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Nef Proteins of Distinct HIV-1 or -2 Isolates Differ in Their Binding Properties for HCK: Isolation of a Novel Nef Binding Factor with Characteristics of an Adaptor Protein

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The Nef gene of the human and simian immunodeficiency viruses HIV and SIV has been implicated in pathogenicity; however, the mechanism by which Nef induces disease is still unknown. An impact on signal transduction in cells has been suggested by the interaction of Nef from an HIV-1 strain and tyrosine kinases like HCK and LCK as well as serine/threonine kinases. We have confirmed the binding of HCK to HIV-1 subtype B Nef and demonstrated an equally strong interaction with a subtype E Nef protein but weaker binding to Nef of HIV-2 subtype A (HIV-2_{D194}). No binding, however, was observed to HIV-2 subtype B Nef (HIV-2_{D205}). Instead, this protein bound to a novel cellular protein, *Nefin1*, with characteristics of an adaptor protein and strong expression in all human hematopoietic tissues. *Nefin1* binds through an amino-terminal domain, which is related to SH3 domains. For interaction of Nef with *Nefin1*, the PxxP motif and the three-dimensional conformation of the molecule appear necessary. In conclusion, this study demonstrates that Nef proteins of divergent strains of HIV-1 and HIV-2 may use different elements of signal transduction pathways for the induction of pathogenicity *in vivo*. © 1998 Academic Press

INTRODUCTION

Nef is one of the early genes of HIV and SIV. Nef deletion mutants of SIV_{mac239} show poor replication *in vivo* and AIDS-like symptoms do not evolve in adult animals (Kestler *et al.,* 1991). For HIV, a reduced pathogenicity of deletion mutants has also been shown (Deacon *et al.,* 1995; Kirchhoff *et al.,* 1995). However, the molecular mechanisms by which nef induces pathogenicity are still unclear.

In cell culture, different effects of Nef have been described (for review see Trono, 1995). Downregulation of CD4 from the cell membrane is dependent on an acidic stretch of amino acids in the amino-terminal region of Nef. This motif is conserved between the Nef proteins of different HIV and SIV strains and the effect seems to be mediated by interaction with a dileucine motif in the carboxy-terminal tail of CD4 (Salghetti *et al.,* 1995). However, the activity of different Nef alleles varies considerably (Mariani and Skowronski, 1993; Michael *et al.,* 1995). In addition to CD4 downregulation an increased infectivity of virions produced from cells in which Nef is present was observed. However, the magnitude of the effect on infectivity varies and may correlate to the activation state of the target cell (Miller *et al.,* 1994, 1995; Chowers *et al.,* 1994; Aiken and Trono, 1995; Schwartz *et al.,* 1995). This effect is dependent on a proline-rich region in Nef (Goldsmith *et al.,* 1995; Saksela *et al.,* 1995), which is also conserved between different isolates and resembles an SH3 domain binding site. The SH3 domain of the human protein tyrosine kinase HCK interacts with this domain of HIV-1_{NL4-3} Nef *in vitro* (Saksela *et al.,* 1995). The association of the proline-rich region of Nef with HCK was shown to promote an increase of the activity of HCK (Moarefi *et al.,* 1997), which results from a displacement of the newly identified intramolecular interaction of the SH3 domain of HCK with the SH2-kinase linker region (Sicheri *et al.,* 1997).

HIV-2_{D205} is a highly divergent HIV-2 isolate (Dietrich *et al.,* 1989) and was the first identified member of the HIV-2 subtype B family, which is not as widespread as the more common HIV-2 subtype A. Because of the genetic divergence even in the SH3 binding motif of this isolate, we investigated the binding properties of HIV-2_{D205} in comparison to more common strains of HIV-2 and different subtypes of HIV-1. While the interaction of Nef from HIV-1_{LAI} with HCK could be confirmed in a two-hybrid system and an HIV-1 subtype E isolate showed equally strong binding, HIV-2_{D205} Nef did not bind to HCK and HIV-2_{D194} Nef (an HIV-2 subtype A isolate with high replication capacity in macrophages, Kühnel *et al.,* 1989) bound only weakly. In addition, a novel Nef interaction partner, *Nefin1*, with characteristics of an adaptor protein, was isolated. This protein inter-

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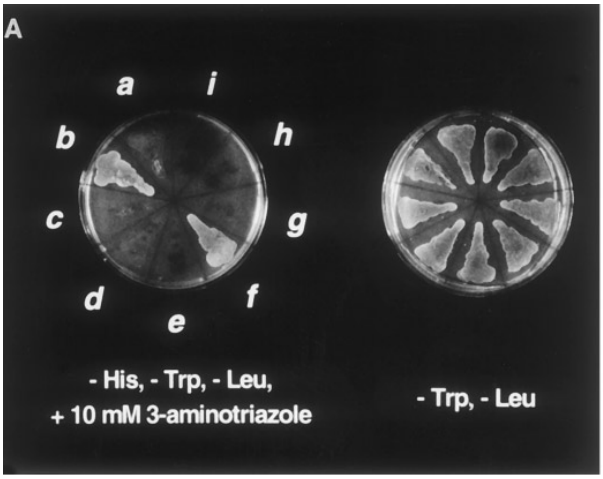

B

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LAI    MGGKWSKSSVVGWPTVRERM-RRAEPAADGVGAASRDLEKHGAITSSNTA   49
D205   ..SAG..KRSERQQGL..KLL.VP.RPYGRLSGER.EQSSRSP-GE.DKD   49

LAI    ATNAACAWL----------EAQEEEEVGFPVTPQVPLRPMTYKAAV      85
D205   KNSPS.EGQNARGAEGGGQQDAD.SD.DD...AY.R.NR....L..      99

LAI    DLSHFLKEKGGLEGLIHSQRRQDILDLWIYHTQGYFPDWQNYTPGPGVRY  135
D205   .M...I.....MY..E..HR...TYFENEE.IVSG.....H...I..    149

LAI    PLTFGWCYKLVPVEPDKVEEANKGENTSLLHPVSLHGMDDPEREVLEWRF  185
D205   .KY...LW.....VP-AATREEE.THC.M..AQISSW..IHG.T.I.Q.  198

LAI    DSRLAFHHVARELHPEYFKNC--------------------          206
D205   ..L..YDY..FNRF..E.GYQSGLPEEEWKARLKARGIPTD          239
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C

Nef:		Construct:	2-Hybrid HCK-SH3 interaction:
1	HIV1-LAI	acidic, PVRPQVPLRP	+ +
2	HIV1-HR004	PVRPQVPLRP	+ +
3	HIV2-D194	PVMPRVPLRE	weak
4	HIV2-D205	YVRPNRPLRP	-

D

Nef:		Construct:	2-Hybrid HCK-SH3 interaction:
1	HIV1-LAI	acidic, PVRPQVPLRP	+ +
2	HIV1-LAI	71, RPQVPLRP	+ +
3	HIV2-D205	YVRPNRPLRP	-
4	HIV2-D205	85, RPNRPLRP	-
5	HIV2-D205$^{LAI\text{-}PxxP}$	85, RPQVPLRP	weak
6	Peptide$^{LAI\text{-}PxxP}$	RPQVPLRP	weak
7	HIV1-LAI$^{205\text{-}PxxP}$	71, RPNRPLRP	-

FIG. 1 Distinct Nef proteins differ in binding of HCK. (A) Interaction of Nef proteins with the protein tyrosine kinase HCK in the two-hybrid system: (a) LexA-HIV-1_{LAI}Nef + VP16, (b) LexA-HIV-1_{LAI}Nef + VP16-HCK-SH3, (c) LexA-HIV-2_{D205}Nef + VP16, (d) LexA-HIV-2_{D205}Nef + VP16-HCK-SH3, (e) LexA-daughterless + VP16, (f) LexA-daughterless + VP16-MyoD, (g) LexA-daughterless + VP16-HCK-SH3, (h) LexA + VP16, (i) LexA + VP16-HCK-SH3. The L40 strain was transformed with two-hybrid plasmids and transformants were streaked on media lacking the indicated amino acids. Growth on medium -His, -Trp, -Leu, +10 mM 3-AT selects for interaction of the fusion proteins. On medium Trp, -Leu as a control no interaction is selected. The addition of 10 mM 3-aminotriazole is neccessary to supress the background activation of the LexA-Nef fusions with the isolated VP16 domain. Only the positive control daughterless + MyoD (f, Hollenberg *et al.,* 1995) as well as HIV-1_{LAI}-Nef + HCK-SH3-domain (b) show an interaction, but not HIV-2_{D205}-Nef + HCK-SH3-domain (d). (B) Comparison of the Nef proteins of HIV-1_{LAI} and HIV-2_{D205}: Amino acid sequences of the Nef proteins of HIV-1_{LAI} and HIV-2_{D205} were aligned using the one letter code. Identical residues were replaced by dots and gaps introduced for optimal alignment are shown as horizontal lines. Verification of our earlier described sequence (Kreutz *et al.,* 1992) of the nef open reading frame of HIV-2_{D205} revealed some discrepancies in the amino-terminal region of the protein. The corrected sequence is used here. The homology of the two proteins is 40% at the amino acid level. Two functional motifs are boxed: The acidic stretch which is critical for CD4 downregulation and the proline-rich region, which was shown to be involved in enhancement of viral infectivity (Goldsmith *et al.,* 1995) and binds *in vitro* to the SH3 domain of the protein tyrosine kinase HCK (Saksela *et al.,* 1995). (C) Differences of subtypes and (D) mutational analysis of Nef binding to HCK: Two-hybrid constructs of the Nef proteins from different HIV isolates and mutants are shown schematically. The locations of the acidic stretch and the proline-rich motif are represented by boxes. The results of these constructs in two-hybrid interaction assays with the SH3 domain of human HCK are given on the right. For the classification of strong or weak interactions see Materials and Methods.

acted specifically with HIV-2_{D205} Nef via an SH3 related domain in a two-hybrid assay as well as in a protein binding assay and was strongly expressed in hematopoetic tissues.

RESULTS

The Nef proteins of divergent HIV-strains differ in their binding properties for HCK

To determine if Nef proteins of highly divergent strains of HIV interact with the same intracellular proteins we analyzed the binding to HCK in a two-hybrid system. While the interaction of HIV-1_{LAI}Nef, which had been described *in vitro* (Saksela *et al.,* 1995) could be confirmed (Fig. 1A), no interaction was seen with the Nef protein of a highly divergent subtype B HIV-2 strain, HIV-2_{D205} (Dietrich *et al.,* 1989). Figure 1B shows the alignment of the Nef protein of HIV-1_{LAI} and the corrected sequence of HIV-2_{D205}. The two proteins have a homology of only 40%. Compared to HIV-1_{LAI} the acidic region and the proline-rich motif (boxed) are principally conserved in HIV-2_{D205} but exhibit several substitutions. Especially a valine residue in the proline-rich motif (PQVP), which is conserved in nearly all other Nef proteins, is

replaced by an arginine (PNRP) in HIV-2_{D205}. In contrast to HIV-2_{D205}, the Nef protein of an HIV-1 subtype E isolate (HIV-1_{HR004}, Ruppach *et al.,* 1996), displayed HCK binding comparable to that of Nef of HIV-1_{LAI} (Fig. 1C, construct 2), while the Nef protein of the HIV-2 subtype A isolate HIV-2_{D194} (Kühnel *et al.,* 1989) only showed a weak interaction with the SH3 domain of HCK (construct 3, Fig. 1C).

To determine which parts of the molecules are essential for a strong interaction, a mutational analysis was carried out (Fig. 1D). The aminoterminal part of HIV-1_{LAI}-Nef is dispensable for a strong interaction with HCK (construct 2). In case of HIV-2_{D205}, both the full length and the core domain of Nef lack the ability to interact with HCK (constructs 3 and 4). A substitution of the proline-rich motif of HIV-2_{D205} (PNRP) by the corresponding amino acids of HIV-1_{LAI} (PQVP) restores a weak interaction with HCK (construct 5), comparable to the signal of HIV-2_{D194} and to the signal of a 10-amino-acid peptide containing this motif (construct 6). Vice versa, the substitution of the PxxP motif in HIV-1_{LAI} Nef with that of HIV-2_{D205} totally abolished binding to HCK (construct 7).

Thus neither HIV-2_{D194} Nef nor the HIV-2_{D205}^{PQVP} mutant protein resemble HIV-1_{LAI} Nef in the property of interacting strongly with HCK when expressed as full-length proteins. Nevertheless the sequence of the PQVP motif of HIV-1_{LAI} Nef seems to be essential for the interaction with HCK, since mutation of the QV doublet in HIV-1_{LAI} Nef to the corresponding NR doublet of the HIV-2_{D205} protein (PNRP) totally abrogates the interaction (Fig. 1D, construct 7 compared to construct 2).

Isolation of HIV-2_{D205}-Nef interacting proteins

Since HIV-2_{D205} Nef differed in its binding properties from HIV-1_{LAI} Nef, we performed a two-hybrid screening in order to isolate proteins which interact with HIV-2_{D205} Nef. A LexA-based two-hybrid system (Hollenberg *et al.,* 1995) with a size-selected cDNA library (Vojtek *et al.,* 1993; Hollenberg *et al.,* 1995) was used for screening with HIV-2_{D205} Nef as a bait. To simplify the screening process, a URA3 marker was additionally introduced into the bait plasmid (pBTM116) and a ura3$^-$ derivate of the L40 strain was used. This addition to the system allows an efficient counterselection against the bait plasmid after short segregation, which reduces the work in verifying the specifity of the isolated clones. Of 5×10^6 transformants plated on selection medium 16 specific clones were obtained which represented six different proteins based on sequence analysis.

To demonstrate direct interaction of the proteins, binding experiments with the Nef protein of HIV-2_{D205} expressed in a baculovirus system and bacterially expressed GST fusions of the potential binding partners were performed. One protein was found to also interact *in vitro* with HIV-2_{D205}-Nef (see Fig. 3A). It represents an unknown protein, which was isolated as six independent clones and was named Nefin1 for "*Nef in*teraction partner-1." As expected, in contrast to HIV-2_{D205} Nef, the Nef protein of HIV-1_{LAI} did not interact with Nefin1 in the two-hybrid system (data not shown). This specifity corresponds to the result with the HCK SH3 domain and confirms that the Nef proteins of HIV-1_{LAI} and HIV-2_{D205} differ in their binding properties. Interestingly, even HIV-2_{D194} Nef did not show an interaction with Nefin1.

Cloning of the complete cDNA of Nefin1

Since only a part of the open reading frame of Nefin1 was isolated in the two-hybrid screen a cDNA library from human thymus was screened to obtain the full-length sequence. Several rounds of screening led to overlapping clones, which resulted in a combined sequence of 3.2 kb with an open reading frame encoding a protein of 923 amino acids, as shown in Fig. 2A. A database search using the Nefin1 sequence resulted in no homology to known sequences, indicating that Nefin1 represents a novel protein. Nevertheless, the Nefin1 sequence displays several motifs possibly involved in protein–protein interactions: A putative leucine zipper is located at amino acids 242 to 264, and 15 tyrosine residues as possible binding sites for SH2 domains are found throughout the protein as well as several PxxP motifs, which could represent binding sites for SH3 domains. The carboxy-terminal region (aa 829–900) of Nefin1 is predicted to form a coiled coil (Lupas *et al.,* 1991). In addition, a potential bipartite nuclear localization sequence is found at aa 201–204 and 215–218.

The Nef binding region of Nefin1 is related to SH3 domains

All six overlapping clones isolated in the two-hybrid screen encompass a 72-amino-acid domain, which resides in the amino-terminal region of Nefin1 (shaded in Fig. 2A), limiting the Nef interaction domain to this region. A more detailed analysis of this domain revealed a clear relationship to SH3 domains. A comparison with several SH3 domains is given in Fig. 2B.

Expression of Nefin1 in human and murine tissues

Northern blot analysis was performed to characterize the mRNA expression of Nefin1. As shown in Fig. 2C a 4.4-kb transcript was hybridized under high stringency with poly(A)$^+$ RNA from several murine (Fig. 2C, lanes a–h) and human (Fig. 2C, lanes i–o) tissues. Particularly, lanes i–o in Fig. 2C revealed a strong expression of Nefin1 mRNA in all human hematopoetic tissues analyzed.

The proline-rich motif but not the acidic stretch of HIV-2_{D205} Nef is necessary for interaction with Nefin1

Figure 3A shows the *in vitro* interaction of a GST fusion protein of the Nef binding domain of Nefin1 with

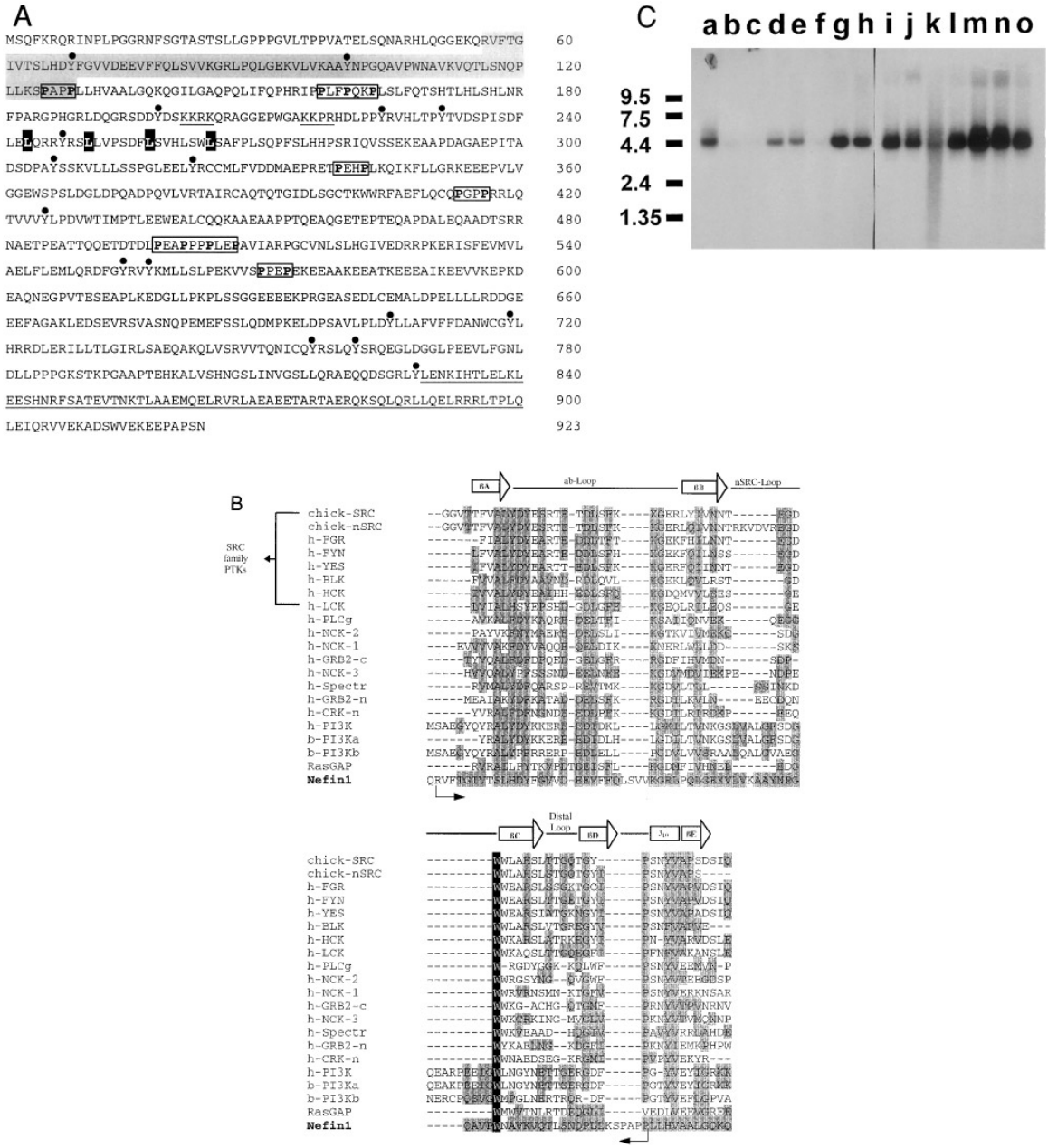

FIG. 2. Characterization of Nefin1 (A) Amino acid sequence of the full-length Nefin1 protein: The sequence is given using the one-letter code for amino acids. The Nef binding domain is shaded and potential tyrosine phosphorylation sites are marked by dots. Putative SH3 binding sites are boxed and the leucine zipper is shown by white letters on black boxes. A potential bipartite nuclear localization signal (aa 201–204/215–218) as well as a predicted coiled coil region at the carboxyl terminus are underlined. (B) Relationship of the Nef binding domain of Nefin1 to SH3 domains: SH3 domains of various proteins were compared with the Nef binding domain of Nefin1 in a multiple sequence alignment using the CLUSTAL-W program (Thompson *et al.,* 1994). The region of Nefin1 which is sufficient for binding is marked by two arrows under the sequence. Above the alignment the position of five β-sheets and loops according the crystal structure of the FYN SH3 domain (Noble *et al.,* 1993) is schematically shown. Amino acid residues are boxed if they are identical with the Nefin1 sequence or correspond to one of the following groups: hydrophobic (M/L/I/V/C), aromatic or ring (F/Y/W/H), small near neutral (A/G/S/T/P), acidic or uncharged polar (D/E/N/Q), and basic (K/R/H). A tryptophane residue (W), which is conserved in all domains, is shown inverted. (C) Expression of Nefin1 in human and murine tissues: Autoradiography of northern blots with different tissues from mice (lanes a–h: heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis) or hematopoetic human tissues (lanes i–o: spleen, lymph node, thymus, appendix, peripheral blood leukocyte, bone marrow, and fetal liver). Both blots were hybridized with single stranded DNA probes of the murine and human Nefin1 respectively and washed under high stringency (0.1 × SSC, 0.1% SDS, 60°C). Size markers in kb are given on the left. Standardization of the blots using a GAPDH probe showed no signal in lane c (murine spleen).

HIV-2_{D205} Nef expressed using a baculovirus system. Since the Nef binding domain of Nefin1 is related to SH3 domains, we next tested which region of the Nef protein is responsible for the interaction with this domain of Nefin1. Two-hybrid analyses of various deletion constructs of HIV-2_{D205} Nef (Fig. 3B) revealed

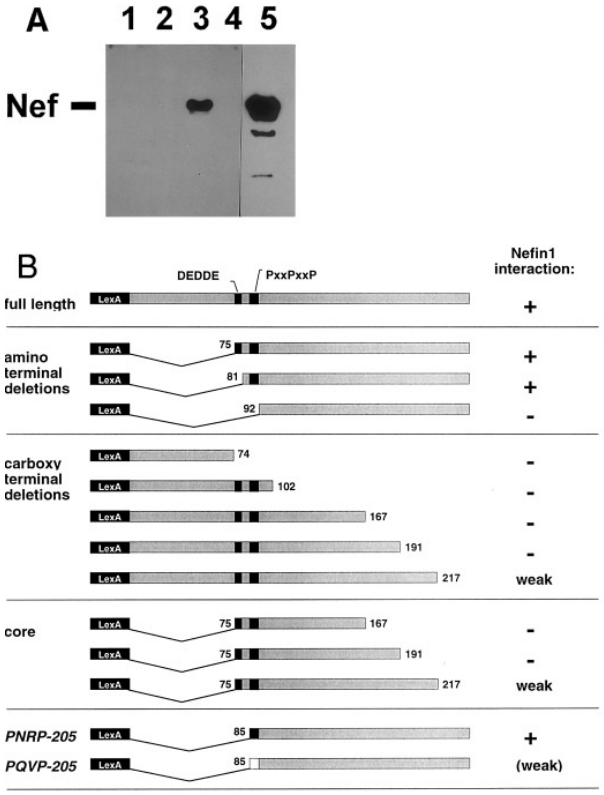

FIG. 3. Interaction of HIV-2_{D205} Nef with Nefin1 (A) *In vitro* interaction of HIV-2_{D205} Nef with Nefin1: 1 μg purified GST (lanes 1 and 2) or GST-Nefin1 (lanes 3 and 4) were either directly loaded (lanes 2 and 4) or incubated with lysate of HIV-2_{D205}-Nef expressing Sf9 cells (lanes 1 and 3) and subsequently resolved on SDS–PAGE. Nef protein recovered from the lysate was detected by Western blotting using a Nef-specific antibody. As a control, 20% of the Sf9 lysate used in the binding assays was directly loaded in lane 5. (B) Two-hybrid interaction analysis of Nefin1 with deletion constructs of HIV-2_{D205} Nef: Several deletion constructs of HIV-2_{D205} Nef as well as one mutated construct containing a PQVP-motif are schematically shown. The locations of the acidic stretch and the proline-rich motif are represented by boxes. The results of two-hybrid interaction assays of the constructs with the Nef binding domain of Nefin1 are given on the right. For scoring the strength of the interaction see Materials and Methods.

that removing the amino-terminal part of Nef including the acidic stretch did not reduce the interaction with Nefin1. In contrast, the deletion of the proline-rich region completely abolished binding. Most carboxy-terminal deletions of Nef also led to complete loss of the interaction. Even a relatively short carboxy-terminal truncation greatly reduced binding. In conclusion, it appears that the interaction of HIV-2_{D205} Nef with the novel protein is dependent on the core domain of Nef, including the PxxP motif as with other nef interacting proteins, and that noncontiguous regions in the primary structure influence the binding as shown for the structures of the HIV-1_{BH10}Nef * HCK SH3 complex (Grzesiek *et al.,* 1996) and the HIV-1_{NL4-3} * FYN^{R96I}SH3 complex (Lee *et al.,* 1996). However, gross structural disturbances due to the carboxy-terminal deletions cannot be ruled out. Replacement of the proline-rich motif (PNRP) of the Nef protein of HIV-2_{D205} with the corresponding motif (PQVP) of HIV-1_{LAI} greatly reduced the interaction with Nefin1 in yeast; however, a residual weak binding was still detectable.

DISCUSSION

In this report we provide evidence that the Nef proteins from distinct HIV isolates are different with respect to their association with cellular proteins. Despite the ability to confirm the interaction of the SH3 domain of human HCK with the Nef protein from the HIV-1 isolate LAI in a cellular environment and to demonstrate binding to the Nef protein of a subtype E isolate of HIV-1, we were unable to detect this interaction using Nef from the divergent isolate HIV-2_{D205}. In addition, even the HIV-2 subtype A isolate HIV-2_{D194} showed only a weak interaction with HCK. Our mutagenesis studies suggest that the inability of HIV-2_{D205} Nef to bind HCK is due to a substitution of the amino acid doublet QV in the proline-rich motif of HIV-1_{LAI} Nef (PQVP) to NR in HIV-2_{D205} (PNRP). Especially the valine residue, which is replaced by an arginine, is highly conserved in nearly all Nef proteins described in the Los Alamos HIV database. Interestingly, HIV-2_{UC1}, the most closely related HIV-2 strain to HIV-2_{D205}, displays an identical sequence in this region (Barnett *et al.,* 1993). In the crystal structure of the HIV-1_{NL4-3} Nef complexed with a mutated FYN^{R961}SH3 domain this valine residue is completely buried at the interface owing to tertiary interactions of the Nef protein (Lee *et al.,* 1996). Nevertheless, a mutation of this sequence in HIV-2_{D205} to the corresponding sequence of HIV-1_{LAI} Nef results only in a weak interaction with HCK comparable to the HIV-2 subtype A isolate HIV-2_{D194}, which is comparable to the interaction of the proline-rich peptide only. In contrast, HIV-1_{LAI} full-length Nef protein shows a considerably stronger interaction (see Fig. 1D and Lee *et al.,* 1995). Thus the C-terminal part of Nef also is important for a strong interaction with cellular proteins.

Several other proteins have been described to show interactions with different Nef isolates: β-COP, a component of non-clathrin-coated vesicles, was isolated in a two-hybrid screen with HIV-1_{LAI} Nef (Benichou *et al.,* 1994) but interacts only weakly with HIV-2 isolates (R. Benarous, personal communication). For LCK, contradictory results were reported but novel data suggest a possible complex of a serine kinase with LCK, which binds to the amino terminus of Nef (Baur *et al.,* 1997). Considerable interest has been generated by a serine kinase activity, which coprecipitates with Nef from HIV-1_{SF2} (Sawai *et al.,* 1994) as well as SIV_{mac239} (Sawai *et al.,* 1995) and phosphorylates a 62-kD protein. Recent data suggest that p62 is the kinase itself and resembles members of the PAK family (Nunn and Marsh, 1996). Very importantly, the inability of mutant Nef to associate with

p62 seems to correlate with a reduced viral load in monkeys (Sawai *et al.,* 1996). However, recent data have also shown that even in the case of this kinase the association is dependent on the viral isolate (Luo and Garcia, 1996) and in another study, interaction of cellular serine/threonine kinases with Nef appeared dispensable for the development of AIDS in rhesus monkeys (Lang *et al.,* 1997).

In this study we isolated Nefin1, a novel protein with characteristics of an adaptor protein as an interaction partner for the Nef protein of HIV-2_{D205}. The interaction is mediated by an SH3-related domain. However, Nefin1 did not show an interaction with the Nef proteins of HIV-1_{LAI} or the HIV-2 subtype A isolate HIV-2_{D194}. In conclusion, it thus appears that several cellular factors associate with the Nef proteins of different HIV/SIV strains and that the composition of these complexes may differ between strains. Another possibility would be that the interaction of Nef with tyrosine kinases would differ between HIV-1 and SIV as well as HIV-2 by the usage of different domains, e.g., SH3 versus SH2 as suggested by Lang *et al.* (1997). If and how this influences the pathogenicity of HIV and SIV remains to be determined.

MATERIALS AND METHODS

Two-hybrid system

For generation of pBTM116^{URA} a 1-kb fragment of the complete *S. cerevisiae* URA3 gene was amplified by PCR and cloned into the *Pvu*II site of the plasmid pBTM116 (Bartel *et al.,* 1993). Nef sequences were amplified by PCR using primers that introduce an *Eco*RI site at the 5′ end and a *Sal*I site at the 3′ end. Subsequently the fragments were cloned into the corresponding restriction sites in plasmid pBTM116^{URA} in frame with the *Escherichia coli* LexA DNA binding domain. Mutant proteins were generated by site-directed mutagenesis (QuikChange, Stratagene) according to the manufacturer's instructions. For LexA peptide fusions sense and antisense oligonucleotides with a 5′ *Eco*RI and a 3′ *Sal*I overhang were annealed and cloned into the corresponding restriction sites of pBTM116. All clones were verified by sequencing. A two-hybrid interaction was scored positive when a streak of yeast containing a bait paired with a VP16 fusion protein showed full growth at a concentration of 3-aminotriazole at least two times higher than the threshold concentration necessary for total inhibition of growth when the bait was paired with the isolated VP16 activation domain. Interactions that did not meet this criterion were scored as weak. Pairings that allowed growth at a 3-amino triazole concentration at least 10× higher than that necessary to suppress background with the isolated VP16 activation domain were scored as strong interactions (symbolized by ++).

Two-hybrid screenings were performed essentially as described (Vojtek *et al.,* 1993) except that an ura3^{-} derivate of the L40 strain was used together with pBTM116^{URA}. To test the specifity of clones, segregation was allowed on plates without leucine followed by a counterselection on plates with 5-FOA (5-fluoroorotic acid). The resulting "bait-cured" strains were again grown on plates without leucine and retransformed in 96-well plates with Nef or unrelated bait constructs, respectively. Library inserts from specific clones were PCR amplified and directly sequenced.

cDNA cloning and sequence analysis

The nef open reading frame from an HIV subtype E isolate was cloned by PCR from genomic DNA of peripheral blood mononuclear cells infected with HIV subtype E (isolate HR004, Ruppach *et al.,* 1996). Sequence determination of the open reading frame revealed a deduced amino acid sequence corresponding to the consensus sequence published (Artenstein *et al.,* 1996). Isolation of the full-length Nefin1 sequence was performed by standard cDNA library screening methods using PCR-generated probes as well as anchored and ligation mediated PCR as described (Karn *et al.,* 1993). Sequencing of double-stranded DNA was done using an ABI 373A sequencer and the dye terminator chemistry according to the protocols of the manufacturer (Applied Biosystems). Sequence comparisons and analysis were performed using the Microgenie software package (Beckmann) as well as the FASTA (Pearson *et al.,* 1988), CLUSTAL-W (Thompson *et al.,* 1994), and COILS (Lupas *et al.,* 1991) programs.

Northern blot analysis

Multiple tissue blots were purchased from Clontech (Palo Alto, CA). A single-stranded cDNA probe corresponding to aa 160 to 300 of Nefin1 was generated by PCR using [α-^{32}P]dCTP (6000 Ci/mmol) as described (Karn *et al.,* 1993). Hybridization was done using QuickHyb-Solution (Stratagene) according to the manufacturer's instructions.

In vitro binding studies

Positive clones from the two-hybrid screen were subcloned as *Not*I fragments in pGEX-4T1 (Pharmacia) followed by expression and purification of the resulting GST fusion proteins in *E. coli* as described by the manufacturer. For expression in Sf-9 cells a cDNA containing the complete open reading frame of a myristylation-deficient version of HIV-2_{D205} Nef in the *Bam*HI site of pAcYM1 (a generous gift from D. Bishop) was used. Recombinant baculoviruses expressing HIV-2_{D205} Nef were prepared by homologous recombination of the plasmid with linearized BaculoGold DNA (Pharmingen) according to the manufacturer's instructions. At 48 h postinfection the Sf-9 cells were washed twice in PBS and lysed with ice-cold lysis buffer (20 mM Tris–Cl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40), freshly supplemented with 10 μg/ml leupeptin and aprotinin, 1 mM phenylmethylsulfonyl fluo-

ride. Cell lysates were prepared by freeze/thaw cycles and clarified by centrifugation for 10 min at 12,000 *g*. Protein concentrations were determined using the BCA method (Pierce). Sf-9 cell extracts were incubated overnight at 4°C with either GST or GST fusion proteins coupled to glutathione Sepharose beads. After washing with ice-cold lysis buffer the proteins were released from the Sepharose by boiling 2 min in SDS sample buffer and electrophoresed on a 12% SDS–polyacrylamide gel. After protein transfer onto Immobilon-P (Millipore), the filters were preincubated overnight with 5% dry milk in PBST (0.05% Tween 20 in PBS) at 4°C, incubated 1 h with the primary antibody at 22°C in reaction buffer (5% dry milk in PBST), washed three times, and incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse antibody (Sigma). Immunoblots were developed using the ECL system (Du Pont, NEN).

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