Rack1 Binds HIV-1 Nef and Can Act as a Nef–Protein Kinase C Adaptor

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INTRODUCTION

Nef proteins of primate immunodeficiency viruses exert pleiotropic effects, such as enhanced endocytosis of CD4 and MHC-I cell surface molecules, perturbation of signal transduction cascades, and virion infectivity enhancement. Nef function intersects that of a number of cell kinases, including C kinases (PKCs) and Src-family kinases. Here the interaction of HIV-1 Nef with Rack1 (receptor for activated C kinase 1) is reported. Nef binds the Rack1 C-terminal moiety in a yeast two-hybrid system and in cell-free pull-down assays and copurifies with in vitro translated Rack1. Nef and Rack1 partially colocalize on the trans-Golgi network and plasma membranes. The presence of Rack1 doubles Nef phosphorylation by PKCs in vitro. Our data agree with the idea that Rack1 acts as a Nef intracellular docking site, bringing Nef and PKCs together. Other signal transduction or endocytosis proteins, in particular Src-like kinases, might meet Nef by intermediation of the Rack1 adaptor.

Nef proteins are encoded by a gene overlapping the 3′ long terminal repeats of human immunodeficiency viruses, types 1 and 2 (HIV-1 and -2), and simian immunodeficiency virus (SIV). Nef is synthesized early after infection and is crucial for the maintenance of high viral loads and for pathogenesis (recent reviews in Cullen, 1998; Marsh, 1999; Oldridge and Marsh, 1998; Harris, 1999). The replication advantage of Nef-positive viruses may be due in part to the enhancement of viral particle infectivity (Aiken and Trono, 1995; Chowers et al., 1995; Miller et al., 1994; Le Gall et al., 1997) and/or virion release (Lama et al., 1999; Ross et al., 1999), in part to Nef-induced release by infected macrophages of CC-chemokines recruiting and activating CD4+ lymphocytes at sites of virus replication (Swingler et al., 1999). However, Nef exerts direct pathogenic effects in the absence of other viral products, as shown by the occurrence of an AIDS-like impairment of the immune system in mice expressing a nef transgene (Skowronski et al., 1993; Hanna et al., 1998; Larsen et al., 1998).

Nef has been credited with diverse molecular partnerships inside the infected cell. One of Nef’s roles is to decrease the cell surface expression of CD4 (and less efficiently of HLA-A/B) by accelerating CD4 endocytosis and routing receptors to lysosomes for degradation. Nef may function as a connector linking CD4 to endocytic adaptor complexes [to the endocytic signals within CD4 and MHC-I cytoplasmic tails; to β/μ adaptins in clathrin adaptor complexes; to PACS-1 in the trans-Golgi network (TGN) (Piguet et al., 2000); to β-COP in coatomer-coated vesicles in the TGN to the lysosome route], although exploiting distinct interaction surfaces in HIV-1 Nef and the cognate SIV protein (reviewed in Piguet et al., 1999; Oldride and Marsh, 1998). Since a mutated CD4 cytoplasmic tail lacking protein kinase C (PKC) target-serines has been shown to be refractory to phorbol esters but sensitive to Nef downregulation (Garcia and Miller, 1991; Aiken et al., 1994), the CD4 endocytosis induced by Nef seems to be independent of CD4 phosphorylation. Conversely, Nef phosphorylation by phorbol-ester-activated PKC enhances the Nef-promoted downregulation of CD4 (Luo et al., 1997). Also, Nef has been proposed to bind theta PKC, an isozyme important for T-cell activation, and to inhibit its function (Smith et al., 1996). Nef is, in addition, a nonsubstrate ligand for various serine/threonine and nonreceptor tyrosine kinases (reviewed in Renkema and Saksela, 2000). Among the latter, Nef interacts with Lck, Hck, and Src kinases, with an essential role in the association played by Nef polyproline helix II binding kinase SH3 domain. Perturbations of the normal functioning of CD4, T-cell receptor, and signaling kinases (Bell et al., 1998; Howe et al., 1998; Fackler et al., 1999; Xu et al., 1999; Wang et al., 2000) most probably underly the reported alterations in cell signaling pathways and gene expression induced by Nef.

These multiple Nef effects reflect the activities of a truly multifunctional viral protein, since they are partly...
mutationally separable (Goldsmith et al., 1995; Le Gall et al., 1997; Iafrate et al., 1997; Mangasarian et al., 1999). Also, the growing number of molecular interactions that are being reported for Nef seemingly reflect the ability of the viral protein to insert into, and modify, the network of dynamic interactions taking place in the endosomal pathway and in signal transduction cascades. Here, we show that HIV-1 Nef can associate with Rack1 (receptor for activated C kinase 1), a known intracellular receptor for PKCs and other C2 domain-containing proteins (Ron et al., 1994). We also show that Rack1 is, like Nef, in equilibrium between a cytosolic and a membrane-bound state and that it partially colocalizes with the viral protein intracellularly. Interestingly, Rack1 enhances Nef phosphorylation by PKCs in vitro.

**RESULTS AND DISCUSSION**

A yeast two-hybrid system (Y2HS) screening, in which HIV-1 Nef was used as a bait for a human cDNA library from HeLa cells (Rossi et al., 1997), identified partial cDNAs for Rack1 as recurrent prey inserts (five independent clones; Fig. 1A). Previous studies exploiting similar yeast screenings have led to the identification of the coatomer component $\beta$-COP (Benichou et al., 1994), of a thioesterase (Liu et al., 1997; Watanabe et al., 1997), of the HsN3 proteasome subunit (Rossi et al., 1997), of a subunit of the vacuolar proton ATPase involved in endosome acidification (Lu et al., 1998), and of Naf, a putative CD4 escort (Fukushi et al., 1999) as Nef partners. All of the Rack1 clones were terminally sequenced

![Diagram](image-url)
and found to cover the 3' 3/7th of Rack1 coding sequence (codons 160/180 through 317). The shortest clone was totally sequenced and found to exactly match the published nucleotide sequence of the human gene. Human Rack1 was originally identified as a homolog of heterotrimeric G–protein β subunits (Guillemot et al., 1989). Like these, Rack1 contains seven tandem repetitions of the WD40 domain (Neer et al., 1994), each including four β strands, and is expected to fold into a toroidal, seven-bladed β propeller structure (Neer and Smith, 1996; Fig. 1A). In resolved structures, propeller stabilization is provided by a "velcro" closure between the N-terminal-most and the C-terminal-most β strands, within the first and seventh WD40 repeat, respectively, and repeats are permuted with respect to the structural repeats (blades) of the β propeller domain (Wall et al., 1995; Fig. 1A). Rack1 lacks the N-terminal α-helical extension essential for G–protein β interaction with the gamma subunits (Wall et al., 1995). From this background, the preliminary inference could be made that Nef binding by Rack1 relies on the C-terminal WD40 repeats 5–7, since the shortest the Rack1 cDNA clone exactly covers these repeats, and that a complete, circularized Rack1 propeller structure is not essential for Nef binding.

We consequently set up experiments aimed at addressing the following questions: (i) whether spurious activation of the ths reporters by Rack1 C-terminal moiety could be ruled out; (ii) whether the interaction could be reproduced in cell-free assays; and (iii) which tracts within Rack1 and Nef were critical for the interaction.

For Y2HS false interaction exclusion, the original Y2HS Rack1(WD5–7) insert within the Gal4-activation domain (AD) plasmid was transformed alone or cotransformed with plasmids expressing the Gal4 DNA-binding domain (DB) either unfused or fused to a Nef-unrelated protein. (The absence of Y2HS activation by Gal4–DB–Nef alone, or crossed with control Gal4–AD constructs, had been previously shown by our and other laboratories). None of the control crosses significantly activated the yeast reporters, thus reassuring us as to the specificity of the Rack1–Nef interaction (Fig. 2).

In order to confirm Rack1–Nef interaction in vitro, a recombinant hexahistidine-tagged Nef purified from bacterial cells or a Nef-unrelated control were pulled down with either Rack1(WD5–7) fused to glutathione S-transferase (GST) or controls (GST alone or fused to a Nef-unrelated protein). The absence of Y2HS activation by Gal4–DB–Nef alone, or crossed with control Gal4–AD constructs, had been previously shown by our and other laboratories). None of the control crosses significantly activated the yeast reporters, thus reassuring us as to the specificity of the Rack1–Nef interaction (Fig. 2).

FIG. 2. Y2HS tests. 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 Leu for double transformants, Trp, Leu, and His to select for Gal4 activity). After 4 days at 30°C, triple selective plates were inspected for colony growth. Colonies from double selection plates were filter assayed for β-gal activity. Alternatively, single colonies were reinoculated into liquid SD-selection medium and shaken at 30°C overnight. Cells were lysed for soluble protein extraction according to the vortexing-glass beads method. Total protein was assayed with the BCA reagent (Pierce) and β-gal was assayed in liquid. β-Gal activity values (average of at least three colonies from at least two independent transformations) are expressed as a percentage of positive control [the activity in cells harboring reference partners, the interacting hybrids Gal4–DB/p53 (amino acids 72–390) and Gal4–A/SV40 TAg (amino acids 84–708)].
it was interesting to verify that a complete, folded Rack1 protein could also interact with Nef and that the observed interaction was not due to the incorrect exposure of amino acid residues normally hidden inside the folded torus. To this purpose, we took advantage of the ability of WD40 proteins to correctly fold when translated in vitro in a rabbit reticulocyte lysate. Folded Rack1 polypeptides produced in this system can be discriminated from incomplete and/or misfolded molecules by gel filtration, since the latter aggregate into high-molecular-weight complexes (Garcia-Higuera et al., 1996). A coupled in vitro transcription–translation system was programmed with a plasmid expressing a full-length rat Rack1 (rat Rack1 is identical at the amino acid level with the cognate human protein; Ron et al., 1994). After completion of the translation reaction, bacterial Nef was added and the mixture was further incubated. The mixture was then loaded onto an analytical gel filtration column and the elution profile of recombinant Rack1 and Nef was revealed by phosphorimaging or by Western blot, respectively, after resolving proteins by SDS–PAGE. Results showed that a proportion of in vitro translated Rack1, as expected, aggregated and was excluded from the column. However, a fraction migrated as a folded monomer and another minor fraction was resolved as a higher molecular weight peak. Nef, on the other hand, eluted mainly as a monomer, but a minor fraction was found to coelute with the higher molecular weight Rack1 peak (Fig. 3B). When the addition of Nef to the Rack1 translation mixture was omitted, the Rack1 heavy peak disappeared; in contrast, on the same column neither Nef run alone (in the presence of molecular weight markers) nor Nef mixed with in vitro translated human G-alpha i produced the higher molecular weight peak (data not shown). We interpret these results as an indication that a folded Rack1 and Nef can interact in vitro, forming a heterodimer or higher order oligomer, which is chromatographically resolved from the monomeric forms of either protein.

We subsequently attempted to more extensively map Rack1–Nef interactions by use of the Y2HS assays (Fig. 2). At first we asked whether Rack1 WD40 repeats 1–4, although not necessary for the interaction, nonetheless could contribute to it, by either establishing additional contacts with the Nef molecule or stabilizing the C-terminal WD40 repeats in the optimal fold. The full-length rat Rack1 or the N-terminal moiety spanning WD40 repeats 1–4 [Rack1(WD5–7)] was expressed in the reporter yeast strain as Gal4–AD fusions, together with Gal4–DB–Nef. While Rack1(WD1–4) did not appreciably activate yeast reporters, full-length Rack1 did, although to a significantly lesser extent than Rack1(WD5–7). This suggests that the WD40 repeats 1–4 do not contribute to Nef binding. Nef binding by the full-length protein, on the other hand, confirms the in vitro results. The apparent reduction in yeast reporter activation observed for the full-length relative to the truncated Rack1(WD5–7) may be a consequence of the experimental setting; for in-
Rack1 BINDS HIV-1 Nef

stance, the complete Rack1 may contain subcellular targeting signals (membrane targeting signals; see below) which counter the nuclear targeting needed for the genetic assay to work; or, due to Rack1 circularization, the N-terminally fused Gal4–A domain may create a steric hindrance on the C-terminal blades, thus partially inhibiting Nef binding. However, among the previously reported Rack1 molecular partners, integrin β subunit (Liliental and Chang, 1998) also displayed preference for an incomplete Rack1 form, prompting the hypothesis that a regulated Rack1 conformational shift may be involved in the association between Rack1 and some partners (see below).

We then attempted to delineate the tract within the Rack1 C-terminal moiety required for Nef binding by creating deletion variants of the initial Rack1(WD5–7) lacking one or two WD repeats and comparing their ability to activate the yeast system as Gal4–A fusions in crosses with Gal4–DB–Nef. Results indicated a general reduction of reporter activation with respect to the reference construct, which was more dramatic (to near background levels) with deletions affecting the sixth WD40. Considering that the sixth WD40 contributes to both the fifth and the sixth Rack1 propeller blades, a conservative interpretation of these data may be that residues within Rack1 blades 5–6 seem particularly important for Nef binding.

Next we looked for Nef regions important for the interaction (Fig. 2). A collection of terminal deletion variants, plus a triple point mutant bearing Ala substitutions at prolines 72–75–78 which are crucial for Nef polyproline helix-II interaction with SH3 domains [Saksela et al., 1995; mutant (Pxx)_3], was analyzed. Results showed that Rack1 binding tolerated deletion of the C-terminal Nef portion including the internal solvent-exposed loop and the C-terminal β strand (Grzesiek et al., 1996; Lee et al., 1996), whereas lesions of the N-terminal unstructured tract and of the central structural core (Grzesiek et al., 1996; Lee et al., 1996) abolished the interaction. However, an intact polyproline tract was not required for Nef–Rack1 association.

It is important to stress that a sample of the Y2HS Nef and Rack1 deletion variants described above was checked by Western blot analysis of total yeast protein. The analysis confirmed that fusions were expressed at similar levels (data not shown); therefore, the two-hybrid readout reflects bona fide interactions, not variations in deletion protein stability in yeast cells.

Rat Rack1 was isolated by interaction cloning as an activated C kinase binding protein, hence the name (Ron et al., 1994). Like other PKC intracellular receptors, it is thought to mediate the relocalization of activated PKCs. Since Nef is a substrate for PKC, a relevant question was whether the Rack1–Nef interaction can influence Nef modification by PKC. To check this point Rack1 was translated in vitro and mixed with bacterial 6His-tagged Nef. The Nef–Rack1 complex as eluted from a gel filtration column, or Nef alone as a control, was exposed to purified α-γ C kinases in a reaction mixture containing Ca²⁺, phosphatydilserine, and diglycerides to activate the enzyme, and [γ-32P]ATP. Subsequently, Nef was recovered by nickel chelation chromatography under denaturing conditions, and washed beads were counted for radioactivity. Results showed that, under the adopted conditions, Nef labeling by PKCs was almost doubled by the presence of Rack1 (Fig. 4A). A comparable result was obtained when Nef copurification with Rack1 by gel filtration was omitted, and Nef was instead mixed in the assay with either GST-Rack1(WD5–7) or control Gst: only the C-terminal Rack1 fusion stimulated Nef phosphorylation by PKC (Fig. 4A). Regardless of Rack1 presence, Nef was labeled mostly on serines, as evidenced by Western blot analysis of the purified Nef with monoclonal antibodies specific to phosphoamino acids (Fig. 4B). This outcome was interesting, since it suggests that Nef and PKCs do not compete for the same binding site(s) on Rack1, although the principal PKC binding site maps within Rack1 WD6 (Ron and Mochly-Rosen, 1995), that is, in a Rack1 tract which may participate in Nef binding, according to our data. Rather, Rack1 seems to facilitate Nef modification by C kinases.

Nef is targeted to the cytosolic face of cell membranes by N-terminal myristoylation and the N-terminal unstructured domain, in particular to rafts (Wang et al., 2000), although a fraction of the protein can be isolated from cells in soluble form, possibly due to a switch in myristic exposure. Membrane-bound Nef is detected by immunofluorescence (IF) analysis in part on the plasma and trans-Golgi network membranes, coherently with its function in endocytosis (Foti et al., 1997). Rack1 has in turn been shown to partition between the cytosol and membrane/cytoskeleton (Chang et al., 1998; Geijsen et al., 1999; Liliental and Chang, 1998; Ron et al., 1999; Yarwood et al., 1999). To compare Nef and Rack1 subcellular localization in cells transiently expressing the viral protein, we investigated it by cell fractionation and IF. 293-HEK cells were dounced in hypotonic buffer and nuclei were sedimented at low gravity (600g). Cell membranes were then pelleted by high-speed centrifugation (100,000g), and the nuclear, microsomal, and cytosolic fractions were analyzed in Western blot with an anti-Rack1 Mab. Cells transfected to express Nef were subjected to the same procedure in parallel (Fig. 5). Most of the Rack1 detected was associated to membranes, with a minor fraction in the cytosolic extract. This distribution was unchanged in Nef-expressing cells, where the viral protein exhibited a similar fractionation profile (a major fraction with membranes, a minor fraction soluble) in accord with many previous reports. Also, preexposure of cells to a PKC agonist [100 ng/ml phorbol myristate acetate (PMA) for 4 h] did not dramatically alter the fractionation profile of either protein. The absence of
PMA effects on Nef subcellular localization confirms previous reports (Coates et al., 1997; Luo et al., 1997).

IF analysis revealed Rack1 on peripheral and intracellular membrane locations, coherent with the protein being on plasma and pericentrosomal membranes. Rack1 signal was found to colocalize in part with that of clathrin, the essential constituent of plasma membrane-coated pits and the TGN vesicle coat, which in turn colocalizes in part with transfected Nef. Both Nef and Rack1 colocalization with clathrin were incomplete, with an apparent variability from cell to cell (Fig. 6 is an example of substantial colocalization). Altogether, data from cell fractionation and IF suggest that Rack1 is in part localized on intracellular membranes, at sites partially coinciding with Nef-positive compartments.

The observations reported above might be relevant with respect to Nef subcellular targeting, to its interaction with PKCs as a substrate, and potentially to its functional interplay with Src kinases and the endocytic apparatus. In summary, we have found that Rack1 is a molecular partner for Nef in both ths and cell-free assays. We also found that the Rack1 is, like Nef, in equilibrium between a cytosolic and membrane-bound state and that it partially colocalizes with the viral protein on the intracellular side of membranes. The interaction is not stable to coimmunoprecipitation (data not shown), a feature shared with many of the proposed Nef–cell protein partnerships (e.g., interactions with AP-1 and AP-2 adaptins and kinases of the Src family). Notably, Rack1 enhances Nef phosphorylation by PKCs in vitro.

A β propeller fold, which creates two extended lateral...
surfaces and a number of grooves between the blades, is ideally suited to proteins establishing multiple or dynamic contacts, as demonstrated in detail for the β-subunits of heterotrimeric G-proteins, and, among non-WD40 propellers, the seven-bladed head of the clathrin heavy chain (ter Haar et al., 1998). It is interesting to recall that Nef has been proposed to exhibit partial homology to G-proteins (Ratner and Niederman, 1995), a hypothesis that subsequent crystallographic and NMR analysis of Nef molecule failed to confirm. Nonetheless, Nef turns out to interact with a G β-like protein. Our interaction mapping analysis suggests that Nef binds the Rack1 propeller in the fifth groove region, at a site close to a tract important for PKC binding, according to previous reports. The Nef binding site should not overlap the PKC binding site, however, since Nef and PKC do not compete for Rack1 binding according to the phosphorylation assay. The Rack1 binding region of PKCs falls within the type I fold-C2 domain of “classical” isozymes (Rotenberg and Sun, 1998). Accordingly, other proteins containing a C2 domain—namely, synaptotagmin I and phospholipase C-gamma1—have been shown to bind Rack1 as well (Disatnik et al., 1994; Mochly-Rosen et al., 1992). On such a basis, theta C kinase, a type II C2 (“novel”) C kinase which has been implicated as a Nef partner, conversely is not expected to bind to Rack1, although the theta isozyme partially colocalizes cytologically with Rack1 in activated lymphocytes (Wang et al., 1999).

FIG. 6. Immunofluorescence analysis of Rack1 and Nef subcellular distribution in HEK-293 cells. Cells grown on glass coverslips were fixed in 4% paraformaldehyde in PHEM buffer and processed for immunofluorescence staining. (A, B) Nef-transfected cells incubated with anti-Nef MAb and anti-clathrin antiserum. (C, D) Untransfected cells incubated with anti-Rack1 MAb and anti-clathrin goat antiserum as the primary antibodies. After the washings the coverslips were stained with TRITC anti-mouse (panIg) plus FITC anti-goat secondary antibodies, mounted in ProLong antifade medium (Molecular Probes), and observed under a Zeiss epifluorescence microscope connected to a charged coupled camera device. (A, C) Fluoresceine channel (clathrin). (B, D) Rhodamine channel (Nef or Rack1, respectively).
ing, by interaction cloning, distinct Rack1 partners: inte-
grin β subunit (Liliental and Chang, 1998), c-Src (Chang
et al., 1998), the constant (βc) chain of macrophage col-
ony-stimulating factor (GM-CSF), interleukin-3 (IL-3) and
interleukin-5 (IL-5) receptors (Geijsen et al., 1999), and
cAMP-specific phosphodiesterase PDE4D5 isoform
(Yarwood et al., 1999). PDE4D5 enzyme exhibits the prop-
erties of a very strong interactor for both soluble and
membrane-bound Rack1. Integrin βc, on the contrary,
seems to interact only upon cell treatment with PMA.
c-Src and other Src family members, like Lck and Yes,
are bound through their N-terminal SH2 domain, and
Rack1 binding exerts an inhibitory effect, maybe locking
the kinases in the basal inactive fold (Chang et al., 1998).
GM-CSF/IL-3/IL-5 receptor βc-chain, finally, seems to be
stably associated to Rack1, while upon PMA stimulation
PKC-β joins to the complex. Based on the dependence
on PMA activation, then, Rack1 partners seem to fall into
two classes: those requiring a PMA-induced conforma-
tional shift (in the partner, e.g., PKC, and possibly in
Rack1, in the case of integrin βc) and those binding Rack1
constitutively, such as PDE4D5, Src-like proteins, cyto-
kine receptor βc-chain, and Nef. Nef, however, exhibits a
preference for a N-terminally truncated Rack1 in ths, so
that in the Nef–Rack1 association a role for a conforma-
tional switch in the WD40 protein cannot be dismissed.
No obvious Rack1-binding motif is shared by Nef and the
growing list of Rack1 partners (data not shown).

In addition, Rack1 turned out to bind in vitro, in a
lipid-independent manner, a subset of pleckstrin-homol-
yogy (PH) domains, among which are spectrin and dy-
namin-1 PH (Rodriguez et al., 1999). The latter is a par-
icularly intriguing partner, as dynamin is a known PKC
target as well as a key player in endocytosis.

The above notions set the framework for a functional
interpretation of our findings. Rack1, in view of its sub-
cellular localization on membranes shown here in adher-
ent human cells, may function as a Nef membrane dock-
ing site. Moreover, in vitro phosphorylation data dem-
strate that the Nef–Rack1 interaction can favor Nef
contacts with activated C kinases of the alpha-gamma
isoenzyme classes, ending up in the kind of viral protein
phosphorylation which has been demonstrated to en-
hance its CD4-downregulation activity (Luo et al., 1997).
In addition, structural and biochemical evidence makes
Rack1 a strong candidate as an adaptor akin to AKAPs,
spatially coordinating the action of multiple signal trans-
duction elements—receptors, kinases, cytoskeletal
components, and possibly endocytic proteins. An entic-
ing hypothesis is that Rack1 may be involved in clustering
of Nef with Src-like kinases, the most obvious can-
didate being the CD4-associated Lck protein. Lck has
been reported to bind Nef via both SH2 and SH3 do-
 mains (Dutarte et al., 1998) and to be displaced from CD4
cytoplasmic domain upon Nef-induced CD4 endocytosis
(Kim et al., 1999; Salghetti et al., 1995) on one hand; on
the other. Src-like proteins associated to the same Rack1
tract—WD40 repeats 5–7—that according to our data
binds Nef. It will be important, then, to ascertain whether
Nef can form a ternary complex with Rack1 and Src-like
proteins or whether it displaces the kinase with a pos-
sibly activating effect.

Another possibility, not mutually exclusive, is that
Rack1 may be involved in the clustering of effector/ regu-
ulatory proteins of the endocytic pathway, among
which modular molecules including C2 domains (e.g., in
the already mentioned sinaptotagmins) linked to other
protein–protein interaction modules (PH, SH3, EH,
ENTH) recur. Nef's ability to contact such dynamic as-
semblages would explain the still-elusive Nef effects,
such as the raising of coated pits number (Foti et al.,
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cytic coat proteins.

Finally, the biological relevance of the Rack1–PKCs
interaction has been demonstrated in part by the use of
peptides mimicking the binding tracts, which inhibit
downstream events like PKC-mediated insulin-induced
maturation of Xenopus oocytes (Mochly-Rosen and Kau-
var, 1998). A similar approach promises to offer further
insights into how Rack1 is involved in Nef functions, and,
more prospectively, to define the potential of peptidomi-
detic compounds blocking Rack1 function as anti-Nef
drugs.

MATERIALS AND METHODS

Cells and antibodies. Human embryonic kidney-293
cells were maintained in Dulbecco's MEM Glutamax
(Gibco), 10% fetal bovine serum, and antibiotics at 37°C.
Transfections were performed according to the calcium
phosphate method, and cells were processed 48 h post-
transfection.

HIV-1 Nef and human/rat Rack1 proteins were de-
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(Chemicon) and with MAb R20620 (IgM) anti-Rack1 C-
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clathrin goat antiserum and anti-phosphoamino acids
MAbs were from Sigma. HRP-conjugated anti-rabbit and
anti-phosphoamino acids MAb receptors, kinases, cytoskeletal
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maturation of Xenopus oocytes (Mochly-Rosen and Kau-
var, 1998). A similar approach promises to offer further
insights into how Rack1 is involved in Nef functions, and,
more prospectively, to define the potential of peptidomi-
detic compounds blocking Rack1 function as anti-Nef
drugs.
histidine-Xpress epitope-tagged Nef in *Escherichia coli*. The control plasmid pTRC-preS expresses the hepatitis B virus preS domain coding sequence, bearing the same N-terminal tag as bacterial Nef.

Similarly, yeast two-hybrid system. All the experiments exploited *Saccharomyces cerevisiae* HF7c strain, carrying HIS3 and lacZ markers under the control of Gal4-responsive sequences. Yeast transformation, selection, filter, and, in liquid, β-galactosidase assays were performed as described previously (Gallina et al., 1999).

**Recombinant proteins.** *E. coli* DH5α cells harboring pGEX–Rack1, pGex–Plk1Cter, or the void vector were induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 3 h, and the corresponding glutathione S-transferase proteins were purified from bacterial lysates by glutathione–Sepharose (Pharmacia) chromatography, according to the manufacturer’s instructions. GST fusions were eluted with 25 mM reduced glutathion in 25 mM Tris–HCl, pH 7.5, and dialyzed against PBS. Hexahistidine-tagged Nef (His–Nef) and His–preS purification has been described (Rossi et al., 1997).

A 35S]methionine-labeled Rack1 protein was synthesized in vitro by programming the TNT-T7 coupled transcription–translation rabbit reticulocyte lysate system (Promega) with pBS–Rack1 plasmid (37°C/45 min). Subsequently, the translation mixture was complemented with nonradioactive 1 mM l-methionine and further incubated for 45 min.

In vitro binding assays. For pull-down assays, purified GST–Rack1 or the GST controls (5 μg each) were reabsorbed onto glutathione–Sepharose resin and incubated with His–Nef or His–preS in binding buffer [25 mM

### Table 1

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Restriction site</th>
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<tr>
<td>Nef5’</td>
<td>TCGCCGGAATTGGCTGGTGGCAAGGTGGTCAAAGAAGTAGT</td>
<td>EcoRI</td>
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<tr>
<td>Nef3’ anti</td>
<td>ACGCCTCTCTCGAGTCGACGGATCTTCTTTGAAGTACCTCGGG</td>
<td>SalI</td>
</tr>
<tr>
<td>NefMut anti</td>
<td>ACGCCTCTAGTAGTTTAAAGAGAACGAGACTTACACAGGGCCAGG</td>
<td>—</td>
</tr>
<tr>
<td>Rack 5’ w/R1</td>
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<td>EcoRI</td>
</tr>
<tr>
<td>Rack 5’ w/R1</td>
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<td>SalI</td>
</tr>
<tr>
<td>Rack 3’ endstopanti</td>
<td>TGTCA GTGACTTAAAGGTGTCGACAATGGTACCTCTCGTG</td>
<td>EcoRI</td>
</tr>
<tr>
<td>Rack 3’ endstopanti</td>
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<td>SalI</td>
</tr>
<tr>
<td>Rack 5’ WD5–Nsense</td>
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<td>EcoRI</td>
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<tr>
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<td>TCTGACGAATTCACCGAGCAAATGACCCTTCG</td>
<td>SalI</td>
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*Underbar, restriction sites introduced for cloning (names in the column at right); boldface, stop codons (antisense) for premature translation termination; boldface italics: residues mutated with respect to natural sequences.*

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**TABLE 1**

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*Underbar, restriction sites introduced for cloning (names in the column at right); boldface, stop codons (antisense) for premature translation termination; boldface italics: residues mutated with respect to natural sequences.*
Hepes, pH 7.4, 50 mM NaCl, 2.5 mM CaCl$_2$, 1 mM MgCl$_2$, 0.1% Triton, 1% BSA. Complete protease inhibitors (Roche) for 1 h at 4°C. The resin was washed four times in the same buffer lacking BSA. Resin-bound complexes were boiled in SDS–PAGE sample buffer and the eluted proteins were resolved in SDS–PAGE (12% acrylamide in running gel) and electroblotted to nitrocellulose (ProBond 83, Schleicher & Schull). After primary antibody and HRP-conjugated secondary antibody incubations, signal was developed with SuperSignal Dura (Pierce) enhanced chemiluminescence substrate.

For gel filtration analysis of the Rack1–Nef interaction, 10 µl of the Rack1 in vitro translation reaction was mixed with 2 µg of recombinant His–Nef in 100 µl of binding buffer (without BSA). After 1 h of incubation at 4°C, the mixture was loaded onto a 15-ml analytical Sephacryl 100 column and the eluted fractions were analyzed by Western blotting with anti-Nef MAb. The dried blot was then exposed to a phosphorimager screen for 24 h.

In vitro phosphorylation experiments. The His–Nef–Rack1 complex as eluted from gel filtration column (approximately 50 ng of recombinant Nef) or His–Nef with/without added GST–Rack1(WD5–7) or GST (50 ng either) was incubated, with or without 40 µU of PKCa-γ (Roche), at 37°C/30 min in PKC buffer: 20 mM Tris–HCl, pH 7.5, 10 mM MgCl$_2$, 0.5 mM CaCl$_2$, 100 ng/ml phosphatidylserine, 250 pM ATP, 100 µCi/ml [γ$^{32}$P]ATP, 5000 Ci/mmole (Amer sham). The reaction was brought to completion by the addition of 100 µM ATP and further incubation for another 10 min. The reaction was stopped with 1 ml of 6 M guanidinium–HCl and 100 mM Tris–HCl, pH 8, and 20 µl/reaction of nickel–NTA agarose (Qiagen) beads were added and shaked end-over-end for 30 min at room temperature. Beads were washed three times in guani dinium buffer and the captured His–Nef radioactivity was determined by scintillation. Alternatively, beads were washed one more time with 100 mM Tris–HCl, pH 8, and boiled in SDS–PAGE sample buffer for Western blot analysis with anti-phosphoamino acid MABs.

Cell transfection, fractionation, and IF analysis. 293-HEK cells were transfected with the standard calcium phosphate protocol, 2 µg plasmid/3 × 10$^6$ cells. For subcellular fractionations, cells were dounced in isotonic buffer and nuclei sedimented at low gravity (600g). Cell membranes were then pelleted by high-speed centrifugation (100,000g), and the nuclear, microsomal, and cytosolic fractions were analyzed in Western blot with anti-Rack1 or anti-Nef MABs. For IF, 1 × 10$^5$ cells were seeded on polylysine-coated glass coverslips. Transfected cells were detached with versene 24 h posttransfection and plated as above. Monolayers were washed twice with PBS containing 0.9 mM CaCl$_2$ and 0.45 mM MgCl$_2$, prewarmed at 37°C, and fixed in freshly prepared, ice-cold 3% (v/v) paraformaldehyde in PBS for 20 min on ice. After being washed three times with ice-cold PBS, residual aldehydes were quenched by immersing each slide in 150 mM ammonium chloride for 30 min. Cells were permeabilized by treatment for 30 min with 0.1% (w/v) saponin in PBS and 10% (v/v) FBS. Incubations with primary antibodies (10 µg/ml) were performed in the same buffer for 30 min in a humid chamber at 37°C. After three washes, cells were incubated simultaneously with FITC-anti-goat and TRITC-anti-mouse secondary antibodies in the same medium [Supplemented with 0.2 µg/ml, 4,6-diamidino-2-phenylindole as a nuclear counterstain]. After the final washes, the coverslips were mounted in ProLong antifade medium (Molecular Probes, Eugene, OR) and examined under a Zeiss epifluorescence microscope connected to a charged-coupled device camera with direct digital image recording.

ACKNOWLEDGMENTS

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