Nef is an accessory protein of human and simian immunodeficiency viruses (HIV and SIV) that is required for efficient viral infectivity and pathogenicity. It decreases the expression of CD4 on the surface of infected cells. V1H is the regulatory subunit H of the vacuolar ATPase (V-ATPase). Previously, the interaction between Nef and V1H has been found to facilitate the internalization of CD4, suggesting that V1H could connect Nef to the endocytic machinery. In this study, we demonstrate that V1H binds to the C-terminal flexible loop in Nef from HIV-1 and to the medium chain (μ2) of the adaptor protein complex 2 (AP-2) in vitro and in vivo. The interaction sites of V1H and μ2 were mapped to a central region in V1H from positions 133 to 363, which contains 4 armadillo repeats, and to the N-terminal adaptin-binding domain in μ2 from positions 1 to 145.

Fusing Nef to V1H reproduced the appropriate trafficking of Nef. This chimera internalized CD4 even in the absence of the C-terminal flexible loop in Nef. Finally, blocking the expression of V1H decreased the enhancement of virion infectivity by Nef. Thus, V1H can function as an adaptor for interactions between Nef and AP-2.
degradation. To this end, we performed binding and internalization studies, which revealed that V1H binds Nef and the μ-chain of AP-2 in vitro and in vivo. In addition, when the C-terminal flexible loop in Nef was mutated or deleted, V1H fused to this truncated Nef protein could perform all the internalization functions of its wild-type counterpart. These findings fulfilled the structural and functional criteria for V1H as an adaptor between Nef and AP-2. Additionally, our results suggest that V1H plays a central role in the enhancement of virion infectivity by Nef.

EXPERIMENTAL PROCEDURES

Cells and Transfections—COS, 293T, and 2×22-1 cells were grown in Dulbecco’s modified Eagle’s medium. Jurkat cells were grown in RPMI 1640 medium. All media were supplemented with 10% fetal calf serum and antibiotics. COS and 293T cells were transfected using LipofectAMINE according to the manufacturer’s instructions (Invitrogen). Jurkat cells were transfected by electroporation with 10 μg of experimental and 20 μg of carrier DNA (salmon sperm DNA from Sigma), respectively, at 250 volts at 960 microfarads. 48 h after the transfection cells were subjected to various analyses.

Plasmid Constructions—All plasmid constructions for cell transfections were made into the pEF-BOS vector system. Plasmids CT, CN, Nef, NefED-AA, and V1H were described and translated using the rabbit reticulocyte system (TNT, Promega, Madison, WI) and [35S]methionine and [35S]cysteine (Amersham Biosciences) according to the manufacturer’s instructions. The quality of translated proteins was verified by SDS-PAGE and autoradiography.

Fluorescence-activated Cell Sorting (FACS) Analyses and CD4 Internalization Assays—COS cells were transfected with the indicated plasmids. 48 h later, the cells were washed three times with cold phosphate-buffered saline, 0.2% bovine serum albumin and incubated with 10 μl of fluorescein isothiocyanate-conjugated anti-CD4 or anti-CD8 antibodies (BD PharMingen) for 30 min on ice. Stained cells were then washed 3 times with wash buffer and 2 μg/ml fluorescein isothiocyanate-conjugated anti-CD4 or anti-CD8 antibodies in wash buffer and 2 μg/ml fluorescein isothiocyanate-conjugated anti-CD4 or anti-CD8 antibodies were incubated for 10 min on ice. Washed cells were then resuspended in 1 ml of PBS and measured by FACS analysis.

In Vitro Binding Assays—For interactions between Nef and V1H, 0.2 μg of Nef was incubated with 20 μl of hybrid GST-V1H protein or glutathione S-transferase (GST) beads and the indicated amounts of recombinant peptides in 50 μl of 50 mM Tris-HCl (pH 8.0), 0.5% Nonidet P-40, 2 mM EDTA, 250 mM NaCl, 10% glycerol, 10 mg/ml aprotinin, 10 mg/ml leupeptin, and 1 mM NaVO3 (kinase buffer) for 2 h. Beads were washed 4 times in the kinase buffer and subjected to SDS-PAGE and autoradiography.

CD4 kinetic internalization assays were performed as described recently (34). 293T cells were co-transfected with 5 μg of a CD4 expressing plasmid together with 5 μg of the respective Nef (Nef), loop mutant Nef (NefED-AA), and Nef-V1H (Nef60V1H) fragment effectors. For evaluation of the kinetic internalization rates, the geometric mean fluorescence of the different time points was measured by FACS analysis. The geometric mean fluorescence of the time point at 0 min was subtracted from the respective values at 5, 10, and 15 min to calculate the level of CD4 internalization.

Protein Purification and in Vitro Translation—GST-V1H fusion proteins were expressed in the Escherichia coli strain DH5α and purified using glutathione-Sepharose beads (Amersham Biosciences) with a modified lysis buffer containing 50 mM Hepes (pH 7.8), 100 mM KCl, 1% Triton X-100, 2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mg/ml lysozyme. Expressed proteins were purified using glutathione-Sepharose beads. The purity of fusion proteins was verified by Coomassie Blue staining of SDS-PAGE and their concentration was determined by a protein assay kit (Bio-Rad).

35S-Labeled hybrid CD8-Nef proteins and the μ2 chain were transcribed and translated using the rabbit reticulocyte system (TNT, Promega, Madison, WI) and [35S]methionine and [35S]cysteine (Amersham Biosciences) according to the manufacturer’s instructions. The quality of translated proteins was verified by SDS-PAGE and autoradiography.

Immunoprecipitation and Metabolic Labeling—A C-terminal His-tagged version of μ2 was expressed from pcDNA3, and HA-tagged V1H was expressed from pEF-Bos in COS cells. For the immune precipitation, mouse monoclonal anti-HA antibody (Roche Molecular Biochemicals) was coupled to tosyl-activated Dynabeads (Dynal Biotech, Lake Success, NY) according to the manufacturer’s protocol and used to precipitate HA-tagged V1H from cell lysates obtained in buffer I (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 5 mM MgCl2, 1% IGEPAL) at 4 °C for 60 min. The beads were washed twice with buffer I and resuspended in SDS sample buffer to elute bound proteins.

293T cells were transfected with 3 μg of plasmid encoding HA-V1H protein. 24 h later cells were starved for methionine and cysteine in methionine- and cysteine-free minimal essential medium (Invitrogen) for 1 h, then labeled by adding 100 μCi of [35S]methionine and [35S]cysteine (100 Ci/mmol; Amersham Biosciences) and incubated for an additional 4 h. After washing 3 times with cold phosphate-buffered saline, the cells were lysed in 1 ml of extraction buffer containing 50 mM Tris-HCl (pH 8.0), 0.5% Nonidet P-40, 2 mM EDTA, 250 mM NaCl, 10% glycerol, 10 mg/ml aprotinin, 10 mg/ml leupeptin, and 1 mM NaVO3 (kinase buffer) for 2 h. Beads were washed 4 times in the kinase buffer and subjected to SDS-PAGE and Western blotting with a mouse anti-HA antibody (27). For the mapping of the interaction sites between V1H and μ2, about 7 μl of the respective GST-V1H protein fragments were incubated with 5 μl of [35S]methionine-labeled μ2 protein fragments for 2–3 h at 4 °C in 500 μl of kinase buffer. Beads were then washed 3 times in the same buffer and subjected to SDS-PAGE and autoradiography.

Viral Production and Infectivity—For virus production in Jurkat cells, 1 μg of proviral DNA of HIV-1NL4-3, and its counterpart deleted in nef gene HIV-1NL4-3ΔNef (a kind gift from J. Guatelli, University of California, San Diego) along with 0–3 μg of AS-V1H plasmid were transfected into 1 × 106 Jurkat cells using DIME (Invitrogen) according to the manufacturer’s instructions. Transfections were balanced to a total of 4 μg with the empty pEF-BOS vector. In 293T cells, 3 μg of the HIV-1NL4-3ΔNef expression plasmid were co-transfected with 3 μg of various Nef or Nef-V1H expression plasmids using LipofectAMINE (Invitrogen). 48 h after the transfection, cellular supernatants were harvested, passed through a 0.45-μm filter (Fisher, Springfield, IL), and stored at −70 °C. The reverse transcriptase activity was measured to calculate the amount of generated virus. The infectivity of viral particles was assayed on infected 5×105 cells grown in 96-well plates (Fisher) by counting the β-galactosidase-positive blue cells 36 h after infection (41). The relative infectivity was calculated as the number of blue cells/ml divided by the reverse transcriptase activity.

RESULTS

Hybrid CD8-Nef-V1H and Nef-V1H Proteins Internalize CD4—Our previous studies demonstrated that the interaction between Nef and V1H facilitates the endocytosis of Nef via CCP, and that Nef binds V1H via its C-terminal flexible loop (27, 34). To determine whether V1H alone could connect Nef and CD4, the hybrid CD8-Nef-V1H proteins, or their derivatives, which contained mutated or deleted Nef proteins (Fig. 1B). On the other hand, the direct internalization assay measured levels of CD4 on cells co-expressing CD4 and Nef, the hybrid CD8-Nef or Nef-V1H proteins, or their derivatives, which contained mutated or deleted Nef proteins (Fig. 1A).

In the case of the direct internalization assay, the expression of CD4 was measured by FACS with the fluorescein isothiocyanate-conjugated anti-CD4 antibody 48 h after each transfection (Fig.
FIG. 1. V1H promotes the indirect internalization of CD4 and the direct internalization of the CD8 chimeras. A, schematic representation of the truncated CD8 protein (CT), Nef (N), V1H, and selected fusion proteins that were used for direct and indirect internalization assays. V1H is equivalent to the yeast VMA13p, and is the regulatory subunit of the V-ATPase. Nef ED-AA contains a mutation of glutamic and aspartic acids to alanine at positions 178 and 179 in Nef from HIV-1SF2. Nef160 contains a deletion from the C terminus to position 160 in Nef. Sizes of proteins are given above the bars: striped bar, CD8; white bar, Nef; black bar, V1H. The transmembrane portion of CD8 (tm) and the N-terminal myristylation (m) of Nef are highlighted.

B, schematic representation of the indirect and direct internalization assays. The indirect internalization assay measures levels of CD4 on the surface. It requires the interaction between CD4 (target) and Nef (effector). Depicted are the four immunoglobulin-like folds of CD4 and the myristylated hybrid Nef-V1H protein. The direct internalization assay measures levels of CD8 on the surface. It examines the ability of Nef itself to be internalized. This assay is studied with the hybrid CD8-Nef-V1H protein (CV1H) or the hybrid CD8-V1H protein (CV1H) that lacks Nef (dotted circle).

C, V1H promotes the indirect internalization of CD4. CD4 was co-expressed with the wild-type Nef and mutant Nef ED-AA proteins as well as other chimeras in COS cells, and levels of CD4 on the surface were measured by FACS. Co-expressed effectors are presented to the left of the bar graph. Bars represent levels of CD4 on the surface (%): white bars, mock; black bars, single effectors; striped bars, hybrid effectors. Error bars reflect S.E. of the mean from three independent experiments.

D, V1H promotes the direct internalization of CD8 chimeras. Fusion proteins were expressed alone in COS cells and levels of CD8 on the surface were measured by FACS. CD8 chimeras are presented to the left of the bar graph. Bar shading and error bars are as in panel C. E, chimeras were expressed at equivalent levels in COS cells. Western blotting was performed with the anti-HA antibody on cellular lysates from the experiments presented in panels C and D.
In contrast, the co-expression of CD4 and the hybrid mutant surface (Fig. 1C, lanes 2–4). The co-expression of CD4 with Nef or hybrid CD8-Nef and Nef-V1H proteins resulted in 4-fold reduced levels of CD4 on the surface (Fig. 1C, lanes 5, 7, and 8). As reported previously (27), the mutation of the diacidic motif of glutamic and aspartic acids at positions 178 and 179 to alanine in Nef (NefED–AA) blocked the internalization of CD4 (Fig. 1C, lane 6).

In contrast, the co-expression of CD4 and the hybrid mutant NefED–AAV1H and deleted Nef160V1H proteins, which contained the mutation of the diacidic motif and the deletion of the C-terminal flexible loop in Nef, respectively, linked to V1H, led to the efficient internalization of CD4 (Fig. 1C, lanes 9 and 10). As presented in Fig. 1E, levels of expression of these chimeras were equivalent. We conclude that the C-terminal flexible loop was dispensable for the interaction between CD4 and Nef and that the physical linkage of V1H to an internalization incompetent Nef restores its appropriate trafficking. Thus, V1H could be the adaptor for the internalization of CD4 by Nef.

Hybrid CD8-Nef-V1H and CD8-V1H Proteins Lead to the Internalization of CD4—To demonstrate that V1H was internalized identically to Nef, direct internalization assays were performed. The hybrid CD8-Nef, CD8-Nef-V1H, and CD8-V1H proteins were expressed on the surface of COS cells, and the levels of CD8 rather than CD4 were measured by FACS (Fig. 1D). The disappearance of CD8 from the cell surface represented the ability of Nef, V1H, or both proteins to engage the endocytic machinery. Thus, if Nef, V1H, or hybrid Nef-V1H proteins internalized CD8 to similar levels in the chimeras, V1H could represent an important target for Nef in CCP and endosomes. Cells expressing a truncated CD8 protein (CT) maintained high levels of CD8 on the surface (Fig. 1D, lane 2).

In contrast, the expression of the hybrid CD8-Nef (CN), CD8-Nef-V1H (CNV1H), and CD8-V1H (CV1H) proteins reduced these levels of CD8 up to 7-fold (Fig. 1D, lanes 3–5). Again, levels of expression of these chimeras were similar (Fig. 1E). Because the hybrid CD8-Nef, CD8-Nef-V1H, and CD8-V1H proteins decreased levels of expression of CD8 equivalently, the interaction between Nef and V1H could account for the intracellular trafficking of Nef.

Kinetic Internalization Rates of CD4 Induced by Hybrid Nef-V1H Proteins—Next, steady-state FACS analyses were confirmed in a kinetic internalization assay. As presented in Fig. 2, the wild type Nef protein promoted the internalization of CD4. Compared with the negative control, 2–3 times more CD4 was internalized in the presence of Nef after 15 min (Fig. 2, compare x symbols to black diamonds; approximately 32 versus 13%). In the presence of the internalization defective Nef mutant NefED–AA protein, the internalization of CD4 was only slightly higher than that observed with the negative control (compare black triangles to black diamonds, approximately 18 versus 13%). However, in the presence of the hybrid mutant Nef(1–160)V1H protein, in which the flexible loop and the C terminus of Nef was substituted by V1H, the internalization of CD4 was at least as high as in the presence of the wild type protein (compare white rectangles to x symbols, approximately 35 versus 32%) after 15 min. Overall, these data confirm the results from the steady-state surface expression. We conclude that V1H can substitute for the function of the flexible loop in Nef for the internalization of CD4.

The C-terminal Flexible Loop Is Required for the Binding of Nef to V1H—The interaction between Nef and V1H was mapped previously to the C-terminal flexible loop in Nef in the yeast two-hybrid system and by co-immunoprecipitation from transfected cells (27, 34). However, whether this interaction occurs directly or requires other cellular components had not been established. To determine whether Nef binds V1H, we performed GST pull-down assays between Nef and V1H. Nef isolated from E. coli or the 35S-labeled hybrid CD8-Nef protein, which was transcribed and translated using the rabbit reticulocyte lysate in vitro, were incubated with the GST-V1H fusion protein coupled to Sepharose beads. Binding proteins were resolved by SDS-PAGE and detected by Western blotting with the mouse anti-Nef antibody or by autoradiography. As presented in Fig. 3A, purified Nef was readily detected with the mouse anti-Nef antibody in the pull-down assays with the hybrid GST-V1H protein but not with GST alone (lanes 4 and 9). Importantly, a 15-residue oligopeptide, SLLHPMSLHGME–DAE (LL/ED), which was derived from the C-terminal flexible loop in Nef and contained both the dileucine and the diacidic motifs, specifically blocked the binding of Nef to the hybrid GST-V1H protein (Fig. 3A, lanes 5–8). In contrast, a scrambled control oligopeptide, GDMSLHSHEMLAEP (Control), which did not contain dileucine or diacidic motifs, did not interfere with this binding (Fig. 3A, lanes 2 and 3). Thus, Nef binds V1H directly via its flexible loop in vitro.

To prove that the hybrid CD8-Nef protein behaves similarly to the wild-type Nef protein, we performed GST pull-down assays between the hybrid CD8-Nef and GST-V1H proteins. As presented in Fig. 3B, the binding patterns between the hybrid CD8-Nef and GST-V1H proteins in the presence of increasing amounts of the LL/ED oligopeptide were indistinguishable from those between Nef and the hybrid GST-V1H protein (Fig. 3B, lanes 2–5, compared with A, lanes 4–8). Importantly, the control oligopeptide also did not interfere with this binding (Fig. 3B, lanes 6 and 7). Thus, the flexible loop in Nef was the major determinant of the binding between Nef and V1H.

V1H Binds AP-2—For the internalization of CD4, the complex consisting of the cytoplasmic tail of CD4, Nef, and V1H must contact the endocytic machinery. To this end we analyzed if V1H could bind to the adaptor protein complex AP-2, the predominant adaptor complex at the plasma membrane. The identification of the regulatory subunit H (43) was preceded by co-purification and co-immunoprecipitation of the medium chain of AP-2 (μ2) with V-ATPases from the bovine brain (44, 45). Although μ2 was not required for the functional activity of
the purified proton pump (46), we wondered if V1H could associate with adaptor protein complexes and thus link the V-ATPase to clathrin-coated pits.

The interaction between V1H and AP-2 was analyzed first in vitro. We co-expressed HA epitope-tagged V1H with C-terminal His epitope-tagged μ2 in COS cells, and immunoprecipitated V1H with mouse monoclonal anti-HA antibodies. As shown in Fig. 4A, a Western blot performed with anti-His antibodies recognized the μ2 in the immunoprecipitation, suggesting that both proteins interact in cells. Of note, the pull down of transfected HA epitope-tagged V1H protein indicated a double band, both proteins interact in cells. Of note, the pull down of transfected HA epitope-tagged V1H protein indicated a double band, suggesting the existence of two specific isoforms of this subunit as observed previously for the bovine V-ATPase (46, 47). Next, we examined the interaction with the whole adaptor protein complex in 35S-labeled lysates. 24 h after the transfection, 293T cells, which expressed HA epitope-tagged V1H protein, were incubated without methionine and cysteine for 1 h, then labeled by adding [35S]methionine and [35S]cysteine to the medium and cultured for an additional 4 h. Cellular lysates were incubated with the mouse monoclonal anti-HA antibody 12CA5. Immunoprecipitated proteins were resolved by SDS-PAGE and subjected to autoradiography. As presented in Fig. 5A, a band for the medium chain (50 kDa), as well as the α chain of AP-2 (17 kDa) were detected by autoradiography.

The structure of the yeast homologue of V1H, VMA13p, was solved by x-ray crystallography recently (49). The all helical structure contains eight armadillo (ARM) repeats that form an elongated molecule. Whereas the 10 N-terminal residues of VMA13p were folded back to the first three ARM repeats, thereby hiding the concave surface of the molecule by a proposed autoinhibition, the last two repeats (7 and 8) were bent apart from the main trunk by the insertion of two helices and form an independent domain. As presented in Fig. 5A, we generated the full-length hybrid GST-V1H protein and four different fragments thereof. These contained the N-terminal six ARM repeats (V1H-A(1–363)), the C-terminal six ARM repeats without the autoinhibitory block (V1H-B(133–483)), the last four ARM repeats of the N-terminal trunk (V1H-C(133–363)), and finally the C-terminal domain of two ARM repeats (V1H-D(362–483)). All GST-V1H fusion proteins were expressed to similar levels and were highly purified (see input control, Fig. 5A). Whereas the μ2 chain was detected in pull-down assays with the full-length hybrid GST-V1H protein and fragments A, B, and C that contained the N-terminal ARM repeats, no binding could be detected with the C-terminal domain (GST-V1H-D) or with GST alone. We conclude that V1H binds to the medium chain of AP-2 via its central ARM repeat region (repeats 3 to 6).

Subsequently, we mapped the site of interaction in μ2 (Fig. 5B). The medium chain protein consists of a two-domain structure with a N-terminal β2-binding domain and a C-terminal domain for the recognition of the tyrosine-based internalization motif (50). Again, full-length μ2 was pulled down efficiently by the hybrid GST-V1H protein and the hybrid GST-V1H-C protein fragments (Fig. 5B, lanes 2 and 3), as well as by the N-terminal μ2-binding segment from positions 1 to 145 (lanes 6 and 7). In sharp contrast, the tyrosine-binding domain of μ2

Mapping of the Interaction Sites in V1H and μ2—Next, to identify the interacting surfaces for both proteins we analyzed the binding between V1H and μ2 in vitro. To this end, we performed GST pull-down assays between μ2, which was transcribed and translated in vitro using the rabbit reticulocyte lysate and wild type and deletion mutant V1H proteins linked to GST, which were expressed in E. coli (Fig. 5). Interacting proteins were resolved by SDS-PAGE, and the 35S-labeled μ2 chain was detected by autoradiography.

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from positions 119 to 435 was not detected in the binding assay with the hybrid GST-V1H protein (lanes 10 and 11). As before, GST alone did not bind any of the three \( \mu 2 \) fragments (lanes 1, 5, and 9). As a control for the integrity of the \( \mu 2 \) fragments, we performed a pull-down experiment with the hybrid GST-SIV-Nef protein from SIV mac239, which contains a functional tyrosine-based sorting motif (28, 34). As expected, the full-length \( \mu 2 \) (50 kDa) binds to ARM repeats 3–6 of V1H (V1H-C) but not to ARM repeats 7 and 8 (V1H-D). All GST-V1H fusion proteins were highly purified and expressed to similar levels (input control). B, the N-terminal domain of \( \mu 2 \) interacts with V1H. Full-length \( \mu 2 \) and its two domains, the \( \beta \)-adaptin-binding domain (1–145) and the YXX\( \Phi \)-binding domain (119–435), were analyzed for their binding to the hybrid GST-V1H(wt) protein and the GST-V1H-C protein fragment (ARM repeats 3–6). As control for \( \mu 2 \) protein fragment functionality, pull-down experiments with GST-SIV-Nef were performed, which contains a tyrosine-based sorting motif.

Antisense V1H Interferes with the Enhancement of Virion Infectivity by Nef—Although significant progress has been made in our understanding of the molecular mechanism of internalization of CD4, the importance of this trafficking for the replication of HIV was not clear. High amounts of CD4 on infected cells can interfere with optimal production and infectivity of HIV (18, 19). However, residues within the flexible loop of Nef that mediate its connection with the endocytic machinery also affect virion infectivity in the absence of CD4 (6). Having identified V1H as a critical component for the internalization of CD4 by Nef, we examined the role that V1H plays in the viral replicative cycle. To this end, we utilized a plasmid that directs the synthesis of the antisense V1H RNA (AS-V1H) and blocks efficiently the expression of endogenous V1H protein (27). Increasing amounts of AS-V1H were co-expressed in Jurkat cells with proviral constructs of HIV-1NL4-3 or HIV-1NL4-3/\( \Delta \)Nef, which contained a deletion of the nef gene, and virions in cell culture supernatants were tested for their relative infectivity in HeLa-CD4 indicator Sx22-1 cells (Fig. 6A). The infectivity of HIV-1 NL4-3 was reduced significantly when particles were produced in the presence of AS-V1H (Fig. 6A, compare white bars in lanes 1–3). In contrast, the infectivity of virus particles synthesized from a provirus lacking the nef gene was unchanged or even slightly increased by AS-V1H (Fig. 6A, compare black bars in lanes 4–6). The presence of AS-V1H had only minor effects on the amounts of HIV particles produced (data not shown). These results suggest that V1H was required for the optimal replication of HIV and that the interaction between Nef and V1H facilitates its positive effects on virion infectivity.

To test this hypothesis directly we investigated whether Nef-V1H fusion proteins were functional and how they influenced virion infectivity. Virions were generated from the HIV-1NL4-3/\( \Delta \)Nef provirus in the absence or presence of various Nef-V1H fusion proteins in 293T cells and subsequently analyzed for their relative infectivity on Sx22-1 cells. In this experimental system, we found that the wild type Nef protein alone increased the infectivity of the Nef-negative virions approximately 2.5-fold when expressed in trans (Fig. 6B).
effect was observed with all Nef-V1H fusion proteins tested, suggesting that these fusion proteins were fully active in enhancing virion infectivity irrespective of the presence of an intact flexible loop in Nef. Together, these results demonstrate that V1H plays an important role for the enhancement of virion infectivity by Nef.

**DISCUSSION**

In this study, we demonstrated that the regulatory subunit H of the V-ATPase binds the C-terminal flexible loop in Nef and the medium chain (μ2) of the adaptor protein complex AP-2. Thus, V1H can connect Nef and CD4 to the endocytic machinery. Direct and specific interactions between Nef and V1H, as well as V1H, and the μ2 chain could be demonstrated in vitro and in vivo. Importantly, binding sites on Nef, which bind CD4 and V1H, were separable so that distinct surfaces on Nef lacking the flexible loop, when fused to V1H, could still internalize CD4. To this end, we performed kinetic internalization assays on the receptor and Nef or fusion proteins between Nef and V1H. Indeed, Nef and V1H were found to traffic similarly to each other, which depended on the flexible loop in Nef. Finally, blocking the expression of V1H and thus the function of the V-ATPase decreased the infectivity of HIV.

Importantly, Nef and V1H decreased steady-state levels and increased rates of internalization of CD8 equivalently (Figs. 1 and 2). In the presence of only the N-terminal 160 residues of Nef, the hybrid mutant Nef160-V1H protein was also able to target CD4 as efficiently as the wild-type Nef protein. Thus, V1H was able to perform the function of the flexible loop in Nef, and the remainder of Nef was sufficient to bind to CD4. This finding is in complete agreement with structural studies between Nef and CD4 (31) and reconciles the severe defect in the internalization of CD4, which was observed with the mutant NefED-AA protein (27). Moreover, as reflected in the steady-state levels of CD4 and CD8, no significant recycling was observed with Nef or V1H fusion proteins.

Binding studies performed with GST-V1H fusion proteins and in vitro translated μ2 demonstrated that a central region of four ARM repeats of V1H interacts with the N-terminal domain in μ2 (Fig. 5). Both protein fragments were stable and expressed to similar levels, which indicated a domain-domain protein interaction. The ARM or HEAT repeat superfold is composed of tandemly arranged helical repeats that form an elongated shaped molecule (51). This fold is known already from other proteins that are involved in intracellular trafficking processes, such as Importin α and β or β-Catenin. Its structural feature allows for sequence motif recognition by its concave surface and simultaneous assembly into multisubunit complexes. As an example, the binding site on Importin β for the small GTP-binding protein Ran does not overlap with its

![FIG. 6. The interaction between Nef and V1H increases viral infectivity. A, antisense (AS) V1H transcripts interfere with increased virion infectivity by Nef. The wild type HIV-1 provirus (HIV-1NL4-3) or its counterpart deleted in the nef gene (HIV-1NL4-3ΔNef) were co-transfected with increasing amounts of AS-V1H (0–3 μg) into Jurkat cells. Virus particles produced 48 h later were examined for their infectivity on the HeLa-CD4 indicator cell line Sx22-1. Plotted are the relative infectivities of particles produced in the absence and presence of AS-V1H, where the infectivity of HIV-1NL4-3 in the absence of AS-V1H was set arbitrarily to 100%. B, Nef-V1H fusion proteins increase virion infectivity. The HIV-1NL4-3ΔNef provirus was co-transfected with the indicated plasmids into 293T cells and the relative infectivity of the resulting virus particles was determined as in A. The relative infectivity of HIV-1NL4-3ΔNef produced in the presence of an empty plasmid vector was arbitrarily set to 100%. Error bars indicate the standard deviation from the mean from three independent experiments.](image)

![FIG. 7. Proposed model of the interactions between AP-2, V-ATPase, and clathrin to stimulate Nef-mediated CD4 internalization. The myristoylated HIV-1 Nef protein interacts with the cytoplasmic tail of CD4 molecules at the plasma membrane and stimulates its internalization. The interaction of AP-2 with clathrin leads to formation of clathrin-coated vesicles (33). Subunit H of the vacuolar (H+) ATPase (V1H) interacts with the peripheral V1 and the integral V0 sector to regulate its proton pump activity (53, 38). V1H binds μ2 of AP-2 to recruit the V-ATPase into clathrin-coated pits. Thus, via the interaction with Nef, V1H enhances the internalization of CD4 to endosomal and lysosomal compartments.](image)
binding sites for the FxFG nucleoporin repeats but may instead generate a conformational change in the molecule (52). Indeed, from structural studies ARM and HEAT repeat containing proteins are known to be very flexible and change its conformation upon variable protein complex formations (51).

By interacting with the N-terminal domain of µ2 (1–145), its ability to bind tyrosine-based sorting motifs for cargo uptake was not blocked. This suggests that a fully functional adaptor protein complex was preserved. However, we cannot exclude at this point that V1H might also substitute for the β-chain of the adaptor protein complex by its binding to µ2, generating thereby a complex with specific trafficking features. Because V1H is supposed to bind both V1 and V0 sectors of the vacular ATPase (47, 53), its interaction with the adaptor protein complexes could also be important for the assembly of two ATPase sectors. Indeed, by its interaction with AP-2, V1H could also connect the V-ATPase to clathrin (32, 44, 45). With its binding to the flexible loop of Nef, V1H could thus function as an adaptor protein to mediate trafficking of CD4 and Nef to endosomes and lysosomes and thereby circumvent the transfer of cargo from adaptor complexes to coatmerns (36). A model that displays the assembly of AP-2 complexes, the V-ATPase and clathrin at the plasma membrane, and the Nef-mediated internalization of CD4 is shown in Fig. 7. The display of the V-ATPase is based on the latest models by electron microscopy (54, 55).

Our results argue for an important role of the interaction between Nef and V1H for the enhancement of virion infectivity. This scenario is similar to that for SIV, where binding of Nef to V1H is also correlated with the increased infectivity of virus particles (34). Importantly, as the involvement of V1H is observed in the absence of CD4 in the virus-producing cells, these results differ from effects that are reported for high levels of CD4 (18, 19). Thus, although the binding of V1H to the flexible loop in Nef facilitated the internalization of CD4, it also affected virion infectivity independently of CD4. What could be the mechanism of this effect of V1H? Consistent with the previous observation that the increase of virion infectivity by Nef is imprinted on the particle in the producing cell, our recent findings suggest that Nef acts as a chaperone of virus production by recruiting the assembly of HIV into lipid rafts (56–58). Because the V-ATPase plays important roles in both endocytic and secretory transport (37, 38), the adaptor function of V1H could facilitate the proper trafficking of a complex between Nef and viral structural proteins to the plasma membrane and their partitioning into lipid rafts, where local rearrangements of the actin cytoskeleton might facilitate particle release (12, 59). As for CD4 down-regulation, it is unclear whether this would occur with or without involvement of the entire V-ATPase and its catalytic activity. Although it might be plausible that the recruitment of the V-ATPase could help to optimize the local pH that is required for the maturation of virus particles, no effect of Nef on maturation per se has been observed (56, 60). Alternatively, Nef and V1H might trigger the internalization of yet unidentified cell surface receptors that counteract virion infectivity by mechanisms similar to CD4 down-regulation. These strategies might represent a variation on a common theme employed by other viruses such as HTLV-I and papillomaviruses that also engage the V-ATPase (16, 61–63). Unraveling further details of the underlying mechanism will not only help us to understand viral pathogenesis, but also yield important insights into the role of the V-ATPase and its individual subunits in intracellular trafficking processes.

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binding of V1H to μ2 promotes nef internalization