The nef gene is required for optimal viral spread of human and simian immunodeficiency viruses. However, the molecular mechanisms underlying the action of the Nef proteins may not be identical for all viral families. Here we investigate the interaction between the Nef protein of human and simian immunodeficiency viruses and SH3 domains from Src family kinases. Using the yeast two-hybrid system and immunoblotting we show that, in contrast to HIV-1 Nef, SIV and HIV-2 Nef poorly interact with Hck SH3 but bind to Src and Fyn SH3 domains. The molecular basis of these differences in SH3 targeting was revealed by sequence analysis and homology modeling of the putative SH3-Nef structures. Three amino acids (Try-113, Thr-117, and Gln-118) that localize in a “hydrophobic pocket” implicated in SH3 binding of HIV-1 Nef, are systematically substituted in SIV/HIV-2 alleles (by Tyr, Glu, and Glu, respectively). We demonstrate that site-directed mutagenesis of these residues in SIVmac239 Nef suffices to restore Hck SH3 binding and co-immunoprecipitation with full-length Hck from transfected cells. Our findings identify fundamental mechanistic differences in targeting of Src family kinases by HIV and SIV Nef. The herein described mechanism of SH3 selection by Nef via a “pocket” proximal to the canonical proline-rich motif may be a common feature for SH3 recognition by their natural ligands.

The nef gene is conserved among all primate lentiviruses and has been implicated in optimal viral replication and disease induction by HIV-1 and SIV (1, 2). Substantial sequence polymorphism has been detected among nef alleles from various cloned isolates of HIV and SIV, suggesting that Nef proteins may have subtype-specific functions or possess different strategies to perform the same task. However, four stretches of residues have been identified that were highly conserved among sequences derived from AIDS patients but also from among sequences of HIV-2 and SIV (3). One of these stretches includes a Pro-Xaa-Xaa-Pro motif, the canonical Src homology (SH) 3 domain binding consensus, indicating that SH3 binding is a common feature of all types of Nef alleles.

An intact PXXP motif is required for the potential of HIV-1 Nef to enhance viral growth and infectivity in cell cultures (4–6). It has subsequently been shown that HIV-1 Nef binds with high affinity to the SH3 domains of the Src family kinases Hck and Lyn, whereas only with a modest affinity to SH3 domains of other members of the Src family (Lck, Fyn, and Src) (7, 8). However, despite the PXXP motif being strictly conserved in all Nef proteins, an HIV-2 Nef allele has been reported to have lost the high affinity for Hck SH3 (9). Controversial data has further been collected for the requirement of the proline-rich motif for AIDS disease progression in infected macaques (10, 11). Especially, it was reported that a PXXP to AXAX mutated SIVmac239 protein could still bind to Src kinase (10), suggesting that SIV might have different strategies of interacting with the Src family of protein tyrosine kinases.

In an effort to find a unifying explanation for the observed discrepancies in SH3 targeting of different Nef alleles, we investigated the interaction of SIV and HIV Nef proteins with four representative of Src family SH3 domains, i.e. Src, Fyn, Lck, and Hck SH3. To establish the molecular basis for our biochemical data, we combined sequence analysis with structural data obtained by homology modeling of HIV-2 and SIV Nef alleles.

**EXPERIMENTAL PROCEDURES**

**Nef Expression Constructs**—The mutation of residues 139 (Tyr to Trp), 148 (Glu to Thr), and 149 (Glu to Gln) in SIVmac239 Nef were generated by PCR using a single mutagenic primer, allowing construction of SIVmac239 WTQ Nef, HIV-1lai Nef, SIVmac239 Nef, SIVmac239 WTQ Nef were cloned in βDNA4, a plasmid construct obtained by the substitution of the CMV promoter from pCDNA3 (Invitrogen) by the chicken β-actin promoter derived from the β-actin-luciferase construct (a kind gift from N. Auffan, CIML, France). HA-tagged constructs were developed by PCR using a 3′-primer which introduced the HA epitope-encoding residues. Each construct was verified by sequencing.

**Transient Transfection in COS-7 Cells**—COS-7 cells were transfected using Fugene6 as recommended by the instructions of the manufacturer (Roche Molecular Biochemicals) with HIV-1lai Nef, SIVmac239 Nef, SIVmac239 WTQ Nef, or mock-transfected. Cells were lysed 24 h after transfection for analysis using detergent buffer (1% Triton X-100, 25 mM Hepes, pH 7.8, 150 mM NaCl, 10 μM EDTA, 1 mM EGTA supplemented with 1 mM PMSF, 0.1 mM Na-vanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin and pepstatin).

**SH3 Affinity Chromatography**—Hck, Lck, Src, and Fyn-GST-SH3 fusion proteins were prepared from Escherichia coli cells, according to

Yves Collette‡§, Stefan Arold‡§‡*, Christophe Picard‡, Katy Janvier‡‡, Serge Benichou‡‡, Richard Benarous‡‡, Daniel Olive†, and Christian Dumas‡

From the ‡U119 INSERM, Université de la Méditerranée, 13009 Marseille, France, the ‡Centre de Biochimie Structurale, UMR C9955 CNRS, U414 INSERM, Université Montpellier I, Faculté de Pharmacie, 34060 Montpellier, France, and the ¶EPI INSERM 9923, Institut Cochin de Génétique Moléculaire, 75014 Paris, France

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4171
the instructions of the manufacturer (Amersham Pharmacia Biotech) and as described previously (12). Lysates from transfected COS-7 cells were reacted with 10 μg of the recombinant GST-SH3 fusion proteins. Glutathione-Sepharose affinity-purified precipitates were analyzed by SDS-PAGE and either HIV-1 (MAT0020 HIV-1 Nef monoclonal antibody, Transgene, France) or SIV Nef immunoblotting (17.2 monoclonal antibody, a kind gift from Drs. K. Krohn and V. Ovod, University of Tampere, Finland), followed by incubation with horseradish peroxidase-conjugated anti-mouse immunoglobulin and development using enhanced chemiluminescence substrate.

Co-immunoprecipitation Assay—To verify in vivo forming stable complexes, transfected cells were lysed in detergent buffer. Clarified supernatants were pre-cleared with protein A-Sepharose and then incubated with anti-Hck polyclonal antibody (1/400 dilution, Santa Cruz, TEBU, France) and with protein A-Sepharose for 2 h at 4 °C on a wheel. Immunoprecipitates were washed in detergent buffer and eluted in sample buffer by boiling, followed by SDS-PAGE and immunoblotting with anti-HA antibody (3F10, Roche Molecular Biochemicals, France) or anti-Hck polyclonal antibody (Santa Cruz, TEBU, France).

Two-hybrid Assay—The vectors for expression of the HIV-1la, HIV-2, and SIVmac239 Nef proteins fused to the Gal4 DNA binding domain (Gal4BD) have been described previously (13, 14). Expression vectors of HIV-1la and SIVmac239 Nef fused to the Gal4BD were constructed by PCR amplification of the respective nef genes, which were then inserted into the BamH-I-SalI restriction sites of the pGBT10 plasmid (15). The PA1 mutant, in which the proline residues in positions 72 and 75 of the HIV-1la Nef protein were replaced by alanines, was provided by G. Cohen (MIT, Cambridge, Massachusetts) (4). This Nef PA1 mutant was amplified by PCR and then subcloned into pGBT10. The construction of the vector for expression of the human Hck SH3 domain fused to the Gal4 activation domain (Gal4AD) was performed by PCR amplification of a 168-base pair fragment (coding from residue Ile-61 to residue Asp-116) using specific primers. This fragment was cloned into the BamHI-XhoI sites of the pGad1318 plasmid (15).

The two-hybrid interaction assays were performed as described previously (13). Briefly, the SFY526 yeast reporter strain, containing the LacZ Gal4-inducible gene, was cotransformed with the Gal4BD and Gal4AD hybrid expression vectors and plated on selective medium. Double transformants were patched on the same medium and then replicated on Whatman 40 filters for β-galactosidase activity. This qualitative assay was monitored for 6 h at 30 °C, and the reaction was then stopped with 1 M Na2CO3. The liquid culture assay for determination of the quantitative β-galactosidase activities was performed in triplicate as described previously (13). The background level was around 4–6 units and corresponds to SFY526 yeast cells expressing the Nef Gal4BD hybrids and the irrelevant Gal4BD-SNF4 hybrid. Interaction between the Gal4BD-SNF1 and Gal4AD-SNF4 hybrids was used as a positive control in both qualitative and quantitative β-galactosidase assays.

Homology Modeling—The sequences of the Nef isolates from SIV and HIV-2 were obtained from the GenBank data base. The conserved core domains (residues 71–148 and 178–203) from these isolates share about 60% identity and do not show any deletions or insertions compared with the sequence of HIV-1la Nef. We used the crystal structure of HIV-1la Nef (16) in complex with the Fyn R96I mutant SH3 domain (Protein Data Bank code 1efn) as molecular template for the modelizations of SIVmac239 and HIV-2la Nef (17) (Protein Data Bank code 1avv). All side chains of conserved residues were frozen in their original conformation in Nef HIV-1. The optimal set of rotamers for the mutated residues was established by cluster analysis of an energy matrix as implemented in the SMD program (18). The modeled complexes containing the new rotamers were then minimized with the AMBER package (19) in vacuo, utilizing a distance-dependent dielectric function ε = r, as an approximation of solvent screening. Energy minimizations were carried out under various constraints that were gradually released. After SMD and AMBER, the minimized Nef proteins showed an root mean square deviation for all atoms compared...
RESULTS

Crystal structures of HIV-1 Nef-SH3 complexes (16, 17) reveal that both the PXXP motif region (i.e. the amino acids that adopt a polyproline type II helix conformation upon binding to an SH3 domain) and tertiary contacts are involved in ligand binding. By analyzing the SH3 binding site of 710 nef alleles (620 HIV-1, 90 HIV-2/SIV) selected in the data base from the Los Alamos National laboratory (20), we noticed the following. All amino acids involved in the Nef-SH3 interface (16, 17) can be clustered into two classes: one that is found conserved in all HIV/SIV Nef isolates, and the other that is systematically characteristic of either SIV/HIV-2 or HIV-1 isolates (Fig. 1), with only very rare exceptions that do not significantly alter the corresponding interface.

The canonical PXXP motif region (residues 71–77 in HIV-1 la Nef, comprising Pro72–Xaa–Xaa–Pro75) is very well conserved in all Nef proteins. Only positions 71 and 73 show some variability. Position 71 is frequently an arginine, a lysine, or a threonine. This variation is already present in HIV-1 Nef isolates and has been shown not to significantly influence SH3 binding (4, 16, 17). Position 73 is a glutamine in HIV-1 Nef and an arginine or lysine in SIV and HIV-2 Nef molecules. The Gln-73 side chain in HIV-1 Nef does not affect SH3 binding but contributes to anchor the PXXP motif region to residues 115 and 116 of the underlying core domain. In SIV and HIV-2, the substitution of Gln-73 by arginine or lysine is counterbalanced by the replacement of the opposite Tyr-115 by a glutamic acid, thus establishing an ion pairing interaction (data not shown). In conclusion, it is very unlikely that differences in SH3 targeting arise from the PXXP motif region of Nef.

In contrast, systematic differences between HIV-1 and HIV-2/SIV Nef proteins were found within the SH3 binding site but sequentially noncontiguous with the PXXP motif. In detail, the following substitutions occur systematically in HIV-2/SIV Nef: M37/L37/L37/L37, Y113W, L114IV, E117T, E118Q, and I120Y (Fig. 1). Because this region of nef overlaps the 3’-long terminal repeat in all primate lentiviruses, we also examined the corresponding nucleotide sequences. Several synonymous substitutions were found in the Los Alamos Compendium (not shown), suggesting that the biological significance of these substitutions might be related to their role in binding SH3-containing proteins rather than a role in the U3 functionality. Computer-aided homology modeling of SIVmac239 and HIV-2rod Nef proteins and their complexes with SH3 domains (see “Experimental Procedures”) show that these amino acids are located on the border of a crevice in the surface of Nef (Figs. 2 and 3). In both modeled and crystal structures (16, 17) of the Nef-SH3 complexes, this crevice interacts with a key residue within the so-called RT-loop of the SH3 domain (Fig. 2). Interestingly, this SH3 residue is a critical component of the Nef-SH3 interface and plays a key role in determining the affinity and specificity of Src family SH3 domains for HIV-1 Nef (7). In HIV-1 Nef, this “specificity pocket” formed between helices αA and αB is dominantly hydrophobic. SH3 domains that have a hydrophobic amino acid (iso-leucine in Hck and Lyn SH3) at the position that interacts with this crevice of Nef consequently display a higher affinity for HIV-1 Nef than SH3 domains that have a charged residue there: arginine in Fyn and Src SH3 (7, 16, 17). Importantly, the amino acid substitutions found consistently in HIV-2/SIV Nef alleles diminish the hydrophobic character of this pocket and introduce negative charges (Fig. 3). As the hydrophobic pocket of HIV-1 Nef is determining its high affinity for Hck SH3 ($K_d = 200–600$ nM) (7, 8), the introduction of charged residues should considerably lower the affinity of SIV/HIV-2 Nef for this SH3 domain.

To test this hypothesis, we probed the Nef-Hck SH3 interaction using the yeast two-hybrid system. The HIV-1la and HIV-1ay2 Nef proteins associated firmly with Hck SH3 (Fig. 4). Consistent with previous reports (4, 10, 11), the double mutant PXXP → AXAA has lost the ability to bind to Hck SH3 (Fig. 4). Using the same technique, we further showed that SIVmac239, SIVsmPBJ, and HIV-2rod Nef proteins do not detectably interact with Hck SH3. The lack of high affinity for Hck SH3 was further confirmed for cell-derived SIVmac239 by SH3 affinity chromatography followed by immunoblotting (Fig. 5a).

We next addressed the question of whether the introduction of charged residues in the specificity pocket of HIV-2/SIV Nef proteins could favor the interaction with Src SH3 domains that have a charged residue in the key position of their RT-loop. Toward that, we first modeled the complex formed between Fyn SH3 domain and HIV-2rod and SIVmac239 Nef molecules. In these model structures, rather than bending away from the hydrophobic pocket of HIV-1 Nef as seen in the crystal structure (17), the Fyn R96 side chain points into the charged pocket of HIV-2/SIV Nef (Fig. 2). The guanidinium moiety is able to form a hydrogen bond with tyrosine 113, and its charge is counterbalanced by glutamic acid 117. Both, HIV-1 Gln-118 and HIV-2/SIV Glu-118 are able to form hydrogen bonds with
the backbone of SH3 A95. The charge of Glu-118 may also play a role by contributing to long range electrostatic interactions prior to the association. In summary, our model studies predict a significantly higher affinity of SIV and HIV-2 Nef alleles for SH3 domains encoding for a positive charged residue at the key position within their RT-loop (8). However, to finally test whether the observed differences in SH3 targeting is effectively because of the triple amino acid substitutions identified in the specificity pocket of Nef, we probed the interaction of an HIV-1-like SIVnefmac239 Nef mutant with Src family SH3 domains. A Y113W/E117T/E118Q triple mutant was generated and tested in SH3 affinity chromatography and immunoblotting experiments (Fig. 6). This mutant expressed a stable protein in transfected cells and exhibited a high affinity for Hck SH3 domain while keeping Fyn SH3 relative affinity unchanged as compared with the wild-type nef SIVnefmac239-encoded product. We next investigated whether these substitutions would have equally significant functional implications in vivo. Similarly to HIV-1 Nef, when co-expressed with Hck, the Y113W/E117T/E118Q triple mutant formed stable complexes with the kinase in transfected cells, as shown by co-immunoprecipitation experiments (Fig. 7). This contrasted to the wild-type nef SIVnefmac239-encoded product which failed to form such complexes, despite similar efficiency in Hck immunoprecipitation (Fig. 7). We concluded that the switch in affinity is dominantly mediated by the above mentioned three amino acids.

**DISCUSSION**

Our experiments establish that SIV/HIV-2 and HIV-1 Nef proteins target different Src family SH3 domains. The specific discrimination between SH3 domains is achieved by a mechanism that is independent from the canonical PXXP motif. This selection mechanism is based on the match or mismatch of three residues of Nef forming a “pocket” for a key residue within the RT-loop of the Src family SH3 domains (Figs. 2 and 3).
It is intriguing that the triple mutant SIV<sup>mac239</sup> WTQ still seems to have a lower relative affinity for Hck SH3 as compared with HIV-1 Nef in our GST pull-down experiments. Correspondingly, the overexposure of the immunoblots for HIV-1 Nef-SH3 domains interactions reveal differences in the relative affinity of HIV-1 Nef for Src or Fyn SH3. We previously observed by isothermal titration calorimetry that the thermodynamic parameters for SH3 binding to the Nef core domain are different from those obtained for binding to full-length Nef (8). The presence of the N terminus increased the affinity for Hck SH3 by a factor of three and did not affect the affinity for Src SH3 (8). Our results presented here may indicate that the N terminus of SIV Nef either contributes differently to SH3 binding than in HIV-1 Nef, or, more likely, does not contribute at all. The latter is consistent with the observation that the affinity of SIV<sup>mac239</sup> Nef for Src and Fyn SH3 is comparable. The absence of a Hck SH3-specific affinity enhancement by the N-terminal of SIV<sup>mac239</sup> Nef would explain why the triple-mutant SIV<sup>mac239</sup> Y113W/E117T/E118Q mutant appears to co-precipitate more efficiently with full-length Hck than HIV-1 Nef in vivo, suggesting some additional mechanisms of binding such as through its SH2 domain. Indeed, although a strong selective pressure was noted in vivo to restore an experimentally disrupted proline motif in SIV Nef (11), this motif appears dispensable for Src binding in intact cells (10). This is in stark contrast to HIV-Nef binding to Hck which requires an intact proline motif (30). Further, conversely to HIV-1 Nef, SIV Nef contains a putative tyrosine-based SH2-interacting motif (31) and indeed can interact with either Hck or Lck SH2 domains (32). That HIV-1 and SIV/HIV-2 have evolved in parallel to target Src family members, yet through distinct mechanisms of binding, highlights the importance to elucidate the role of these cellular kinases in the primate lentiviruses replicative cycle. Elucidation of incongruences in functional aspects between SIV and HIV-1 Nef molecules is indispensable for the use of SIV Nef as a model for drug design. As an alternative to mutational analysis in the proline motif (4, 10, 11, 26), we now propose to design mutations that selectively inactivate specific Nef functions to assess their effect on viral replication and pathogenesis in vivo.

The striking similarity between the modes of interaction of SH3 domains with Nef and with peptide ligands leads us to propose that the viral Nef protein engages in molecular mimicry, having incorporated a motif derived from cellular proteins. A similar pocket may therefore be a common feature for SH3 recognition by their natural ligands. This hypothesis is also supported by mutational studies that reveal the importance of the SH3 key residues for ligand recognition (33).

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Addendum—During the preparation of this manuscript, Craig et al. (Craig, H. M., Pandori, M. W., Riggs, N. L., Richman, D. D., and Guatelli, J. C. (1999) Virology 262, 55–63) reported on partial functional defects introduced by mutations in the prolinepyrrole helix and the hydrophobic pocket of HIV-1 Nef.
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