelling performed, as described previously (Olivetta *et al*., 2000), using phycoerythrin (PE)-conjugated Leu3A monoclonal antibody (MAb) (Becton Dickinson) on 293/CD4 cells co-transfected with both GFP- and Nef-expressing vectors (molar ratio 1:5). Labelled

cells were assessed by FACS analysis (Becton Dickinson). Assays for CD4 internalization and recycling were performed, as reported previously (Mangasarian *et al.*, 1997; Piguet *et al.*, 1998), on transfected 293/CD4 cells. Briefly, cells harvested 48 h after transfection were labelled with PE-conjugated anti-CD4 MAb for 30 min at 4 °C and then washed extensively. Afterwards, cells were incubated at 37 °C and, after 15 and 30 min (the time-points at which the triggering of accelerated CD4 internalization by Nef is more clearly distinguishable) (Mangasarian *et al.*, 1997; Piguet *et al.*, 1998, 1999), washed with a 7-fold excess of cold PBS (pH 2). Fractions of internalized CD4 were calculated as the product of the mean fluorescence intensity (MFI) value recorded after the pulse at 37 °C and acid washes, subtracted of the background MFI value recorded after incubation at 4 °C and acid washes, all divided by the total (i.e. surface plus internalized) MFI value recorded after the incubation of cells at 37 °C and before acid washes. Rates of internalization of CD8Nef chimeras were measured in the same way, except that the Leu3A PE-conjugated anti-CD8 MAb was used. To measure the rate of CD4 recycling (Piguet *et al.*, 1998; Mangasarian *et al.*, 1999), fractions of cells from the endocytosis assay were warmed for 15 and 30 min and washed again in cold, acidic PBS. The remaining fluorescence was measured by FACS analysis. Percentages of recycled CD4 were calculated as 1−(MFI rew/MFI fp), where the numerator refers to the MFI values after the re-warming and acid washes and the denominator refers to the MFI values measured after the first pulse at 37 °C and acid washes. In all of the analyses, dead cells were excluded by means of side and forward scatter parameters during FACS acquisition.

■ **p62NAK activation assay.** 293T cells transfected with vectors expressing diverse CD8Nef chimeras were lysed 48 h after transfection in 0·5 ml of extraction buffer containing 50 mM Tris–HCl (pH 8·0), 0·5% NP40, 2 mM EDTA, 250 mM NaCl, 10% glycerol, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM $NaVO_3$. Cell lysates were incubated with 100 μl of anti-CD8-coupled beads (Dynal) for 4 h at 4 °C, washed three times with extraction buffer and once with kinase activation buffer (KAB) containing 50 mM HEPES, 150 mM NaCl, 5 mM EDTA, 0·02% Triton X-100 and 10 mM $MnCl_2$. Immunoprecipitated proteins were incubated in 50 μl KAB containing 0·37 MBq of [γ-^{32}P]ATP for 10 min at room temperature and finally washed three times with lysis buffer. Phosphorylated proteins were resolved by 10% SDS–PAGE and detected by autoradiography.

Results

F12-HIV antiviral effect depends on the contemporary presence of three rare amino acid substitutions

We reported previously that the expression of the F12-HIV*nef* allele blocks HIV-1 release (D'Aloja *et al.*, 1998; Olivetta *et al.*, 2000). F12-HIV*nef* bears three amino acid substitutions, $G^{140}E$, $V^{153}L$ and $E^{177}G$, which are rarely detectable in *nef* alleles sequenced from both laboratory-adapted and clinical HIV-1 isolates. As an example, among 148 *nef* alleles cloned from lymphocytes of both progressing and non-progressing HIV-1-seropositive patients (Brambilla *et al.*, 1999), neither $G^{140}E$ nor $V^{153}L$ substitutions were detected and only a single *nef* allele was found to carry the $E^{177}G$ substitution. Attempting to separate Nef functions that are involved specifically in the F12-HIVNef-induced antiviral effect, we analysed three F12-HIVNef back mutants, i.e. *nef* alleles in which each of the three typical F12-HIVNef amino acid substitutions was reversed separately in the Nef sequence of NL4-3, a clade B replication competent HIV-1 strain tightly correlated to the F12-HIV genome (Carlini *et al.*, 1992).

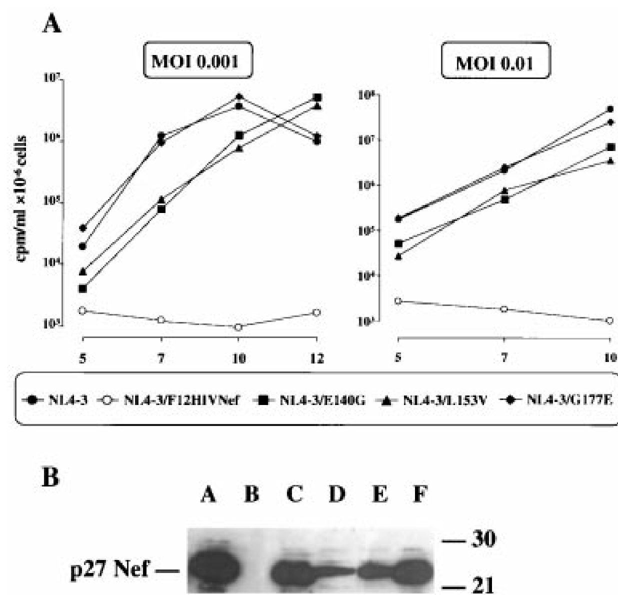

Fig. 1. The expression of F12-HIVNef back mutants fails to negatively affect virus replication. (A) CEMss cells were challenged at an m.o.i. of 0·001 or 0·01 with NL4-3 HIV-1 chimeric viruses expressing each F12-HIVNef back mutant and, as a control, with wt or NL4-3/F12-HIVNef virus strains. The activity of RT was measured at different days after infection as c.p.m./ml and normalized for 10^6 cells after the subtraction of background values. Data from one representative of two independent experiments are reported. (B) Western blot analyses performed on 20 μg of protein from 293 cell lysates obtained 2 days after transfection with pNL4-3-based molecular clones expressing either wt Nef (lane A), F12-HIVNef (lane C) or $E^{140}G$ (lane D), $L^{153}V$ (lane E) or $G^{177}E$ (lane F) F12-HIVNef back mutants. As a control, cell lysate from 293 cells transfected with a *nef*-defective NL4-3 HIV-1 provirus (lane B) was analysed. Protein was detected using a polyclonal anti-Nef mouse serum. Molecular size markers (kDa) are given on the right.

In order to evaluate the effects of the three amino acid substitutions in terms of HIV-1 replication, human lymphoblastoid CEMss cells were infected with virus preparations yielded upon transfection of 293 cells with pNL4-3 molecular clones expressing each of the F12-HIVNef back mutants. As a control, CEMss cells were infected with an equal m.o.i. of either wt (NL4-3) or NL4-3/F12-HIVNef strains. Clearly, each back mutation led the NL4-3 HIV-1 to regain the productive phenotype (Fig. 1A). The apparent stability of diverse Nef proteins was proven by Western blot analyses on lysates of 293 cells transfected with the respective infectious molecular clones (Fig. 1B). Overall, similar results were obtained by challenging pools of HeLaCD4 cell clones stably expressing each of the F12-HIVNef back mutants (data not shown).

Our data unambiguously demonstrate that the antiviral effect induced by F12-HIVNef expression is the result of the simultaneous presence of the $G^{140}E$, $V^{153}L$ and $E^{177}G$ amino acid substitutions.

F12-HIVNef expression does not induce accelerated CD4 internalization

Cells expressing F12-HIVNef are defective for CD4 down-regulation (D'Aloja *et al.*, 1998; Olivetta *et al.*, 2000). Nevertheless, F12-HIVNef interacts with the CD4 intracytoplasmic tail, whose presence is required for the antiviral effect (Olivetta *et al.*, 2000). Considering that high levels of membrane CD4 lead to a decreased production of infectious HIV-1 by means of impaired viral envelope protein incorporation (Lama *et al.*, 1999; Ross *et al.*, 1999), it was conceivable that unaltered levels of CD4 in the membrane are indispensable for the antiviral phenotype of F12-HIVNef. The measurement of steady-state CD4 levels does not exhaustively describe the effects of Nef on CD4. This is a receptor that constitutively undergoes internalization and recycling (Marsh & Pelchen-Matthews, 1996). Two genetically and functionally distinguishable events had been identified as the result of the CD4–Nef interaction (Piguet *et al.*, 1998, 1999), i.e. the triggering of a strong acceleration in the rate of CD4 internalization and the inhibition of recycling by routing internalized CD4 to the lysosome compartment. Attempting to correlate the mechanics of the F12-HIVNef-induced antiviral action with its effects on CD4, we investigated the functional defect(s) of F12-HIVNef that underlie the lack of CD4 down-regulation. Steady-state CD4 levels were measured in 293/CD4 cells by co-transfecting GFP- and F12-HIV*nef*-expressing vectors in a 1:5 molar ratio. CD4 FACS analysis of GFP-positive cells carried out 48 h post-transfection demonstrated that, as reported previously (D'Aloja *et al.*, 1998; Olivetta *et al.*, 2000), F12-HIVNef expression did not alter the steady-state exposition of CD4 (Fig. 2A). Both CD4 internalization and recycling assays were performed by transfecting 293/CD4 cells with the vector expressing pcDNA3/F12-HIVNef. Results were considered exclusively in the presence of transfection efficiencies $> 70\%$, as monitored by scoring GFP-positive cells in parallel conditions. As shown in Fig. 2(B), F12-HIVNef expression failed to accelerate CD4 internalization, as the endocytosis rate was similar to that detected in cells transfected with the control vector. As expected, results from recycling assays did not increase the significance of the overall CD4 analyses (data not shown). We conclude that F12-HIVNef expression does not induce CD4 down-regulation as a consequence of a defect in the acceleration of CD4 endocytosis.

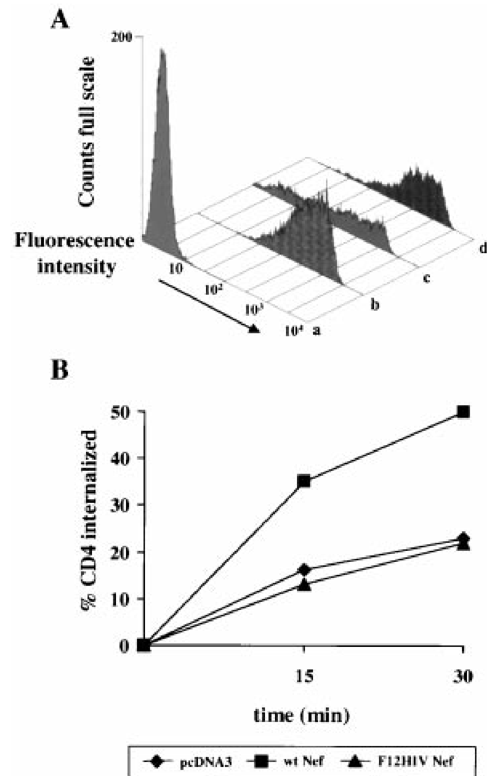

Fig. 2. The lack of CD4 down-regulation in cells expressing F12-HIVNef depends on an impaired rate of CD4 internalization. (A) Effects on CD4 steady-state levels of cells expressing F12-HIV or wt Nef. 293/CD4 cells were co-transfected with vectors expressing GFP and F12-HIV or wt Nef (molar ratio 1:5), and membrane CD4 levels were measured 48 h thereafter. FACS analyses of 293/CD4 cells transfected with pcDNA3 (b) or vectors expressing wt (c) or F12-HIV (d) Nef and labelled with PE-conjugated anti-CD4 MAb are shown. As a control, 293/CD4 cells transfected with pcDNA3 labelled with PE-conjugated, non-specific mouse immunoglobulin (Ig) G isotype (a) was included. Only GFP-positive cells were considered. (B) Rate of CD4 internalization in 293/CD4 cells transfected with either pcDNA3 or vectors expressing wt or F12-HIV Nef. At 48 h after transfection, cells were labelled with anti-CD4 PE-conjugated MAb for 1 h at 4 °C. Afterwards, cells were washed, pulsed at 37 °C and, after 15 and 30 min, washed with acidic buffer and analysed by FACS. The efficiency of transfection ranged from 70 to 90% throughout all of the experiments. Results from one representative of three independent experiments are reported.

Analysis of CD4 internalization in cells expressing F12-HIVNef back mutants

We were interested in establishing whether the lack of accelerated CD4 internalization correlated with the block of HIV-1 release observed in F12-HIVNef-expressing cells. Thus, the effects of F12-HIVNef back mutants on CD4 were analysed by reproducing the assays for both steady-state levels and internalization rates. We found that both $E^{140}G$ and $L^{153}V$ Nef back mutants reverted to a wt-like phenotype in terms of both CD4 steady-state levels and endocytosis rates (Fig. 3). Conversely, and similarly to F12-HIVNef, the expression of the $G^{177}E$ Nef back mutant did not induce CD4 down-regulation due to an impaired rate of CD4 internalization (Fig. 3). We conclude that, even if an effective CD4 down-regulation is not compatible with the inhibitory effect of F12-HIVNef, additional Nef functions seem to be involved.

CD4-independent Nef internalization is impaired in cells expressing either F12-HIVNef or the $G^{177}E$ back mutant

Here we demonstrate that F12-HIVNef and its $G^{177}E$ back mutant behave similarly in terms of CD4 internalization, but

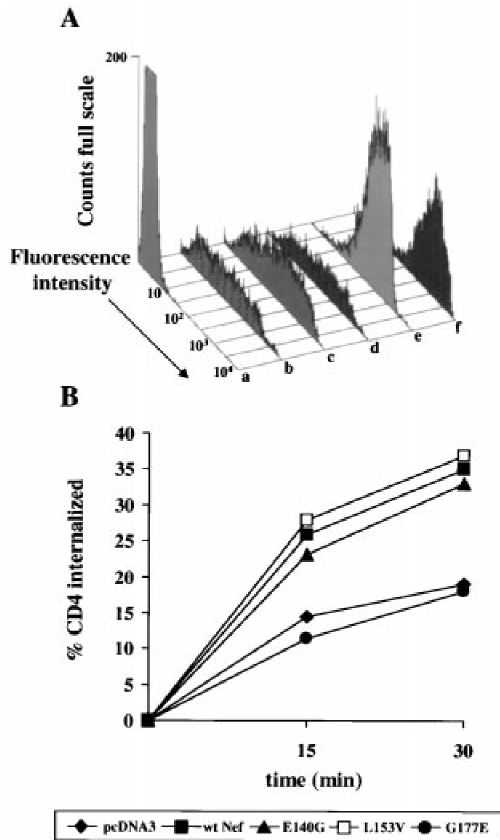

Fig. 3. Steady-state levels and internalization rates of CD4 in cells expressing F12-HIVNef back mutants. (A) Effects on CD4 steady-state levels in cells expressing either wt (b), $E^{140}G$ (c), $L^{153}V$ (d) or $G^{177}E$ (e) back mutants. 293/CD4 cells were co-transfected with vectors expressing GFP and Nef mutants (molar ratio 1:5) and the effects on the CD4 receptors were analysed as described in Fig. 2. As a control, 293/CD4 cells transfected with the empty pcDNA3 vector were labelled with PE-conjugated non-specific IgG (a) or with PE-conjugated anti-CD4 MAb (f). (B) Rate of CD4 internalization in 293/CD4 cells transfected with either pcDNA3 or vectors expressing wt or F12-HIV Nef back mutants. Assays were carried out as described in Fig. 2. The efficiency of transfection ranged from 70 to 90% throughout all of the experiments. Results from one representative of three independent experiments are reported.

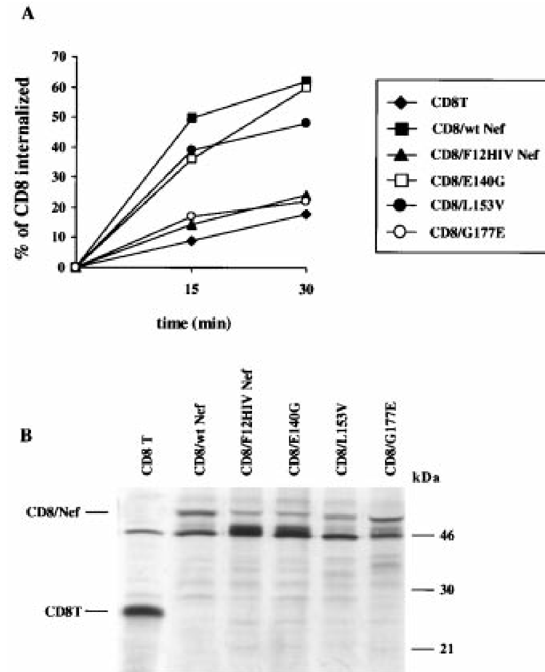

Fig. 4. Both F12-HIVNef and its $G^{177}E$ back mutant fail to accelerate auto-internalization. (A) 293T cells were transfected with vectors expressing CD8-based chimeras carrying F12-HIV*nef* or its back mutants. After 48 h, CD8-internalization assays were performed by labelling cells with PE-conjugated anti-CD8 MAb. As a control, cells were transfected with either pEF-BOS-CD8T, which expresses a CD8 molecule truncated in its intracytoplasmic domain (CD8T), or the CD8wtNef chimera. Assays were carried out as described in Fig. 2. Results from one representative of two experiments are reported. (B) SDS–PAGE of anti-CD8-immunoprecipitated lysates from ^{35}S-labelled cells 48 h after transfection with the indicated molecular constructs. Products of CD8-based constructs are indicated on the left. Molecular size markers (kDa) are given on the right.

differ in terms of antiviral effect. Possible differences in the mechanism of CD4 internalization may help to find additional insights into the mechanics of F12-HIVNef-induced antiviral effects. It has been reported that Nef possesses an intrinsic (i.e. not CD4-mediated) ability to induce auto-internalization through interaction with clathrin adaptor molecules (AP-2) (Lu *et al.*, 1998; Mangasarian *et al.*, 1997). We investigated the CD4-independent internalization activity of F12-HIVNef and its back mutants in the context of CD8Nef molecular chimeras by measuring the rate of CD8 internalization upon transfection on 293T cells. As shown in Fig. 4(A), impaired internalization activities have been detected in cells expressing either F12-HIVNef or the $G^{177}E$ back mutant. Conversely, either the $E^{140}G$ or the $L^{153}V$ amino acid substitutions led to a reversion towards the wt phenotype. The amount and stability of the products of each chimeric construct were evaluated by PAGE analysis of anti-CD8-immunoprecipitated cell lysates after ^{35}S-labelling of a fraction of the transfected cell cultures utilized for the internalization assay (Fig. 4B).

These data suggest strongly that the lack of accelerated CD4 internalization observed in cells expressing either F12-HIVNef or its $G^{177}E$ back mutant depends on a similarly impaired involvement with endocytotic machinery rather than on a defect in the interaction with CD4.

F12-HIVNef expression does not induce activation of p62NAK and rescue by the $G^{177}E$ back mutation

As the expression of the $G^{177}E$ F12-HIVNef back mutant did not inhibit either CD4 membrane expression or HIV-1 release, we conclude that the F12-HIVNef antiviral effect could not be merely the consequence of unaltered levels of membrane CD4 exposition. For these reasons, additional markers of Nef functions were investigated. Among the cellular proteins known already to associate with and/or to be activated by Nef, the most informative results were obtained by analysing

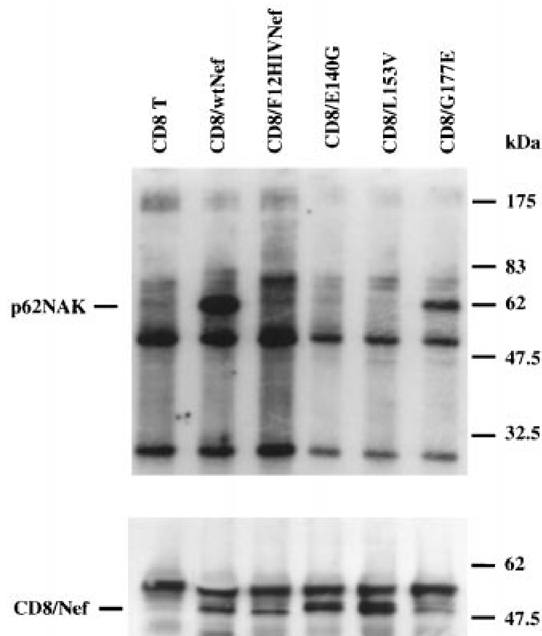

Fig. 5. Analysis of p62NAK activation in cells expressing F12-HIVNef or its back mutants. A kinase assay performed on lysates of 293T cells transfected with vectors expressing different CD8Nef chimeras or, as a negative control, the CD8 receptor truncated in its intracytoplasmic domain (CD8T), is shown. At 48 h after transfection, cells were lysed and immunoprecipitated with anti-CD8-coupled beads. Reaction products obtained after the incubation with [γ-^{32}P]ATP were resolved by 10% SDS–PAGE and revealed by autoradiography. In the lower panel, the anti-Nef Western blot assay of immunoprecipitated cell lysates, analysed also in the kinase assay, is shown. Protein detection was performed by using a polyclonal anti-Nef mouse serum. Molecular size markers (kDa) are given on the right.

p62NAK activation. This is a serine/threonine kinase capable of auto-phosphorylation (Sawai *et al.*, 1996) and is both immunologically and functionally related to the p21-activated kinases (PAK) (Nunn & Marsh, 1996). Of note, the activity of p62NAK has been correlated positively with the process of HIV-1 release (Lu *et al.*, 1996), the step where F12-HIVNef acts specifically in blocking HIV-1 replication.

No p62NAK activation was found in F12-HIVNef-expressing cells, where, conversely, the apparent phosphorylation of the, as yet, unidentified protein with a molecular mass of ≅ 75 kDa could be appreciated (Fig. 5). Similarly, both the $E^{140}G$ and the $L^{153}V$ back mutants failed to activate p62NAK, whereas the $G^{177}E$ back mutation was sufficient to rescue p62NAK activation (Fig. 5). This indicates that the lack of p62NAK activation is not sufficient per se to explain the F12-HIVNef antiviral phenotype. Both the amount and the integrity of CD8Nef chimeric proteins after immunoprecipitation were checked by anti-Nef Western blot analysis (Fig. 5).

The upstream effector of p62NAK has been identified in the product of the protooncogene *vav* (Fackler *et al.*, 2000). Of note, we did not observe differences in Vav activation between cells expressing wt or F12-HIVNef (data not shown).

F12-HIV Nef antiviral effect depends on both the lack of accelerated CD4 internalization and an, as yet, still unidentified function

We demonstrated that the NL4-3/F12-HIV*nef* genome regains the infectious phenotype upon reversion of any of the three F12-HIVNef typical amino acid substitutions (Fig. 1). Furthermore, the $G^{177}E$ back mutant does not accelerate the rate of CD4 internalization. Both the $E^{140}G$ and the $L^{153}V$ back mutants are defective for p62NAK activation, whereas parental F12-HIVNef lacks both functions (Table 1). In order to define more stringently the role of CD4 down-regulation in the F12-HIVNef-inhibitory phenotype, we recovered a 293 cell population that stably expresses a CD4 molecule mutated in the intracytoplasmic dileucine motif ($LL^{413}AA$). In this way, CD4 becomes defective for its physiological internalization activity, thus resisting the Nef-induced accelerated internalization (Aiken *et al.*, 1994).

We asked whether the expression of the $E^{140}G$ and $L^{153}V$ Nef back mutants could lead to a phenotype similar to F12-HIVNef in cells where they can no longer induce CD4 down-regulation. 293 cells stably expressing the mutated CD4

Table 1. Summary of results

			Block of HIV-1 release	
nef allele	Accelerated CD4 endocytosis	p62NAK activation	CD4-expressing cells	$LL^{413}AA$ CD4-expressing cells
NL4-3	+	+	−	−
F12-HIV	−	−	+	+
$E^{140}G$	+	−	−	−
$L^{153}V$	+	−	−	+
$G^{177}E$	−	+	−	−

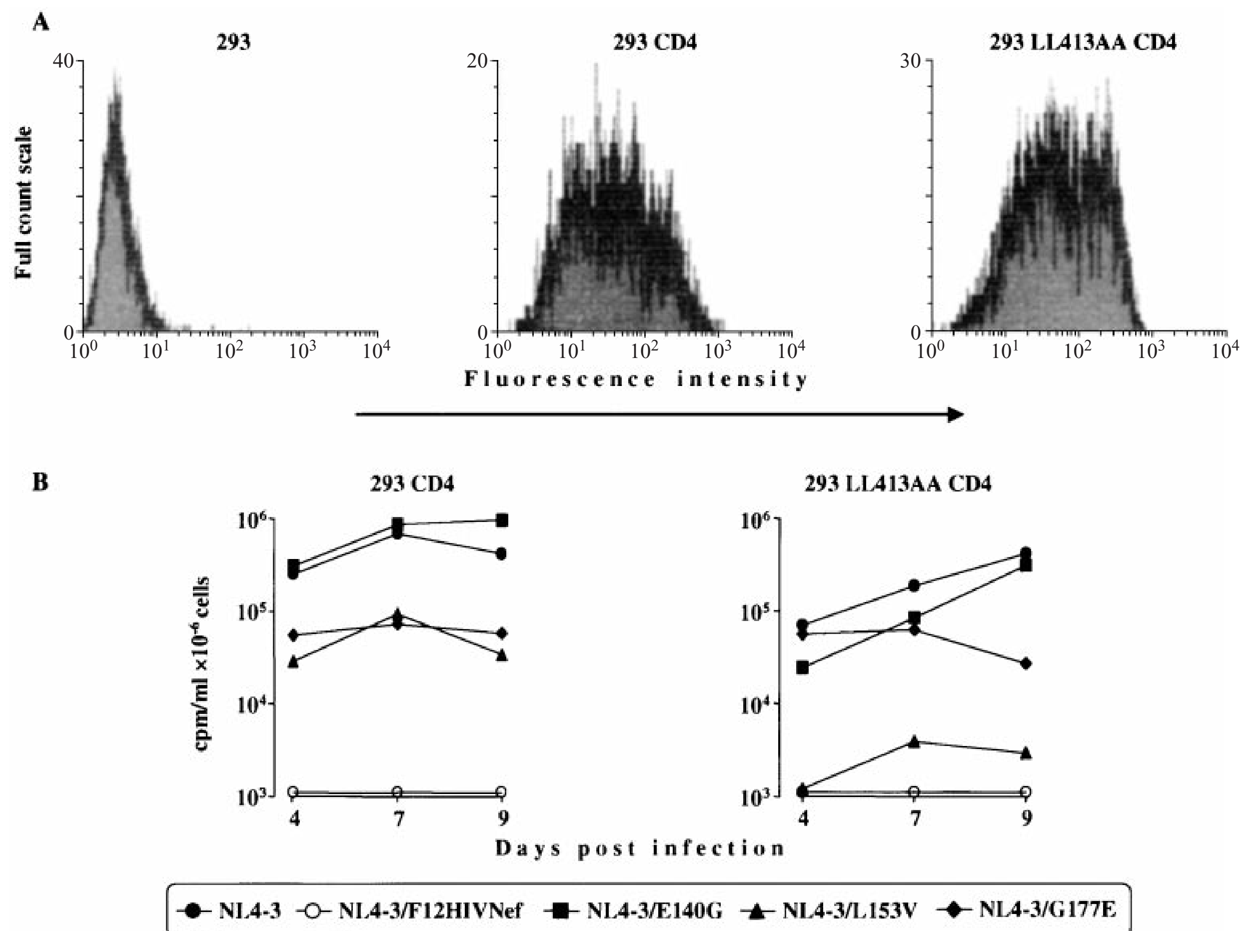

Fig. 6. HIV-1 expressing the $L^{153}V$ back mutant shows a non-producer phenotype upon infection of cells expressing Nef-resistant CD4. (A) CD4 FACS analysis of 293 cell populations stably expressing the wt or the $LL^{413}AA$-mutated CD4 receptor. Parental 293 cells served as a negative control. Cells were labelled with a PE-conjugated anti-CD4 MAb and analysed by FACS. In either cell population, the MFI value after labelling with a non-specific PE-conjugated mouse IgG overlapped that from anti-CD4-labelled 293 cells. (B) 293 cells stably expressing the human CD4 receptor or its $LL^{413}AA$ mutant were infected with the wt or the NL4-3 chimeric viruses expressing F12-HIVNef or its back mutants (m.o.i. of 1). The activity of RT was measured at different days after infection as c.p.m./ml and normalized for 10^6 cells after subtraction of the background values. Data from one representative of three independent experiments are reported.

showed $>95\%$ positivity by FACS analysis, with an MFI value slightly (1·5-fold) exceeding that from 293 cells expressing parental CD4 (Fig. 6A). This was not surprising considering that the $LL^{413}AA$ mutation leads to an impaired constitutive endocytosis of CD4 molecules. Then, we infected 293 cells expressing either the $LL^{413}AA$ mutant or, as a control, the parental CD4 with NL4-3 HIV-1 chimeric viruses expressing F12-HIVNef or its back mutants and assayed RT levels in supernatants at different days post-infection. Interestingly, and differently to that observed through the infection of cells expressing wt CD4 (see Fig. 1), NL4-3 HIV-1 viruses expressing the $L^{153}V$ back mutant re-acquired the non-producer phenotype that is detected typically in F12-HIVNef-expressing NL4-3 (Fig. 6B). The very low levels of RT detected in later time-points should relate to a small leakage in the Nef resistance effect induced by the mutations in the CD4 dileucine motif. Conversely, the expression of the mutated CD4 molecule did not induce strong modifications in the phenotype of NL4-3 expressing wt, F12-HIV, $E^{140}G$ or $G^{177}E$ *nef* alleles (Fig. 6B). The opposite phenotypes of HIV-1 expressing either the $E^{140}G$ or the $L^{153}V$ Nef back mutants reveal that, aside from the lack of accelerated CD4 internalization, an additional and, as yet, still unidentified function is required for the F12-HIVNef antiviral effect.

Discussion

We isolated previously a *nef* allele (F12-HIV*nef*) whose expression fails to induce CD4 down-regulation and, more intriguingly, blocks HIV-1 replication (D'Aloja *et al.*, 1998; Olivetta *et al.*, 2000). Here we demonstrate that this antiviral effect depends on the contemporary presence of three rare

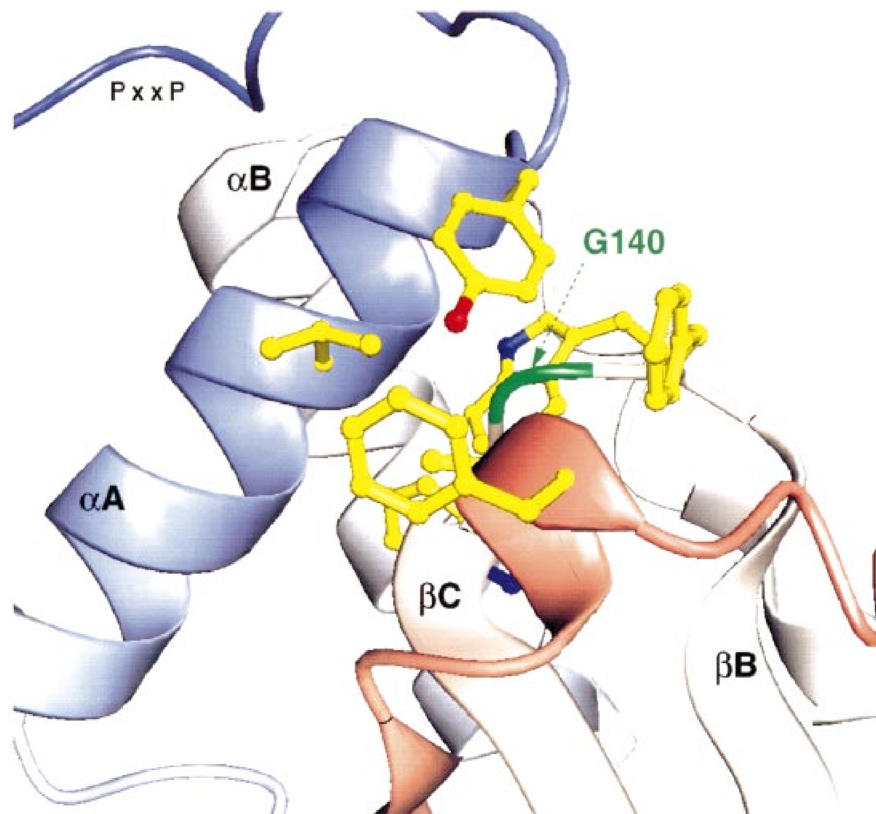

Fig. 7. A structural model of HIV-1 Nef in the region surrounding the 140 amino acid position, as deduced from crystallization studies. The figure was created using the MOLOSCRIPT and RASTER3D programs.

amino acid substitutions. Furthermore, the effects of both F12-HIVNef and its three back mutants in terms of both CD4 down-regulation and p62NAK activation were analysed. These are well-characterized markers of Nef activity and both influence the efficiency of HIV-1 replication (Lama *et al*., 1999; Lu *et al*., 1996; Ross *et al*., 1999).

The absolute requirement of the CD4 intracytoplasmic tail for the F12-HIVNef-induced antiviral effect has been demonstrated previously (Olivetta *et al*., 2000). In this paper, we provide data that point out the lack of Nef-induced accelerated CD4 internalization as an additional requisite for the antiviral effect. However, HIV-1 expressing the $G^{177}E$ back mutant is a replication competent virus, despite its inability to accelerate CD4 endocytosis, whereas HIV-1 expressing the $E^{140}G$ back mutant maintains the producer phenotype seen also in Nef-resistant CD4-expressing cells (Table 1). This indicates that the lack of Nef-induced accelerated CD4 internalization is not sufficient to explain the F12-HIVNef phenotype.

We show that F12-HIVNef does not activate p62NAK and, among the back mutants analysed, only the $G^{177}E$ back mutant re-acquires such a function. p62NAK is a serine/threonine kinase identified recently as PAK-1 (Fackler *et al*., 2000) or PAK-2 (Arora *et al*., 2000; Renkema *et al*., 1999). It has been demonstrated that the Nef-dependent p62NAK activation induces cytoskeleton rearrangement, which facilitates the virus release process (Fackler *et al*., 1999) and, in general, seems to be involved in the progress of AIDS pathogenesis (Khan *et al*., 1998; Sawai *et al*., 1996).

By matching data from CD4 and p62NAK analyses with infection experiments (Table 1), we conclude that the simultaneous lack of both functions correlates with the Nef-induced block of virus release. However, the productive phenotype resulting from the infection of 293 $LL^{413}AA$ CD4 cells with HIV-1 expressing the $E^{140}G$ back mutant indicates that still unknown factors/functions are also involved. Among the large array of Nef protein-binding partners we analysed, neither the Nef-binding protein-1, identified as the catalytic subunit of the vacuolar ATPase (Lu *et al*., 1998), the p85 regulatory subunit of the phosphatidylinositol-3-kinase (Andreas Baur, unpublished observations), nor Vav seemed to play a role in the F12-HIVNef phenotype. In the kinase assay reported here, a protein with a molecular mass of $\cong$ 75 kDa is detectable in lysates from cells expressing F12-HIVNef. Further investigations aimed to establish whether this product represents a novel F12-HIVNef-binding partner are required.

It is conceivable that the strong antiviral effect of F12-HIVNef is not solely the consequence of the lack of Nef-specific functions, but also of an active negative function. The

existence of such a function could be deduced by the opposite phenotypes of the $E^{140}G$ and $L^{153}V$ back mutants in $LL^{413}AA$ CD4-expressing cells. This function seems to correlate with the presence of the E^{140} residue and acts only in the absence of both CD4 accelerated internalization and p62NAK activation.

Our findings allow us to speculate on the structural basis of the F12-HIVNef phenotype. Wt Nef protein has been crystallized, although not in its full-length form (Franken *et al.*, 1997; Grzesiek *et al.*, 1997; Lee *et al.*, 1996). Thus, we have the possibility to predict the structural consequences of each F12-HIVNef amino acid substitution. At position 140, F12-HIVNef displays an E residue instead of a G residue, which is normally present in replication-competent HIV-1 strains. This position resides in the turn between the B and C β-strands (Fig. 7). The introduction of the E side chain may cause both steric (lack of space for the long side chain of E) and charge problems (the environment, formed by V^{85}, Y^{81}, F^{139}, W^{141} and A^{190}, is rather hydrophobic, as opposed to E, which is negatively charged). It is thus conceivable that the $G^{140}E$ mutation could generate a partially unfolded protein. Of note, we observed that such an amino acid substitution effectively co-operates with the $V^{153}L$ mutation in failing to trigger accelerated CD4 endocytosis.

Both L^{153} and G^{177} reside within a long solvent-exposed loop of the Nef C terminus that was not included in the crystallized structure of Nef (Franken *et al.*, 1997; Grzesiek *et al.*, 1997; Lee *et al.*, 1996). Considering that this loop seems to be unstructured, these mutations would not affect protein folding. However, mutations in the C-terminal region that profoundly influence Nef functions have been reported. In particular, the conserved LL motif (aa 164–165) acts as a specific endocytotic signal (Craig *et al.*, 1998) by addressing clathrin-coated pit adaptor complexes (AP-2). In addition, the 154–155 EE diacidic motif has been demonstrated to be critical for the β-COP recruitment of Nef–CD4–AP2 complexes before lysosome degradation (Piguet *et al.*, 1999). In contrast, the $V^{153}L$ amino acid substitution in F12-HIVNef, mapping immediately beside the EE diacidic motif, appears to influence CD4 down-regulation at the internalization step, but only in co-operation with the $G^{140}E$ substitution.

The Nef regions specific for p62NAK binding have been mapped in the Nef core domain, in particular, in both the polyproline and the highly conserved arginine motifs (Manninen *et al.*, 1998; Sawai *et al.*, 1995). Here, we show that the amino acid at position 177 is important also for p62NAK activation. This could be deduced considering that both the $E^{140}G$ and the $L^{153}V$ Nef back mutants as well as the parental F12-HIVNef do not activate p62NAK and that only the $G^{177}E$ mutant reverts towards the wt phenotype. This result appears to be consistent with the data from Luo *et al.* (1997), who highlighted the importance of the C-terminal region of Nef in p62NAK activation.

It has been reported already that both accelerated CD4 endocytosis and p62NAK activation favour virus spread (Fackler *et al.*, 1999; Lama *et al.*, 1999; Ross *et al.*, 1999). Here, we strengthen these observations by describing a complete block of release in HIV-1 expressing a Nef mutant lacking both functions. This is additional evidence to support the fact that interactions of Nef with appropriated cell targets could be critical for virus replication.

We were able to define both the genetic and the functional markers of the antiviral action of F12-HIVNef by the analysis of its back mutants. This system represents a potent tool for gaining even more detailed insight into the antiviral action mechanism of F12-HIVNef and also for revealing Nef functions that are still uncharacterized.

This study was supported by Professor B. M. Peterlin and Dr O. Fackler, University of California, San Francisco, CA, USA, who also critically read the manuscript. We are grateful to C. Aiken, Vanderbilt University School of Medicine, Nashville, TN, USA, who generously provided the CMX/CD4 $LL^{413}AA$ expression vector. We also acknowledge F. M. Regini, A. Lippa and D. Pino for excellent editorial assistance. This work was supported by grants from the AIDS project of the Ministry of Health, Rome, Italy.

References

Aiken, C., Konner, J., Landau, N. R., Lenburg, M. E. & Trono, D. (1994). Nef induces CD4 endocytosis: requirement for a critical dileucine motif in the membrane-proximal CD4 cytoplasmic domain. *Cell* **75**, 853–864.

Arora, V. K., Molina, R. P., Foster, J. L., Blakemore, J. L., Chernoff, J., Fredericksen, B. L. & Garcia, J. V. (2000). Lentivirus Nef specifically activates Pak2. *Journal of Virology* **74**, 11081–11087.

Bell, I., Ashman, C., Maughan, J., Hooker, E., Cook, F. & Reinhart, T. A. (1998). Association of simian immunodeficiency virus Nef with the T-cell receptor (TCR) ζ chain leads to TCR down-modulation. *Journal of General Virology* **79**, 2717–2727.

Brambilla, A., Turchetto, L., Gatti, A., Bovolenta, C., Veglia, F., Santagostino, E., Gringeri, A., Clementi, M., Poli, G., Bagnarelli, P. & Vicenzi, E. (1999). Defective *nef* alleles in a cohort of hemophiliacs with progressing and nonprogressing HIV-1 infection. *Virology* **259**, 349–368.

Bryant, M. L., Ratner, L., Duronio, R. J., Kishore, N. S., Devadas, B., Adams, S. P. & Grodon, J. I. (1991). Incorporation of 12-methoxydodecanoate into the human immunodeficiency virus 1 Gag polyprotein precursor inhibits its proteolytic processing and virus production in a chronically infected human lymphoid cell line. *Proceedings of the National Academy of Sciences, USA* **88**, 2055–2059.

Carlini, F., Federico, M., Equestre, M., Ricci, S., Ratti, G., Zibai, Q., Verani, P. & Rossi, G. B. (1992). Sequence analysis of an HIV-1 proviral DNA from a non-producer chronically infected Hut-78 cellular clone. *Journal of Virological Diseases* **1**, 40–55.

Chowers, M. Y., Spina, C. A., Kwoh, T. J., Fitch, N. J. S., Richman, D. D. & Guatelli, J. C. (1994). Optimal infectivity *in vitro* of human immunodeficiency virus type 1 requires an intact *nef* gene. *Journal of Virology* **68**, 2906–2914.

Cohen, G. B., Gandhi, R. T., Davis, D. M., Mandelboim, O., Chen, B. K., Strominger, J. L. & Baltimore, D. (1999). The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells. *Immunity* **10**, 661–671.

Collins, K. L., Chen, B. K., Kalams, S. A., Walker, B. D. & Baltimore, D. (1998). HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes. *Nature* **391**, 397–401.

Craig, H. M., Pandori, M. W. & Guatelli, J. C. (1998). Interaction of HIV-1 Nef with the cellular dileucine-based sorting pathway is required for CD4 down-regulation and optimal viral infectivity. *Proceedings of the National Academy of Sciences, USA* **95**, 11229–11234.

D'Aloja, P., Olivetta, E., Bona, R., Nappi, F., Pedacchia, D., Pugliese, K., Ferrari, G., Verani, P. & Federico, M. (1998). *gag*, *vif*, and *nef* genes contribute to the homologous viral interference induced by a non-producer human immunodeficiency virus type 1 (HIV-1) variant: identification of novel HIV–1-inhibiting viral protein mutants. *Journal of Virology* **72**, 4308–4319.

Fackler, O. T., Luo, W., Geyer, M., Alberts, A. S. & Peterlin, B. M. (1999). Activation of Vav by Nef induces cytoskeletal rearrangements and downstream effector functions. *Molecular Cell* **3**, 729–739.

Fackler, O. T., Lu, X., Frost, J. A. A., Geyer, M., Jiang, B., Luo, W., Abo, A., Alberts, A. S. & Peterlin, B. M. (2000). p21-activated kinase 1 plays a critical role in cellular activation by Nef. *Molecular and Cellular Biology* **20**, 2619–2627.

Federico, M., Taddeo, B., Carlini, F., Nappi, F., Verani, P. & Rossi, G. B. (1993). A recombinant retrovirus carrying a non-producer human immunodeficiency virus (HIV) type 1 variant induces resistance to superinfecting HIV. *Journal of General Virology* **74**, 2099–2110.

Franken, P., Arold, S., Padilla, A., Bodeus, M., Hoh, F., Strub, M. P., Boyer, M., Jullien, M., Benarous, R. & Dumas, C. (1997). HIV-1 Nef protein: purification, crystallization, and preliminary X-ray diffraction studies. *Protein Science* **6**, 2681–2683.

Greenberg, M. E., Bronson, S., Lock, M., Neumann, M., Pavlakis, G. N. & Skowronski, J. (1997). Co-localization of HIV-1 Nef with the AP-2 adaptor protein complex correlates with Nef-induced CD4 down-regulation. *EMBO Journal* **16**, 6964–6976.

Greenberg, M. E., Iafrate, A. J. & Skowronski, J. (1998). The SH3 domain-binding surface and an acidic motif in HIV-1 Nef regulate trafficking of class I MHC complexes. *EMBO Journal* **17**, 2777–2789.

Grzesiek, S., Bax, A., Hu, J. S., Kaufman, J., Palmer, I., Stahl, S. J., Tjandra, N. & Wingfield, P. T. (1997). Refined solution structure and backbone dynamics of HIV-1 Nef. *Protein Science* **6**, 1248–1263.

Khan, I. H., Sawai, E. T., Antonio, E., Weber, C. J., Mandell, C. P., Montbriand, P. & Luciw, P. A. (1998). Role of the SH3-ligand domain of simian immunodeficiency virus Nef in interaction with Nef-associated kinase and simian AIDS in rhesus macaques. *Journal of Virology* **72**, 5820–5830.

Lama, J., Mangasarian, A. & Trono, D. (1999). Cell-surface expression of CD4 reduces HIV-1 infectivity by blocking Env incorporation in a Nef- and Vpu-inhibitable manner. *Current Biology* **9**, 622–631.

Lee, C. H., Saksela, K., Mirza, U. A., Chait, B. T. & Kuriyan, J. (1996). Crystal structure of the conserved core of HIV-1 Nef complexed with a Src family SH3 domain. *Cell* **85**, 931–942.

Le Gall, S., Erdtmann, L., Benichou, S., Berlioz-Torrent, C., Liu, L., Benarous, R., Heard, J. M. & Schwartz, O. (1998). Nef interacts with the mu subunit of clathrin adaptor complexes and reveals a cryptic sorting signal in MHC I molecules. *Immunity* **8**, 483–495.

Lu, X., Wu, X., Plemenitas, A., Yu, H., Sawai, E. T., Abo, A. & Peterlin, B. M. (1996). CDC42 and Rac1 are implicated in the activation of the Nef-associated kinase and replication of HIV-1. *Current Biology* **6**, 1677–1684.

Lu, X., Yu, H., Brodsky, F. M. & Peterlin, B. M. (1998). Interactions between HIV-1 Nef and vacuolar ATPase facilitate the internalization of CD4. *Immunity* **8**, 647–656.

Luo, T., Livingston, R. A. & Garcia, J. V. (1997). Infectivity enhancement by human immunodeficiency virus type 1 Nef is independent of its association with a cellular serine/threonine kinase. *Journal of Virology* **71**, 9524–9530.

Mangasarian, A., Foti, M., Aiken, C., Chin, D., Carpentier, J. L. & Trono, D. (1997). The HIV-1 Nef protein acts as a connector with sorting pathways in the Golgi and at the plasma membrane. *Immunity* **6**, 67–77.

Mangasarian, A., Piguet, V., Wang, J. K., Chen, Y. L. & Trono, D. (1999). Nef-induced CD4 and major histocompatibility complex class I (MHC-I) down-regulation are governed by distinct determinants: N-terminal α-helix and proline repeat of Nef selectively regulate MHC-I trafficking. *Journal of Virology* **73**, 1964–1973.

Manninen, A., Hiipakka, M., Vihinen, M., Lu, W., Mayer, B. J. & Saksela, K. (1998). SH3-domain binding function of HIV-1 Nef is required for association with a PAK-related kinase. *Virology* **250**, 273–282.

Marsh, M. & Pelchen-Matthews, A. (1996). Endocytic and exocytic regulation of CD4 expression and function. *Current Topics in Microbiology and Immunology* **205**, 107–135.

Miller, M., Warmerdam, M. T., Gaston, I., Greene, W. C. & Feinberg, M. B. (1994). The human immunodeficiency virus-1 *nef* gene product: a positive factor for viral infection and replication in primary lymphocytes and macrophages. *Journal of Experimental Medicine* **179**, 101–113.

Nunn, M. F. & Marsh, J. W. (1996). Human immunodeficiency virus type 1 Nef associates with a member of the p21-activated kinase family. *Journal of Virology* **70**, 6157–6161.

Olivetta, E., Pugliese, K., Bona, R., D'Aloja, P., Ferrantelli, F., Santarcangelo, A. C., Mattia, G., Verani, P. & Federico, M. (2000). *cis* expression of the F12 human immunodeficiency virus (HIV) Nef allele transforms the highly productive NL4-3 HIV type 1 to a replication-defective strain: involvement of both Env gp41 and CD4 intracytoplasmic tails. *Journal of Virology* **74**, 483–492.

Palm, G. J., Zdanov, A., Gaitanaris, G. A., Stauber, R., Pavlakis, G. N. & Wlodawer, A. (1997). The structural basis for spectral variation in green fluorescent protein. *Nature Structural Biology* **4**, 361–365.

Piguet, V., Chen, Y. L., Mangasarian, A., Foti, M., Carpentier, J. L. & Trono, D. (1998). Mechanism of Nef-induced CD4 endocytosis: Nef connects CD4 with the mu chain of adaptor complexes. *EMBO Journal* **17**, 2472–2481.

Piguet, V., Gu, F., Foti, M., Demaurex, N., Gruenberg, J., Carpentier, J. L. & Trono, D. (1999). Nef-induced CD4 degradation: a diacidic-based motif in Nef functions as a lysosomal targeting signal through the binding of β-COP in endosomes. *Cell* **97**, 63–73.

Renkema, G. H., Manninen, A., Mann, D. A., Harris, M. & Saksela, K. (1999). Identification of the Nef-associated kinase as p21-activated kinase 2. *Current Biology* **9**, 1407–1410.

Ross, T. M., Oran, A. E. & Cullen, B. R. (1999). Inhibition of HIV-1 progeny virion release by cell-surface CD4 is relieved by expression of the viral Nef protein. *Current Biology* **9**, 613–621.

Rossi, G. B., Verani, P., Macchi, B., Federico, M., Orecchia, A., Nicoletti, L., Buttò, S., Lazzarin, A., Mariani, G., Ippolito, G. & Manzari, V. (1987). Recovery of HIV-related retroviruses from Italian patients with AIDS or AIDS-related complex and from asymptomatic at-risk individuals. *Annals of the New York Academy of Sciences* **511**, 390–400.

Saksela, K. (1997). HIV-1 Nef and host cell protein kinases. *Frontiers of Bioscience* **2**, 606–618.

Sawai, E. T., Baur, A., Struble, H., Peterlin, B. M., Levy, J. A. & Cheng-Mayer, C. (1994). Human immunodeficiency virus type 1 Nef associates with a cellular serine kinase in T lymphocytes. *Proceedings of the National Academy of Sciences, USA* **91**, 1539–1543.

Sawai, E. T., Baur, A. S., Peterlin, B. M., Levy, J. A. & Cheng-Mayer, C. (1995). A conserved domain and membrane targeting of Nef from HIV

and SIV are required for association with a cellular serine kinase activity. *Journal of Biological Chemistry* **270**, 15307–15314.

Sawai, E. T., Khan, I. H., Montbriand, P. M., Peterlin, B. M., Cheng-Mayer, C. & Luciw, P. A. (1996). Activation of PAK by HIV and SIV Nef: importance for AIDS in rhesus macaques. *Current Biology* **6**, 1519–1527.

Schwartz, O., Maréchal, V., Le Gall, S., Lemonnier, F. & Heard, J. M. (1996). Endocytosis of major histocompatibility complex class I molecules is induced by the HIV-1 Nef protein. *Nature Medicine* **2**, 338–342.

Spina, C. A., Kwoh, T. J., Chowers, M. Y., Guatelli, J. C. & Richman, D. D. (1994). The importance of *nef* in the induction of human immunodeficiency virus type 1 replication from primary quiescent CD4 lymphocytes. *Journal of Experimental Medicine* **179**, 115–123.

Taddeo, B., Carlini, F., Verani, P. & Engelman, A. (1996). Reversion of a human immunodeficiency virus type 1 integrase mutant at a second site restores enzyme function and virus infectivity. *Journal of Virology* **70**, 8277–8284.

Wigler, M., Sweet, R., Sim, G. K., Wold, B., Pellicer, A., Lacy, E., Maniatis, T., Silverstein, S. & Awel, R. (1979). Transformation of mammalian cells with genes from procaryotes and eucaryotes. *Cell* **16**, 758–777.

Xu, X. N., Laffert, B., Screaton, G. R., Kraft, M., Wolf, D., Kolanus, W., Mongkolsapai, J., McMichael, A. J. & Baur, A. S. (1999). Induction of Fas ligand expression by HIV involves the interaction of Nef with the T cell receptor ζ chain. *Journal of Experimental Medicine* **189**, 1489–1496.

Received 22 March 2001; Accepted 1 August 2001