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HsN3 Proteasomal Subunit as a Target for Human Immunodeficiency

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HIV-1 Nef protein is important for pathogenicity, but its biochemical function remains obscure. To clarify its role, a yeast two-hybrid system (ths) screening was utilized to identify Nef cellular partners. Of 79 yeast clones harboring cDNAs for putative Nef binding proteins, 27 (34%) contained the coding region for HsN3 proteasomal subunit. HsN3 behaved as *bona fide* Nef partner in ths control crosses. Nef–HsN3 interaction was confirmed by *in vitro* binding experiments. In particular, recombinant Nef was able to capture the HsN3 subunit from a natural proteasome preparation. In Nef, the interacting region was mapped within aa 34–143, which span the structured portion of the protein, including the SH3-binding domain. In HsN3, Nef-binding portion was restricted to aa 73–249, and the tract 219–249 — reminiscent of SH3 domain N-terminal 3/5ths — was shown to be essential, though not sufficient. Attempts to purify a Nef–HsN3 complex from transfected COS7 cells were unsuccessful. However, Nef was found to markedly downregulate intracellular levels of both a coexpressed HsN3 and the endogenous simian homologue. These results suggest that Nef, by binding to a subunit, might alter proteasome function in infected cells.

INTRODUCTION

Primate retrovirus *nef* genes code for a 24- to 34-kDa protein synthesized abundantly at an early stage of viral replication (Cullen, 1994; Ratner and Niedermann, 1995; Trono, 1995; Harris, 1996). Nef is dispensable for replication *in vitro,* but contributes substantially to viral pathogenicity in animal models (Kestler III *et al.,* 1991; Jamieson *et al.,* 1994), with the notable exception of neonatal macaques (Baba *et al.,* 1995). Defects in the *nef* gene account for some of the described cohorts of long-term nonprogressors among HIV-1-infected humans (Deacon *et al.,* 1995). In agreement with *in vivo* data, *in vitro nef* product stimulates viral growth via a direct enhancement of virion infectivity (Aiken and Trono, 1995; Chowers *et al.,* 1995; Miller *et al.,* 1995; Schwartz *et al.,* 1995), which seems to involve incorporation of Nef into viral particles and its processing by HIV-1 protease (Pandori *et al.,* 1996; Welker *et al.,* 1996; Bukovsky *et al.,* 1997). Another well established Nef effect is the downregulation of CD4 exposure on the cell surface (Garcia and Miller, 1991), induced by endocytosis enhancement (Aiken *et al.,* 1994). In view of CD4's role both as T cell receptor (TCR) accessory component in healthy T cells, and as the main HIV-1 receptor, CD4 downregulation might either impair CD4 signalling, or prevent cell superinfection, or both. Recently, Nef has also been shown to trigger endocytosis of MHCI molecules (Schwartz *et al.,* 1996). Infectivity enhancement and CD4 downregulation are separable Nef activities, since some mutations abolishing the former — namely, those altering a PxxP repeat domain — leave CD4 downregulation unaffected (Goldsmith *et al.,* 1995; Saksela *et al.,* 1995).

In addition, Nef expression modulates T-cell activation events (Skowronski *et al.,* 1993; Baur *et al.,* 1994; Rhee and Marsh, 1994). In some experimental settings it has been reported to interfere with the induction of transcription factors NF-kB and AP1, which participate in immune, inflammatory, and antiviral responses (Niederman *et al.,* 1992, 1993).

How Nef can produce such an array of disparate effects remains elusive in molecular terms. Clues have come from studies attempting to identify cellular Nef-binding proteins. Concordant results from Nef–CD4 coexpression experiments in insect cells (Harris and Neil, 1994) or yeast two-hybrid system (Rossi *et al.,* 1996) and from nuclear magnetic resonance studies (Grzesiek *et al.,* 1996) indicate that Nef binds at low affinity the cytoplasmic tail of CD4. This suggests that a transient Nef–CD4 physical interaction might be one of the events routing CD4 to endosomes. Nef PxxP motifs have been shown to interact with the SH3 domain of members of the Src tyrosine-protein kinase family, albeit discrepancies exist as to the preference for Hck, Lyn, or Lck (Sak-

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sela *et al.,* 1995; Collette *et al.,* 1996; Greenway *et al.,* 1996). Since (i) Lck constitutively associates to the cytoplasmic domain of CD4, (ii) Lck or related kinases are involved in the signal cascades emanating from TCR, and (iii) Nef SH3 interacting domain is essential for infectivity enhancement, one could conclude that Lck or related proteins have the potential for participating in all Nef activities mentioned above. Serine/threonine protein kinases have also been implicated as Nef partners. Nef interferes in transfected cells with protein kinase C theta isozyme (Smith *et al.,* 1996), the one specifically involved in T-cell activation. Furthermore, Nef copurifies with, and activates, some p21-activated kinase (PAK) family members (Nunn and Marsh, 1996; Sawai *et al.,* 1996; Lu *et al.,* 1996), and mutations impairing copurification also block virus pathogenicity in the SIV/*rhesus* macaque model (Sawai *et al.,* 1996). Finally, virion incorporation of a Ser/Thr kinase needs Nef and might account for HIV-1 matrix protein phosphorylation (Swingler *et al.,* 1997).

Nef can assume different cell localizations and oligomerize (reviewed in Ratner and Niederman, 1995 and Harris, 1996), thus it might contact different proteins in distinct compartments and function as an adapter linking many partners at once at a given site. In a search for additional Nef cellular partners, we exploited the yeast two-hybrid system (ths; Fields and Song, 1989) to directly isolate genes coding for HIV-1 Nef binding proteins. An earlier report adopting this approach identified β-COP as a Nef interacting protein (Benichou *et al.,* 1994).

In our screening, the gene for HsN3 proteasomal subunit and for the deubiquitinating protease pDFFRX emerged as frequent isolates. The proteasome is the principal multicatalitic complex responsible for nonlysosomal protein breakdown in eukaryotic cells (Coux *et al.,* 1996). Misfolded and damaged proteins are targeted to the proteasome for degradation. Proteasome is also involved in the generation of peptides for class I MHC. Finally, proteasome has been implicated in many cellular regulatory mechanisms that depend on specific proteolytic modifications, the regulation of NF-kB and AP1 among them. The 20S proteasome core associates to additional factors, the principal being the 19S factor, which generates 26S proteasome and confers ubiquitin dependence on the proteasomal enzymes. Ubiquitination at lysine residues, beyond being a degradation signal, is emerging also as an activating signal, e.g., to turn on the IkB kinase complex which activates NF-kB, and to trigger the endocytosis of surface receptors (reviewed in Hochstrasser, 1996). In view of the pervasive role played by ubiquitination and proteasome activities, targeting by Nef of two players in these phenomena might partly explain Nef pleiotropic action in the cell, in particular NF-kB inhibition.

Nef–HsN3 interaction was confirmed by *in vitro* binding assays. Transfection experiments showed that Nef induces a selective depletion of intracellular HsN3. Thus, Nef apparently joins Tax and pX proteins in the list of retroid virus products committed, *inter alia,* to alter proteasome function.

MATERIALS AND METHODS

Plasmid constructions

HIV-1 (BH10 strain) *nef* gene, minus the natural ATG, was amplified from the cloned provirus DNA using *Pfu* polymerase (Stratagene) and the primer pair 5′-TCGCCGGAATTCGGTGGCAAGTGGTCAAAAAGTAGT (upstream) and 5′-ACGCCTCTGCAGTCAGCAGTTCTTGAAGTACTCCGG3′ (downstream). The amplification product was digested with *Eco*RI and *Pst*I (underlined in the primers) and the 618-bp *nef* fragment cloned downstream from, and in frame with, GAL4DB coding region into pGBT9 vector (Clontech), to create pGBT9-Nef. The same *nef* insert was introduced into pGEX-4T-1 (Pharmacia) and pTrc-His-B (Invitrogen) *Escherichia coli* expression vectors, generating pGEX-Nef and pTRC-Nef, respectively.

For pMTNef plasmid construction, the full-length *nef* gene (ATG conserved) was amplified from HIV-1 BH10 DNA with the primer pair 5′GCTTGGCTGCAGTTTTGCTATAAGATGGGTGGC (upstream) and 5′GTAGAATTCTCGATGTCAGCAGTTCTTGAAG (downstream). The product was cut with *Pst*I and *Eco*RI (underlined in the primers) and cloned into the same sites of pMT-2 eukaryotic expression vector (a generous gift of P. Comoglio). Similarly, to generate pMTHA-Nef plasmid *nef* sequence was amplified with the primers 5′GCTATAACCCGGGTGGCAAGTGG (upstream) and 5′GCTAAGCTTCAGCAGTTCTTGAAG (downstream) and cloned into *Sma*I–*Hin*dIII sites of pBSHA plasmid (a gift of L. Cartegni), downstream from a stretch encoding influenza Hemagglutinin HA epitope (YPYDVPDYA). The segment encoding HA–Nef fusion was then reexcised as a *Pst*I–*Hin*dIII (blunted) fragment and ligated into the *Pst*I–*Sma*I-linearized pMT-2 vector.

pBS-HsN3 plasmid was created by subcloning into pBlueScriptSK+ vector the sequence encoding full-length HsN3 protein, excised as a 940-bp *Eco*RI–*Xho*I fragment from one of the pGAD-GH-HsN3 clones isolated with THS. Since this cDNA lacks the natural HsN3 ATG, the RNA transcript driven by pBS-HsN3 T3 promoter is assumed to be translated *in vitro* (see below) as a HsN3 version with a shortened propeptide, initiated from the internal ATG12. The identical *Eco*RI–*Xho*I fragment was inserted into pGEX-4T-3 vector (Pharmacia), creating pGEX-HsN3. Finally, pMTHsN3 construct was obtained by amplifying HsN3 coding sequence from pGAD-GH-HsN3. The 5′ primer (5′-GCTTGGCTGCAGAAGATGGAAGCGTTTTTGGGGTC) reintroduces natural HsN3 ATG (in bold face). As the 3′ primer, an oligonucleotide matching to the pGADGH vector downstream from the polylinker

(see below) was used. The amplified fragment was cut with *Pst*I and *Sph*I endonucleases (the latter one cleaving once within the amplification product), and the 5′ fragment was cloned into *Pst*I–*Kpn*I-restricted pMT-2 vector, along with the *Sph*I–*Kpn*I fragment from pBSHsN3, thus reconstituting a complete HsN3 coding sequence.

To obtain Nef and HsN3 deletion derivatives, natural restriction sites were exploited to remove portions of the coding sequences and reinsert the shortened genes into pGBT9 or pGAD-GH vectors, respectively.

All the amplified inserts mentioned above have been fully sequenced prior to use in subsequent experiments.

Yeast two-hybrid system

Saccharomyces cerevisiae HF7c strain, carrying *HIS3* and *lacZ* markers under the control of Gal4 binding sequences, was transformed with pGBT9-Nef according to the lithium acetate protocol and selected for leucine prototrophy on SD-Leu$^-$ plates. Alternatively, yeast cells were double transformed with pGBT9-Nef and either pGAD424 or pTD1 plasmids, driving the synthesis of Gal4-A alone or Gal4-A fused to aa 84–708 of SV40 TAg, respectively. Cells were selected for leucine and triptophan prototrophy on SD plates lacking both amino acids. Yeast colonies were then subjected to a filter test for β-galactosidase expression, as described (Rossi *et al.,* 1996). Both pGBT9-Nef single transformants and pGBT9-Nef/pGAD424 and pGBT9-Nef/pTD1 double transformants were found to be negative for β-galactosidase production, which suggested that Nef fusion could neither activate Gal4 responsive reporter alone (mimicking an activation domain) nor bind spuriously Gal4-A or Gal4-A fusions. A commercial cDNA library from HeLa cells, cloned into pGAD-GH vector (Clontech) was amplified in *E. coli,* and DNA was purified with a double CsCl gradient. The library was overtransformed into yeast harboring pGBT9-Nef, and double transformants were plated for selection on SD medium w/o Trp, Leu, and His, supplemented with 10 m*M* 3-aminotriazole; an aliquot was plated on SD-Leu$^-$Trp$^-$ to estimate the number of screened double transformants. Colonies grown on triple selective medium were counterscreened for β-galactosidase expression by the standard filter assay. Positive clones were then analyzed to identify clones sharing a cDNA insert. Single colonies were picked from plates and dispersed in 100 μl of sterile distilled water. The cDNAs in the library plasmid were amplified by use of thd primers 5′-CTAGAACTAGTGGATCCCCCGGGCTGC3′ (upstream) and 5′-GCGCGCGTAATACGACTCACTATAGGG3′ (downstream), annealing to pGAD-GH polylinker on either side of the insert. The PCR products were then restricted with either *Hae*III or *Hin*fI. A representative from each group of similar restriction patterns was radiolabeled (MegaPrime kit, Amersham) and used to probe all the amplified inserts immobilized on nitrocellulose filters, to check the homogeneity of the restriction groups. Two to five clones per each recurring insert and all single clones were rescued into *E. coli* as described and subjected to standard false positive exclusion tests. Inserts were sequenced and compared to DNA and protein data banks by use of the FASTA and BLAST algorithms.

To quantify β-gal reporter activation in yeast double transformants expressing one of the DB-Nef $\times$ A-HsN3Δ or DB-NefΔ $\times$ A-HsN3 combinations, cell extracts were assayed in liquid for β-gal (Ausbel *et al.,* 1991) and for total protein concentration, and reporter enzyme activity was calculated.

Recombinant proteins

E. coli DH5-α cells harboring pGEX-Nef or pGEX-HsN3 were induced with 1 m*M* isopropyl-β-D-thiogalctopyranoside (IPTG) for 3 hr, and the corresponding glutathione *S*-transferase (Gst)–Nef and Gst–HsN3 hybrid proteins were purified from cleared bacterial lysates by glutathione–Sepharose (Pharmacia) chromatography, according to manufacturer instructions, in the presence of protease inhibitors (10 μg/ml each of leupeptin, pepstatin, aprotinin, and 1 m*M* phenyl-metyl-sulfonyl fluoride and benzamidine). GST fusions were either left adsorbed to the resin or eluted with 10 m*M* reduced glutathione in 25 m*M* Tris–HCl, pH 7.5, and dialyzed against PBS.

Hexahistidine-tagged Nef (His-Nef) synthesis was induced in *E. coli*-DH5-α (pTrc-Nef) cells with 1 m*M* IPTG, for 3 hr. The protein was directly extracted from bacterial pellet with buffered 6 *M* Guanidinium–HCl and subsequently purified on Ni-NTA-agarose resin (Qiagen) under denaturing conditions, according to manufacturer instructions. His-Nef was renatured while still bound to the resin by sequential washes in decreasing urea concentrations, eluted with 300 m*M* imidazole, and extensively dyalized against PBS-protease inhibitors.

A L-[^{35}S]methionine-labeled HsN3 protein was synthesized *in vitro* by programming the TNT-T3 coupled transcription-translation rabbit reticulocyte lysate system (Promega) with pBS-HsN3 plasmid. The radiolabeled protein was used unpurified in subsequent assays.

20S proteasome particles were purified from HeLa cells following a published procedure (Rivett *et al.,* 1994).

Electrophoresis and Western blotting

In experiments exploiting L-[^{35}S]methionine-labeling (see below), proteins were resolved by SDS–PAGE (12% polyacrylamide in the resolving gel) and visualized/quantified by use of a phosphorimager (445 SI, Molecular Dynamics). In nonradioactive experiments, proteins were electroblotted onto nitrocellulose membrane. This was saturated first in 1% bovine casein in 50 m*M* Tri–HCl,

pH 7.5, 50 m*M* NaCl, 0.15% Tween 20 (WB buffer), at room temperature for 1 hr, and subsequently in the same buffer containing 5% Skimmilk (Difco) in place of casein, for another hour. Then it was incubated at room temperature for 2 hr with appropriate dilutions of one of the following antibodies: monoclonal antibody (MAb) 2001 anti Nef (Chemicon); MAb 12CA5 anti-HA (Boehringer); MAb 6630 anti-vimentin (Sigma); MAb MCP205 anti-HsN3, MAb MCP257 anti-HC9, MAb MCP102 anti-C10 (a generous gift of K. Hendil). After three washes in WB-Skimmilk buffer, membranes were incubated for 1 hr at room temperature with horseradish peroxidase (HRP)-conjugated anti-mouse, and revealed with an HRP chemiluminescent substrate (Super Signal, Pierce). Blots were exposed to X-ray film, and signals within film linear range were quantified with an imaging densitometer (GS670, Bio-Rad).

In vitro binding assays

For pull-down assays, Gst-Nef, Gst-HsN3, or controls, bound to glutathione–Sepharose resin, were incubated with prey protein for 1 hr at 4° in binding buffer (25 m*M* Hepes, pH 7.4, 50 m*M* NaCl, 2.5 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 0.1% Triton, 1% BSA, added with the protease inhibitor cocktail), and washed four times in the same buffer lacking BSA. Resin-bound complexes were boiled in SDS–PAGE sample buffer and analyzed in Western blot. Alternatively, Gst-HsN3 or the unfused Gst control were immobilized on nitrocellulose filter using a vacuum slot-blot device. After preliminary saturation in binding buffer, the membrane was incubated with His-Nef in the same buffer, at 4° for 1 hr. Then the membrane was washed four times in binding buffer and processed as for Western blot, using anti-Nef MAb as the primary antibody.

Cell transfection and analysis

COS-7 cells were grown in Dulbecco's MEM Glutamax (Gibco), 10% fetal bovine serum. In a typical experiment, 2.5 μg of each pMT-2 plasmid derivative (individually or in pairs) has been used to transform a 70% confluent 25-cm^2 flask, using the standard calcium phosphate procedure. Cells were harvested 48 hr after transfection and directly lysed in prewarmed (95°) SDS–PAGE sample buffer. Alternatively, after transfection, cells were washed twice with ice-cold PBS plus the protease inhibitor cocktail, scraped with a rubber policeman in the same buffer, briefly spun at 4°, and resuspended in cold lysis buffer (20 m*M* HEPES, pH 7.4, 50 m*M* NaCl, 2.5 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, protease inhibitors) added with either 0.01 or 0.5% Nonidet P-40. After a 15-min incubation on ice, cell suspension was forced three times through a 24-gauge syringe needle and centrifuged in a minifuge (13 krpm/15 min, 4°). Comparable amounts of pellet and supernatant fractions were boiled in SDS–PAGE sample buffer, loaded onto 12% SDS–PAGE gels, and analyzed in Western blot. In a third approach, at 45 hr cells were starved for 30 min in methionine and serum-free medium and labeled for 2 hr in 3 ml of the same medium containing 200 μCi L-[^{35}S]methionine (Pro-Mix, >1000 Ci/mmol, Amersham). They were then dissolved in 0.5% Nonidet P-40-lysis buffer and processed as described above. The centrifugation supernatants were immunoprecipitated with rabbit polyclonal antibody H847 anti-human proteasome (gift of K. Hendil).

To analyze endogenous N3 gene transcription in the presence of various pMT-2 derivatives, transfectants were processed for Northern blotting. Cells were lysed and total RNA purified according to the RNeasy kit (Qiagen) specifications. Per each transfectant, 6 μg of purified RNA was electrophoresed in 1.5% denaturing agarose (in 6% formaldehyde, 20 m*M* sodium phosphate, pH 7.00), blotted onto Hybond N+ (Amersham) nylon membrane, and hybridized with ^{32}P-labeled HsN3 cDNA insert (1.5×10^6 cpm/ml hybridization solution). Wash stringency was kept moderate (2× SSC, 0.05% SDS at 50°) to allow simian transcript recognition. The blot was rehybridized with actin cDNA probe for HsN3 signal normalization. Bands were quantified by use of the Phosphorimager.

RESULTS

A yeast two-hybrid system screening (Fields and Song, 1989) for cellular proteins that interact with HIV-1 Nef was performed. Yeast expressing a fusion protein comprising the DNA binding (DB) domain of Gal4 transcriptional activator and Nef (BH10 isolate) was transformed with a library of human cDNAs from HeLa cells, fused to the sequence encoding the Gal4 activation (A) domain. Due to the C-terminal position with respect to Gal4-DB, fused Nef is no longer myristoylated. The bait fusion was preliminarily tested to rule out that it could spuriously activate the ths reporters (Fig. 1).

Of $\approx 2 \times 10^7$ independent transformants, 79 were selected on the basis of their ability to activate transcription of both *HIS3* (selectable) and *lacZ* (screenable) reporter genes under the control of Gal4 binding sites. Using a combined PCR/dot-blot hybridization analysis (see Materials and Methods) the clones were divided in classes of related cDNA inserts. Partial sequences of several inserts from each class were then obtained to confirm class homogeneity and aligned to DNA and protein sequences in data banks. This analysis showed that 27 clones (34% of positives) contained the cDNA for HsN3 β-type proteasome subunit (Nothwang *et al.,* 1994). All five sequenced HsN3 clones contained the complete coding sequence (including the N-terminal propeptide, with the exception of the N-terminal Met codon), fused in frame to Gal4-A.

A second recurrent isolate was found to coincide with

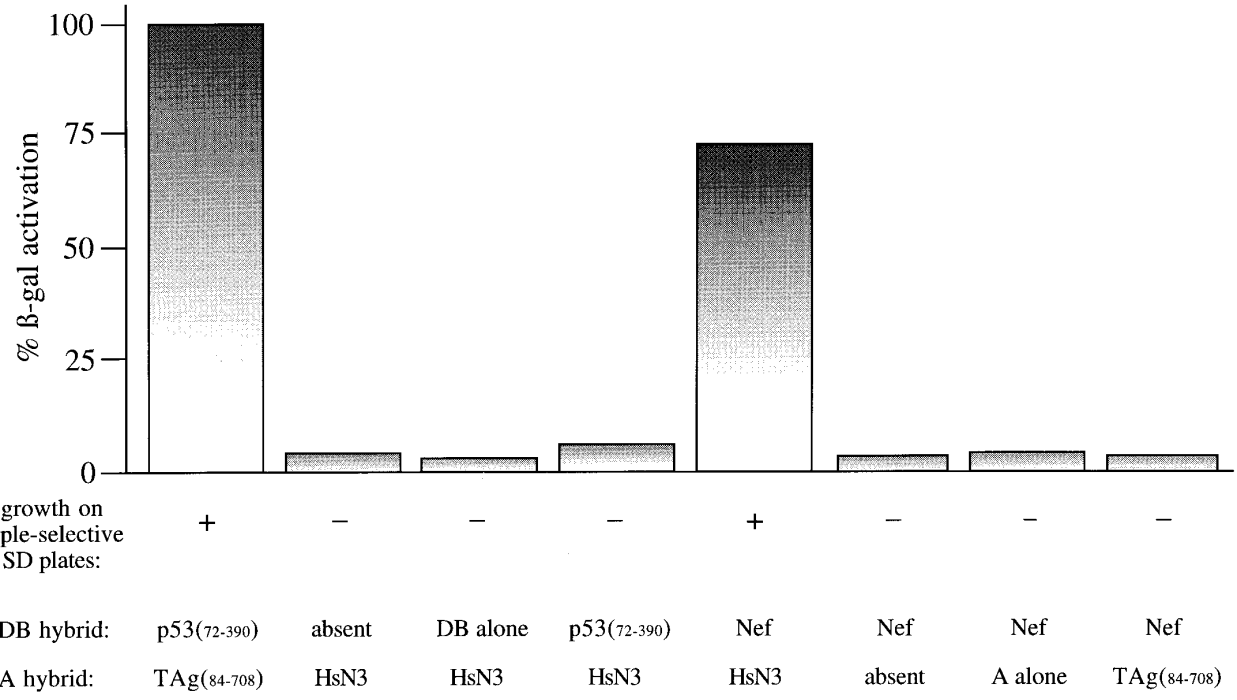


FIG. 1. Summary of yeast THS tests implicating HsN3 proteasome subunit as a Nef partner. HF7c yeast cells were transformed with plasmid combinations to express the indicated proteins. Cells were plated onto SD synthetic medium-agar plates, lacking the selection amino acids (Trp and/or Leu for single or double transformants, Trp, Leu, and His to select for Gal4 activity). After 4 days at 30°, plates were inspected for colony growth. Single colonies from single/double selection plates were reinoculated into liquid SD-selection medium and shaken at 30° overnight. Cells were lysed for soluble protein extraction according to the vortexing-glass beads method. All the above procedures followed Clontech Matchmaker kit protocols. Total protein was assayed with the BCA reagent (Pierce), and β-gal was assayed after Ausubel *et al.* (1991). Activity values (average of at least three colonies from at least two independent transformations) are expressed as percentage of positive control (the activity in cells harboring the strongly interacting hybrids Gal4-DB/p53 and Gal4-A/SV40 TAg).

the DFFRX gene (Jones *et al.,* 1996), a human homologue of *Drosophila fat facets* gene, encoding a deubiquitinating hydrolase (Huang *et al.,* 1995). The DFFRX fusion was also in frame with Gal4-A, but covered only the C-terminal 1/6th of the gene (codons 2058–2547 in the shortest ths clone).

Both Gal4–A/HsN3 (Fig. 1) and Gal4–A/DFFRX (data not shown) fusions in the original cross with Gal4–DB/Nef were strong activators of the *lacZ* reporter (60–70% activation relative to a standard ths interaction control, Gal4–DB/p53 × Gal4–A/TAg). Both HsN3 (Fig. 1) and DFFRX (not shown) passed standard tests for false positive exclusion, showing that neither protein could spuriously activate the reporter. Thus, both proteins appear as *bona fide* Nef partners in the yeast system.

These results suggested the possibility that Nef could bind two distinct components of the ubiquitin-proteasome pathway. Subsequent work, reported hereafter, was aimed at a more extensive characterization of Nef interaction with the most frequent isolate, HsN3. Nef binding properties of the pDFFRX protein, whose complete cDNA sequence has been recently published (Jones *et al.,* 1996), are presently under scrutiny.

Nef/HsN3 interaction was confirmed by *in vitro* binding experiments (Fig. 2) exploiting recombinant or natural Nef and HsN3 proteins in various combinations.

In one pull-down assay, a GST–Nef fusion and either GST fused to a Nef-unrelated protein or unfused GST were compared for their ability to capture an *in vitro*-translated, [^{35}S]methionine-labeled HsN3 protein. The GST proteins were captured with a Sepharose–glutathione resin, and the amount of copurifying HsN3 was revealed by fluorography after SDS–PAGE. Results of such experiments (Fig. 2A) showed that the Nef–GST fusion has an affinity for HsN3 much higher than the controls.

In a reverse approach, a GST–HsN3 fusion or a control GST were incubated with increasing amounts of a bacterially produced Nef, bearing a six histidine tag at its N-terminus (His-Nef; data not shown). Alternatively, increasing amounts of either GST–HsN3 or GST were incubated with a fixed amount of His-Nef (Fig. 2B). After adsorption to Sepharose–glutathione, the captured Nef was revealed in Western blot with an anti-Nef monoclonal antibody. In both cases, the HsN3 fusion, contrary to GST alone, bound Nef very efficiently, in a dose-dependent manner. The same combinations were assayed in a far-Western format (Fig. 2C), in which increasing amounts of either GST–HsN3 or GST were immobilized

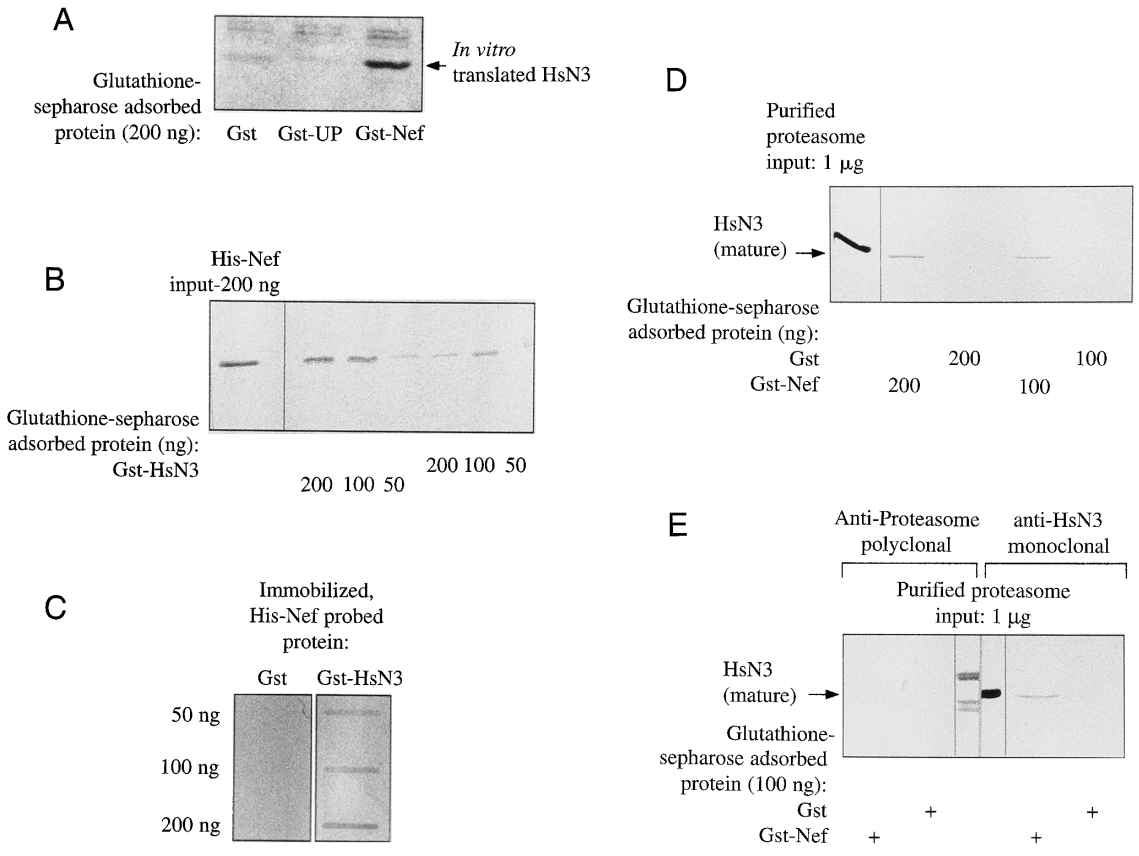


FIG. 2. Recombinant Nef binds both recombinant and natural HsN3 subunit *in vitro.* The indicated Gst fusions, adsorbed to glutathione–Sepharose resin, were incubated with *in vitro* translated HsN3 (A), bacterially expressed, purified His-Nef (B), or a purified human proteasome preparation (D, E), and analyzed, as described under Materials and Methods. Gst-UP: Gst-fused, Nef-unrelated protein (hepatitis B virus L surface protein, PreS domain). In C, Gst-HsN3 or the unfused Gst control dotted on nitrocellulose were serially incubated with His-Nef, anti-Nef MAb, and HRP-anti-mouse antibody.

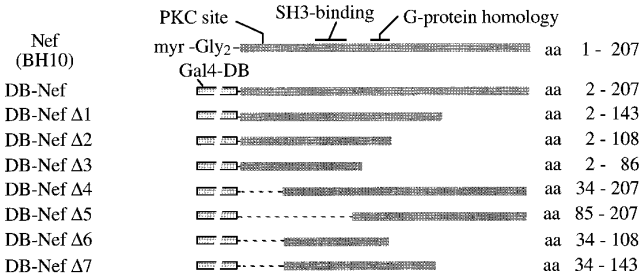
onto a nitrocellulose membrane and incubated with His-tagged Nef. Also in this case, HsN3 fusion, but not GST, retained Nef, and did it in proportion to the quantity of immobilized protein.

To ascertain the ability of Nef to interact with natural HsN3, the assay utilizing Gst–Nef fusion as the bait was repeated using as the prey a purified proteasome preparation instead of an *in vitro*-translated HsN3 subunit. Pulled-down proteins were detected in Western blot with either an anti-HsN3 monoclonal antibody or a rabbit anti-human proteasome antiserum (which apparently included negligible amounts of anti-HsN3 specific antibodies). In agreement with previous results, GST–Nef, but not unfused GST, captured the mature HsN3 subunit present in purified proteasomes, as shown by the specific monoclonal (Figs. 2D and 2E). By contrast, the antiserum revealed trace amounts of the total proteasome subunit ladder with either GST or GST–Nef bait (Fig. 2E). The opposite results obtained with the HsN3-specific antibody and the antibodies against total proteasome suggest that the fused Nef moiety could specifically bind HsN3 among spontaneously dissociated subunits in the proteasome preparation, or, alternatively, that it could bind HsN3 still incorporated into 20S particles, which were consequently dissociated.

In order to define the interacting regions in Nef and HsN3, a series of directional deletion mutants were created for both proteins, and each mutant was then tested against the intact partner in ths. Nef deletion variants fused to Gal4–DB were coexpressed in yeast with Gal4–A/HsN3, and the extent of reporter gene activation was measured with a liquid β-gal assay. As shown in Fig. 3, deletion analysis allowed restriction of the HsN3-interacting portion to Nef amino acids 34–143.

Conversely, a set of HsN3 deletion mutants fused to Gal4-A was expressed in yeast cells together with DB-Nef. Results from these experiments (Fig. 4) localized the Nef-binding region between HsN3 amino acids 73–249. A C-terminal stretch (aa 219–249) appeared particularly important, since its removal abolished Nef interaction. However, this C-terminal region alone was not sufficient for Nef-binding.

To verify that Nef and HsN3 interact also in intact mammalian cells, COS7 cells were transiently trans-



Proteins expressed in yeast double-transformants	Growth on triple-selective SD plates	Colony phenotype in ß-gal filter assay	In liquid ß-gal assay (% activation)
DB-Nef X A-HsN3	+	blue	
DB-Nef Δ1 X A-HsN3	+	blue	
DB-Nef Δ2 X A-HsN3	-	white	
DB-Nef Δ3 X A-HsN3	-	white	
DB-Nef Δ4 X A-HsN3	+	blue	
DB-Nef Δ5 X A-HsN3	-	white	
DB-Nef Δ6 X A-HsN3	-	white	
DB-Nef Δ7 X A-HsN3	+	blue	

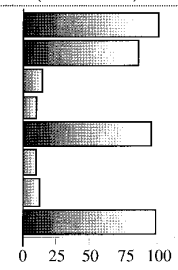
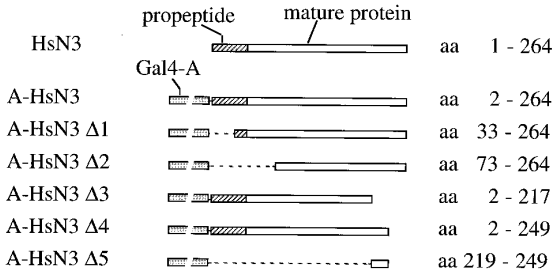


FIG. 3. Deletional mapping of the Nef portion which binds HsN3. Nef or Nef deletions were expressed in the yeast system as Gal4-DB fusions (top), in combination with Gal4-A-HsN3, as originally cloned in ths. Over the diagrammatic representation of wt Nef, the position of some relevant sequence tracts (Ratner and Niederman, 1995) is signalled. Selectable (*HIS3*) or screenable (*lacZ*) reporter activation was assessed as described under Materials and Methods and in the legend to Fig. 1 (bottom). The in liquid β-gal activity is reported as percentage of the intact partners cross (Gal4-DB/Nef X Gal4-A/HsN3).

fected either singly or doubly with plasmid-based expression vectors for Nef and HsN3, the former either unfused or carrying an epitope tag from influenza Hemagglutinin fused at the N-terminus (designated HA-Nef).

Preliminary immunoblot analysis with monoclonal antibodies to Nef (N-terminal epitope), HA or HsN3 revealed that cells singly transfected with the appropriate plasmid produced the corresponding protein in easily detectable amounts (Fig. 5A). Since COS7 cells constitutively express the simian homologue of HsN3, a baseline amount of simian subunit, electrophoretically indistinguishable from its human counterpart, was detected in controls that



Proteins expressed in yeast double-transformants	Growth on triple-selective SD plates	Colony phenotype in ß-gal filter assay	In liquid ß-gal assay (% activation)
A-HsN3 X DB-Nef	+	blue	
A-HsN3 Δ1 X DB-Nef	+	blue	
A-HsN3 Δ2 X DB-Nef	+	blue	
A-HsN3 Δ3 X DB-Nef	-	white	
A-HsN3 Δ4 X DB-Nef	+	blue	
A-HsN3 Δ5 X DB-Nef	-	white	

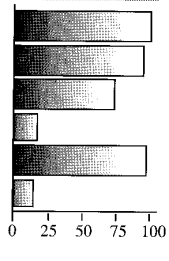


FIG. 4. Delineation of the HsN3 portion which binds Nef. HsN3, as originally cloned in ths or HsN3 deletions were expressed in the yeast system as Gal4-A fusions (top), in combination with Gal4-DB-Nef, and *HIS3* and *lacZ* reporter activation was assayed (bottom).

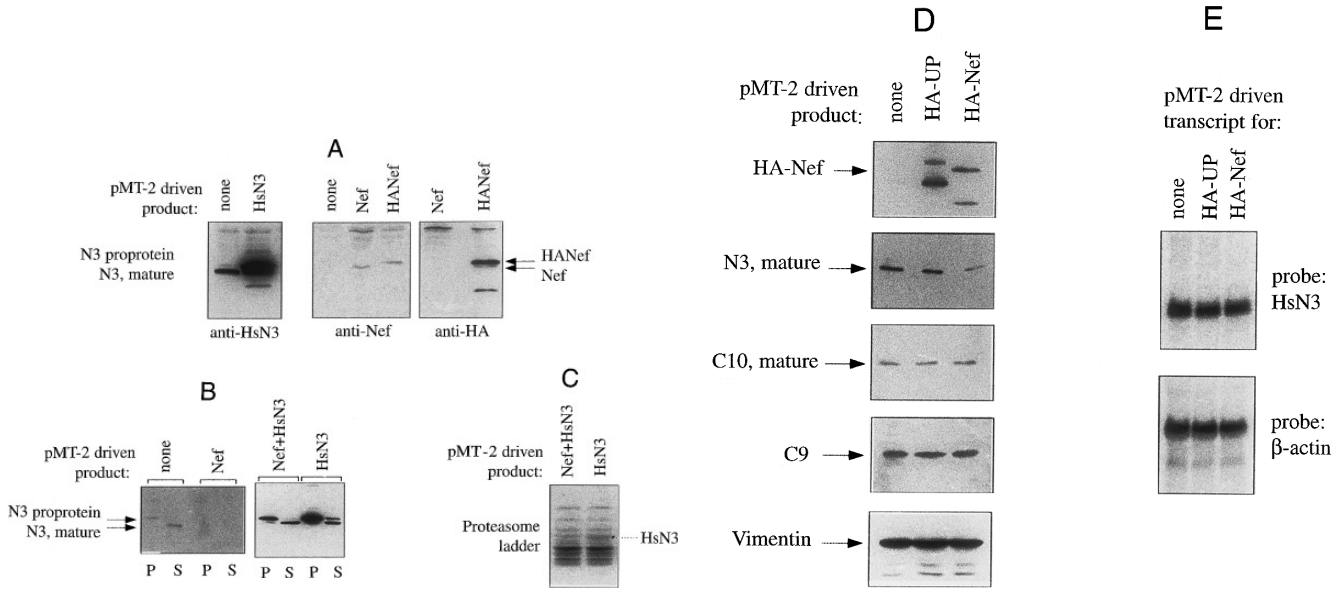


FIG. 5. Nef-induced downregulation of endogenous and transfected N3 subunits in COS-7 cells. Cells transfected to express the indicated products were analyzed in Western blot (A, B, D), in radioimmunoprecipitation (C), or in Northern blot (E), as described under Materials and Methods. In A and D, whole cell lysates, probed with distinct antibodies, were analyzed. In B, pellet (P) and supernatant (S) of fractionated cells, analyzed with anti-HsN3 MAb, are shown. In C, the soluble extract from a replica of transfectants in B-right was analyzed by immunoprecipitation with rabbit anti-human proteasome serum. In E, total RNA extracted from the same transfectants in C was analyzed in Northern blot using HsN3 cDNA as the probe and subsequently rehybridized with β-actin probe (a residue of HsN3 signal is still visible below the actin signal in the lower panel). In A–C, N3 indicates the position of human HsN3 and/or simian HsN3 homolog, which comigrate. In D and E, HA-UP indicates transfectants in which vector drives the synthesis of a HA-tagged, Nef-unrelated protein (a human HOX family protein).

received the empty vector (pMT-2). Comparison between pMT-2 transfected cells and HsN3 transfectants showed that the natural simian subunit was present mainly in the processed 28-kDa form, typical of the assembled proteasome, and in lesser amounts in the 32-kDa form still bearing the N-terminal propeptide (Thomson and Rivett, 1996). By contrast, in HsN3 transfectants the 32-kDa form was predominant, arguably because the proportion synthesized in excess relative to the other subunits could not be incorporated into 20S particles and processed consequently. (However, that human N3 subunit was partly coassembled with other simian subunits was proven by radioimmunoprecipitation experiments using anti-HsN3 MAb, in which a ladder of coprecipitating endogenous proteasome subunits was revealed along with HsN3; data not shown.) The unprocessed and processed forms were separated when cells were subjected to a mild fractionation protocol (0.01% nonionic detergent in the extraction buffer). While the processed, proteasome-associated form was found principally in the soluble fraction, the precursor form remained in the particulate fraction (see Fig. 5B). Under similar conditions, both Nef and HA-Nef were found in the particulate fraction (data not shown). On the other hand, when harsher extraction conditions were adopted (0.5% nonionic detergent), Nef and both HsN3 forms were largely solubilized (data not shown).

In an attempt to detect a stable Nef–HsN3 interaction, we then performed immunoprecipitation experiments from cotransfected cells. Lysates in 0.5% nonionic detergent from cells doubly expressing HsN3 and Nef or HA-Nef were immunoprecipitated with monoclonal antibodies against Nef, HA epitope, or HsN3, and immunoprecipitates were examined by immunoblot with anti-HsN3 or anti-Nef. While each antibody captured the corresponding antigen, this analysis failed to reveal appreciable amounts of coprecipitating partner (data not shown).

Nef–HsN3 interaction in cotransfected cells might have escaped our analysis because it was transient or was disrupted by the lysis procedure. We then looked for an indirect proof of Nef–HsN3 interaction, by verifying whether Nef coexpression altered the subcellular distribution and/or the extent of conversion of precursor to mature HsN3. A relative increase in HsN3 proprotein could be expected if Nef interfered with HsN3 incorporation into proteasome. To our surprise, immunoblot analysis demonstrated that Nef induced, on the contrary, a dramatic downregulation of HsN3 levels. This effect was observed both for the overexpressed human subunit, in Nef + HsN3 (Fig. 5B) and HA-Nef + HsN3 (not shown) double transformants, and for the endogenous simian subunit (N3), in Nef (Fig. 5B) and HA-Nef (Fig. 5C) single transformants. This implies in particular that the effect was not discriminating between human and simian N3. Also, the effect was selective to HsN3 β subunit or its simian homolog, since the levels of other proteasomal

proteins, as visualized in immunoprecipitation with anti-proteasome antiserum, were unaffected by Nef in both Nef single transfectants (data not shown) and Nef plus HsN3 double transfectants (Fig. 5D). In the last experiment, we could observe an interpretable HsN3 signal only with a long (2 hr) radioactive pulse. The reduction of plasmid-driven HsN3 in the presence of Nef was already appreciable at the 0-hr chase point (Fig. 5D) and did not change appreciably through a 12-hr chase (data not shown). Thus, to the best of our knowledge, HsN3 down-regulation is either a translational or an early posttranslational effect.

In mammals, three proteasomal subunits, LMP2, LMP7, and MECL-1, are inducible by γ-interferon and replace other constitutive subunits (LMP2 and LMP7 are important for class I T antigens presentation). Proteasome subunits are modulated at the transcriptional level also in other settings, for instance during insect development, in mitogen-stimulated cells, and in muscle atrophy in mammalians (reviewed in Coux *et al.,* 1996). In addition, Nef has been known to influence transcription of a number of genes. Thus, proteasome subunit genes can undergo transcriptional modulation, and Nef might in principle induce it. To ascertain whether Nef was turning off transcription from the HsN3 promoter on the vector and/or from the endogenous simian gene promoter, Nef or HA-Nef transformants and Nef/HA-Nef plus HsN3 double transformants were analyzed in Northern blot for their content in HsN3 mRNA. No appreciable Nef-dependent decrease in HsN3 or natural simian homolog mRNAs was revealed by this analysis in repeated experiments. A recapitulative experiment is shown in Figs. 5D and 5E, where equal amounts of either total protein or total RNA from COS-7 cells expressing the empty vector, a HA-tagged, Nef-unrelated protein, or HA-Nef were compared for content in simian proteasome subunits N3, C10 (another β-type subunit), and C9 (an α-type subunit) (Fig. 5D) and N3 transcript content (Fig. 5E), in Western blot and Northern blot, respectively. While the level of N3 protein in HA-Nef transfectants was approximately 1/15th relative to controls, the level of N3 mRNA was apparently unaffected by HA-Nef expression, and the levels of distinct proteasome subunits C9 and C10 appeared unchanged as well.

DISCUSSION

A number of virus proteins are tailored to accomplish multiple tasks, in agreement with viruses' commitment to genetic economy. Lentiviral Nef proteins warrant a special mention in this context, since the list of events they can trigger in infected cells is steadily growing, and at least the two best documented ones, CD4 receptor downregulation and virion infectivity enhancement, seem to reflect separable activities. The list is further extended by the finding, reported here, that HIV-1 Nef protein can bind proteasomal subunit HsN3 and determine its selective downmodulation.

20S proteasome (Coux *et al.,* 1996) is formed by four heptameric rings stacked to form a hollow cylinder. Seven α subunits form each of the outer rings, and seven β subunits form each of the inner rings. The β subunits — which include the proteolytically active ones — are coded for by 7 related genes in yeast and by more than 10 genes in mammalians. They are synthesized as inactive proproteins, carrying an N-terminal propeptide that chaperones them into the assembling 20S complex (Chen and Hochstrasser, 1996; Schmidtke *et al.,* 1996) and is removed upon the sealing of the catalytic chamber, thus preventing the premature activation of the proteases. HsN3 is a constitutive proteasome β subunit (Kopp *et al.,* 1995), but is thought to be proteolytically inactive (Schmidtke *et al.,* 1996).

In our study, HsN3 was isolated as the most frequent Nef partner in a ths screening covering 2×10^6 independent clones. Next, the interaction was confirmed by *in vitro* experiments, indicating that Nef could bind free HsN3, not only in recombinant versions but also as the mature protein dissociated from a proteasome preparation either spontaneously or after Nef binding.

In Nef, the region essential for interaction was mapped to aa 34–143. This tract covers almost completely the most extended structured portion in a Nef crystal model, aa 71–148, including, from the N-terminus, PPII helix, α helixes A and B, and β-sheets A–C, up to the boundary of the solvent-exposed loop 149–177 (Lee *et al.,* 1996). Tract 34–143 includes the Arg105–Arg106 motif essential for copurification with, and activation of, PAKs by Nef (Sawai *et al.,* 1996; Lu *et al.,* 1996), as well as all the residues involved in tyrosine kinase SH3 binding (Lee *et al.,* 1996), with the exception of the conserved Pro147, which enhances SH3 binding *in vitro* (Saksela *et al.,* 1995). More generally, deletion/domain-swapping studies have shown that the integrity of this central portion of the protein is important for Nef activity in the principal assays, Nef-induced CD4 downregulation (Goldsmith *et al.,* 1995; Garcia and Foster, 1996), infectivity enhancement (Goldsmith *et al.,* 1995), and PAK copurification (Garcia and Foster, 1996). Truncation at position 134, on the other hand, deletes the loop charged residues found to be important for CD4 downregulation (Aiken *et al.,* 1996).

The interacting HsN3 region could be restricted to region 73–249, with aa 219–249 being essential, though not sufficient. It is worth noting that the C-terminal portion of proteasome β-type subunits is, together with the N-terminal propeptide, the least conserved and a probable mediator of subunit-specific functions (Coux *et al.,* 1996). More interestingly, HsN3 C-terminal aa 219–264 can be aligned to SH3 domains from Src-related tyrosine kinases

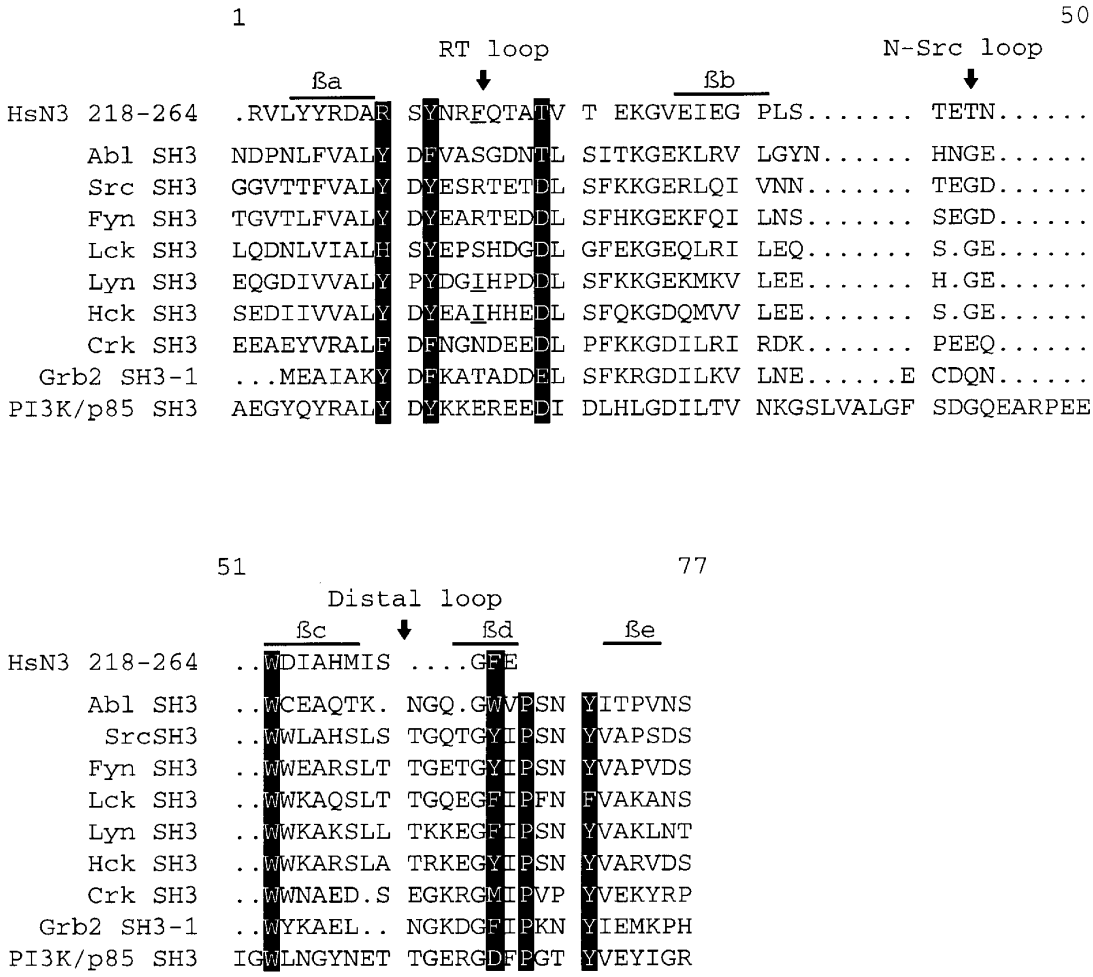


FIG. 6. HsN3 C-terminus is homologous to the SH3 protein module. HsN3 sequence was compared with SwissPir data bank, using the BlastP algorithm. A significant alignment was found between HsN3 C-terminus and Src SH3 domain. HsN3 C-terminal 47 amino acids were then aligned to a choice of SH3 domains from different proteins, by use of the Clustal algorithm. The amino acid residues involved in PxxP motifs binding by SH3s (Mayer and Eck, 1995) and (hypothetically) HsN3 have been boxed. The Ile residue in Hck and Lyn sequence important for Nef PxxP repeat binding (according to *in vitro* experiments, Saksela *et al.,* 1995; and structural studies, Grzesiek *et al.,* 1996; Lee *et al.,* 1996), and the Phe residue similarly positioned in HsN3, are underlined.

and other proteins (Fig. 6). SH3 domains are approximately 60 aa long and fold into five β-sheets interrupted by loops. Crystallographic and mutational analysis have shown that seven aromatic, hydrophobic, or charged residues with conserved location and spacing in the aligned SH3s (highlighted in Fig. 6) are crucial for PxxP repeat recognition (Mayer and Eck, 1995). HsN3 sequence is homologous to SH3 β-sheets 1–4, with the first five critical residues identical or homologous to at least one known SH3. Also, a conservative substitution (Phe232) aligns to the Ile residue in Hck and Lyn SH3 RT loop (Fig. 6) found to give an essential contribution to the interaction with Nef PxxP (Saksela *et al.,* 1995; Grzesiek *et al.,* 1996; Lee *et al.,* 1996). Thus, Nef binding might be mediated by recognition of Nef proline-rich motifs by a HsN3 stretch resembling an incomplete SH3 domain. Since our truncation analysis has indicated that the last 15 HsN3 aa are dispensable for Nef binding, the proteasome subunit might use for recognition only the N-terminal 3/5 of the SH3-like stretch. The fact that sequences in the upstream 73–217 tract are also required for Nef binding might mean that they either participate in binding or expose in the optimal conformation the crucial 219–249 tract. A more extensive mutational analysis of both partners is required to substantiate this hypothesis.

In order to demonstrate that Nef–HsN3 interaction can actually take place inside mammalian cells, *nef* and HsN3-coding genes were transfected into COS7 cells. While attempts to copurify Nef–HsN3 complex were fruitless, a dramatic downregulation of intracellular HsN3 protein levels was observed in Nef transfectants, in face of maintained HsN3 mRNA levels. Nef-induced downmodulation appeared to affect HsN3 in both the proprotein and the mature form.

Our favored interpretation of the above observations is that also within the cell HIV-1 Nef interacts with HsN3 proteasomal subunit, possibly prior to its processing inside mature proteasomes. Since Nef binding does not require per se the N-terminal propeptide, Nef preference for HsN3 proprotein might descend from a common subcellular localization and/or the masking in the 20S particle of HsN3 tracts essential for the interaction (see Groll *et al.,* 1997, for yeast proteasome structure). Nef binding would divert HsN3 to a breakdown pathway. Alternatively, it might induce a negative feedback at a posttranscriptional stage in HsN3 expression. HsN3 downregulation might imply the replacement by a distinct β-type subunit into assembled 20S particles, although this has not yet been experimentally proven. Admittedly, this model is speculative in nature, since the essential point of a Nef–HsN3 physical interaction in intact cells could not be demonstrated, and the downregulation effect might be indirect. Even in this case one must admit that the effect is HsN3-specific.

The HsN3 downregulation effect is produced with equal efficiency by wild-type, myristoylated Nef and by a N-terminally tagged, nonmyristoylated Nef mutant. This is a striking point, since the other most extensively analyzed Nef effects are abolished or strongly attenuated by mutational suppression of N-terminal myristoylation. This may be an indication that HsN3 downregulation reflects indeed a distinct Nef activity, not related to, e.g., a membrane targeting dependent activation of a peripheral signal cascade. On the other hand, it could unveil a function of a naturally soluble Nef form: the proportion of myristoylated Nef which is cytosolic in cell fractionation assays; the variant initiated at *nef* 5′-most internal ATG (Kaminchik *et al.,* 1991); or the proteolytic Nef fragment (lacking the N-terminal 1–57 tract) generated by HIV-1 protease inside HIV-1 virions (Pandori *et al.,* 1996; Welker *et al.,* 1996; Bukovsky *et al.,* 1997), which might exert its effects after introduction into the recipient cells.

Targeting of a proteasome subunit, and HsN3 in particular, by viral proteins is not unprecedented. Human T-cell leukemia virus type 1 Tax protein has been reported to bind both HsN3 and the α-type subunit HC9 (Rousset *et al.,* 1996). Hepatitis B virus pX protein binds XAPC7, a novel α-type subunit (Fischer *et al.,* 1995; Huang *et al.,* 1996). Interestingly, the pX region that mediates XAPC7 binding shows homologies to a Nef helical stretch after aa 100 (Samuel *et al.,* 1991). Functional parallelism between Nef, Tax, and pX goes further, because also Tax (Lillehoj and Alexander, 1992) and pX (Wu *et al.,* 1990) have been detected as virion constituents, and like Nef might influence virion infectivity. Both Tax and pX affect intracellular signalling, and stimulate in particular NF-kB responsive transcription. For both, proteasome subunit binding has been hypothesized to trigger NF-kB activation (Rousset *et al.,* 1996; Huang *et al.,* 1996), which depends on proteolysis by the proteasome (Palombella *et al.,* 1994). It is tempting to speculate that HsN3 might play a dedicated role in NF-kB activation, and that Nef-induced downregulation of HsN3 might in turn be related to Nef negative effect on NF-kB, reported by some groups (Niederman *et al.,* 1992, 1993). More generally, by binding to a proteasome component, Nef, as well as Tax and pX, might interfere in a complex way with degradative processes, enhancing or depressing the half-life of transcription factors, of other regulatory proteins or of peripheral receptors. This could explain in part their markedly pleiotropic effects in the cell.

Another intriguing possibility is that Nef, by interfering with proteasome function, may depress the presentation of viral T cell antigens by class I MHC, to the advantage of viral infection establishment or persistence. Such an effect might synergize with MHCI surface downregulation.

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