Infectivity Enhancement by Human Immunodeficiency Virus Type 1 Nef Is Independent of Its Association with a Cellular Serine/Threonine Kinase

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Nef proteins from human immunodeficiency virus type 1 isolate SF2 (HIV-1_{SF2}) and simian immunodeficiency virus isolate mac239 (SIVmac239) have been found to associate with a cellular serine/threonine kinase designated NAK. We have recently shown that the association of Nef with NAK is isolate dependent. To identify the structural basis for Nef-kinase association, several chimeric molecules were constructed between SF2 Nef (binding NAK) and 233 Nef (a primary isolate not binding NAK) and stably expressed in HuT-78 human T cells via retrovirus-mediated gene transfer. The Nef 233/SF2/SF2 chimera in which the N-terminal 37 amino acids of SF2 Nef were replaced by those of 233 Nef showed the same ability as SF2 Nef to bind NAK. The Nef 233/SF2/233 chimera in which the N-terminal 37 amino acids and the C-terminal 72 amino acids of SF2 Nef were replaced by corresponding sequences from 233 Nef completely lost the ability to associate with the kinase activity. Furthermore, replacement of the C-terminal 72 amino acids of 233 Nef with the equivalent SF2 sequence (chimera 233/233/SF2) fully restored kinase association to 233 Nef. These results suggest that (i) the core of Nef is not sufficient for NAK binding, (ii) the C terminus of SF2 Nef contains structural determinants important for association with NAK, and (iii) the failure of 233 Nef to bind NAK is due to a defect in its C terminus. Taking advantage of the C terminus of 233 Nef being nonfunctional and using an infectious clone of HIV-1_{SF2}, we show that association with NAK is not required for Nef-mediated infectivity enhancement. While the strong and reproducible association of some Nef isolates with NAK has been clearly established, the role of NAK in Nef function remains to be fully elucidated.

The *nef* genes of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) encode a 27- to 34-kDa myristoylated phosphoprotein (4, 7, 14, 21, 37, 53). Nef, together with Tat and Rev, is expressed early during viral replication (26, 30, 41, 46). Nef plays a crucial role in the maintenance of high virus load and subsequent development of AIDS in adult macaques infected with SIV (25). The significance of Nef in virus replication has also been highlighted in studies using a SCID-hu mouse model of HIV infection (3, 23). Consistent with an essential role for Nef in HIV pathogenesis, several long-term nonprogressors have been documented to be infected with *nef*-defective viruses (16, 28). All of these *in vivo* findings suggest an important role for Nef in HIV replication and the development of AIDS.

In vitro studies have shown that Nef downregulates cell surface expression of CD4, the main receptor for both HIV and SIV (8, 15, 24, 29, 31, 38). Nef downregulates cell surface CD4 expression and decreases total cellular CD4 levels by accelerating its endocytosis and its subsequent degradation in lysosomes (1, 5, 6, 10, 17, 19, 21, 35, 39, 45). Nef also enhances viral infectivity and viral growth *in vitro* (40, 55). Upregulation of viral growth by Nef has been reported to be mediated through an increase in proviral DNA synthesis (2, 51). The observation that virions produced by a *nef*-containing provirus are more infectious than those produced by a *nef*-deleted provirus suggests that intrinsic differences between these viruses exist (2, 40, 51, 55). Several independent findings have addressed the possible effect of Nef on virion assembly and struc-

ture. First, a cellular kinase activity found in purified virions is important for transport of the preintegration complex to the nuclei (13); second, Nef specifically associates with a cellular serine/threonine kinase (NAK) (48); and third, recent evidence suggests that Nef itself is incorporated into virions (12, 44, 58). Based on these and other observations, models postulating that Nef may directly modify key viral components or may facilitate the incorporation of a cellular factor (kinase?) into the virion have been proposed (56, 57, 59).

We have previously shown that association with NAK is not a highly conserved property among different Nef isolates and that association with NAK is not required for Nef-induced CD4 cell surface downregulation (36). In this report, several chimeras from Nef isolates that either bind or do not bind NAK were used to determine the structural requirements for Nef-NAK association. Our results suggest that the C-terminal region of Nef is essential for association with NAK. Furthermore, we show that the association with this kinase activity is not an absolute requirement for Nef-mediated viral infectivity enhancement.

MATERIALS AND METHODS

Cell lines and culture conditions. Human HuT-78 T cells were transduced to express only the neomycin phosphotransferase (*neo*) gene or the different Nefs and *neo* essentially as described previously (5, 19, 35). HuT-78 cells were cultured in RPMI 1640 medium with 25 mM HEPES supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, Utah), 50 IU of penicillin, and 50 µg of streptomycin per ml, 2 mM L-glutamine, and 1 mM sodium pyruvate (complete medium). 293T and HeLa-CD4-LTR-β-Gal (HeLa-MAGI) cells were cultured in Dulbecco modified Eagle medium supplemented with 50 IU of penicillin and 50 µg of streptomycin per ml, and with 10% fetal bovine serum. Cell lines were maintained at 37°C in a humidified incubator with 5% (HuT-78 cells) or 10% CO₂ (293T and HeLa-MAGI cells).

Construction of Nef chimeras and proviruses. To construct chimeras between the SF2 and 233 Nef isolates, SF2 or 233 *nef* coding sequences adapted by PCR to be flanked by *EcoRI* sites were cloned into pcDNA (Invitrogen, Carlsbad,

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FIG. 1. Diagrammatic representation of HIV-1 SF2 and 233 Nefs and their chimeras. The amino acid sequences of SF2 and 233 Nef are indicated by white and black boxes respectively. In the 233/SF2/SF2 chimera, the N terminus of SF2 Nef has been replaced by that of 233 Nef. In the 233/SF2/233 chimera, the core of 233 Nef (amino acids 34 to 134) was replaced by that of SF2 Nef. In the 233/233/SF2 chimera, the last 72 amino acids of 233 Nef were replaced by those from SF2 Nef (see Materials and Methods for details).

Calif.). Two unique restriction enzyme sites in *nef* (*Xho*I and *Eco*RV) were used to exchange corresponding regions between SF2 and 233 *nef* in frame (Fig. 1). The *Eco*RI fragment containing chimeric *nef* coding sequence was then cloned into the retrovirus vector pLXSN (5, 6) and pcDNA. The nomenclature used reflects the N terminus, core, and C terminus of the chimeric molecule. Thus, the 233/SF2/SF2 Nef chimera contains the N-terminal 33 amino acids of 233 Nef in front of the core and C terminus of SF2 Nef. The 233/SF2/233 chimera contains the core of SF2 Nef flanked by the N and C terminus of 233 Nef. The 233/233/SF2 chimera contains the N terminus and core of 233 Nef in front of the C terminus of SF2 Nef. The specific locations of the different junctions are indicated in Fig. 1.

Production of wild-type HIV-1_{SF2} and HIV-1_{SF2} with Nef chimeras. The different proviruses containing chimeric *nefs* were constructed by removing a *Xho*I-*Bsp*EI fragment from the pcDNA-based constructs described above (Fig. 1) and replacing the corresponding fragment in the HIV-1_{SF2} provirus clone p9B18 (kindly provided by P. Luciw). In all constructs, the N terminus (from the ATG to the *Xho*I site) and the last six amino acids of SF2 Nef (from the *Bsp*EI to the stop codon) were maintained intact. These last six amino acids are identical in both SF2 and 233 Nef. The nomenclature described above was also used to name the Nef chimeras in these proviruses (see Fig. 4).

Infectious viruses were prepared in 293T cells (10^6) transfected with p9B18 or its derivatives containing chimeric Nefs (3 μ g), using Lipofectamine-mediated transfection as instructed by the manufacturer (Life Technologies). Virus was harvested 48 h posttransfection, filtered through a 0.45- μ m-pore-size filter, and stored frozen in aliquots at -70°C . Lysates from the transfected cells were used for analysis of Nef expression by Western blot analysis or immunoprecipitation and for NAK activity by *in vitro* kinase assay (see below).

Flow cytometry analysis. For analysis of cell surface CD4 levels, cells (5×10^5) were stained with phycoerythrin (PE)-conjugated monoclonal antibody to human CD4 or an isotype-matched control (both from Exalpha, Boston, Mass.) diluted in phosphate-buffered saline-5% calf serum-0.1% NaN_3 (PBS-CS- NaN_3) for 20 min on ice. Stained cells were washed twice in 2 ml of ice-cold PBS-CS- NaN_3 and analyzed on a Becton Dickinson FACScan instrument with LYSYS II software. All fluorescence data were collected in log mode.

Western blot and immunoprecipitation analysis. Nef expression was determined directly or after immunoprecipitation with a rabbit polyclonal anti-Nef antiserum by Western blot analysis using alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG) (Promega, Madison, Wis.) or horseradish peroxidase-conjugated anti-rabbit IgG (Amersham, Arlington Heights, Ill.). To visualize the immunocomplexes, 5-bromo-4-chloro-3-indolyl-1-phosphate and nitroblue tetrazolium were added to the alkaline phosphatase conjugates; horseradish peroxidase conjugates were visualized by using enhanced chemiluminescence (ECL kit; Amersham).

***In vitro* kinase assay.** The assay for the cellular kinase activity associated with Nef was performed essentially as described by Sawai et al. (48), using retrovirus-transduced Nef-expressing HuT-78 cells (4.5×10^6) or transfected 293T cells (10^6).

Infectivity assay. HeLa-MAGI cells (6×10^4) were plated in 12-well plates and infected 24 h later in triplicate with 5 ng of p24 of virus stock. Cells were infected with wild-type HIV-1_{SF2}, HIV-1_{SF2} Δ *Xho*I (frameshift in Nef open reading

frame), or viruses containing *nef* chimeras in 400 μ l of cell culture medium containing 20 μ g of DEAE-dextran (Sigma, St. Louis, Mo.) per ml at 37°C in an incubator with 8% CO_2 . Two hours after inoculation, 1.6 ml of culture medium was added, and the cells were incubated for an additional 36 h. Cells were then fixed and stained as described previously (27). Blue cells were counted as an indicator of infected cells.

RESULTS

Nef encoded by the 233 primary isolate does not bind NAK.

SF2 and SIVmac239 Nef bind to and activate a cellular serine/threonine kinase (NAK) (48). We have previously reported that the ability to coimmunoprecipitate NAK is Nef isolate dependent and identified the 233 Nef primary isolate as negative for this function (36). Two possibilities could account for this phenotype: a lack of binding or a failure to activate NAK. To distinguish between these two possibilities, two control experiments were performed. First, we confirmed that 233 Nef is efficiently immunoprecipitated by rabbit anti-Nef polyclonal antibodies (data not shown); second, we determined that the amount of NAK in the cell is limiting since increasing the amount of Nef expression does not increase the amount of NAK immunoprecipitated by saturating amounts of anti-Nef antibodies (data not shown). We then proceeded to determine if 233 Nef binds NAK. For this purpose, a competition analysis for NAK between 233 and SF2 Nef was performed. Cells were transfected with a SF2 Nef expression construct alone or in combination with increasing amounts of a 233 Nef expression construct. Lysates from the transfected cells were split into two samples. One sample was used for Western blot analysis to determine Nef expression. The second sample was immunoprecipitated with saturating amounts of HIV-1 Nef-specific antibodies, and the absorbed proteins were assayed for kinase activity. It should be noted 233 Nef has a faster mobility in sodium dodecyl sulfate-polyacrylamide gel electrophoresis than SF2 Nef, and the two proteins can be readily distinguished on this basis (Fig. 2A). This difference in mobility has been previously reported and is also observed with NL4-3 Nef (6, 36, 43). As expected, in the samples containing the competitor 233 Nef plasmid, there were significantly higher levels of 233 Nef than SF2 Nef (Fig. 2A). Interestingly, immunoprecipitates from the cells expressing SF2 Nef alone showed the same level of phosphoproteins as those containing excess of 233 Nef (Fig. 2B). Similar results were obtained in assays using several different preparations of anti-Nef antibodies or Nef-specific monoclonal antibodies (data not shown). These results indicate 233 Nef does not compete for binding with NAK and that even when expressed at high levels, it does not interfere with SF2 Nef activation of NAK. Therefore, the failure to detect NAK in 233 Nef immunoprecipitates is likely due to its inability to bind this kinase.

The C terminus of SF2 Nef is required for association with NAK and is sufficient to complement 233 Nef. To determine the molecular basis for the lack of association between 233 Nef and NAK, we constructed chimeras between SF2 and 233 Nef (Fig. 1). These chimeras were stably expressed via retrovirus-mediated gene transfer in HuT-78 cells as determined by Western blot analysis (Fig. 3A). As expected from the difference in electrophoretic mobility between SF2 and 233 Nef, the chimeras also showed corresponding differences in their electrophoretic mobilities depending on how the chimera was constructed. These chimeras were not only stably expressed but also found to be functional, as determined by their ability to induce CD4 cell surface downregulation (Fig. 3B). *In vitro* kinase assays were performed with immunoprecipitates obtained with saturating amounts of rabbit polyclonal anti-Nef antibodies to determine the levels of NAK associated with

FIG. 2. Nef encoded by the 233 primary isolate does not bind NAK. 293T cells were transfected with pcDNA.Nef.SF2 (0.7 μg) alone or with the same amount of pcDNA.Nef.SF2 and increasing amounts of pcDNA.Nef.233 (3.5 and 5.3 μg). The cell lysates were split into two samples, one for analysis of Nef expression and the other for kinase assay. All samples were transfected with a total of 6 μg of DNA. The amounts of the different plasmids are indicated at the top. (A) Western blot analysis for Nef expression. Lane 1, cells transfected with pcDNA control vector; lane 2, cells transfected with pcDNA.Nef.SF2 and pcDNA control vector; lanes 3 and 4, cells transfected with pcDNA.Nef.SF2 and increasing amounts of pcDNA.Nef.233. The molecular mass marker (in kilodaltons) is indicated on the left. SF2 and 233 Nef are indicated by arrows. The band with the faster mobility as previously reported corresponds to 233 Nef (6, 36, 43). (B) In vitro assay for kinase activity in Nef immunoprecipitates. The lane assignments are the same as in panel A. The phosphoproteins designated by Sawai et al. as P62, P72 doublet (48–50) are indicated by arrows. In our experimental setting, the apparent molecular masses of these proteins are 62, 84, and 88 kDa. We also observe an additional 70-kDa phosphoprotein. Molecular mass markers (in kilodaltons) are indicated on the left.

each of these chimeras. The results from the kinase assay showed that replacement of the N terminus of SF2 Nef with that of 233 Nef (233/SF2/SF2 chimera) did not affect NAK binding, suggesting that the N-terminal regions of SF2 and 233 Nef are interchangeable (Fig. 3C, lane 4). Similar chimeras constructed between SF2 and NL4-3 or Bru or YU2 Nef also bound NAK (data not shown). These data can be interpreted as suggesting that (i) the N terminus of Nef is not involved in direct binding to NAK or (ii) the N termini of all of these different Nefs are functional. The fact that chimera 233/SF2/233 completely lost NAK binding ability suggests that the core of SF2 Nef is not sufficient for NAK binding (Fig. 3C, lane 5). Interestingly, the 233/233/SF2 Nef chimera bound NAK quite efficiently (Fig. 3C, lane 6). However, the reciprocal SF2/SF2/233 chimera did not bind NAK (Fig. 4 and 5B). These results suggest that the failure of 233 Nef to bind NAK is due to a defect at its C terminus. The fact that the C terminus of SF2 Nef is able to confer kinase association activity to 233 Nef suggests that there are no other defects in the molecule.

Association with NAK is not required for Nef-induced virus infectivity enhancement. It has been suggested that association of Nef with NAK is required for high virus production, viral infectivity enhancement, and possibly pathogenesis in SIV-infected macaques (34, 50, 59). To address if there is a correlation between kinase association and infectivity enhancement, we took advantage of the fact that the lack of NAK binding by 233 Nef maps to its C terminus. We introduced 233 Nef se-

quences into an infectious proviral clone of HIV-1_{SF2} (Fig. 4) and evaluated the ability of the Nef chimeras to bind NAK and to enhance HIV infectivity. Virus infectivity enhancement was determined by a simple and reliable single-round infectious assay previously used by us and others for this purpose (2, 20, 27, 36, 59). To facilitate cloning and to make as few changes as possible into the provirus, *XhoI*-*BspEI* fragments from 233 Nef and two Nef chimeras shown in Fig. 1 were used to replace the corresponding *nef* sequences in the SF2 provirus. These fragments correspond to amino acids 38 to 204 and 34 to 200 for SF2 and 233 Nef, respectively. Three proviruses with Nef chimeras containing the 233 Nef C terminus (SF2/SF2/233), the 233 Nef core (SF2/233/SF2), or both (SF2/233/233) were directly compared to wild-type HIV-1_{SF2} or HIV-1_{SF2}Δ*XhoI*, a provirus with a frameshift in the *nef* open reading frame (Fig. 4). Nef was efficiently expressed from all of the proviruses except HIV-1_{SF2}Δ*XhoI*. In addition, all of the different Nefs were efficiently immunoprecipitated by the rabbit polyclonal anti-Nef antiserum (Fig. 5A). The kinase binding phenotype in the context of the HIV provirus for each of these chimeras was determined (Fig. 5B). Consistent with the results in Fig. 3C, chimeric Nefs encoded by proviruses containing the C terminus from 233 Nef did not bind NAK, while those containing the C terminus from SF2 Nef did bind NAK (Fig. 5B). We then proceeded to determine the ability of each of the different chimeras to enhance infectivity. HIV-1_{SF2} and the HIV-1_{SF2}Δ*XhoI* frameshift mutant *nef* were used as controls. Wild-type HIV-1 SF2 was approximately 50-fold more infective than the virus defective for Nef (Fig. 6). Surprisingly, even though only one of the three viruses containing the Nef chimeras (SF2/233/SF2) showed NAK binding (Fig. 5B), all three showed infectivity enhancements similar to the wild-type virus. These results indicate that the association of Nef with NAK is not an absolute requirement for Nef's enhancement of viral infectivity.

DISCUSSION

In vivo studies have demonstrated the importance of Nef in virus replication and pathogenesis (23, 25). In vitro, Nef has been shown to have at least two independent functions: downregulation of cell surface CD4 and enhancement of viral infectivity (19–21, 40, 55). In addition, it has been suggested that Nef also suppresses major histocompatibility complex I cell surface expression and interferes with T-cell activation (9, 11, 22, 33, 52, 54). However, the precise mechanisms underlying all of these functions of Nef are largely unknown. Interaction with host factors is likely to mediate Nef's functions. Screens for cellular proteins which may be involved in Nef functions have yielded little information. Solid and convincing evidence is available only for the in vivo association of Nef with a cellular serine/threonine kinase activity, NAK (48). Preliminary information suggests that NAK has similarities with PAK kinases (34, 42, 50). Although the role of a PAK-related kinase in Nef function has not been well delineated, it has been suggested that NAK plays a direct role in viral infectivity enhancement, virus production, and pathogenesis (34, 50, 59).

We have previously shown that association with NAK is not a property conserved among different Nef isolates (36). We and others have also shown that the association with NAK is not required for Nef-mediated CD4 cell surface downregulation (36, 59). In this study, we used several chimeric Nefs to investigate the structural basis for the interaction between Nef and NAK. We also used these chimeras to identify the region(s) of Nef responsible for the lack of kinase association observed with some Nef isolates (36). Our results suggest that

FIG. 3. The core region of Nef is not sufficient for association with NAK, and the C terminus of SF2 Nef confers 233 Nef the ability to associate with NAK. (A) Western blot analysis of cell lysates for Nef expression. Lane 1, HuT-78/LN; lane 2, HuT-78/LNefSN.SF2; lane 3, HuT-78/LNefSN.233; lane 4, HuT-78/LNefSN.233/SF2/SF2; lane 5, HuT-78/LNefSN.233/SF2/233; lane 6, HuT-78/LNefSN.233/233/SF2. Molecular mass markers (in kilodaltons) are indicated on the left. Nef proteins are indicated by arrows on the right. (B) Flow cytometry analysis of CD4 cell surface expression. Graph a, HuT-78/LN control cells stained with PE-control antibody (dotted line) or PE-CD4 antibody (solid line). Graphs b to f, cells stained with PE-CD4 antibody: HuT-78/LNefSN.SF2 (b), HuT-78/LNefSN.233 (c), HuT-78/LNefSN.233/SF2/SF2 (d), HuT-78/LNefSN.233/SF2/233 (e), and HuT-78/LNefSN.233/233/SF2 (f). (C) In vitro assay for kinase activity associated with Nef. A portion of the samples used for panel A was used for these assays; the samples are in the same order as in panel A. The 62-, 84-, and 88-kDa phosphoproteins (formerly designated by Sawai et al. as P62, P72 doublet [48-50]) are indicated by arrows on the right. Molecular mass markers (in kilodaltons) are on the left.

the N-terminal and core regions of SF2 Nef are not sufficient for association with NAK. Interestingly, the C-terminal region of SF2 Nef was found to play a critical role in the association of Nef with NAK. Sawai et al. reported that the core region of SF2 Nef (from amino acids 45 to 127) is required for binding to the kinase by CD8-Nef chimeras (49). Our data do not contradict this previous report but suggest that a functional C terminus is necessary for association of NAK with intact Nef. So far there is no evidence suggesting that the core region of Nef is sufficient for association with NAK. Interestingly, in a study by Sawai et al. three CD8-Nef fusion proteins with C-terminal truncations (CD8-Nef 1-127, CD8-Nef 1-145, and CD8-Nef 1-167) were found to be unstable (49). This finding is consistent with our previous report indicating that a C-terminal

deletion in SIV Nef renders the protein highly unstable (18). However, similar studies have not been conducted with SF2 Nef. The previous observations together with those presented here suggest that the C-terminal region of Nef is not only critical to stabilize the general structure of Nef but also likely to be important for the interaction between Nef and NAK.

The importance of the C-terminal region of Nef in structural stabilization and function is supported by its crystal structure (32). The C-terminal 20 amino acids of Nef (amino acids 185 to 204 for SF2 Nef) form one β sheet and two α helices (β C, α C, and α D) (32). This C-terminal region partially covers the distal surface of the β strands which together with N-terminal helices form the bulk of the hydrophobic core (32). At this time, the points of contact between Nef and NAK and whether the tail of Nef is part of these interactions are not known. Based on the crystal structure, the tail might play a role in fixing the conformation of Nef so that it can interact with NAK. A clear answer

FIG. 4. Diagram illustrating the HIV-1_{SF2} genome and the different Nef chimeras (indicated below). The portions of 233 *nef* are indicated in black. Positions of the junctions where amino acids were substituted are indicated above (number for SF2 Nef) and below (number for 233 Nef) each bar (see Materials and Methods for details of provirus construction). LTR, long terminal repeat.

for these questions will have to wait until the structure of the Nef-NAK complex is resolved.

In spite of the significant diversity in amino acid sequence among different Nef isolates, there are several highly conserved regions. The PXXP motif (amino acids 73 to 82 for SF2 Nef) is one of them. In *in vitro* experiments, this motif was shown to be required for binding to the SH3 domain of a

subset of Src kinases (47). Mutational analysis suggested that three of the four prolines in this region are important for association with NAK (59). In addition, one arginine residue at position 110 (for SF2) was found to be essential for association with NAK (49, 59). The failure of 233 Nef to associate with NAK cannot be attributed to a defect in either of these two regions, as 233/233/SF2 binds NAK (Fig. 3C). The fact that the C-terminal region of SF2 Nef is sufficient to confer 233 Nef kinase binding indicates that it is the C-terminal region of 233 Nef that is defective. This defect affects only NAK binding, as 233 Nef induces cell surface CD4 downregulation and infectivity enhancement. Even though the C terminus of Nef is required, it itself is not sufficient to associate with NAK (49). Therefore, other regions such as the PXXP motif are likely to be involved in kinase association (49, 59). There are 12 of 72 amino acid differences between the C-terminal tails of SF2 and 233 Nef. These 12 amino acids fall within a 38-amino-acid stretch. Extensive site-directed mutagenesis will be required for identification of the residues in the C terminus of 233 Nef responsible for the lack of binding to NAK.

A comparison of the infectivities of wild-type virus, SF2 Δ XhoI, or viruses containing chimeric Nefs showed that only the SF2 Δ XhoI Nef had significantly impaired infectivity. In addition, the two Nef chimeras SF2/233/233 and SF2/SF2/233 enhanced viral infectivity even though they do not bind NAK. The viral infectivity enhancement mediated by Nefs lacking NAK binding could not be attributed to the changes made in the 3' long terminal repeat of the chimeric viruses, as transcription activities were similar for all constructs tested (data not shown). In addition, the SF2/233/233 hybrid was 35-fold more infective than its Nef frameshift mutant (data not shown). These results show that these chimeras are functional and suggest that the association of Nef with NAK is not absolutely required for Nef-mediated infectivity enhancement. These results also clarify the apparent discrepancy with our

FIG. 5. Analysis of Nef expression and NAK activity in virus-producing cells. Proviruses were transfected into 293T cells. After harvesting of virus supernatant, cell lysates were used directly for immunoprecipitation followed by Western blot analysis for Nef expression (A) and *in vitro* kinase assay (B) as described in Materials and Methods. (A) Immunoprecipitation-Western blot analysis for Nef expression. Proviruses transfected 293T cell lysates were immunoprecipitated by a rabbit anti-Nef antiserum; the immunocomplexes were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. The separated proteins were probed with the same rabbit anti-Nef antiserum and detected by goat anti-rabbit IgG (Fc specific) conjugated with alkaline phosphatase. Lane 1, cells transfected with the wild-type SF2 provirus; lane 2, cells transfected with SF2 provirus with a frameshift in *nef*; lane 3, cells transfected with the provirus containing the SF2/233/233 chimera; lane 4, cells transfected with the provirus containing the SF2/SF2/233 chimera; lane 5, cells transfected with the provirus containing the SF2/233/SF2 chimera. Rabbit Ig heavy chain and Nef proteins are indicated by arrows on the right. Molecular mass markers (in kilodaltons) are indicated on the left. (B) *In vitro* assay for kinase activity associated with Nef and chimeras. The lane assignments are the same as in panel A. The 62-, 84-, and 88-kDa phosphoproteins associated with Nef (48–50) are indicated by arrows on the right. Molecular mass markers (in kilodaltons) are indicated on the left.

FIG. 6. The association of Nef with NAK is not required for Nef-mediated virus infectivity enhancement. Equal amounts (5 ng of p24) of the viruses produced with the indicated Nef chimeras were used to infect HeLa-MAGI cells. Cells were stained 48 h postinfection with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal), and blue cells were counted. Each bar represents the average of three determinations. The experiment was performed twice with similar results.

previous suggestion that there might be a correlation between NAK binding and Nef-mediated infectivity enhancement (36). In light of the information presented here and the previous reports showing that NAK binding is not required for CD4 downregulation (36), the function of the NAK-Nef association is unclear. It is reasonable to assume that the interaction between Nef and NAK which is reproducible in many different cell types and by many different laboratories must not be merely artifactual. However, the relevance of the interaction between Nef and NAK in the context of known functions of Nef remains to be established.

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