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 Baur2 and Maurizio Federico1

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, G140E, V153L and E177G. 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-HIVNef 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 G177E 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 E140G and L153V 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-HIVNef 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. 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, C140E, V132L and E177G (Carlini et al., 1992). We reported recently that the in cis expression of F12-HIVNef transforms the highly productive NL4-3 HIV-1 release (D’Aloja et al., 1999). 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 (LL133AA) 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 (Vigler 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<sup>6</sup> cells after back ground subtraction. Virus titrations were carried out either by scoring the number of syncitia 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-Bgal 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 F12Nef chimeric proteins was assessed either by Western blot or by anti-CD8 immunoprecipitation of lysates from 35S-labelled cells (D’Aloja et al., 1998) followed by 12% PAGE.

- **Mutagenesis, cloning and subcloning.** To introduce site-specific mutations into the F12-HIVNef allele, the overlapping PCR technique was carried out as described previously (Taddeo et al., 1996). A first amplification step using F12-HIVNef as the template served to generate two half fragments of nef. Internal primer couples carried the back mutations. The external primers contained HindIII (‘5’) and EcoRI (‘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<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 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 HindIII/EcoRI 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<sub>nef</sub> constructs as a template and a pair of oligoprimer carrying the MluI (‘5’ end) and Clal (‘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 MluI (whose restriction site was present in a linker inserted between the env stop and nef start codons) and Clal (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 oligoprimer carrying BamHI (forward) and SalI (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/CD4LL133AA 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 dyeoxy 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 NaN3. 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 MnCl2. Immunoprecipitated proteins were incubated in 50 µl KAB containing 0.37 MBq of [γ-32P]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-HIVNef bears three amino acid substitutions, G140E, V152L, and E177G, 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 G140E nor V152L substitutions were detected and only a single nef allele was found to carry the E177G 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).

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 virus strains. The activity of RT was measured at different days after infection as c.p.m./ml and normalized for 106 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 E140G (lane D), L153V (lane E) or G177E (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.

![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 106 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 E140G (lane D), L153V (lane E) or G177E (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.](image-url)
F12-HIV Nef expression does not induce accelerated CD4 internalization

Cells expressing F12-HIV Nef are defective for CD4 down-regulation (D’Aloja et al., 1998; Olivetta et al., 2000). Nevertheless, F12-HIV Nef 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-HIV Nef. 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-HIV Nef-induced antiviral action with its effects on CD4, we investigated the functional defect(s) of F12-HIV Nef 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-HIV Nef 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-HIV Nef. 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-HIV Nef 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-HIV Nef expression does not induce CD4 down-regulation as a consequence of a defect in the acceleration of CD4 endocytosis.

Analysis of CD4 internalization in cells expressing F12-HIV Nef 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-HIV Nef-expressing cells. Thus, the effects of F12-HIV Nef back mutants on CD4 were analysed by reproducing the assays for both steady-state levels and internalization rates. We found that both E140G and L153V 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-HIV Nef, the expression of the G177E 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-HIV Nef, additional Nef functions seem to be involved.

CD4-independent Nef internalization is impaired in cells expressing either F12-HIV Nef or the G177E back mutant

Here we demonstrate that F12-HIV Nef and its G177E back mutant behave similarly in terms of CD4 internalization, but
Mutants from an HIV-1-inhibiting nef allele

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), E140G (c), L153V (d) or G177E (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.

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 its back mutant. Conversely, either the E140G or the L153V 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 35S-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 G177E back mutant depends on a similarly impaired involvement with endocytic machinery rather than on a defect in the interaction with CD4.

Fig. 4. Both F12-HIVNef and its G177E back mutant fail to accelerate auto-internalization. (A) 293T cells were transfected with vectors expressing CD8-based chimeras carrying F12-HIVNef 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 35S-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.

F12-HIVNef expression does not induce activation of p62NAK and rescue by the G177E back mutation

As the expression of the G177E 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
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.

Table 1. Summary of results

<table>
<thead>
<tr>
<th>nef allele</th>
<th>Accelerated CD4 endocytosis</th>
<th>p62NAK activation</th>
<th>Block of HIV-1 release</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD4-expressing cells</td>
<td>LL413AA CD4-expressing cells</td>
<td></td>
</tr>
<tr>
<td>NL4-3</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>F12-HIV</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>E148G</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>L153V</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>G177E</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

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 \( \cong 75\) kDa could be appreciated (Fig. 5). Similarly, both the E148G and the L153V back mutants failed to activate p62NAK, whereas the G177E 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 G177E back mutant does not accelerate the rate of CD4 internalization. Both the E148G and the L153V 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 (LL413AA). 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 E148G and L153V Nef back mutants could lead to a phenotype similar to F12-HIVNef in cells where they can no longer induce CD4 downregulation. 293 cells stably expressing the mutated CD4
Mutants from an HIV-1-inhibiting nef allele

Fig. 6. HIV-1 expressing the L153V 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 LL413AA-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 LL413AA 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 LL413AA mutation leads to an impaired constitutive endocytosis of CD4 molecules. Then, we infected 293 cells expressing either the LL413AA 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), NL4-3-4 HIV-1 viruses expressing the L153V 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, E140G or G177E nef alleles (Fig. 6B). The opposite phenotypes of HIV-1 expressing either the E140G or the L153V 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
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 G177E back mutant is a replication competent virus, despite its inability to accelerate CD4 endocytosis, whereas HIV-1 expressing the E140G 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 G177E 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 LL143AA CD4 cells with HIV-1 expressing the E140G 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 ≈ 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 E140G and L153V back mutants in LL143AA CD4-expressing cells. This function seems to correlate with the presence of the E140 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 V85, Y86, E139, W141 and A196, is rather hydrophobic, as opposed to E, which is negatively charged). It is thus conceivable that the G140E mutation could generate a partially unfolded protein. Of note, we observed that such an amino acid substitution effectively co-operates with the V153L mutation in failing to trigger accelerated CD4 endocytosis.

Both L153 and G177 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 V153L 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 G140E 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 E140G and the L153V Nef back mutants as well as the parental F12-HIVNef do not activate p62NAK and that only the G177E 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 LL143AA 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


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


Received 22 March 2001; Accepted 1 August 2001