HIV-1 Nef-Induced Secretion of the Proinflammatory Protease TACE into Extracellular Vesicles Is Mediated by Raf-1 and Can Be Suppressed by Clinical Protein Kinase Inhibitors

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ABSTRACT Chronic immune activation is an important driver of human immunodeficiency virus type 1 (HIV-1) pathogenesis and has been associated with the presence of tumor necrosis factor- α converting enzyme (TACE) in extracellular vesicles (EVs) circulating in infected individuals. We have recently shown that activation of the Src-family tyrosine kinase hematopoietic cell kinase (Hck) by HIV-1 Nef can trigger the packaging of TACE into EVs via an unconventional protein secretion pathway. Using a panel of HIV-1 Nef mutants and natural HIV-2 and simian immunodeficiency virus (SIV) Nef alleles, we now show that the capacity to promote TACE secretion depends on the superior ability of HIV-1-like Nef alleles to induce Hck kinase activity, whereas other Nef effector functions are dispensable. Strikingly, among the numerous Src-family downstream effectors, serine/threonine kinase Raf-1 was found to be necessary and alone sufficient to trigger the secretion of TACE into EVs. These data reveal the involvement of Raf-1 in regulation of unconventional protein secretion and highlight the importance of Raf-1 as a cellular effector of Nef, thereby suggesting a novel rationale for testing pharmacological inhibitors of the Raf-MAPK pathway to treat HIV-associated immune activation.

IMPORTANCE Chronic immune activation contributes to the immunopathogenesis of human immunodeficiency virus type 1 (HIV-1) infection and is associated with poor recovery of the immune system despite potent antiretroviral therapy, which is observed in 10% to 40% drug-treated patients depending on the definition of immune reconstitution. We have previously shown that the HIV pathogenicity factor Nef can promote loading of the proinflammatory protease TACE into extracellular vesicles (EVs), and the levels of such TACE-containing EVs circulating in the blood correlate with low CD4 lymphocyte counts in HIV patients receiving antiretroviral therapy. Here, we show that Nef promotes uploading of TACE into EVs by triggering unconventional secretion via activation of the Hck/Raf/mitogen-activated protein kinase (MAPK) cascade. We find that several pharmaceutical inhibitors of these kinases that are currently in clinical use for other diseases can potently suppress this pathogenic deregulation and could thus provide a novel strategy for treating HIV-associated immune activation.

KEYWORDS ADAM17, HIV-1, Hck, Nef, Raf, TACE, extracellular vesicles, kinase inhibitor

Nef is a multifunctional accessory protein carried by all primate lentiviruses (human immunodeficiency virus type 1 [HIV-1], HIV-2, and simian immunodeficiency viruses [SIVs]). Nef acts as a pathogenicity factor that interacts with numerous host cell proteins in order to promote virus replication and immune evasion (1–4). Although HIV and SIV lacking a functional *nef* gene are replication competent, infections with such Nef-defective viruses are associated with low viral loads and delayed or no disease pathogenesis (5).

Citation Zhao Z, Fagerlund R, Baur AS, Saksela K. 2021. HIV-1 Nef-induced secretion of the proinflammatory protease TACE into extracellular vesicles is mediated by Raf-1 and can be suppressed by clinical protein kinase inhibitors. *J Virol* 95:e00180-21. <https://doi.org/10.1128/JVI.00180-21>.

Editor Frank Kirchhoff, Ulm University Medical Center

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Received 3 February 2021

Accepted 10 February 2021

Accepted manuscript posted online 17 February 2021

Published 12 April 2021

A cellular function of HIV-1 Nef that is well understood at the molecular and mechanistic level is its interaction with hematopoietic cell kinase (Hck), a member of the Src-family tyrosine kinases (SFKs). Nef binds tightly to the SH3 domain of Hck, thereby disrupting an intramolecular autoinhibitory contact and pushing Hck into a catalytically active conformation (6, 7). Hck is the principal SFK controlling activation and effector functions of the macrophage lineage, the second main target cell population of HIV-1 besides CD4⁺ lymphocytes (8, 9).

We have recently shown that activation of Hck by Nef can induce packaging of the proinflammatory protease tumor necrosis factor- α converting enzyme (TACE; also known as ADAM17) into extracellular vesicles (EVs) (10) via a process involving an unconventional protein secretion pathway (11). These findings link Hck activation to the immunopathogenesis of HIV-1 infection since the levels of TACE-containing EVs correlate with markers of immune activation and low CD4 cell counts in the blood of HIV-1-infected individuals (12).

In this study, we have examined the cellular signaling events that lead to packaging of TACE into EVs upon Nef-induced Hck activation and discovered a critical role for the rapidly accelerated fibrosarcoma (Raf) kinase in triggering the unconventional secretion pathway that mediates this process.

RESULTS

Capacity of lentiviral Nef proteins to activate vesicle secretion of TACE. To examine the mechanism of Nef-induced secretion of TACE into EVs, we first tested a panel of Nef variants carrying disrupting mutations in the potentially most relevant functional motifs of Nef. One was a double alanine substitution (SF2 Nef-P76A;P79A, named “Nef-AxxA”) targeting the core SH3 domain binding site in Nef (the PxxP motif), thereby disrupting all SH3 domain-mediated protein complexes and indirectly affecting many more cellular interactions and functions of Nef (4). Another SH3-related mutation tested was a phenylalanine-to-arginine substitution (SF2 Nef-F94R, named “Nef-F94R”) disrupting a hydrophobic pocket that is specifically required for high-affinity binding to the Hck SH3 domain (6). In addition, we tested an alanine substitution of phenylalanine-195 (SF2 Nef-F195A, named “Nef-F195A”) because mutation of this residue (13) specifically abrogates another prominent and extensively studied protein kinase interaction of Nef, namely, association with the serine/threonine kinase PAK2 (14), which is important for host cell manipulation by Nef via altered actin dynamics (15).

Finally, two well-characterized Nef mutants that abrogate protein interactions enabling Nef to hijack host cell membrane trafficking were tested. These mutations included disruption of a di-acidic sequence motif (SF2 Nef-E178A;D179A, named “Nef-EDAA”) that prevents binding of Nef to the clathrin adaptor protein complex AP-2, thereby blocking downregulation of cell surface proteins, such as CD4 (16, 17), and an tetra-alanine substitution of an acidic cluster preceding the PxxP motif of Nef (SF2 Nef-E66A;E67A;E68A;E66A, named “Nef-E4A”) that is required for Nef-mediated MHC-I downregulation (18) by stabilizing a Nef/MHC-I/AP-1 complex (19) and by binding to the sorting proteins PACS-1 and PACS-2 (20).

Expression vectors encoding these SF2-Nef alleles and TACE were cotransfected into HZ1 cells together with paxillin, a prominent substrate for phosphorylation by Src-family tyrosine kinases (SFKs). HZ1 is an HEK293-derived cell line (11) that mimics the macrophage lineage in constitutively expressing hematopoietic cell kinase (Hck), an SFK preferably activated by Nef via SH3 domain displacement (6, 21, 22). Supporting our earlier findings (10, 11), transfection of wild-type Nef into HZ1 cells efficiently induced tyrosine phosphorylation of paxillin and triggered secretion of TACE into EVs, whereas Nef-AxxA containing a disrupted SH3-binding motif was defective in both of these functions (Fig. 1A). Here, as well as in the other experiments described below that involved EVs, these vesicle preparations were also blotted with an antibody against α -tubulin endogenously present in them to control for the uniform quality of EV collection and sample loading.

FIG 1 Capacity for Hck activation and triggering of TACE secretion by wild-type or rationally mutated Nef proteins from different primate lentiviruses. HZ-1 cells stably expressing Hck were cotransfected with vectors for TACE and paxillin alone (control) or together with Nef from the canonical HIV-1 M-group strain SF2 (WT) or the indicated functionally compromised versions of it described in the text (A), or Nef alleles from an HIV-1 O-group strain (13127k4), three HIV-2 strains (CBL-23, 310319k2, and 310328k2), two chimpanzee SIV strains (GAB2c148 and CAM3K1), or three monkey SIV strains (mac239, smmFFM1, and smmFWR1) (B). For clarity, all HIV-1/SIVcpz-type Nef alleles are indicated with an empty circle and all HIV-2/SIVsmm alleles with a solid circle. Forty-eight hours after transfection, extracellular vesicles (EVs) and total cell lysates (cells) prepared from these cultures were examined by Western blotting using antibodies against the indicated proteins.

In contrast to Nef-AxxA, the mutations disrupting interactions of Nef with proteins involved in membrane trafficking (Nef-EDAA and Nef-E4A) did not substantially interfere with Nef-induced TACE secretion nor with tyrosine phosphorylation of paxillin. Likewise, although PAK kinases have the potential to modulate the effects of Nef on TACE secretion (23), the PAK2 binding-deficient mutant Nef-F195A did not behave differently compared with wild-type SF2 Nef. In contrast, Nef-F94R that has a specific defect in selectively binding to Hck SH3 with high affinity (6, 24) failed to promote paxillin phosphorylation and TACE secretion, similar to the Nef-AxxA mutant that is lacking any SH3 binding capacity. Quantification of TACE detection in EVs in the Western blot in Fig. 1 and blots from two other independent experiments are shown in Fig. S1A in the supplemental material. Likewise, quantification of the relevant read-out protein signals from three or more comparable Western blots corresponding to data shown in the other figures is provided in Fig. S1B to F. Thus, based on the above studies with SF2 Nef, we conclude that SH3 binding, and in particular Hck SH3 binding, but not an association with PAK2 or capacity to modulate host cell membrane trafficking, is required for inducing TACE secretion into EVs triggered by Nef.

The PxxP motif is highly conserved in all lentiviral Nef alleles, providing them with a capacity for at least a basal level of binding to many different SH3 domain-containing and -associated cellular proteins (4). Whereas the Phe94 residue discussed above can also be found in most lentiviral Nef proteins, many other residues contributing to the hydrophobic pocket that stabilizes high-affinity binding of Hck SH3 to Nefs from classical HIV-1 strains (such as SF2) are conserved only in HIV-1/SIVcpz- but not in HIV-2/SIV-type Nef alleles (6). We therefore also included some HIV-2/SIV-type as well as more divergent HIV-1-type Nef alleles in our analyses (Fig. 1B). In agreement with their anticipated lower Hck SH3 binding affinity (6, 25), Nef proteins encoded by all three HIV-2 Nef alleles tested (CBL-23, 310319k2, and 301248k2) as well as all three monkey SIV alleles tested (SIVmac239, SIVsmmFFM1, and SIVsmmFWR1) were unable to stimulate paxillin phosphorylation and to trigger vesicle secretion of TACE. In contrast, the HIV-1 O-group Nef allele 13127k4 as well both of the chimpanzee SIV Nef alleles tested (GAB2c148

FIG 2 The effects of Nef-induced Hck activation on MAPK-regulated gene expression and on triggering of TACE secretion are mediated by Raf-1. (A) HZ-1 cells were transfected with an AP-1 transcription factor-dependent luciferase reporter alone (control) or together with wild-type SF2 Nef (WT), Nef-AxxA (AxxA), or wild-type Nef plus the Raf-suppressor RKTG (WT+RKTG), and either left untreated (no) or supplemented 30 min after transfection with 1 μ M the Raf inhibitor LY3009120 (LY30) as indicated. Shown are the luciferase activity values measured from lysates prepared from these cells 24 h after transfection normalized to the corresponding control sample that was set to 1.00. (B) SF2 Nef and TACE were cotransfected into HZ-1 cells alone (three first lanes) or together with RKTG (rightmost lane), and 48 h later, extracellular vesicles (EVs) and total cell lysates (cells) prepared from these cultures were examined by Western blotting using antibodies against the indicated proteins. When indicated, the cultures were treated for 45 h before harvesting of the EVs/lysates with 1 μ M LY3009120 (LY30) or 20 μ M the MEK inhibitor U0126 (U01).

and CAM3k1) resembled the canonical HIV-1 M-group Nef allele SF2 used in Fig. 1A in being active in both of these functions (Fig. 1B and Fig. S1B).

These results suggest that the lack of Hck-activated TACE secretion into inflammation-promoting vesicles is a characteristic difference between infection by HIV-1 and HIV-2-like viruses and could be related to the differences in the severity of the immunopathogenesis associated with these infections.

Role of Raf-1 as a critical cellular effector of Nef in TACE secretion. Because these data indicated that the capacity of different Nef alleles to induce TACE secretion was determined by their potential to activate Hck via SH3 domain displacement, we decided to focus on understanding the downstream effectors of Hck in this signaling.

SFKs are ubiquitously involved in a myriad of cellular signaling pathways and phosphorylate a vast array of substrates (21, 26, 27). One mediator of SFK signaling is the Raf family of serine/threonine kinases (A-Raf, B-Raf, and C-Raf/Raf-1), which are activated by SFKs via the Ras GTPases as well as by direct tyrosine phosphorylation (28, 29). Raf kinases are also known as mitogen-activated protein kinase kinase kinase (MAP3Ks) because of their dedicated role as initiating enzymes of the MAPK cascades (28, 29).

In the early days of Nef research, we showed that in macrophages, Nef-induced Hck activity led to upregulation of gene expression driven by the MAPK-regulated transcription factor activator protein-1 (AP-1) (30). To validate the HZ1 cell line as a macrophage model, we transfected these cells with an AP-1-responsive luciferase reporter plasmid with or without Nef. As shown in Fig. 2A, wild-type Nef but not the SH3 binding-deficient Nef-AxxA mutant caused a robust activation of AP-1-driven gene expression in HZ1 cells. In agreement with the role of Raf-1 as an SFK effector and an upstream activator of MAPK signaling, we found that Nef-induced AP-1 transcription could be efficiently abolished by treating the cells with the potent pan-Raf inhibitor LY3009120 (31). In addition to this pharmacological inhibitor, we also tested the effect of coexpressing Raf kinase trapping to Golgi (RKTG), a Golgi transmembrane protein that can suppress Raf-MAPK signaling by sequestering Raf-1 (32). In support of the

FIG 3 The dominant role and phosphotyrosine-dependent activation of Raf-1 in mediating Nef/Hck-induced TACE secretion. (A) HEK239 cells were transfected with TACE together with Hck-p59 or Raf-BXB and left untreated or supplemented 3 h after transfection with 200 ng/ml of brefeldin A (BFA) as indicated. Forty-eight hours after transfection, extracellular vesicles (EVs) and total cell lysates (cells) prepared from these cultures were examined by Western blotting using antibodies against the indicated proteins. (B) Stably Hck-expressing HZ-1 cells were cotransfected with TACE and Nef together with two different amounts (10 μ g or 5 μ g) of vectors encoding wild-type Raf-1 kinase (Raf-WT) or its mutated derivative that cannot be tyrosine phosphorylated by Src-family kinases (Raf-Y/F), as indicated. Proteins in extracellular vesicles and total cell lysates prepared from these cultures were examined as in A.

results obtained using LY3009120, cotransfection of RKTG also blocked the ability of Nef to stimulate AP-1-dependent reporter gene expression (Fig. 2A).

To examine if the Hck-Raf signaling axis might also contribute to Nef-induced TACE secretion, we tested the effect of pharmacological (LY3009120 treatment) and genetic (RKTG coexpression) inhibition of Raf-1 on TACE secretion by Nef-transfected HZ1 cells (Fig. 2B and Fig. S1C). We found that both of these strategies to inhibit Raf-1 activity completely prevented Nef from promoting TACE secretion into EVs. Likewise, Nef-induced TACE secretion could also be efficiently blocked by inhibiting the MAPK pathway immediately downstream of Raf-1 with the widely used experimental MEK inhibitor U0126 (33).

The strict Raf dependence of Nef-activated Hck signaling led us to test if Raf-1 activation might alone be sufficient to trigger TACE secretion. To this end, we cotransfected HEK293 cells with TACE and Raf-BXB, a constitutively activated form of Raf-1 lacking its N-terminal regulatory domain (34). Strikingly, we found that Raf-BXB expression could fully substitute Nef and Hck and triggered TACE secretion into EVs just as efficiently as Hck overexpression (Fig. 3). Similar to TACE secretion triggered by augmented Hck activity (11), Raf-BXB-stimulated routing of TACE into EVs was mediated via an unconventional secretion pathway because it was not blocked by inhibition of endoplasmic reticulum (ER)-to-Golgi trafficking with brefeldin A (BFA). However, as in the case of Hck activation (11), ER-to-Golgi blockage by BFA did prevent furin-mediated proteolytic maturation of TACE directed to EV secretion by Raf-BXB, as indicated by the disappearance of the lower molecular weight (80 kDa) species of TACE from the secreted EVs (Fig. 3A).

These results clearly show that Raf-1 was not only necessary but also surprisingly sufficient to trigger secretion of TACE into EVs. Thus, activation of the signaling pathway downstream of Raf-1 can alone mediate the effect of Nef-induced Hck activation, and no tyrosine phosphorylation of any Hck-ligand proteins in the secretory apparatus itself or elsewhere in the cell is required for this process.

This central role of Raf-1 as a downstream mediator of Nef/Hck signaling led us to investigate the role of the Raf-1 tyrosine residues Y340 and Y341. These residues are known to be phosphorylated by Src-family kinases, which control the capacity of Raf-1 to be activated by Ras (35, 36). Whereas cotransfection of wild-type Raf-1 together

with Nef into the Hck-expressing HZ1 cell line did not significantly alter the triggering of TACE secretion into EVs, a mutated version of Raf-1 (termed Raf-Y/F) in which these two tyrosines had been replaced with phenylalanine residues inhibited Nef-activated TACE secretion in a dose-dependent manner (Fig. 3B and Fig. S1D). Thus, stimulation of Raf-1 signaling by Nef is mediated via tyrosine phosphorylation of the Raf-1 at residues 340/341 by catalytically activated Hck.

Interestingly, in 1998, Hodge et al. reported that Nef could bind directly to Raf-1 (37). However, no functional role for this interaction was shown, and no follow-up studies on this finding have been reported. We tried to confirm such an interaction, but we could observe only minimal or no coimmunoprecipitation even when Nef and Raf-1 were highly overexpressed together in HEK293 cells (data not shown). Moreover, as shown in Fig. 1A, we found Nef-EDAA to be fully able to trigger TACE secretion (Fig. 1A), although substitution of the Nef residues E178 and D179 disrupt an acidic cluster that Hodge et al. reported as the Raf-binding site in Nef (37) (and subsequently shown by Bonifacino and colleagues to mediate AP-2 recruitment [16]). Thus, even if a weak Nef/Raf-1 complex might assemble under some conditions, we conclude that such an interaction is not required for Hck/Raf-1-mediated activation of TACE secretion by Nef.

Clinical inhibitors. Incomplete immune reconstitution despite potent antiretroviral therapy remains a major problem in clinical management of HIV/AIDS and is associated with persistent immune activation (38, 39). Considering the correlation between TACE-containing plasma vesicles, low CD4 counts, and markers of immune activation (12) and the role of the MAPK signaling in vesicle secretion of TACE reported here, the possibility of treating HIV-associated immune activation with pharmacological MAPK inhibitors therefore emerges as an attractive idea.

Although the experimental compounds LY3009120 and U0126 applied in Fig. 2 for suppression of Nef-induced TACE secretion are useful research tools, several Raf-MAPK inhibitors are already in clinical use in oncology (40). We therefore collected a panel of such pharmaceuticals and characterized their potency in inhibition of TACE secretion triggered by Nef.

One of these drugs was sorafenib, a multikinase currently used for treatment of several cancers. It was originally discovered as a Raf-1 inhibitor, and unlike the clinically approved BRAF-V600E inhibitors, can suppress wild-type Raf kinase activity (41). In addition, we tested four clinically approved allosteric MEK inhibitors acting downstream of Raf-1 in the MAPK pathway, namely, trametinib, binimetinib, cobimetinib, and selumetinib (42). In addition, we tested a potent Hck antagonist, namely, dasatinib, a Bcr-Abl and Src-family tyrosine kinase inhibitor currently in clinical use to treat chronic myeloid leukemia (CML) and acute myelogenous leukemia (AML) (43).

We first tested all six of these drugs in a single clinically relevant concentration (maximum concentration of drug in serum [$C_{\max}$], i.e., the maximal serum concentration achieved after administration of the recommended dose of the drug) to study their ability to block TACE secretion into EVs triggered by Nef via Hck-Raf-MAPK activation. With the exception of sorafenib that showed only modest inhibitory potential, all tested drugs effectively reduced the amount of TACE found in the EVs (Fig. 4A and Fig. S1E).

To examine in more detail the relative potency of these compounds as inhibitors of Nef-activated Raf signaling in the HZ-1 cell model, we tested them over a wide range of concentrations (from 5 nM to 50 μ M) for their capacity to inhibit AP-1-dependent gene expression induced by Nef expression (Fig. 4B). Potent and dose-dependent suppression of Nef/Hck-induced AP-1 activity was observed with all tested MEK inhibitors. Of these inhibitors, trametinib showed an especially favorable inhibitory profile with strong AP-1 suppression already at the 50 nM concentration and no signs of toxicity at the 50 μ M concentration. Dasatinib also showed a promising inhibitory potential. Although at 500 nM and higher concentrations it showed apparent toxicity, as measured by suppression of basal gene expression directed by a minimal promoter, at lower concentrations, it strongly inhibited Nef/Hck-induced MAPK activation without associated toxicity. In contrast, the Raf/multikinase inhibitor sorafenib could not significantly inhibit Nef-induced AP-1 activity at concentrations that were not toxic to the cells.

FIG 4 Inhibition of Nef/Hck-activated secretion of TACE into EVs can be blocked by clinically approved kinase inhibitors. (A) TACE and Nef were cotransfected into HZ-1 cells that were either left untreated (–) or supplemented 3 h after transfection with the indicated drugs at their C_{max} concentrations, corresponding to 1.16 μ M sorafenib (Sor), 1.48 μ M binimetinib (Bin), 513 nM cobimetinib (Cob), 4.86 μ M selumetinib (Sel), 35.75 nM trametinib (Tra), and 0.12 μ M dasatinib (Das). Forty-eight hours after transfection, extracellular vesicles (EVs) and total cell lysates (cells) prepared from these cultures were examined by Western blotting using antibodies against the indicated proteins. (B) HZ-1 cells were cotransfected with HIV-1 Nef plus an AP-1-regulated firefly luciferase reporter plasmid and a constitutively expressed minimal promoter-driven renilla luciferase plasmid. The indicated drugs were added to the transfected cultures at concentrations ranging from 5 nM to 50 μ M, and the luciferase activity in the transfected cells was measured 24 h later. Shown are firefly luciferase values in these cells after normalization for transfection efficiency based on the corresponding renilla luciferase counts and setting the values in untreated cells to 100% for each series. The data shown are based on three independent experiments. Renilla luciferase activity values less than half of the untreated controls were considered drug toxicity, and these samples were excluded from further analysis (*). (C) M1-like macrophages differentiated from THP-1 cells lentivirally transduced with wild-type Nef (WT), Nef AxxA mutant (AxxA), or a control vector (–) were treated for 2 days with dasatinib (Das), LY3009120 (LY30), or binimetinib (Bin) at their C_{max} concentrations or left untreated (–) before they were harvested and examined by Western blotting for expression of Nef, total ERK1/2, and phosphorylated ERK1/2.

To further validate the above results from transfected HZ-1 cells, we examined Nef-induced activation of Hck-Raf-MAPK signaling and opportunities for its pharmacological inhibition in M1 macrophage-like cells differentiated from the human monocytic cell line THP-1 infected with a Nef-encoding HIV-derived lentiviral vector. Macrophage differentiation of THP-1 cells is a widely used model for studying human macrophage function and is associated with establishment of a macrophage-like gene expression pattern, including upregulation of Hck (44, 45). When THP-1 cells transduced with wild-type Nef were differentiated toward an M1 macrophage phenotype, a clear induction of ERK1/2 (MAPK1/2) phosphorylation indicative of Hck-Raf-MAPK pathway activation was observed (Fig. 4C and Fig. S1F). In contrast, no MAPK activation was seen in similarly differentiated THP-1-derived macrophages lacking Nef expression or transduced with the Nef-AxxA mutant that is unable to bind and activate Hck. In agreement with the data from the HZ-1 cell model, Nef-induced MAPK activation in these *in vitro*-differentiated macrophages could be completely blocked by using clinically relevant concentrations (C_{max}) of inhibitors for Hck, Raf, or MEK, namely, dasatinib, LY3009120, or binimetinib.

DISCUSSION

In this study, we have investigated the detailed role of Hck activation in Nef-induced triggering of TACE secretion into extracellular vesicles. Our previous studies have

established such a role for Nef-activated Hck also *in vivo* and correlated the abundance of TACE-containing plasma vesicles with the immunopathology of HIV-1 infection (10, 12).

Interestingly, our current data show that HIV-2-like Nef alleles are unable to similarly trigger TACE EV secretion via Hck activation. Previous studies by Schindler et al. have suggested that the differential capacity of HIV-1 and HIV-2 Nef proteins to suppress T cell receptor (TCR) signaling (46) plays a role in contributing to the more severe pathogenicity and higher level of immune activation associated with HIV-1 infections than those of HIV-2 and natural SIV infections (46). It is tempting to speculate that the ability to promote EV secretion of TACE via Hck activation could represent a similar pathogenicity determinant differing between HIV-1 and HIV-2.

A key finding of the current study was that Raf-1 has a much more important role in mediating the cellular effects of Nef than has been appreciated. While it was not surprising that the effect of Nef-induced Hck activation on AP-1-dependent gene expression was found to be mediated via Raf-1, it was unexpected that triggering of vesicle secretion of TACE via regulation of the unconventional protein secretion (UPS) machinery was also entirely Raf mediated. Of note, although direct binding between Nef and Raf-1 has been previously reported (37), such an interaction could not be confirmed in this study, and the proposed Raf-binding residues in Nef were found to be dispensable for activation of TACE secretion.

The capacity of a dominantly active Raf-1 mutant to fully recapitulate the effects of Nef-activated Hck indicated that, surprisingly, no tyrosine phosphorylation of substrates of Hck are involved in activation of the UPS pathway that selects TACE as its vesicle cargo. The substrate specificity of Raf kinases is restricted, and MEK1 and MEK2 (also known as MAP2K1 and MAP2K2) are their only well-established substrates (28, 47). Indeed, Nef/Hck/Raf-induced TACE secretion could be fully suppressed with various experimental and clinical MEK inhibitors. The critical MAPK ligands in the UPS machinery that mediate this regulation remain to be identified, but based on the literature, they could include at least the Golgi-stacking GRASP proteins, which are also involved in many forms of UPS (48) and known to be phosphorylated by ERK1/2 MAP kinases (49), as well as the ER-to-Golgi traffic-coordinating scaffold protein Sec16, which is also involved in UPS (50) and is a notable target for MAPK phosphorylation in the early secretory apparatus (51).

In any case, independent of the identity of the downstream target molecule(s) of the Hck/Raf/MAPK cascade in the cellular protein sorting and membrane trafficking machinery, the ability to block Nef-induced secretion of TACE into inflammatory vesicles by clinically approved kinase inhibitors could have therapeutic utility in treating HIV-associated immune activation. Although the relative contribution of TACE-containing EVs under this multifactorial condition remains to be defined, our data provide a rationale for testing Src-family and MEK inhibitors, such as dasatinib, trametinib, cobimetinib, and binimetinib (40), especially in the subgroup of HIV-positive patients that show poor immune recovery despite effective antiretroviral therapy (52).

MATERIALS AND METHODS

Reagents and cell lines. Raf inhibitor LY3009120; multikinase inhibitor sorafenib; tyrosine kinase inhibitor dasatinib; and MEK inhibitors U0126, cobimetinib, trametinib, selumetinib, and binimetinib were all from Selleck Chemicals. Mouse anti-Myc (sc-40), mouse anti-GFP (sc-9996), rabbit anti-paxillin (sc-5574), and rabbit anti-Hck (sc-72) were from Santa Cruz Biotechnology. Metalloprotease inhibitor 1, 10-phenanthroline was from Sigma-Aldrich. Mouse anti-TACE cyto-domain (MAB2129) was from R&D Systems, mouse anti-pY31 paxillin from BD Biosciences, mouse anti- α -tubulin (T9026) from Sigma-Aldrich, and sheep anti-Nef from Targeted Affinity. Streptavidin IRDye680CW, streptavidin IRDye800CW, IRDye680CW, goat anti-mouse IgG, and IRDye800CW goat anti-rabbit IgG were from Li-Cor Biotechnology. HEK293 and THP-1 cells were obtained from ATCC (CRL-1573 and TIB-202, respectively). The derivation and characterization of the Hck-expressing macrophage model cell line HZ-1 have been described elsewhere (11). Both cell lines were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (FBS), 0.05 mg/ml penicillin, and 0.05 mg/ml streptomycin.

Plasmids. The pRK5M-TACE expression vector was obtained from Addgene (plasmid number 31714 provided by Rik Derynck). Human RKTG (GenBank accession no. [NP_001035292](#)) cDNA was cloned as a GFP gene fusion by inserting it into the pEGFP-N1 vector (Clontech). Nef alleles from HIV-1M SF2 ([P03407.3](#)), HIV-1O 13127k4 ([AY536904.1](#)) (53), HIV-2 CBL-23 ([DQ222472.1](#)) (54), HIV-2 310319k2

(DQ092766.1) (54), SIVcpz GAB2c148 (AY536912.1) (53), SIVmac 239 (QFD59953.1), SIVssm FFm1 (DQ092762.1) (54), and 301248k2 (DQ092765.1), as well as the cDNAs for human Raf-1 (NP_001341618.1), paxillin (NP_002850.2), and Hck p59 (NP_001165600), were cloned into the expression vector pEBB (for Hck) or its derivatives (55) containing a C-terminal Myc-tag (for Nef alleles) or a 123-amino acid (aa) biotin acceptor domain (for Raf-1). Raf-1 mutants Y340F/Y341F, Raf-BxB (an in-frame deletion of amino acids 26 to 302), and SF2 Nef mutants (P76A, D178A/E179A, F195A, F94R, and E66A/E67A/E68A/E69A) were created with the same vector backbone using standard PCR-assisted mutagenesis.

EVs and cell lysate preparations. EV pellets were purified via a typical 3-step centrifugation protocol as previously described (11). Briefly, 2 days posttransfection, 12 ml of supernatants was harvested and sequentially centrifuged for 10 minutes at $1,000 \times g$ and 30 minutes at $10,000 \times g$ and then ultracentrifuged for 90 minutes at $100,000 \times g$. The latter pellets were resuspended in 100 μ l of Laemmli sample buffer as EV preparations. Cells were collected and lysed on ice for 10 minutes in lysis buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.4], 1% NP-40, and 10 mM 1, 10-phenanthroline), plus Pierce protease and phosphatase inhibitor tablets as recommended by the supplier (Thermo Fisher Scientific), and then cells were centrifuged at $16,000 \times g$ at 4°C to obtain postnuclear supernatants.

Immunoblots. Proteins from cell lysates and EVs were analyzed by standard SDS gel electrophoresis and Western blotting using IRDye-labeled detection reagents detailed above. Odyssey infrared imaging system and Image Studio v3.1 software (Li-Cor Biosciences) were employed to visualize the blots and to quantify the specific signals for detection of the relevant proteins graphed in Fig. S1 as mean values $\pm$ SD of at least three independent experiments.

AP-1 luciferase reporter assay. HZ1 cells were transfected using the TransIT-2020 reagent (Mirus) with 50 ng of an SF2 Nef expression vector together with 50 ng of a reporter plasmid containing tandem copies of consensus AP-1 binding motifs inserted in the minimal promoter-containing pFLUC plasmid (56) to drive the AP-1 inducible expression of firefly luciferase, plus 50 ng of the plasmid pRL-TK (Promega) expressing low and constitutive levels of renilla luciferase from a minimal herpes simplex virus (HSV)-thymidine kinase promoter.

Cells were collected and lysed with lysis buffer (Promega) on ice. A dual-luciferase reporter assay system from Promega was utilized to determine luciferase activities following the manufacturer's protocol using Berthold Sirius single-tube luminometer detection. Renilla luciferase activity (when at least 50% of untreated cells; lower counts considered drug toxicity) was used as an internal control to normalize AP-1 luciferase counts for variation in the transfection efficiency, whereas lower renilla counts were considered drug toxicity.

Lentiviral transduction of Nef. To produce Nef-expressing HIV-like pseudoviruses, HEK293 cells cultured in 6-well plates were cotransfected with cocktail of 3 μ g pDelta8.9 packaging plasmid, 1.5 μ g vesicular stomatitis virus G (VSV-G) envelope vector, and 3 μ g pWPI-derived subgenomic construct expressing wild-type or AxxA mutant HIV-1 SF2 Nef plus 12 μ g polyethylenimine (PEI). Medium was refreshed after 5 hours, collected 1 day later, and passed through a 0.22- μ m filter. A total of 0.5 ml of these lentiviral supernatants were added onto 1×10^6 THP1 cells (ATCC TIB-202) growing in 2 ml of RPMI 1640 plus 10% FBS, 2% L-glutamine, 0.05 mg/ml penicillin, and 0.05 mg/ml streptomycin per well of a 6-well plate.

Macrophage differentiation. A total of 1×10^7 THP1 cells were seeded into each well in a 6-well plate and treated with 10 ng/ml of phorbol myristate acetate (PMA) for 2 days. Adherent cells (M0 macrophages) were further cultured for 2 days in the presence of 10 ng/ml PMA and 10 ng/ml of granulocyte-macrophage colony-stimulating factor (GM-CSF) (ThermoFisher) to differentiate them toward an M1 macrophage phenotype.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.04 MB.

ACKNOWLEDGMENTS

We thank Virpi Syvälahti for expert technical assistance and Frank Kirchhoff for the panel of lentiviral Nef alleles used in this study.

This work was supported by grants to K.S. from the Helsinki University Central Hospital Research Council and the Jane and Aatos Erkko Foundation.

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