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Natural variation of the *nef* gene in human immunodeficiency virus type 2 infections in Portugal

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Human immunodeficiency virus type 2 (HIV-2) infections cause severe immunodeficiency in humans, although HIV-2 is associated frequently with reduced virulence and pathogenicity compared to HIV-1. Genetic determinants that play a role in HIV pathogenesis are relatively poorly understood but *nef* has been implicated in inducing a more pathogenic phenotype *in vivo*. However, relatively little is known about the role of *nef* in HIV-2 pathogenesis. To address this, the genetic composition of 44 *nef* alleles from 37 HIV-2-infected individuals in Portugal, encompassing a wide spectrum of disease associations, CD4 counts and virus load, has been assessed. All *nef* alleles were subtype A, with no evidence of gross deletions, truncations or disruptions in the *nef*-encoding sequence; all were full-length and intact. HIV-2 long terminal repeat sequences were conserved and also indicated subtype A infections. Detailed analysis of motifs that mediate *nef* function in HIV-1 and simian immunodeficiency virus, such as CD4 downregulation and putative SH2/SH3 interactions, revealed significant natural variation. In particular, the central P^{104}xxPLR motif exhibited wide interpatient variation, ranging from an HIV-1-like tetra-proline structure $(PxxP)_3$ to a disrupted minimal core motif (P^{104}xxQLR). The $P^{107}\rightarrow$Q substitution was associated with an asymptomatic phenotype (Fisher's exact test, $P=0{\cdot}026$) and low virus loads. These data indicate that discrete differences in the *nef* gene sequence rather than gross structural changes are more likely to play a role in HIV-2 pathogenesis mediated via specific functional interactions.

INTRODUCTION

Human immunodeficiency virus type 2 (HIV-2) is the second human retrovirus to be causally linked to AIDS (Clavel *et al.*, 1986). HIV-2 has remained restricted largely to west Africa (reviewed by Schim van der Loeff & Aaby, 1999; Bock & Markovitz, 2001), unlike HIV-1, which has spread rapidly to all parts of the world. HIV-2 has been associated especially with former Portuguese colonies, particularly Guinea Bissau, which reports the highest prevalence of HIV-2 in west Africa: up to 8 % of the population is infected with HIV-2 but is accompanied by relatively low rates of excess mortality (Poulsen *et al.*, 1997; Berry *et al.*, 2002). In Europe, Portugal reports the highest prevalence of HIV-2 infections, with 4 % of notified AIDS cases due solely to HIV-2 (National AIDS Commission, 2002; Soriano *et al.*, 2000).

Differences in the natural history between HIV-1 and HIV-2 are well documented (Marlink *et al.*, 1994; Whittle *et al.*, 1994), although the mechanism(s) that governs the maintenance of an attenuated phenotype for extended time-periods in a large proportion of HIV-2-infected individuals is not fully understood. Differences in virus subtype may provide one explanation, in particular of HIV-2 variants that resemble simian immunodeficiency virus (SIV_{SM}) infections, which appear apathogenic (Chen *et al.*, 1997; Gao *et al.*, 1994; Wolf *et al.*, 2001; Yamaguchi *et al.*, 2000), but these are few in number and distinct from infections due to the more pathogenic subtypes A and B. Isolation of virus from asymptomatic HIV-2-infected individuals is more difficult than for HIV-1 (Schulz *et al.*, 1990; Simon *et al.*, 1993); many strains are characterized by low levels of cytopathogenicity and reduced virulence. HIV-2 strains, however, exhibit the capacity to utilize a broad spectrum of co-receptors and do not appear to be restricted in their cell tropism (reviewed by Reeves & Doms, 2002).

The failure of HIV-2 to develop into a pandemic and the more attenuated phenotype *in vivo* is correlated with low levels of plasma HIV-2 RNA (Berry *et al.*, 1998, 2002; Andersson *et al.*, 2000; Ariyoshi *et al.*, 2000; Popper *et al.*, 1999) and when associated with high CD4 counts, low

plasma viraemia is predictive of normal survival (Berry *et al.*, 2002). Conversely, advanced HIV-2 infections and disease are associated frequently with high virus load (Ariyoshi *et al.*, 2000; Berry *et al.*, 1998, 2002), with HIV-2 plasma RNA levels representing an independent predictor of disease progression. Hence, host or viral genetic factors that increase virus replication/virus load are likely to have a direct impact on the pathogenesis of HIV-2. Unlike HIV-1 and SIV, the role of *nef* in the differing phenotypes of HIV-2 infections has not been studied as extensively.

Nef, an auxiliary protein that is highly conserved among different primate lentiviruses, regulates virus replication and acts as an immune modulator (Collette, 1997). Genetically and structurally, HIV-2 *nef* is more similar to $SIV_{MAC/SM}$ *nef* than to HIV-1 *nef* and is approximately 260 amino acids in length. *In vivo* studies of SIV_{MAC} in Asian macaques indicate that an intact *nef* gene is essential for full disease induction and maintenance of high virus load (Kestler *et al.*, 1991). Virus populations attenuated due to the disruption of *nef* have been demonstrated in both SIV-infected macaques (Whatmore *et al.*, 1995) and long-term survivors with HIV-1 (Deacon *et al.*, 1995; Michael *et al.*, 1995; Geffin *et al.*, 2000; Learmont *et al.*, 1999). However, no consistent relationship between *nef* disruption and prolonged survival in HIV-1-infected individuals has been shown (Carl *et al.*, 2000a, b; Kirchhoff *et al.*, 1999; Mourich *et al.*, 1999), suggesting a complex role for *nef* in the development of human AIDS. Studies of transgenic mice have also indicated a major role for *nef* in inducing a pathogenic phenotype (Hanna *et al.*, 1998, 2001). One previous report based on a mixture of HIV-2 subtypes indicated a higher proportion of truncated *nef* alleles in asymptomatic HIV-2 infections than expected for HIV-1, an observation that might explain differences in pathogenesis (Switzer *et al.*, 1998) and which, therefore, warrants further investigation.

Nef accomplishes different functions for virus infectivity and virulence (reviewed by Piguet & Trono, 1999; Fackler & Baur, 2002). HIV-1 *nef* downregulates cell surface CD4 expression and probably MHC class I expression (Greenberg *et al.*, 1997; Mangasarian *et al.*, 1999), connecting the viral receptor and cellular components by adaptor protein (AP)-forming complexes with clathrin-coated pits (Foti *et al.*, 1997), which mediate endocytosis (Piguet *et al.*, 1998). Di-leucine-based motifs in the C-terminal part of *nef* are important in this process (Craig *et al.*, 1998). In SIV *nef*, both tyrosine- and leucine-based motifs are involved in CD4 downmodulation (Bresnahan *et al.*, 1999). Although AP interactions vary for different virus–host interactions among different primate lentiviruses (Greenberg *et al.*, 1997; Karn *et al.*, 1998; Lock *et al.*, 1999), combined data from functional studies indicate the same net effect of *nef*-induced CD4 downregulation. Other cellular partners such as V-ATPase and β-COP (Piguet *et al.*, 1999) may also be involved in these complex processes.

Nef augments virus infectivity *in vivo* by activating target cells through T-cell signalling and signal transduction pathways (Bell *et al.*, 1998; Simmons *et al.*, 2001; Manninen *et al.*, 1998; Fackler *et al.*, 2001; Fackler & Baur, 2002). Canonical proline-rich (PxxP) regions in *nef* (Saksela *et al.*, 1995) mediate interactions with Src homology region 3 (SH3)-binding domains for Src family kinases such as Hck, Lck and Fyn (Cheng *et al.*, 1999; Lee *et al.*, 1995; Collette *et al.*, 2000). In HIV-2 and SIV *nef*, the core SH3-binding domain is usually represented by a minimal binding consensus motif (P^{104}xxPLR), although this is typically a tetra-proline structure $(PxxP)_3$ in HIV-1.

In this study of HIV-2 infections, we describe in detail HIV-2 *nef* sequence variability in Portugal and its relation to putative *nef* function and pathogenesis.

METHODS

Study population. Whole blood was collected between 1994 and 2000 from 37 individuals living in Lisbon, Portugal. These individuals were identified as having HIV-2 infection using a range of serological and genome detection methods and represented a broad cross-section of HIV-2 infections in this country. A wide range of epidemiological and clinical data was collected, including age, gender, ethnic status, route of transmission and country of infection (summarized in Table 1). Of the 37 individuals studied, only seven received anti-retroviral therapy, three (117, 281 and 956) to treat progressive HIV-2 infection and the other four (1227, 1570, 1428 and 1378), who received transient anti-retroviral therapy, to prevent vertical transmission around the time of birth. All four women receiving therapy to prevent HIV transmission were asymptomatic. HIV-2 viral RNA load was only measured subsequently in three women who had received transient drug therapy (1227, 1570 and 1428) and from whom plasma was available.

Amplification of HIV-2 *nef* and *nef*/LTR. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll–Hypaque density gradient centrifugation. Cellular DNA was prepared by proteinase K treatment, phenol/chloroform purification and ethanol precipitation. DNA resuspended in nuclease-free water was quantified by spectrophotometry. HIV-2 proviral sequences were amplified initially using primers located in the LTR and *pol* (integrase), as described previously (Berry *et al.*, 1994). *nef* and *nef*/U3 LTR regions were amplified using different primer combinations. HIV-2 *nef* was amplified using outer primers [sense, 5′-GAGCTCGGTACCC-GGGATGGGTGCGAGTGGATCCAA-3′ (nt 8503–8538); antisense, 5′-TCTAGAGTCGACCTGCAGGGTATCCCTCTTGCTTTC-3′ (nt 9262–9287)], numbering based on $HIV\text{-}2_{ISY}$ (GenBank accession no. J04498), and the *nef*/LTR to the R region was amplified using a nested PCR protocol with outer [sense, 5′-AAGGGC-TATAGGCCWGTDTTCT-3′ (nt 8234–8254); antisense, 5′-TGGTGAGAGTCTAGCAGGG-3′ (nt 9534–9552)] and inner [sense, 5′-GCCCAACTGCAATATGGGTGCGAGT-3′ (nt 8506–8530); antisense, 5′-AACACCCAGGCTCTACCTGCT-3′ (nt 9513–9533)] primers.

Amplifications were performed in 100 mM KCl, 20 mM Tris/HCl (pH 9), 0·1 mM EDTA, 1 mM DTT, 0·5 % Tween 20, 50 % (v/v) glycerol, 1·5 mM $MgCl_2$, 200 μM of each dNTPs (dATP, dTTP, dCTP and dGTP), 300 nM of each internal primer pair and either 2·5 units of Ampli*Taq* Gold DNA polymerase (Perkin-Elmer) or 2·6 units of *Taq*/*Pwo* using the Expand High Fidelity PCR system enzyme mixture (Roche). Primary amplifications were performed as follows: 94 °C for 10 min for 1 cycle and 94 °C for 30 s, 53 °C for 45 s and 72 °C for 1 min 30 sec for 35 cycles. Nested reactions with inner primers were 94 °C for 15 s, 50 °C for 45 s, 72 °C for 1 min 30 s for 35 cycles with a final extension step at 72 °C for 7 min. Products were visualized on 1·75 %

Table 1. Epidemiological, immunological and virological characteristics of the 37 HIV-2-infected individuals studied

NA, Not available.

Reference (individual)	Country of infection	Race	Sex	Age	Route of infection	Clinical symptoms	Anti-retroviral therapy	Plasma HIV-2 viral RNA load (copies ml^{-1})	CD4 count (cells mm^{-3})	Additional information
268	Cape Verde	B	M	55	Heterosexual	Yes	No	200	340	Lung cancer
423	Guinea Bissau	B	M	47	Heterosexual	Yes	No	<200	966	Tuberculosis
379	Portugal	W	M	37	Heterosexual	Yes	No	62 950	366	Tuberculosis
546	Portugal	W	F	65	Blood transfusion	Yes	No	<200	297	Infected 1987/Died 2000
1069	Africa	B	F	67	Blood transfusion	Yes	No	34 750	NA	Infected 1979/Died 1999
138	Africa	B	M	44	Heterosexual	Yes	No	NA	NA	Tuberculosis
984	Portugal	W	F	45	Blood transfusion	Yes	No	8 830	195	Infected in 1978
117	Guinea Bissau	B	M	8	Blood transfusion	Yes	Yes	NA	199	Infected in 1986
281	Guinea Bissau	B	M	42	Heterosexual	Yes	Yes	NA	46	Tuberculosis
956	Guinea Bissau	B	F	32	Heterosexual	Yes	Yes	NA	NA	Died in 1999
280	Guinea Bissau	W	M	48	Heterosexual	Yes	No	NA	NA	
120	Angola	B	F	32	Blood transfusion	No	No	NA	NA	
MP	Portugal	B	F	30	Heterosexual	No	No	<200	610	Prostitute
741	Portugal	W	F	27	Heterosexual	No	No	<200	NA	
1227	Africa	B	F	NA	Heterosexual	No	Yes	<200	NA	Study of HIV vertical transmission
1268	Guinea Bissau	B	F	21	Heterosexual	No	No	3 730	529	
BL	Guinea Bissau	B	M	48	Heterosexual	No	No	<200	288	
794	Cape Verde	B	F	30	Heterosexual	No	No	<200	NA	
293	Guinea Bissau	B	M	32	Heterosexual	No	No	NA	519	Diabetic
483	Africa	B	F	24	Heterosexual	No	No	200	NA	
1139	Portugal	W	F	NA	Heterosexual	No	No	<200	NA	
1147	Cape Verde	B	M	53	Heterosexual	No	No	2 259	407	
1570	Guinea Bissau	B	F	NA	Heterosexual	No	Yes	<200	427	Study of HIV vertical transmission
1215	Portugal	W	F	NA	Heterosexual	No	No	<200	430	
1320	Africa	B	F	NA	Heterosexual	No	No	200	444	
1544	Cape Verde	B	M	63	Heterosexual	No	No	NA	730	
1428	Portugal	W	F	NA	Heterosexual	No	Yes	<200	NA	Study of HIV vertical transmission
292	Portugal	W	F	45	Heterosexual	No	No	<200	NA	
1096	Portugal	W	M	16	Blood products	No	No	<200	902	Haemophiliac
1543	Guinea Bissau	B	F	28	Heterosexual	No	No	<200	993	
LF	Portugal	W	F	44	Heterosexual	No	No	400	437	
EP	Portugal	W	F	52	Heterosexual	No	No	<200	490	
1567	Portugal	W	F	26	Heterosexual	No	No	3 491	1346	IV drug user
PL	Guinea Bissau	B	M	45	Blood transfusion	No	No	6 920	127	Infected in 1973
511	Portugal	W	F	36	Heterosexual	No	No	500	473	
223	Guinea Bissau	B	F	40	Heterosexual	No	No	NA	161	
1378	Africa	B	F	NA	Heterosexual	No	Yes	NA	NA	Study of HIV vertical transmission

agarose gel containing ethidium bromide (0·5 μg ml^{-1}). Aliquots (2–10 μl) containing 0·6 μg extracted genomic DNA were used in primary amplifications and 2 μl of first-round product was used as template for second-round PCR.

Purification of PCR fragments and sequencing. PCR products were purified for sequencing reactions by precipitation with 20 % PEG_{8000} in 2·5 M NaCl. Alternatively, oligonucleotide primers were separated from the PCR product by gel filtration using a Sepharose CL4B column (Pharmacia) under gravity (Almond *et al.*, 1992). Direct sequence analysis of both strands was determined using a modified Sequenase (Amersham) protocol and T7 polymerase (Pharmacia). Terminations were carried out at 50 °C. ABI PRISM Big Dye Terminator kits were used with a cycling profile recommended by the manufacturer. Additional sequencing primers to those used to generate the product were at conserved sites within *nef* in either the sense orientation [5′-GGGCTACGAGAGAGACTCTT-3′ (nt 8561–8580), 5′-GCGAGTGGATCCAAGAAG-3′ (nt 8525–8542), 5′-GATAAGGGGGGACTGGAAGGGATG-3′ (nt 8891–8914), 5′-CAGCAAGGAGACTTTATG-3′ (nt 8700–8717) and 5′-TGGCT-ATGGAAGCTAGT-3′ (nt 9035–9051)] or the antisense orientation [5′-TGCTTTCAGTTTTGCCTTC-3′ (nt 9250–9268), 5′-GTACTAG-CTTCCATAGCC-3′ (nt 9036–9053), 5′-CATCATCTGAATCTACAT-3′ (nt 8785–8801) and 5′-GTTCTCCATGGGGTATTC-3′ (nt 8716–8733)], also numbered according to HIV-2_{ISY}. Sequence contigs were generated using the Gap4 program (Staden software package; Bonfield *et al.*, 1995) from a consensus generated from both sense and antisense strands and analyses were performed using the GCG software package and the CLUSTAL W program to generate multiple sequence alignments (Thompson *et al.*, 1994).

Cloning of *nef* alleles. PCR products from a selected number of individuals were cloned into the pGEM-T plasmid vector with a TA Cloning kit (Promega). Ligated DNA was transfected into JM109 cells (Promega) and plasmid DNA purified using QIAprep kits (Qiagen). Clones were sequenced as described above.

Phylogenetic analysis. Nucleotide sequences were aligned and evaluated with CLUSTAL W and phylogenetic trees were constructed from the matrix built by the neighbour-joining method (NEIGHBOR, version 3.5c; Felsenstein, 1989). Nucleotide substitution rates per site were estimated for each pairwise sequence comparison on the basis of the Kimura two-parameter model using DNADIST. Bootstrap analysis was performed with SEQBOOT and CONSENSE with 100 re-samplings. Trees were rooted with SIV_{MM239} and drawn with NJPLOT; reference sequences were obtained from the Los Alamos database. Nucleotide sequences reported in this study have been submitted to the EMBL nucleotide sequence database (accession nos AJ344369–AJ344415). Epidemiological and clinical aspects were evaluated statistically with SPSS (statistical package for social science).

Quantification of HIV-2 RNA levels in plasma. EDTA-treated plasma samples were stored at −80 °C until levels of HIV-2 viral RNA were assessed using a quantitative RT-PCR assay (Berry *et al.*, 1998). In brief, RNA was extracted according to the method of Boom *et al.* (1990) and RT-PCR performed, in duplicate, with HIV-2-specific LTR oligonucleotide sequences in single-tube assays using Titan reagents (Roche). Amplification products were quantified by chemiluminescence using an internal HIV-2 probe linked to alkaline phosphatase (Oswel). The reliable level of sensitivity of the assay was 200 copies ml^{-1} using an input plasma volume of 100 μl. For statistical analyses, plasmas with <200 copies ml^{-1} were assigned an arbitrary value of 20 copies ml^{-1}.

RESULTS

Epidemiological, clinical and virological characteristics of the study population

Of the 37 HIV-2-infected individuals studied (Table 1), 24 (64·9 %) were female and 13 (35·1 %) were male, with a mean overall age of 39·4 years ($n=37$, range 8–67 years). The main route of transmission was heterosexual (30/37, 81·2 %), the remaining seven being transfusion-associated infections. HIV-2 infection was acquired in Portugal in 14 (37·8 %) individuals; the other 23 (62·2 %) infections were most likely acquired in Africa: Guinea Bissau ($n=12$, 32·4 %), Cape Verde ($n=4$, 10·8 %), Angola ($n=1$, 2·7 %) or an unidentified African country ($n=6$, 16·2 %). Following clinical examination, 11/37 (29·7 %) were symptomatic (HIV-related illness/AIDS) and 26 (70·3 %) were asymptomatic, including three individuals (1069, PL and 280) who had been infected for 20 years or more. CD4 lymphocyte counts and HIV-2 viral RNA loads were available for the majority of, but not all, individuals. In the asymptomatic group, mean CD4 counts were 548 ($n=17$, range 161–1346 cells mm^{-3}) and mean HIV-2 RNA levels were 817 copies ml^{-1} ($n=21$, range <200–6920). In the symptomatic group, mean CD4 counts were correspondingly lower at 328 ($n=8$, range 46–966 cells mm^{-3}) and the mean HIV-2 viral RNA load was 20 times higher than that in the asymptomatic group at 17 051 copies ml^{-1} ($n=7$, range <200–62 950).

HIV-2 *nef* sequences in Portugal are subtype A and full-length

A total of 44 *nef*/LTR proviral sequences spanning the entire *nef* gene were amplified from 37 HIV-2-infected individuals (792 bp, Fig. 1). Consensus sequences derived from each of the 37 individuals from bulk product were substantially full-length with little evidence of deletions, truncations or other disruptions to *nef*. Predicted amino acid sequences (see below) suggested a functional HIV-2 Nef protein to be present in all individuals studied, irrespective of any other factor. Subsequent bleeds from six individuals yielded a *nef* sequence with few nucleotide or amino acid differences from the first sequence; hence, comparisons of *nef* variation were made on the first, bulk amplification of each patient. This observation was confirmed further by dilution experiments of template DNA. Phylogenetic relationships between Portuguese *nef* sequences were compared with HIV-2 subtype A (HIV-2_{ROD}, HIV-2_{BEN}, HIV-2_{ALI}, HIV-2_{ISY} and HIV-2_{ST}), subtype B (HIV-$2_{D205/ALT}$, HIV-2_{UC1} and HIV-2_{EHO}) and SIV_{MM239} (Fig. 1). All Portuguese sequences were HIV-2 subtype A, with no other HIV-2/SIV_{SM} subtypes (B–G) identified. No phylogenetic relationship was established between *nef* sequence and disease status and no statistical link between HIV-2 *nef* sequence and route of transmission, either sexual or blood transfusion, was apparent (data not shown).

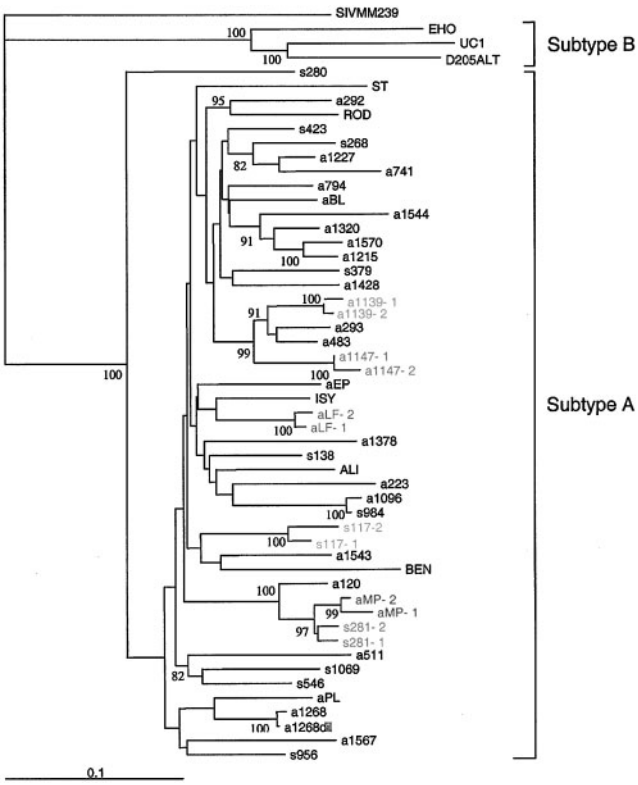

Fig. 1. Phylogenetic tree based on 44 *nef*/LTR sequences derived from proviral DNA fragments amplified by PCR from PBMCs of 37 individuals, five reference HIV-2 subtype A sequences (HIV-2_{BEN}, HIV-2_{ALI}, HIV-2_{ISY}, HIV-2_{ROD} and HIV-2_{ST}) and three reference sequences for subtype B (HIV-2_{EHO}, HIV-$2_{ALT/D205}$ and HIV-2_{UC1}). SIV_{MM239} was used as the outgroup sequence. A second sequence obtained from each of six individuals is indicated (1139-1/1139-2, 1147-1/1147-2, LF-1/LF-2, MP-1/MP-2, 281-1/281-2 and 117-1/117-2). Prefixes of 'a' and 's' indicate asymptomatic and symptomatic, respectively. Samples suffixed 'dil' indicate that the sequence was derived from a 1/10 dilution of the original DNA material. Bootstrap values above 80 %, based on 100 replicates, are indicated at the branching nodes. Evolutionary distances were estimated using the Kimura two-parameter model and genetic relationships were determined using the neighbour-joining method. Bar, 10 % sequence divergence.

Analysis of LTR sequences

Sequence data for the non-overlapping U3 region of the LTR was available for 31 individuals, indicating no disruptions to the structure of the LTR. Comparisons of the core promoter and enhancer elements important in the transcriptional activity of the LTR, including PuB-1, PuB-2 p-*ets*, peri-κB and NF-κB sites up to the Sp1 sites and TATA box, indicated further sequences to be subtype A with high levels of sequence conservation *in vivo*. Sequences are not shown but have been filed in GenBank.

HIV-2 *nef* amino acid variation

Alignment of predicted amino acid sequences for the entire *nef* ORF for all 37 Portuguese HIV-2-infected individuals is shown in Fig. 2; the first sequence obtained for each individual is presented. The Portuguese HIV-2 *nef* consensus sequence was also compared with other lentivirus *nef* sequences, including a prototypic HIV-1 group M virus (HIV-1_{LAI}), SIV_{CPZ} (Gab), from chimpanzees, and SIV_{MM239}, an experimental infection of Asian macaques derived originally from SIV_{SM}. Predicted coding sequences were compared for 10 putative regions considered important for HIV-1, HIV-2 or SIV *nef* function (Fig. 2). Throughout *nef*, a number of key regions were conserved among the Portuguese sequences, irrespective of clinical status, virus load or CD4 count, and deletions that might disrupt such sites were not evident. Moreover, only one patient showed evidence of a deleted *nef* sequence: patient 379 (symptomatic/AIDS) exhibited a *nef* deletion of 8 aa downstream of a suggested N-terminal RNA-binding motif (Echarri *et al.*, 1997).

The myristoylation signal (aa 1–6, HIV-2 consensus GASGSK) was 100 % conserved and GPG(V/T) sequences predictive of a β-turn were also conserved, with a T present in one AIDS patient (281) and in six asymptomatic individuals. Comparative analyses of other regions identified as being relevant to *nef* function exhibited a more complex pattern of variation, particularly the SH3-binding PxxP motif, the di-leucine-based region important for downregulation of CD4 (in HIV-1 the ENTSLL motif) and sites for putative SH2 interactions based on tyrosine-sorting signals.

(i) Analysis of HIV-2 *nef* for putative tyrosine-based sorting motifs. The canonical tyrosine-based sorting motif Yxxϕ, present as Y^{28}GRL and Y^{39}SQS in SIV_{MAC239} and Y^{39}SRF in HIV-2_{ROD}, is Y^{39}LQS in these Portuguese sequences, with changes to this basic configuration infrequent. Patient 984, an AIDS patient with a CD4 count of 195 and viral RNA levels of 8830 copies ml^{-1}, exhibited a R→Y mutation at position 24; patients LF and PL exhibited C→Y mutations at position 28. However, additional tyrosine-based motifs in HIV-2, such as YE *nef* described for SIV_{MAC239}, were not present in any HIV-2 sequences, including those in the symptomatic group. Interestingly, the most conserved tyrosine residue at position 39 was mutated in two AIDS patients (117-1 and 1378) to phenylalanine, serine or cysteine. There was no apparent relationship between disease status and tyrosine-based motifs.

(ii) Di-leucine-based motifs for CD4 downregulation. Cellular di-leucine-based sorting pathways required for CD4 downregulation in HIV-1 are characterized by a core hexameric motif ENTSLL (ExxxLL), which conforms to the consensus E/DXXXLϕ found in cellular transmembrane proteins (Craig *et al.*, 1998). HIV-2 *nef* sequences analysed for a comparable motif ($L^{198}L^{199}$) indicated variation at only L^{199}, present as leucine, valine or

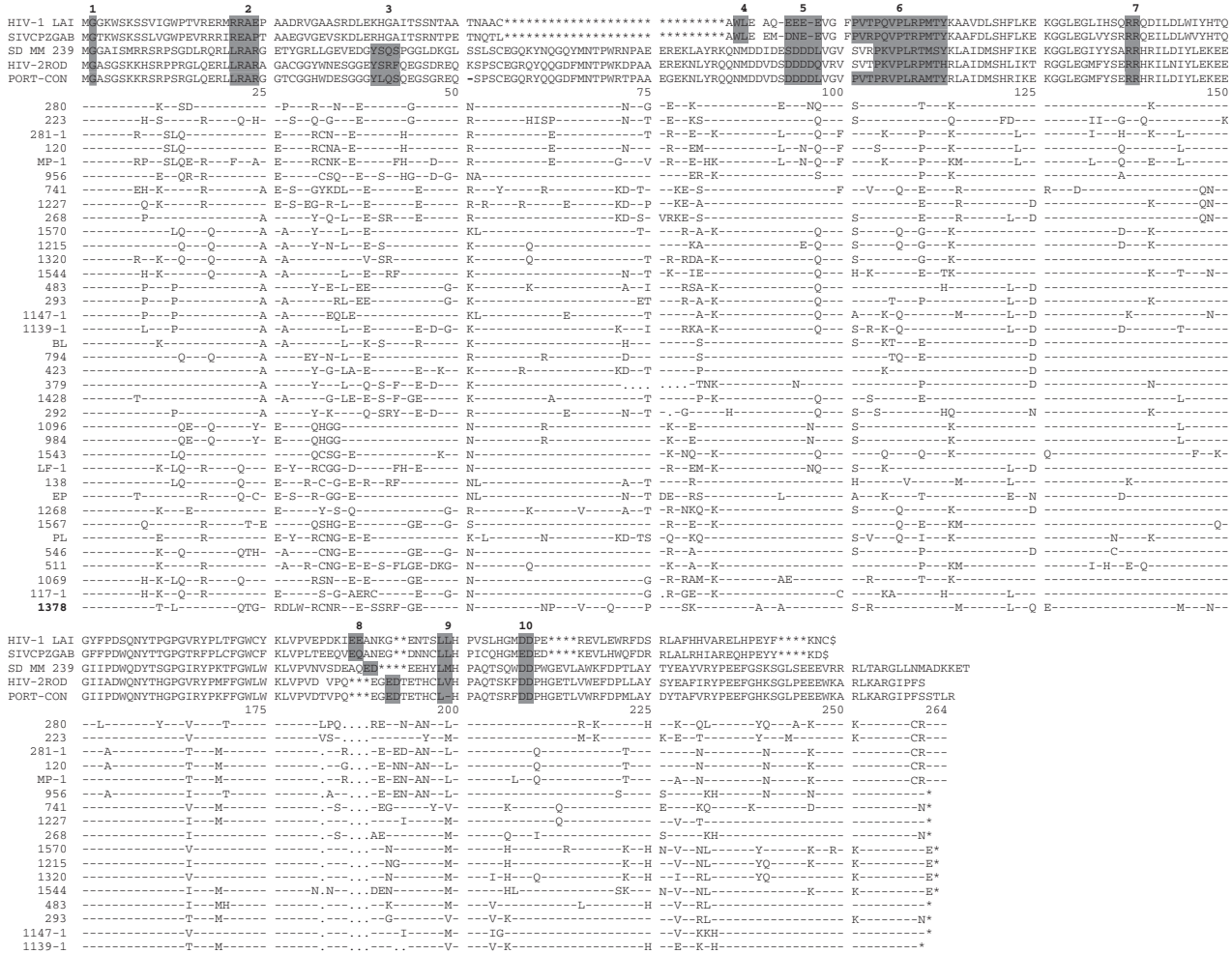

Fig. 2. Alignment of the *nef* gene amino acid sequence of 37 HIV-2 infected individuals in the Portuguese cohort related to HIV-1, SIV_{CPZ}, SIV_{MAC}, $HIV\text{-}2_{ROD}$ and the Portuguese HIV-2 *nef* consensus sequence. Sites important for *nef* function from the N- to the C-termini are highlighted for the different groups as follows: 1, myristoylation site; 2, N-terminal helix and putative MHC-I downregulation site; 3, tyrosine-based adaptor protein (AP) recruitment motif; 4, CD4-binding site (as characterized for HIV-1); 5, acidic cluster (MHC-I downregulation); 6, proline-based repeat sequence (MHC-I and SH3 binding); 7, PAK binding; 8, COP-1 recruitment; 9, di-leucine-based AP recruitment (HIV-1 *nef*); and 10, V-ATPase and Raf-1 binding.

methionine, with L^{198} conserved throughout. Interestingly, patients 280, 120, 281, MP and 956 grouped into an EANCLL configuration, three of which (281, MP and 956) had tetra-proline $(PxxP)_3$ motifs. Overall, 15/37 (40·5 %) individuals had the $L^{198}L^{199}$ motif, with seven (19 %) substituting the leucine at position 199 for valine and 15 (40·5 %) for methionine. The number of LL motifs was distributed evenly between asymptomatic and symptomatic groups at eight and seven, respectively, forming an asymptomatic consensus of LM and symptomatic consensus of LL. Interestingly, the region immediately preceding this was relatively heterogeneous across all

individuals (aa 185–195, Fig. 2), covering a putative site for β-COP recruitment. However, there was no apparent relationship between this variability and disease status.

(iii) Putative SH3-binding domains characterized by proline-rich (PxxP) motifs. The proline-rich motif (P^{104}xxPLR) was assessed for variation in more detail. Within this core region, $R^{105}\rightarrow$K was observed frequently among these Portuguese sequences to form the sequence P^{104}KVPLR, with the arginine residue at position 109. This adds to the observation that this region, which mediates interaction with SH3 ligands, is evolutionarily conserved among primate lentiviruses and is present as a minus-orientation binding consensus. Moreover, HIV-2 *nef* sequences generated in this study exhibited a broad range of PxxP configurations (Fig. 2, Table 2). An HIV-1-like $(PxxP)_3$ configuration was observed for six individuals (16·2 %), three asymptomatic (MP, 511 and 293) and three symptomatic (281, 956 and 379). Conversely, disrupted minimal PxxP motifs were associated exclusively with an asymptomatic profile (11/26, 42·3 %), where the P^{107} residue was mutated to glutamine in nine individuals (PL, 1567, 1215, 1570, 1147, 794, 1268, 741 and 1139) and the P^{104} residue to serine in two (120 and 292), the only individuals not preserving the proline at position 104. Disruption of the core P^{104}xxPLR region, $P^{107}\rightarrow$Q ($n=9$) and $P^{104}\rightarrow$S ($n=2$) was associated strongly with an asymptomatic phenotype, although patient PL who had been infected for 24 years had a relatively low CD4 count in 1997 (127 cells mm^{-3}). The tyrosine (Y^{145}) forming part of a putative hydrophobic pocket (Y^{145}LEKE^{149}E^{150}) distal to the PxxP region implicated in SH3 binding in HIV-1 *nef* (Collette *et al.*, 2000) was absolutely conserved, with minimal changes to other residues associated with this motif, with E^{149} mutated to either a glutamine or a lysine in patients 1543 and 1567 and E^{150} mutated to lysine in patient 223; all three individuals were asymptomatic. In addition, there was an $I^{144}\rightarrow$L substitution in six individuals (281, MP, 120, 984, 1428 and 1096) immediately prior to Y^{145} and similar mutations in three other individuals: $I^{144}\rightarrow$T ($n=2$) and $I^{144}\rightarrow$M ($n=1$), although the significance of these changes is not known. Other sequences associated with the $(PxxP)_3$ region in HIV-1 is a phenylalanine residue (F^{122}). In HIV-2, this appeared in four individuals (281, 120, MP and 741), although the consensus of the comparable residue is a valine. No additional PxxP motifs in other regions of *nef* were identified in any of these HIV-2 sequences. Variability in the central PxxP region was investigated further for associations with disease status and virus load.

Relationship between PxxP sequence and plasma virus load

The spectrum of proline configurations observed in these patients was related to HIV-2 viral RNA levels determined for 27 different individuals at the first time-point (Table 2). Overall, HIV-2 virus load was low, reflecting the high proportion of asymptomatic persons, with 77·7 % (21/27) having $\leqslant$500 copies ml^{-1} and 59·3 % (16/27) having $\leqslant$200 copies ml^{-1}. Only 3 of 21 individuals with $\leqslant$500 copies plasma RNA ml^{-1} were symptomatic (patients 423, 546 and 268). Of these, patient 423 had a CD4 count of 996 cells mm^{-3} and patients 546 and 268 had counts of 297 and 340 cells mm^{-3}, respectively. There was a non-significant trend for increasing levels of HIV-2 RNA with additional prolines, although the number of individuals with high virus load was small, with only three individuals having relatively high levels of HIV-2 RNA (patients 379, 1069 and 984), with 62 950, 34 750 and 8830 copies ml^{-1}, respectively; all three were symptomatic. Of four individuals who possessed the tetra-proline motif and for whom virus load data were available, two (patients 379 and 117-2) had high virus loads; the other two individuals (511 and MP) had low/undetectable virus loads and were asymptomatic, with CD4 counts of 473 and 610 cells mm^{-3}, respectively.

Of the 20 individuals with an intact P^{104}xxPLR motif but not a tetra-proline configuration, eight (117-1, 423, 280, 268, 984, 138, 1069 and 546) had evidence of symptomatic infection/AIDS. The second sample taken from patient 117 at 5 years later indicated an $A^{107}\rightarrow$P change, forming a $(PxxP)_3$ motif, and an $A^{106}\rightarrow$V change. The Nef protein of all 20 would seem to be capable of interacting with SH3 in this region. Further subdivisions of this group indicate that 11 individuals have only the minimal P^{104}xxPLR sequence ($n=3$ symptomatic: 268, 984 and 138; $n=8$ asymptomatic: 1378, 223, EP, 1096, 1544, 1320, BL and LF); eight ($n=4$ symptomatic: 280, 1069, 423 and 117; $n=4$ asymptomatic: 1543, 1428, 483 and 1227) had an additional P^{101} (P^{101}xxPxxPLR) and one symptomatic patient (546) had a PxxPLRP^{110} configuration.

Finally, 11 individuals had a disruption in the central region of the PxxP motif, mostly as a $P^{107}\rightarrow$Q mutation ($n=9$), all of which were asymptomatic; this was statistically significant (Fisher's exact test, $P=0{\cdot}026$). There was also a non-significant trend for lower virus loads with fewer prolines present (Table 2). Levels of viral RNA correlated more closely to clinical status, with a significantly higher plasma virus load in symptomatic than asymptomatic individuals (mean HIV-2 RNA levels were 17 051 and 817 copies ml^{-1}, respectively; unpaired Student *t*-test, $P=0{\cdot}0026$), whereby all symptomatic individuals possessed an intact core P^{104}xxPLR motif. Conversely, disruptions to the P^{104}xxPLR core were associated with lower virus loads, which only exceeded 5000 copies ml^{-1} in one asymptomatic individual (PL) who had been infected for 24 years and who had a low (127 cells mm^{-3}) CD4 count.

Longitudinal changes in *nef* and cloned *nef* sequences

A second sample obtained from each of six individuals, two symptomatic (281-2 and 117-2) and four asymptomatic (LF-2, MP-2, 1147-2 and 1139-2), allowed comparison with *nef* sequences from earlier time-points. The close genetic

Table 2. Variation of the PxxP region of HIV-2 *nef* in the 37 HIV-2-infected individuals related to levels of plasma virus load (27 individuals) and CD4 lymphocyte counts (25 individuals)

Mean virus load and CD4 counts are indicated for three groups categorized according to the configuration of proline residues ranging from a tetra-proline configuration to a disrupted minimal core $P^{104}xxP^{107}$ motif. NA, Not available.

Reference	HIV-2 *nef* sequence variation of PxxP motif										Viral RNA load (copies ml^{-1})	CD4 count (cells mm^{-3})	Clinical status
Consensus	P^{101}	V	T	P	R	V	P	L	R	A^{110}			
Tetra-proline configuration $(PxxP)_3$													
281-1	**P**	V	T	**P**	K	V	**P**	L	R	**P**	NA	46	**Symptomatic**
379	**P**	V	T	**P**	R	V	**P**	L	R	**P**	62 950	366	**Symptomatic**
956	**P**	V	T	**P**	R	V	**P**	L	R	**P**	NA	220	**Symptomatic**
MP-1	**P**	V	T	**P**	K	V	**P**	L	R	**P**	<200	610	Asymptomatic
293	**P**	V	T	**P**	R	T	**P**	L	R	**P**	NA	519	Asymptomatic
511	**P**	V	T	**P**	R	V	**P**	L	R	**P**	500	473	Asymptomatic
Mean ($n=6$)											21 156 ($n=3$)	372 ($n=6$)	
Intact $PxxP^{107}$ sequence													
138	H	V	T	**P**	R	V	**P**	V	R	A	NA	NA	**Symptomatic**
984	S	V	T	**P**	R	V	**P**	L	R	A	8 830	195	**Symptomatic**
268	S	V	T	**P**	R	V	**P**	L	R	E	200	340	**Symptomatic**
BL	S	V	T	**P**	K	T	**P**	L	R	E	<200	288	Asymptomatic
EP	A	V	T	**P**	K	V	**P**	L	R	T	<200	490	Asymptomatic
LF-1	S	V	T	**P**	K	V	**P**	L	R	A	400	437	Asymptomatic
223	S	V	T	**P**	R	V	**P**	L	R	A	NA	161	Asymptomatic
1096	S	V	T	**P**	R	V	**P**	L	R	A	<200	902	Asymptomatic
1320	S	V	T	**P**	R	V	**P**	L	R	G	200	444	Asymptomatic
1378	S	V	R	**P**	R	V	**P**	L	R	A	NA	NA	Asymptomatic
1544	H	V	K	**P**	R	V	**P**	L	R	E	NA	730	Asymptomatic
117-1	**P**	V	T	**P**	K	A	**P**	L	R	A	NA	199	**Symptomatic**
280	S	V	T	**P**	R	V	**P**	L	R	T	NA	NA	**Symptomatic**
423	**P**	V	T	**P**	K	V	**P**	L	R	A	<200	966	**Symptomatic**
1069	**P**	V	R	**P**	R	V	**P**	L	R	T	34 750	NA	**Symptomatic**
483	**P**	V	T	**P**	R	V	**P**	L	R	A	200	NA	Asymptomatic
1227	**P**	V	T	**P**	R	V	**P**	L	R	E	<200	NA	Asymptomatic
1428	**P**	V	S	**P**	R	V	**P**	L	R	E	<200	NA	Asymptomatic
1543	**P**	V	T	**P**	Q	V	**P**	L	R	Q	<200	993	Asymptomatic
546	S	V	T	**P**	R	V	**P**	L	R	**P**	<200	297	**Symptomatic**
Mean ($n=20$)											3 195 ($n=14$)	495 ($n=13$)	
Disrupted minimal motif P^{104}/P^{107}													
PL	S	V	V	**P**	R	V	Q	L	R	I	6 920	127	Asymptomatic
741	**P**	V	V	**P**	R	V	Q	L	R	E	<200	NA	Asymptomatic
794	**P**	V	T	**P**	R	T	Q	L	R	E	<200	NA	Asymptomatic
1139-1	S	V	R	**P**	K	V	Q	L	R	A	<200	NA	Asymptomatic
1147-1	A	V	T	**P**	K	V	Q	L	R	A	2 259	407	Asymptomatic
1215	S	V	T	**P**	R	V	Q	L	R	G	<200	430	Asymptomatic
1268	S	V	T	**P**	R	V	Q	L	R	A	3 730	529	Asymptomatic
1567	**P**	V	T	**P**	R	V	Q	L	R	E	3 491	1 346	Asymptomatic
1570	S	V	T	**P**	R	V	Q	L	R	G	<200	427	Asymptomatic
120	**P**	V	T	S	R	V	**P**	L	R	**P**	NA	NA	Asymptomatic
292	S	V	T	S	R	V	**P**	L	R	A	<200	NA	Asymptomatic
Mean ($n=11$)											1 650 ($n=10$)	544 ($n=6$)	

relationship between the two sequences (Fig. 1) indicated no difference in *nef* sequence measured at two distinct time-points. However, some evidence for increased nucleotide distances was observed, relating to sampling times that ranged from 0·6 to 1·5 % for 6 months to 1 year (sequences 1147-1/1147-2, 1139-1/1139-2 and 281-1/281-2), 1·6–2·4 % at 3 years (LF-1/LF-2 and MP-1/MP-2) and 3·4 % for a 5 year break between sampling (117-1/117-2). These findings (approximately 0·5–1 % per year) are similar to values estimated previously (Shankarappa *et al.*, 1999; Pieniazek *et al.*, 1999).

Clones isolated from three patients with disparate *nef* sequences were also sequenced: one AIDS patient (sample 117-2), one asymptomatic individual (sample MP-2) and one asymptomatic individual (1268) with disruptions in both proline-rich and di-leucine regions. No major variation in *nef* sequence, either synonymous or non-synonymous, was identified in individual clones compared to bulk sequence derived from a 'swarm' of proviruses and overall, the cloned data confirmed the observed changes in *nef* sequence to be present as majority sequence (Fig. 3).

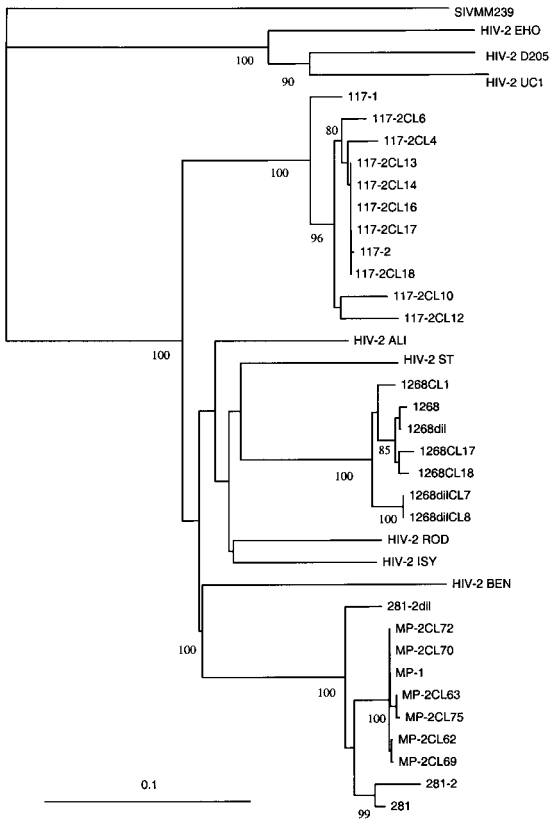

Fig. 3. Phylogenetic tree of *nef* sequences derived from 20 individual clones (CL) obtained from three HIV-2-infected individuals (117, 1268 and MP), five reference HIV-2 subtype A sequences (HIV-2_{BEN}, HIV-2_{ALI}, HIV-2_{ISY}, HIV-2_{ROD} and HIV-2_{ST}) and three reference sequences for subtype B (HIV-2_{EHO}, HIV-$2_{ALT/D205}$ and HIV-2_{UC1}). SIV_{MM239} was used as the outgroup sequence. Data obtained from bulk sequence analysis (117-1, 281-1, 1268 and MP-1). The suffix 'dil' indicates sequences derived from an original 1/10 dilution of template DNA. The majority of clones from patients 117 and MP were obtained from the second time-point (117-2 and MP-2). Bootstrap values above 80 %, based on 100 replicates, are indicated at the branching nodes. Evolutionary distances were estimated using the Kimura two-parameter model and genetic relationships were determined using the neighbour-joining method. Bar, 10 % sequence divergence.

DISCUSSION

The natural variability of *nef* and its relation to putative *nef* function in HIV-2-infected individuals, where there is a higher proportion of long-term survivors compared to HIV-1, is not well documented. One previous study of HIV-2 *nef* sequences reported a higher frequency (10–15 %) of disrupted *nef* alleles in asymptomatic HIV-2-infected individuals (Switzer *et al.*, 1998), implying a role for *nef* in the pathogenesis of HIV-2 infection. In this cross-sectional analysis of HIV-2-infected individuals living in Portugal, we found little evidence of major *nef* disruption, akin to the situation in HIV-1, whereas more discrete changes in *nef* sequence likely to impact on specific aspects of *nef* function were identified. All HIV-2 *nef* sequences were of a single subtype (A), reflecting the demographical links with Guinea Bissau (Grassly *et al.*, 1998) and contrasting with the wide spectrum of *nef* sequences from HIV-1 group M (Jubier-Maurin *et al.*, 1999). Interestingly, two of the six disrupted sequences described by Switzer *et al.* (1998) were HIV-2 subtype B infections, not subtype A, originating from the Ivory Coast where HIV-2 subtype B is prevalent (Pieniazek *et al.*, 1999). Hence, truncation as a feature of *nef* disruption per se seems unlikely to account for the higher proportion of attenuated HIV-2 phenotypes observed frequently, particularly if the infecting virus belongs to subtype A. Similarly, LTR structures containing core promoter and enhancer elements important in gene regulation and virus expression (Clark *et al.*, 1995; Leiden *et al.*, 1992; Markovitz *et al.*, 1992) were also intact with a subtype A identity and conserved *in vivo* (Berry *et al.*, 2001).

Studies of SIV_{MAC} have indicated that subtle differences in *nef* can alter dramatically the *in vivo* characteristics of virus replication (Whatmore *et al.*, 1995) and a broad body of data exists relating the structure of HIV-1 *nef* with functional characteristics (Geyer *et al.*, 2001; Piguet & Trono, 1999). Sequence-specific motifs involved in key functions of *nef*, such as enhancement of virus infectivity, downregulation of CD4 and interaction with T-cell signalling pathways, have the potential to invoke a variety of cellular pathways that can have an impact on pathogenesis. Hence, we embarked on a more systematic study of the HIV-2 *nef* sequence based on known properties of HIV-1 or SIV *nef*.

Key features of *nef* function, such as the myristoylation signal, were absolutely conserved among HIV-2 sequences, re-enforcing the notion that *N*-myristoylation is critical for the biological activity of all *nef* proteins (Harris, 1995).

Analysis of motifs in HIV-2 *nef* indicated no difference in N-terminal tyrosine-based sorting signals, with no additional tyrosine residues, such as RQ→YE (YE *nef*), which, under certain circumstances, have been linked to acute lymphocyte activation and pathogenicity of some strains of SIV involving SH2 interactions (Luo & Peterlin, 1997). Interestingly, interactions of SIV *nef* with human Lck or Hck do not seem to be mediated via the consensus proline motif, as is the case for HIV-1 *nef* (Collette *et al.*, 1996), as SIV *nef* demonstrates the ability to bind Lck and Hck SH2 domains, suggesting the multiple mechanisms by which *nef* is able to bind to and regulate Src kinases (Greenway *et al.*, 1999). The role of Src family kinases and the interaction with either SH2 or SH3 remains to be clarified for HIV-2.

In this respect, the natural variability of proline sequences that could mediate putative SH3 interactions was an important finding in this study. This variability was wider than that recognized previously and appeared to be related, at least in part, to disease status. There was a significant correlation between a P^{107}→Q mutation at the centre of the P^{104}xxPLR core and an asymptomatic phenotype, which formed one end of a wide spectrum of proline configurations. Though numbers were relatively small, an increased number of proline residues was accompanied by increased virus load. However, tetra-proline $(PxxP)_3$ sequences in three asymptomatic individuals were also identified and RNA levels in 5/6 patients with $(PxxP)_2$ motifs were below the level of detection. Moreover, the wide spectrum of PxxP configurations encompassed all theoretical combinations ranging from $(PxxP)_3$ to P^{104}xxQ. This situation contrasts markedly with HIV-1 where, even in studies of long-term survivors, the consensus tetra-proline motif remains preserved, with no difference in proline motif configurations between rapid progressors and long-term survivors (Kirchhoff *et al.*, 1999).

Interestingly, in studies of SIV-infected macaques, there appears to be a strong selection pressure for a functional SH3-binding ligand *in vivo*, associated with kinase interactions (Khan *et al.*, 1998), although other studies cast doubt on the functional significance of the PxxP motif in leading to disease induction during the acute phase (Lang *et al.*, 1997). However, the latter study seems unlikely to be representative of the broader picture of disease development in simian AIDS. Moreover, studies in the TgCD4 mouse model for HIV-1 *nef* have identified the PxxP region to be a crucial determinant of pathogenicity (Hanna *et al.*, 2001). The natural variability of proline-rich sequences in these HIV-2-infected individuals is compelling, given the potential for this region to interact with a variety of cellular partners and to modulate virus replication at the level of the T-cell receptor signalling environment (Fackler *et al.*, 2001). The fact that a P^{107}→Q mutation was found only in asymptomatic individuals who also had low levels of peripheral viral RNA suggests a role for PxxP–SH3 interactions in influencing pathogenesis. Hence, one could envisage a situation where the secondary structure of the polyproline type II helix is stabilized by the additional proline residues, with the critical P^{104}xxP^{107} providing the important hydrophobic contacts with binding of the SH3 domain. The trend for increasing plasma virus load with increasing numbers of proline residues may reflect this and disruption of the binding consensus would have a major impact on putative SH3 interactions.

Immunological control also seems more effective for HIV-2 than HIV-1 (Whittle *et al.*, 1998), with lower levels of immune activation and reduced levels of apoptosis (Michel *et al.*, 2000) reflecting the low virus steady-state of many HIV-2 infections (Andersson *et al.*, 2000; Ariyoshi *et al.*, 2000; Berry *et al.*, 1998, 2002; Popper *et al.*, 1999). Recent data have indicated a role for *nef* in apoptotic regulatory pathways (Geleziunas *et al.*, 2001; Wolf *et al.*, 2001; Fackler & Baur, 2002) and aspects of cell signalling that may have a bearing on virulence (Simmons *et al.*, 2001). Functional data are required to determine which cellular signalling pathways may be involved and define a more precise role for HIV-2 *nef* PxxP–SH3 interactions. Whether changes in a single region or gene such as *nef* might be expected to influence pathogenicity directly in the face of multiple virus–host interactions, including immunological considerations, is an interesting question. Certainly, the natural variation observed in the PxxP region was unexpected and would suggest that this region may be one of the more relevant regions for HIV-2 *nef* function *in vivo*. Molecular clones derived from this study are currently being taken forward to address some of these issues in order to provide a fuller understanding of the role of *nef* in the pathogenesis of HIV-2 infections.

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