Activation of p21-activated Kinase 2 and Its Association with Nef Are Conserved in Murine Cells but Are Not Sufficient to Induce an AIDS-like Disease in CD4C/HIV Transgenic Mice*

Received for publication, November 28, 2005 Published, JBC Papers in Press, December 28, 2005, DOI 10.1074/jbc.M512710200

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A well conserved feature of human immunodeficiency virus, type 1 (HIV-1) and simian immunodeficiency virus (SIV) Nef is the interaction with and activation of the human p21-activated kinase 2 (PAK2). The conservation of this interaction in other species and its significance for Nef pathogenesis in vivo are poorly documented. In the present study, we measured these parameters in Nef-expressing thymocytes, macrophages, and dendritic cells of a transgenic (Tg) mouse model of AIDS (CD4C/HIV). We found that Nef binds to and activates PAK2, but not PAK1 and -3, in these three cell subsets. Nef associates with only a small fraction of PAK2. The Nef-PAK2 complex also comprises β-PIX-COOL. The impact of the Nef-PAK2 association on disease development was also analyzed in Tg mice expressing 10 different Nef mutant alleles. CD4C/HIV Tg mice expressing Nef alleles defective in Nef-PAK2 association (P69A, P72A/P75A, R105A/R106A, Δ56–66, or G2A [myristoylation site]) failed to develop disease of the non-lymphoid organs (kidneys and lungs). Among these, only Tg mice expressing NefP69A and NefG2A showed some depletion of CD4+ T cells, although, given the down-regulation of the CD4 surface protein was documented in all these Tg lines, except those expressing NefΔ56–66. Among other Tg mice expressing Nef mutants having conserved the Nef-PAK2 association (RD35AA, D174K, P147A/P150A, Δ8–17, and Δ25–65), only Tg mice expressing NefA8–17 develop kidney and lung diseases, but all showed partial CD4+ T cell depletion despite some being defective for CD4 down-regulation (RD35AA and D174K). Therefore, Nef can activate murine PAK2 and associate with a small fraction of it, as in human cells. Such activation and binding of PAK2 is clearly not sufficient but may be required to induce a multiorgan AIDS-like disease in Tg mice.

Nef is an accessory protein of HIV3 and SIV, which has been found to be essential for high levels of viral replication and disease progression.

The importance of Nef as a key factor for viral pathogenesis emerges from clinical and experimental observations. Isolated viruses from long term non-progressor HIV-infected individuals were found to harbor mutated nef genes (1–5). Rhesus macaques infected with Nef-deleted SIV failed to develop disease (6–8). Finally, more recent studies showed that both HIV-1 and SIV Nef were necessary and sufficient to cause an AIDS-like disease in Tg mice (9, 10).

Although the role of Nef for the viral life cycle and pathogenesis is not debatable, its function at the molecular level are less understood. Several functions of Nef, discovered mainly from in vitro studies, have been reported (for reviews see Refs. 11–16). Nef was shown to enhance viral infectivity, a function involving its incorporation into the virus particle, where Nef is cleaved by the viral protease (17, 18). Nef has also been shown to mediate down-regulation of the cell-surface expression of CD4 molecules (19, 20), a function apparently independent of its role in enhancing viral infectivity (21). This down-regulation of CD4 expression by Nef prevents superinfection and accelerates viral infectivity by enhancing HIV-1 replication (22–24). In addition, CD4 down-modulation facilitates increased release and spread of HIV in vivo with the consequent establishment of high virus loads (25–28). Additionally, it has been reported that Nef down-regulates the expression of major histocompatibility complex class I, CD3, and CD28 surface molecules, up-regulates Fas-ligand expression, and alters the production of several cytokines, thus affecting the host immune responses (29–33). Finally, Nef has been shown to modulate cellular activation of T cell signaling pathways (9, 34–37). However, the importance and the physiological relevance of each of these individual functions are poorly documented in lymphocytes, macrophages, and dendritic cells (DCs), the natural targets of HIV-1 infection. Thus, Nef pathogenesis in vivo still remains unclear and requires further investigation.

Nef has been found to interact with a number of cellular protein kinases, including members of the Src family of tyrosine kinases (38–42), as well as yet unidentified serine/threonine kinase (43, 44) and another serine/threonine kinase (45, 46) known as Nef-associated kinase (NAK) identified as a member of the p21-activated protein kinases (PAKs) family (47–49). Activation of PAK has been implicated in several cellular processes, including reorganization of the cell cytoskeleton, mitogen-activated protein kinase signaling cascades (50–52), and pro- as well as anti-apoptotic effects (53–55). The Nef-NAK association has also been implicated in increased virion infectivity (56). This interaction of PAK with Nef is a highly conserved feature of primary HIV-1 and SIV Nef alleles (57, 58), suggesting that this association is important for Nef functions. Moreover, targeting Nef to the plasma

* This work was supported by grants from the Canadian Institutes of Health Research, HIV/AIDS Research Program (to P. J. and Z. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus; Tg, transgenic; DC, dendritic cell; PAK, p21-activated kinase; NAK, Nef-associated kinase; GST, glutathione S-transferase; IVKA, in vitro kinase assay; Ab, antibody; LN, lymph node; FACS, fluorescence-activated cell sorting; 7AAD, 7-amino-actinomycin D.

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membrane is critical to allow NAK association and its autophosphorylation (59–61). Among the PAK family members, PAK2 appears to preferentially associate with Nef (47, 48), although PAK1 has also been described as the preferred binding partner (49). In addition, several studies, mostly using the SIV Nef allele, have linked this association to efficient pathogenesis and enhancement of viral infectivity (62, 63). However, these observations were challenged by other studies showing efficient pathogenesis and enhancement of viral infectivity (62, 63). Northern Blot Analysis—Northern blot analysis was carried out on 10 µg of total RNA from different tissues, using 32P-labeled HIV-1 probe, as previously described (9).

Antibodies—Rabbit anti-HIV Nef antisera were raised against purified GST-HIV Nef fusion protein, as described (9). Anti-β-PIX-COOL and p95PKL were produced in rabbits immunized with purified GST/β-PIX150–154 and GST/p95PK147–949, respectively. Anti-actin and rabbit anti-αPAK (C-19) or goat anti-γPAK antibodies (V-19) were from Sigma and Santa Cruz Biotechnology, respectively. The latter were used to immunoprecipitate PAK2. Specific antibodies against PAK1, PAK2, and PAK3 have previously been described (47).

Western Blot Analysis—Protein expression was assessed by Western blot analysis of lymphoid organs from different founders using rabbit anti-Nef antisera as described previously (9). Briefly, cells were extracted in radiolabeled precipitation assay buffer (0.5% sodium deoxycholate, 1% Triton X-100, 0.1% SDS in phosphate-buffered saline) containing protease inhibitors aprotinin (2 µg/ml), pepstatin (1 µg/ml), N-p-tosyl-l-lysine chloromethyl ketone (50 µg/ml) and leupeptin (2 µg/ml). Cell extracts (∼100 µg) were loaded onto SDS-10% polyacrylamide gel and blotted on polyvinylidene difluoride Immobilon-P membrane (Millipore). Membranes were blocked overnight in 5% skim milk-0.1% Tween 20 (Sigma) in Tris-buffered saline at 4 °C. Blocked membranes were incubated with the anti-Nef antisera (1:1000), anti-actin (1:2000), or anti-PAK2 (V-19) antibodies, for 2 h at room temperature and washed with 5% skim milk-0.1% Tween 20. Proteins were visualized by incubating the membranes with secondary antibodies coupled to Alexa 680 fluorochrome followed by scanning with Odyssey® infrared imaging system (Licor) for all experiments except the one shown in Fig. 2B. In this latter experiment, the membrane was incubated for 1 h with the secondary reagent rabbit IgG TrueBlot™ (1:1000, Bioscience), and the Nef protein was subsequently revealed using enhanced chemiluminescent horse-radish peroxidase substrate ECL (PerkinElmer Life Sciences), as described previously (9).

Preparation of Peritoneal Macrophages—Peritoneal macrophages were harvested by injecting 10 ml of fresh media (Iscove’s medium supplemented with 10% fetal bovine serum) into the peritoneal cavity of the animals followed by peritoneal lavage at 3 h. The attached macrophages were washed three times with phosphate-buffered saline and lysed with ice-cold lysing buffer for subsequent assays.

In Vitro Kinase Assay—Cells were lysed in kinase extraction buffer (KEB) (1% Nonidet P-40, 50 mM Tris-HCl, pH 8.0, 10% glycerol, 2 mM EDTA, and 137 mM NaCl) containing protease (aprotinin (2 µg/ml) and leupeptin (2 µg/ml)) and phosphatase (sodium orthovanadate (0.37 µg/ml) and sodium fluoride (0.04 µg/ml)) inhibitors. Equal amounts of proteins were used for all the in vitro kinase assay (IVKA) reactions, and quantification of proteins was done using the colorimetric microBCA assay. For immunoprecipitation, anti-Nef or anti-PAK antibodies were added to 250 µg of thymocyte lysates or 100 µg of macrophage lysates and incubated overnight. Protein G-coupled agarose beads (Amersham Biosciences, 50 µl) were then added, and the pellets were washed three times with the KEB buffer and once with kinase activation buffer (1% Triton, 100 mM NaCl, 50 mM Tris-HCl, pH 8.0, 5 mM MgCl2, and 2 mM sodium orthovanadate). The IVKA reaction were performed in 50 µl containing 10 µCi of [γ-32P]ATP for 5 min at room temperature, and the pellets were washed once with KEB and resuspended in 2X SDS-
Flow Cytometry and Cell Sorting—Flow cytometry was performed on a BD Biosciences FACScan using antibodies against various cell surface markers, as described previously (9, 68, 69, 72): CD4, CD8, CD38, and Thy1.2 for T cells and B220 and Mac-1 for B cells and macrophages, respectively, and CD44, CD25, CD69, CD45RB, and CD62L for T cell naive/activation phenotype. Apoptosis was assessed by 7-amino-actinomycin D (7AAD) and annexin V (fluorescein isothiocyanate-labeled)/propidium iodide staining as previously described (73).

Histological Analysis—A group of 15–20 Tg animals and an equivalent number of non-Tg littermates were generated from each line and observed for signs of disease (hypoactivity, ruffled hair, and respiratory problems). Mice were sacrificed, and lymphoid and non-lymphoid organs were collected and embedded in paraaffin blocks. Sections (5 μm) were processed for microscopic and histological assessment, essentially as previously described (9, 68, 69). Semiquantitation assessment of the histological phenotypes was done as previously described (67).

RESULTS

NAK Activity Is Present in Thymocytes, Macrophages, and DCs of CD4C/HIVNef Tg Mice—We evaluated the association of Nef with NAK and its subsequent activation by an in vitro kinase assay (IVKA) performed on total lysates from thymocytes, macrophages, and DCs. These three populations of cells express Nef in CD4C/HIVNef Tg mice (Fig. 1A). Nef was immunoprecipitated with associated proteins using polyclonal anti-Nef serum followed by IVKA, and phosphorylated substrates were visualized by SDS-PAGE and autoradiography. This analysis showed the presence of major phosphorylated proteins of ~62, 85, and 95 kDa in lysates from Tg cells, whereas these were not detectable in extracts from non-Tg cells (Fig. 1B). Some phosphorylated proteins were sometimes more apparent in specific cell types. For example, the p62 species, most likely a PAK member (see below), was best detected in Tg DCs and macrophages, although with variations among experiments (Fig. 1B), but was barely detectable in Tg thymocytes. The amount of phosphorylated p62 in Tg tissues was low in comparison to that previously observed in cell culture studies (48, 74, 75). Phospho-amino acids analysis demonstrated that the majority of the phosphorylation of the p62/p95/p85 species occurred on serine residues (Fig. 1C), indicating that murine NAK, like its human and simian homologues (47, 48, 56, 59, 64, 65, 74), is a serine-threonine kinase. These results indicated that Nef binds to a murine serine kinase in primary immune cells of Tg mice. By analogy to the human protein kinase interacting with Nef, which was previously reported to be PAK, the murine NAK detected in Tg cells share several similarities with PAK. However, we have been unable to demonstrate an association of Nef with PAK, after immunoprecipitation with anti-Nef Ab followed by Western blot analysis with anti-PAK Ab (data not shown), suggesting that this association is transient or that only a small fraction of PAK may bind to Nef (see below). Such negative data are consistent with results of other groups, which have experienced similar findings, even in conditions of PAK overexpression (47, 63, 76).

PAK2 Is Selectively Activated by Nef in Target Cells of CD4C/HIV Tg Mice—Contradictory evidences have been reported as to whether human PAK1 or PAK2 interacts with Nef (47–49). The identity of the mouse PAK family member associated by Nef in vivo was examined. IVKA were performed on lysates from Nef-expressing thymocytes, macrophages, and DCs after immunoprecipitation with antibodies interacting specifically with each PAK1 family member, PAK1, PAK2, and PAK3 (47). Only PAK2 was found to be activated by Nef in the three Tg cell populations tested (thymocytes, macrophages, and DCs) (Fig. 2A). The profiles of phosphorylated proteins immunoprecipitated with anti-PAK2 Ab were similar in each cell population. The majority of the phosphorylated protein was represented by the p95 and p85 species. In addition, these profiles were similar to those obtained when IVKA was carried out following anti-Nef Ab immunoprecipitation (Fig. 2A, lane 1). Notably, the expression of Nef in Tg thymocytes, macrophages, and DCs resulted in a significantly increased phosphorylation level of the kinase substrates following IVKA, compared with the levels observed in non-Tg cells (compare lanes 1, 6, 12, and 19 versus 2, 5, 11, and 20). These results show that Nef expression in primary mouse immune cells leads to PAK2 activation and suggest that the serine-threonine kinase interacting with Nef is mouse PAK2.

To determine the fraction of activated NAK bound to Nef in Tg thymocytes and macrophages, we performed IVKA on cell extracts preceded by two or three cycles of immunodepletion with anti-Nef Ab. Controls for efficiency of immunodepletion by Western blotting
showed that Nef was barely detectable after the first cycle of immunodepletion (Fig. 2B). Despite this pre-treatment, subsequent immunoprecipitation with anti-PAK2 Ab revealed readily detectable increase of activated PAK2 in Tg compared with non-Tg cells (Fig. 2C). Semi-quantitation of radioactive signals from these IVKA experiments revealed that a substantial portion of the total activated PAK2 is not bound to Nef compared with the bound one (not bound: ∼75% for thymocytes and ∼60% for macrophages). The PAK activity was barely detectable in non-Tg cells, suggesting that this activation was specifically due to the expression of Nef in major immune subsets of cells from CD4C/HIV-NefWT Tg mice. Total protein extract from thymocytes (250 μg), macrophages (100 μg), and DCs (100 μg) from 3-month-old CD4C/HIV-NefWT Tg and non-Tg littermate mice were incubated with an anti-Nef polyclonal serum overnight and subjected to an IVKA using 10 μCi of [γ-32P]ATP for 5 min at room temperature. The phosphorylated proteins were next separated by SDS-PAGE and visualized by autoradiography. The data obtained from thymocytes and macrophages are representative of at least 20 independent experiments, and those obtained from DCs are representative of three independent experiments, each involving cells from pooled Tg (n = 3) and non-Tg (n = 3) mice in each experiment. C, phospho-amino acids analysis of the 32P-labeled p62/p95/p85 species. IVKA was performed on thymocyte extracts using an anti-Nef serum. The samples were run on SDS-PAGE and transferred onto membrane, and autoradiography was performed. The p62, p85, and p95 phosphoproteins found in the Nef immunoprecipitates were excised from the membrane and incubated in 5.7 N HCl, and the released amino acids were subjected to one-dimensional chromatography, on a thin layer cellulose plate.

Characterization of the Nef-NAK Complex—Several investigators have identified the Nef-interacting p62 phosphoprotein as PAK2, a human PAK family member (46–48, 74). PAK2 has been shown to associate with β-PIX-COOL (77, 78), whereas the p85 phosphorylated NAK substrate was identified as PIX in Nef immunoprecipitates (74). However, the identity of the phosphorylated substrates of NAK in murine primary cells is not known. To determine the identity of the phosphoproteins present in the Nef-NAK complex, which serve as substrates of NAK, we performed immunoprecipitations with antibodies raised against β-PIX-COOL proteins known to be present in PAK complexes (77–79), followed by IVKA on thymocyte or macrophage extracts from Tg and non-Tg mice. Similar phosphorylation profiles to those seen with the anti-Nef or anti-PAK-2 Abs were observed with anti-β-PIX-COOL Ab (Fig. 3, A and B). In addition, as observed with the anti-PAK2 and anti-Nef Ab immunoprecipitations, the levels of phosphorylated proteins immunoprecipitated with the anti-β-PIX-COOL Ab were much higher in Tg than in non-Tg cells (Fig. 3, A and B), suggesting that the kinase associated with β-PIX-COOL in Tg thymocytes and macrophages is activated. Furthermore, an immunodepletion experiment carried out using anti-Nef Ab showed that a significant proportion of β-PIX-COOL not associated with Nef was present in the remaining supernatant of Tg thymocytes (82%) and macrophages (40%) (Fig. 3C). This result strongly suggests that the majority of the β-PIX-COOL proteins are not stably associated with Nef in Tg cells. A reverse experiment involving immunodepletion with anti-β-PIX-COOL Ab followed by immunoprecipitation with anti-Nef Ab, showed that a minor proportion (∼14%) of β-PIX-COOL was associated with Nef-PK2 in Tg macrophages (Fig. 3C).

To further characterize the p85- and p95-phosphorylated substrates, a limited digestion with the Staphylococcus aureus V8 peptidase or chymotrypsin was performed on these two 32P-labeled purified substrates produced by IVKA from the anti-Nef or anti-β-PIX immunoprecipitates. Comparison of the digestion patterns of both p85 and p95 substrates from the anti-Nef or anti-β-PIX immunoprecipitation revealed similarities except for one major digestion product observed in the
V8-digested p85 substrate (Fig. 3D). In addition, comparison of the digestion patterns from the immunoprecipitation with an anti-Nef and anti-β-PIX-COOL sera shows strong similarity of the digested fragments from each immunoprecipitate. Together, these results suggest that the p85 and p95 substrates represent the same protein species (β-PIX-COOL) being differently phosphorylated and present in both anti-Nef and anti-β-PIX-COOL complexes. Our finding, that the same substrate is present in both anti-Nef and anti-β-PIX-COOL complexes, supports the notion that β-PIX-COOL is a member of the Nef-NAK complex.

Mutations of Nef Abolishing PAK2 Binding and Activation Also Impair Their in Vivo Pathogenicity—Contradictory studies on the role of the Nef-associated PAK activation in AIDS pathogenesis have been published. In the SIV model, several studies showed that this association was dispensable (59, 64–66), while others claimed that it was critical (62, 63), for the development of simian AIDS in rhesus macaques. Given this controversy and to get a better understanding of the impact of PAK2 activation in the development of the AIDS-like phenotypes in CD4C/HIV-Tg mice, we studied Tg mice expressing two different Nef mutations (NefP69A and NefR105A/R106A), previously reported to disrupt Nef-PAK binding and to abolish PAK activation (46, 60). The NefP69A mutation was previously shown to abolish PAK binding without affecting other Nef functions, notably its binding to Hck (60). However, the R105/R106 residues have been found to be involved in Nef dimerization/oligomerization (80), and in CD4 down-regulation and in enhancement of viral infectivity (56, 81). We also included an additional mutant, NefP147A/P150A, harboring a conserved proline-rich motif originally thought to contribute to SH3 binding (41).

FIGURE 2. PAK2 is selectively activated in the immune cells of CD4C/HIV-NefWT Tg mice. A, total extracts of thymocytes (250 μg), macrophages (100 μg), and DCs (100 μg) from 3-month-old CD4C/HIV-NefWT Tg and non-Tg littermate mice were immunoprecipitated with specific serum raised against PAK1, PAK2, and PAK3 (47) followed by an IVKA. The samples were then run on SDS-PAGE and visualized by autoradiography. These data are representative of six independent experiments done with a group of four or five pooled Tg and non-Tg littermate animals in each experiment. Membranes were cut, and protein loading in each lane was determined by Western (W) blotting with anti-PAK (C-19) Ab or with Ab specific for PAK1. B, immunodepletion of Nef. The anti-Nef immunoprecipitates from the total thymocyte extracts (TE) or from supernatants (sup) were run on SDS-PAGE, and Western blotting was done with anti-Nef Ab. The right panel is a control with immunoblotting without immunoprecipitation. C, immunodepletion experiments. Total protein extract (TE) from thymocytes (250 μg) or macrophages (100 μg) of CD4C/HIV-NefWT Tg and non-Tg mice were Nef-depleted by two or three successive rounds of immunoprecipitation with an anti-Nef serum and the subsequent supernatant (sup) was immunoprecipitated with anti-PAK2 (V-19) antibodies. The reverse experiment was also carried out for macrophages: PAK2-depletion, followed by immunoprecipitation with anti-Nef on the subsequent supernatants. An IVKA was performed on each immunoprecipitate, and the samples were run on SDS-PAGE. The phosphorylated proteins were detected by autoradiography.
CD4C/HIV-NeF$^{p69A}$, CD4C/HIV-NeF$^{R105A/R106A}$, and CD4C/HIV-NeF$^{P147A/P150A}$ Tg mice, respectively (Fig. 4A). Independent Tg lines from each Nef mutant (NeF$^{p69A}$, F62331, F62335, and F62337; NeF$^{R105A/R106A}$, F98769 and F95139; and NeF$^{P147A/P150A}$, F61937 and F61937) were established by breeding on the C3H background.

Transgene expression, evaluated by Northern (Fig. 4B) and Western (Fig. 4C) blot analysis, revealed specific expression of NeF RNA in the lymphoid tissues (with more expression in thymus than in LN and spleen), but not in non-lymphoid tissues (e.g. kidney, and data not shown). Levels of NeF protein expression in the thymus correlate with RNA expression levels: F62337 > F62335 > 62331 for NeF$^{p69A}$ Tg mice, F95139 > F98769 for NeF$^{R105A/R106A}$, and F59063 > F61937 for NeF$^{P147A/P150A}$. Mice from founders that expressed mutated NeF at comparable (F62331, F62335, F98769, and F61937) or higher (F62337, F95139, and F59063) protein levels than NeFWT of CD4C/HIVNeF Tg mice were selected for comparative studies, because both the incidence and progression of the AIDS-like disease in CD4C/HIVNeF Tg mice was previously shown to correlate well with the level of NeF expression (9).
We first confirmed that the Nef-NAK association was disrupted and that PAK activation was abolished as expected in two different sub-populations (thymocytes and macrophages) of these NefP69A and NefR105A/R106A Tg mice but not in those of the NefP147A/P150A Tg mice (Fig. 5A). We then confirmed that PAK activation was abolished, by using IVKA on PAK2 immunoprecipitates of lysates from both Tg thy-
mocytes and macrophages (Fig. 5B). To confirm that the anti-Nef polyclonal Ab could immunoprecipitate the mutant Nef proteins showing no binding to NAK, we performed anti-Nef immunoprecipitations on [35S]methionine-labeled thymocytes. This experiment showed that the Nef mutant proteins in Tg thymocytes had retained their ability to be recognized by the anti-Nef Ab used in these IVKA experiments (Fig. 5C).

The progeny of each Tg line were routinely monitored for the presence of signs of disease (wasting, edema, diarrhea, ruffled hair, hypoactivity, and early death). None of these clinical features developed in these NefP69A-, NefR105A/R106A-, and NefP147A/P150A-expressing Tg lines during the 12-month observation period.

Total cell numbers in lymphoid organs of CD4C/HIV-NefP69A Tg mice were decreased, whereas those of CD4C/HIV-NefR105A/R106A Tg mice were in the range of control non-Tg mice (Table 1). In CD4C/HIV-NefP147A/P150A Tg mice, total thymocytes were also depleted significantly, but the peripheral lymphoid cells were not (Table 1). FACS analysis, performed on thymocytes and on cells from peripheral lymphoid organs (spleen and LN), using T-cell (CD4, CD8, Thy1.2, and Tcrσ-), B-cell (B220)-, and macrophage (Mac-1)-specific markers showed that cells from CD4C/HIV-NefP147A/P150A Tg mice appeared normal. However, this analysis revealed a lower number of CD4+ T cells in the thymus and peripheral organs of CD4C/HIV-NefP69A Tg mice but only in the thymus of CD4C/HIV-NefR105A/R106A Tg mice compared with non-Tg mice (Fig. 6A and B) and Tables 1–3). Depletion of CD4+ T cells correlated with the level of Nef protein expression. This analysis also demonstrated a partial down-regulation of cell surface CD4, compared with non-Tg controls, both on thymocytes and on CD4+ T cells of peripheral organs. This effect was mainly seen in CD4C/HIV-NefP69A and CD4C/HIV-NefP147A/P150A Tg mice but was less obvious in CD4C/HIV-NefR105A/R106A Tg mice (Fig. 6A and B) and Tables 1–3).

**TABLE 1**

| Cell number in lymphoid organs of control and CD4C/HIV-Nefmut Tg mice |
|-----------------|-----------------|-----------------|
| Mouse line      | Number of cells (×10⁶/organ) |
|                 | Thymus | Spleen | Mesenteric lymph nodes |
| Non-Tg          | 111.1 ± 22.3 | 94.4 ± 44.0 | 23.8 ± 7.4 |
| CD4C/HIV-NefWT  | 17.7 ± 11.7 |
| CD4C/HIV-NefP69A| 60.6 ± 18.4 |
|                 | 68.8 ± 24.6 |
|                 | 13.4 ± 4.4 |
| F62330          | 58.6 ± 14.9 |
|                 | 66.9 ± 13.2 |
|                 | 18.6 ± 2.8 |
| F62335          | 32.6 ± 18.1 |
|                 | 43.4 ± 27.7 |
|                 | 17.8 ± 9.7 |
| CD4C/HIV-NefP147A/P150A | 85.3 ± 24.1 |
|                 | 90.2 ± 33.7 |
|                 | 19.4 ± 8.9 |
| F95139          | 100.4 ± 13.9 |
|                 | 96.6 ± 16.2 |
|                 | 17.9 ± 6.6 |
| CD4C/HIV-NefR105A/R106A | 60.6 ± 15.5 |
|                 | 85.3 ± 26.5 |
|                 | 28.0 ± 13.6 |
| F59063          | 96.7 ± 17.6 |
|                 | 94.8 ± 24.8 |
|                 | 30.9 ± 7.4 |

* A minimum of nine mice (3–9 months-old) of each founder line were analyzed. 

* The non-Tg control values were obtained by pooling the results of all of the non-Tg littermates from different lines. 

* p < 0.05 using Student’s t test.
FIGURE 6. Immunophenotypic analysis of lymphocytes from CD4C/HIV-NefP69A, CD4C/HIV-NefR105A/R106A, and CD4C/HIV-NefP147A/P150A Tg mice. FACS analysis of cell populations from thymus (A) and lymph nodes (B–D) from representative CD4C/HIV-NefP69A, CD4C/HIV-NefR105A/R106A, CD4C/HIV-NefP147A/P150A, and CD4C/HIV-NefWT Tg mice and their non-Tg littermates. A and B, T cells were labeled for the CD4, CD8, and TcRαβ markers. The percentage of cells found in each quadrant is indicated. A dotted line is drawn across the

| RATIO: (Tg/Non-Tg) | Non-Tg | P69A | RR105/106AA | P147A/P150A | WT |
|-------------------|--------|------|-------------|-------------|-----|
| CD69+             | 1      | 1.3 (± 0.3)* | 1.0 (± 0.1) | 1.4 (± 0.1)* | 2.5 (± 0.2)**  |
| CD25+             | 1      | 1.5 (± 0.2)* | 1.0 (± 0.1) | 1.5 (± 0.1)* | 2.7 (± 0.4)*** |
| CD44+             | 1      | 1.9 (± 0.4)* | 1.1 (± 0.2) | 1.6 (± 0.2)* | 1.6 (± 0.2)*** |
| CD45RB+           | 1      | 2.0 (± 0.3)** | 0.9 (± 0.1) | 1.6 (± 0.1)*** | 1.6 (± 0.1)*** |
| CD62L-            | 1      | 1.6 (± 0.2)** | 0.8 (± 0.1) | 1.5 (± 0.2)** | 1.5 (± 0.2)**  |

| Apoptotic/ (dead cells (%) | Non-Tg | P69A | RR105/106AA | P147A/P150A | WT |
|----------------------------|--------|------|-------------|-------------|-----|
| 9 ± 1.7                    | 14 ± 2.0* | 8 ± 0.8 | 12.1 ± 1.6 | 32 ± 6.3*** |
Because Tg CD4+ T cells expressing NefWT exhibit an activated phenotype (72) and because the interaction between Nef and NAK may be important for this Nef-mediated cellular activation, we asked whether the abrogation of Nef-NAK activation would affect T cell activation. The activation status of the peripheral Tg CD4+ T cells was analyzed by FACS, by staining for CD69, CD4, CD45RB, CD62L, and CD25 activation markers. Expression of these markers on CD4+ T cells expressing NefR105A/R106A mutant was comparable to that expressed on non-Tg CD4+ T cells (Fig. 6C), suggesting that disruption of Nef-NAK association may affect their expression. However, a higher proportion of Tg CD4+/NefR105A/R106A mutant T cells constitutively expressed CD69 and CD25, as compared with those of non-Tg littermate controls (Fig. 6C). A larger proportion of these Tg CD4+/NefR105A/R106A mutant T cells also appeared to exhibit an activated/memory-like phenotype, being CD45RBhi-, CD45RBlo-, and CD62Llow-positive, thus indicating that T cell activation is independent from Nef-NAK interaction. A similar analysis of the mice expressing NefP147A/P150A showed similar activation (Fig. 6C).

We have shown that CD4+ T cell depletion is associated with enhanced apoptosis in CD4C/HIV Tg mice (73). PAK2 phosphorylation of Bad has been reported to abrogate its pro-apoptotic effect and increase cell survival (54). To determine whether the loss of Nef/PAK2 association would affect T cell death, we measured apoptosis of lymphoid T cells by FACS, staining with annexin V/propropidium iodide or 7AAD. The proportion of 7AAD+ (apoptotic/dead cells) CD4+ T cells from mice expressing NefR105A/R106A mutant did not show the enhanced apoptotic/death phenotype (Fig. 6D) usually associated with expression of NefWT (73).

Next we examined organs of Tg mice for signs of pathological changes. Histological examination of non-lymphoid organs (kidney, pancreas) to show the down-regulation of the cell surface CD4, C. analysis of surface activation markers. Three-color staining of LN cells. T cells were labeled for CD4 and TCRβ as well as for CD25, CD44, CD45RB, CD62L, or CD69. Results from a representative experiment on cells gated on CD4+, TCRβ+, and CD69+ are shown. The percentage of cells found in each quadrant is indicated. At the bottom, a summary of the results for additional cell activation markers is presented as a ratio (Tg/non-Tg). The data were pooled from four non-Tg and four Tg mice from each mutant. The statistical analysis was done by the Student's t test, *p < 0.05; **p < 0.01; and ***p < 0.001. D, quantitation of apoptotic/dead CD4+ T cells. LN cells of Tg or non-Tg mice were analyzed by FACS after staining with anti-CD4-APC mAbs and 7AAD. The data pooled from three non-Tg and four Tg mice of each indicated line represent percentages of apoptotic/dead cells among the CD4+ T cell subpopulation. Statistical analysis was performed by the Student's t test.
intestine, liver, and lungs) of CD4C/HIV-NefP69A, CD4C/HIV-
NefR105A/R106A, and CD4C/HIVP147A/P150A Tg mice showed no signifi-
cant differences between the Tg and non-Tg animals (data not shown).
In CD4C/HIV-NefP69A Tg mice, a partial disruption of the cortico-
medullary junction of the thymus (4/16 and 4/17 Tg mice, respec-
tively), a disturbed architecture of spleen (3/16 and 1/17 Tg mice,
respectively), and LN (3/16, 4/17 Tg mice, respectively) were
observed (data not shown). Such changes in lymphoid organs were
only rarely noted and were very mild in CD4C/HIV-NefR105A/R106A
Tg mice. Therefore, these results suggest that the PAK2 association
may be required for the development of the multiorgan disease of
CD4C/HIV Tg mice but not for CD4 down-regulation. Regarding
the CD4+ T cell loss, results from one mutant (P69A) indicated that
the Nef-NAK interaction was not necessary for at least partial loss,
whereas those from the other mutant (R105A/R106A) showed that it
might be required.

PAK2 Activation Is Not Sufficient for the Development of a Multiorgan
Disease in CD4C/HIV Tg Mice—Although the previous results from Tg
mice expressing NefP69A or Nef with point mutations at G2A, P72AaspP75A, RD35AA, and D174K (A), or Nef with an internal deletion at Δ8–17, Δ25–65, and Δ56–66 (B), were precipitated with
anti-Nef serum followed by IVKA. The phosphorylated samples were separated by SDS-PAGE and visualized by autoradiography. The star indicates that these control lanes are the same as those shown in Fig. 5, because all these samples were loaded on the same gel. These data are representative of at least three independent experiments done with a group of 3 Tg and non-Tg littermate animals in each experiment. C, thymocytes from non-Tg or Tg mice expressing NefP69A or Nef with mutation at G2A and P72AaspP75A were incubated with [35S]methionine, and their extracts were immunoprecipitated with anti-Nef serum. The35S-labeled Nef proteins were detected as described in the legend to Fig. 5C. Note the capacity of this anti-Nef serum used to immunoprecipitate the mutated Nef proteins effectively. D, thymocyte and macrophage extracts from non-Tg or Tg mice expressing NefG2A, NefRD35AA, and NefD174K were immunoprecipitated with anti-PAK2 (V-19) antibody followed by IVKA (top). The numbers above the lanes show semiquantitative values of PAK2 activity for the
mutants relative to non-Tg samples, as described in the legend to Fig. 5B. Western blot with anti-PAK2 (V-19) Ab was performed to detect the amount of immunoprecipitated PAK2 in the thymocyte extracts (bottom).

FIGURE 7. Characterization of Nef-NAK interaction in Tg mice expressing additional Nef mutants. Thymocyte (250 μg) or macrophage (100 μg) extracts from Tg mice expressing control NefWT or Nef with point mutations at G2A, P72AaspP75A, RD35AA, and D174K (A), or Nef with an internal deletion at Δ8–17, Δ25–65, and Δ56–66 (B), were precipitated with
anti-Nef serum followed by IVKA. The phosphorylated samples were separated by SDS-PAGE and visualized by autoradiography. The star indicates that these control lanes are the same as those shown in Fig. 5, because all these samples were loaded on the same gel. These data are representative of at least three independent experiments done with a group of 3 Tg and non-Tg littermate animals in each experiment. C, thymocytes from non-Tg or Tg mice expressing NefP69A or Nef with mutation at G2A and P72AaspP75A were incubated with
[35S]methionine, and their extracts were immunoprecipitated with anti-Nef serum. The35S-labeled Nef proteins were detected as described in the legend to Fig. 5C. Note the capacity of this anti-Nef serum used to immunoprecipitate the mutated Nef proteins effectively. D, thymocyte and macrophage extracts from non-Tg or Tg mice expressing NefG2A, NefRD35AA, and NefD174K were immunoprecipitated with anti-PAK2 (V-19) antibody followed by IVKA (top). The numbers above the lanes show semiquantitative values of PAK2 activity for the
mutants relative to non-Tg samples, as described in the legend to Fig. 5B. Western blot with anti-PAK2 (V-19) Ab was performed to detect the amount of immunoprecipitated PAK2 in the thymocyte extracts (bottom).
Nef-NAK Association in HIV Tg Mice

**TABLE 4**

Summary of the phenotypes observed in CD4C/HIV\textsuperscript{Nef} mutants Tg mice

| Nef mutation | NAK association | Non-lymphoid organ disease\textsuperscript{a} | CD4 down-regulation\textsuperscript{b} | CD4\textsuperscript{+} T cell depletion\textsuperscript{b} |
|--------------|-----------------|-------------------------------------------|---------------------------------|---------------------------------|
|              |                 |                                           | Thy | LN | Thy | LN |
| WT           | Yes             | Severe                                    | + + + | + + + | + + + | + + + |
| G2A          | No              | ND                                        | + + + | + | + | + |
| P72A/P75A    | No              | ND                                        | + | + | + | + |
| P69A         | No              | ND                                        | + + + | + + + | + + + | + + + |
| R105A/R106A  | No              | ND                                        | + | + | + | + |
| R256A        | Yes             | Moderate                                  | + | + | + | + |
| D174K        | Yes             | Severe                                    | + | + | + | + |
| \(\Delta 8-17\) | Yes         | Severe                                    | + | + | + | + |
| \(\Delta 25-65\) | Yes       | Severe                                    | + | + | + | + |
| \(\Delta 56-68\) | No          | ND                                        | + | + | + | + |
| P147A/P150A  | Yes             | ND                                        | + | + | + | + |

\textsuperscript{a} Mean score was calculated for all mice of the group, as described under "Materials and Methods."

\textsuperscript{b} The symbols "+" and "-" reflect the percentages of CD4 down-regulation or CD4\textsuperscript{+} T cell depletion according to the following scale: the score "-" was given for <15%, "+" for 15–40%, "++" for 40–60%, "+++" for 60–80%, and "++++" >80%. Percentages were calculated relative to non-Tg control mice. The data shown are for the founder lines expressing the highest levels of each Nef mutant.

\textsuperscript{c} ND, no disease.

Nef\textsuperscript{A25–65}, Nef\textsuperscript{D35A}, Nef\textsuperscript{D174K}, and Nef\textsuperscript{P147A/P150} did (Fig. 7, A and B). The results for the P72A/P75A and G2A Nef mutants confirmed previous studies in human (60–62) and rodent (82) cell lines. Again, to confirm that the absence of Nef-NAK interaction did not result from the failure of the anti-Nef Ab to immunoprecipitate these mutant Nef proteins, we performed immunoprecipitation on extracts from \[^{35}S\]methionine-labeled thymocytes (Fig. 7C). This experiment confirmed that the immunoprecipitations of two of the Nef mutants, expressed in Tg thymocytes, were comparable to that of Nef\textsuperscript{WT} in these conditions (Fig. 7C). For each Nef mutant, the activation of PAK2 was also examined after immunoprecipitation with an anti-PAK2 Ab and IVKA (Fig. 7D). The mutants capable of associating with NAK showed a comparable level of PAK2 activity as the one seen in Nef\textsuperscript{WT} Tg mice (Fig. 7D). Semiquantification assessments of radioactive signals from these IVKA experiments showed that the levels of phosphorylated substrates in the Nef\textsuperscript{RD35AA}, Nef\textsuperscript{D174K}, and Nef\textsuperscript{WT} Tg thymocytes were 6-, 12-, and 5-fold higher than those found in non-Tg controls, respectively (Fig. 7D). This indicates that the increased PAK2 activity is due to the expression of Nef in Tg thymocytes. In contrast, PAK2 activity was not enhanced in Tg cells expressing one of the mutant (Nef\textsuperscript{G2A}), which did not associate with NAK (Fig. 7, A and D).

Interestingly, among the Nef mutants having conserved NAK2 activation capability, some Tg mice (CD4/HIV-Nef\textsuperscript{A8–16} and CD4C/HIV-Nef\textsuperscript{D174K}) develop lung and kidney diseases, while others (CD4/HIV-Nef\textsuperscript{A25–65}, CD4C/HIV-Nef\textsuperscript{RD35A}, and CD4C/HIV-Nef\textsuperscript{P147A/P150}) did not (67, 68), indicating that the presence of NAK in T cells and macrophages is not sufficient by itself to elicit organ disease in Tg mice.

Attempts to correlate the Nef-NAK interaction with CD4\textsuperscript{+} T cell depletion were also made. This analysis indicated that such interaction, absent in Nef\textsuperscript{RD35A}-expressing cells, was not required for CD4\textsuperscript{+} T cell depletion observed in these Tg mice. However, in each mutant Tg line in which NAK activity was present (Nef\textsuperscript{D1A–17}, Nef\textsuperscript{A25–65}, Nef\textsuperscript{RD35A}, Nef\textsuperscript{D174K}, and Nef\textsuperscript{P147A/P150}) CD4\textsuperscript{+} T cell depletion also occurred, suggesting that complex formation between Nef and NAK may have a negative impact on CD4\textsuperscript{+} T cell fate. A summary of the phenotypes observed in these mutants is shown in Table 4.

**DISCUSSION**

**Nef/PAK2 Association in Murine Cells**—We used a mouse model of AIDS, the CD4C/HIV Tg mice, to study the binding of Nef to the PAK family members and their activation. Our data demonstrate that Nef expression in all murine primary Tg cells tested (thymocytes, macrophages, and DCs), specifically activates PAK2, but not PAK1 nor PAK3.

In addition, we showed that Nef associates, in these Tg target cells, with a serine kinase that is likely to be PAK2. The association is non-stoichiometric as only a small fraction of PAK2 was found to bind to Nef, thus preventing its unambiguous identification by a co-immunoprecipitation approach. The difficulty in detecting a Nef-PAK association has also been a recurring problem in human cells, even in conditions of overexpression of tagged proteins (47, 48, 75). This suggests that the Nef-PAK interaction is transient or weak or, alternatively, that only a small subpopulation of PAK2 molecules can bind to Nef. In fact, Renkema et al. (75) have demonstrated in an established cell line that NAK represents a distinct subpopulation of PAK2 with high kinase activity. This group recently showed that the Nef-PAK2 association was more stable with a kinase-dead PAK2 mutant, suggesting that the PAK2 kinase activity is involved in destabilizing the complex (76). Considering that Nef binds and activates PAK2 in mouse and human cells, the catalytic activity of PAK2 is also likely to promote dissociation of the Nef-PAK2 complex in murine cells, as it was reported in human cells (76).

However, several lines of evidence suggest that the detected Nef-associated kinase (NAK) in Tg murine cells is indeed PAK2. First, NAK primary phosphorylates serine residues, as PAK2 (83). Second, following anti-Nef or anti-PAK2 immunoprecipitations, the substrates of the IVKA not only show a similar pattern of phosphorylation but also have a similar phospho-peptide fingerprint. Third, these phosphorylated substrates (obtained by IVKA following immunoprecipitation with anti-Nef or anti-PAK2) are also similar to those obtained after immunoprecipitation with anti-β-PIX-COOL, a known partner of PAK (77–79, 84) and a known phosphorylated partner of the Nef-PAK complex in human cells (74). Fourth, the Nef mutants, which have been shown to disrupt the association of Nef with human PAK2, were also found to disrupt the association with murine NAK. Therefore, Nef appears to activate PAK2 and to associate with a small fraction of it. It still remains to be determined whether Nef binding to PAK2 requires activation of PAK2, as recently shown in human cells (76), or whether binding itself of Nef to PAK2 leads to PAK2 activation and how.

Our findings confirm previous studies with Nef expressed in primary and established human cell lines (47, 48, 74, 76), and extend them to murine cells. It remains unclear why some investigators identified PAK1 as a partner of Nef in human cells (49). It is of interest to note that the association of Nef with only one of the several members of the PAK family is a conserved property in human and mouse cells. In fact, sequence comparison of the mouse and human PAK2 showed that both molecules are highly homologous with only a few distinct changes. Among these amino acid substitutions is the absence of the caspase-3
cleavage site in the murine sequence (53), indicating that cleavage of PAK2 at this site by caspase-3 is not essential for the induction of Nef-mediated phenotypes in murine cells.

After IVKA following immunoprecipitation with anti-Nef, the levels of autophosphorylated p62 PAK2 are low, compared with what has been reported by some investigators (48, 74, 75). The reason for this is not clear. One explanation could be that most of the PAK2 phosphoacceptor sites susceptible to autophosphorylation may already be phosphorylated in Tg mice. Second, the cell type studied may influence this phosphorylation pattern. Our study was performed on primary T cells, macrophages, and DCs, in contrast to previous work performed with established immortalized cell lines. We already noticed that the intensity of the p62 band was generally stronger in macrophages than in thymocytes. Finally, the Nef allele used here (NL4–3) is already known to give a weaker autophosphorylation signal relative to other Nef alleles in human cells. In these studies, Nef association with the serine/threonine kinase was found to be viral isolate-dependent. In particular, the p62-phosphorylated species (presumably resulting from autophosphorylation) in the anti-Nef immunoprecipitate from cells infected with the NL4–3 strain of HIV-1 was weaker than that from cells infected with other strains (SF2, 248, 8161, and 13127) (48, 65, 76, 85, 86). Nevertheless, two major phosphorylation substrates could be recognized in these anti-Nef precipitates subjected to IVKA. These proteins are part of the Nef complex with PAK2. Our results suggest that they could represent β-PIX-COOL, a protein that has been reported to interact with PAK2 (77, 78) and other PAK family members (79, 84) and to be part of the Nef-PAK complex (74) in human cells. Thus, Nef appears to be part of a multiprotein complex containing at least PAK2 and β-PIX-COOL. This complex may also contain pp95 Paxillin kinase linker (PKL), which has been found in the PAK2/β-PIX complex (77, 78). Using rabbit anti-PKL antibodies followed by IVKA, we indeed observed a similar pattern of phosphorylated substrates in Thymocytes, although of lesser intensity, as those generated with anti-Nef, anti-PAK2, or anti-β-PIX-COOL Ab (data not shown). The assembly of this complex appears to be dependent on Nef targeting to the plasma membrane and may require other N-terminal Nef domain(s) (amino acids 57–66) for interaction, because NAK activity was completely absent in thymocyte lysates from other N-terminal Nef domain(s) (amino acids 57–66) for interaction, dependent on Nef targeting to the plasma membrane and may require Ab (data not shown). The assembly of this complex appears to be some cell populations, it does not appear to be the case for murine CD4C/CD8− T cells, which showed enhanced apoptosis (73), including those expressing NefP69A (Fig. 6D), a mutant that has lost the ability to bind to and to activate PAK2.

**Nef Activation of PAK2 Is Not Sufficient for the Development of Nef-induced AIDS-like Disease—Previews in vitro work on the effects of Nef-PAK association on Nef-induced infectivity enhancement has been controversial (56, 65). Similarly, in vivo studies in rhesus macaques infected with SIV strain carrying a mutation in the PXXP motif led to discordant results regarding the role of NAK-Nef association in disease progression (59, 62, 63, 66). Because the P72A/P75A mutation affects not only the binding of Nef to PAK2 but also is known to abrogate its association with the Src family members, in particular Hck (41), conclusions about the involvement of PAK activity in HIV or SIV pathogenesis with this single mutant may be difficult.**

To evaluate the contribution of enhanced Nef-induced PAK2 activity in the pathogenesis of the AIDS-like disease in CD4C/HIV Tg mice, we studied several mutants of Nef, some of them defective in PAK2 association. In addition, we correlated the ability of these Nef mutants to activate and bind to PAK2 with their ability to induce different phenotypes in vivo in Tg mice. Regarding the T-cell phenotype, this mutational analysis clearly indicated that PAK2 activation was dispensable for the Nef-mediated down-regulation of CD4 cell-surface molecule, some mutants (NefG2A, NefP72A/P75A, and NefP69A) with no PAK2-inducing capacity still remaining active at causing CD4 down-regulation. This conclusion is in agreement with results in human cells showing that Nef association with p62 kinase does not correlate with its effect on CD4 down-regulation (81).

This study also demonstrated that CD4+ T-cell loss occurred in each Tg line expressing a Nef mutant competent in binding PAK2 (NefG2A, NefP72A/P75A, NefR105A/R106A) but not in CD4C/HIV-NefP69A Tg mice defective in PAK2 activation, suggesting a positive contribution of PAK2 association in CD4+ T-cell loss. However, results from another mutant (NefP69A) indicated that CD4+ T-cell activation, depletion, and apoptosis can occur in the absence of Nef-induced PAK2 activation. Because of the nature of this latter mutation (P69A), it is possible that conformational changes favor binding to new effectors, thus bypassing the requirement for PAK2 activation in cells expressing this NefP69A mutant. In fact, different Nef alleles may induce CD4+ T-cell loss through distinct mechanisms, some of which may be dependent on PAK2 activation, whereas others may be independent. Further studies will be needed to test this hypothesis.

Second, regarding a possible correlation between the organ disease in Tg mice and the Nef-induced PAK2 activation, our data with 10 Nef mutants strongly suggest that PAK2 association by Nef is not sufficient to induce kidney and lung diseases, because three mutants able to bind PAK2 (NefG47A/P150A, NefP72A/P75A, and NefR105A/R106A) nevertheless failed to induce these organ diseases. However, results with other mutants suggest that PAK2 association may be required for lung and kidney diseases. Indeed, none of the five mutants having lost PAK2 binding capability among those studied (NefG2A, NefP72A/P75A, NefP69A, NefR105A/R106A, and NefP57–66) induced kidney and lung diseases. But the problem with this interpretation is the evidence that some of these mutations are known to affect other functions of Nef, in addition to its binding to PAK: namely abrogating its binding to Hck through indirect structural changes for the P72A/P75A mutation (41, 90, 91) and disrupting CD4 down-regulation and CD3 signaling for the R105A/R106A mutation (56, 81, 92, 93). It therefore appears that PAK2 binding to Nef may be required for induction of kidney and lung diseases in Tg mice but is clearly not sufficient.

Finally, this mutational analysis established that the kidney/lung organ diseases and the T-cell phenotype segregated independently. Indeed, three mutants of Nef described here (NefP69A, NefP105A/R106A, and NefP147A/P150A) and others previously reported (NefG2A and NefR25–65 (68)), were inactive at inducing kidney and lung diseases while remaining competent for causing CD4+ T-cell loss. This suggests that these organ diseases and CD4+ T-cell loss represent distinct phenotypes, most likely resulting from expression of Nef in distinct cell populations, respectively, CD4+ T cells and possibly DCs and macrophages.
In conclusion, our findings provide, for the first time, insights into the role of Nef-mediated PK2 activation in the pathogenesis of various abnormalities associated with the development of an AIDS-like disease in a small animal model. Because Nef binds to and activates PK2 in both human and primary murine cells and that human and mouse PK2 are highly conserved, the finding on Nef-mediated PK2 activation in this mouse model may be applicable and relevant to human AIDS.

Acknowledgments—We thank Mathieu Arcand and Sylvain Meloche previously from our Institute for the help in the phospho-amino acid analysis experiment, Pascale Jovert, Valerie Cote, Karina Lamarre, Jean-Renee Sylvestre, and Rita Gingras, Monique Villani, Chantal Gagnon, and Chantal Guertin for typing the manuscript.

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