Human Immunodeficiency Virus Types 1 and 2 and Simian Immunodeficiency Virus Nef Use Distinct but Overlapping Target Sites for Downregulation of Cell Surface CD4

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Although the Nef proteins encoded by human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIV) are known to induce the efficient internalization and degradation of cell surface CD4, it remains unclear whether this process involves a direct interaction between Nef and CD4. Here, we report that CD4 downregulation by HIV-1 and SIV Nef requires distinct but overlapping target sites within the CD4 intracytoplasmic domain. In particular, mutation of a glutamic acid residue located at CD4 residue 405 or of arginine and methionine residues located, respectively, at residues 406 and 407 results in a mutant CD4 protein that is efficiently downregulated by HIV-1 Nef but refractory to downregulation by SIV Nef. However, both HIV-1 and SIV Nef require an isoleucine located at residue 410 and the dileucine motif found at CD4 residues 413 and 414. CD4 downregulation induced by the Nef protein encoded by HIV-2 is shown to require a CD4 target sequence that is similar to, but distinct from, that observed with SIV Nef. These data explain the previous finding that the murine CD4 protein, which has an alanine at residue 405, is refractory to downregulation by SIV, but not HIV-1, Nef (J. L. Foster, S. J. Anderson, A. L. B. Frazier, and J. V. Garcia, *Virology* 201:373–379, 1994). In addition, these observations provide strong genetic support for the hypothesis that the Nef-mediated downregulation of cell surface CD4 requires a direct Nef-CD4 interaction.

It is now well-established that the Nef protein encoded by the known primate immunodeficiency viruses is essential for efficient virus replication, and hence pathogenesis, in the infected host (20–22). However, the precise role(s) of Nef in the replication cycles of human immunodeficiency virus types 1 and 2, (HIV-1 and -2), and in the related simian immunodeficiency viruses (SIVs), remains to be fully established (reviewed in reference 10). While data from several groups have demonstrated that Nef can enhance the infectivity of HIV-1 virions released by some Nef-expressing cells (2, 8, 28, 31, 36), Nef has been found to be dispensable for efficient virus replication in other culture settings (21, 23, 24, 30). A wide range of Nef proteins, derived from laboratory and primary isolates of HIV-1 and from the pathogenic Mac239 isolate of SIV, have also been shown to induce the efficient internalization and degradation of the cell surface CD4 receptor for HIV-1 (1, 3, 4, 6, 7, 13, 15, 16, 27, 33). This Nef activity, which renders cells resistant to viral superinfection (6), has the potential to increase virus replication by facilitating release of progeny virions (10). However, mutational analysis of Nef has demonstrated that infectivity enhancement and CD4 downregulation can be functionally segregated (16, 31), thus strongly suggesting that Nef has at least two distinct biological activities. In addition, several groups have presented data to support the theory that Nef can affect the activation state of infected cells (5, 11, 12, 35). It remains unclear whether this represents a separate, third biological phenotype or whether the effects of Nef on cell activation are instead correlated with effects on virion infectivity and/or CD4 internalization.

Although the importance of the Nef-induced internalization of CD4 for efficient HIV and SIV replication *in vivo* remains to

be established, this effect is a readily reproducible, and highly specific, consequence of Nef expression. Specificity is conferred by sequences located in the 38-amino-acid (aa) intracytoplasmic domain of CD4, including a dileucine motif that is critical for Nef-mediated endocytosis (1, 3, 32). With the possible exception of major histocompatibility complex class I molecules, whose responsiveness to Nef has been controversial (6, 34), none of the many other types of cell surface molecules that have been examined thus far has been shown to be internalized in response to Nef expression (6, 26). It is therefore apparent that CD4 downregulation must involve the sequence-specific recognition of the CD4 intracytoplasmic domain by Nef, by a Nef-induced cellular factor, or by a combination of both.

If the CD4 intracytoplasmic domain is recognized exclusively by a cellular Nef cofactor(s), then the target sequence for Nef function is predicted to be highly conserved among all primate immunodeficiency viruses. However, if the CD4 target is recognized by Nef directly, then the possibility exists that different Nef isolates display distinct target specificities. We were therefore intrigued by the report (13) that while HIV-1 Nef could induce the efficient internalization of both human and mouse CD4, the Nef protein encoded by SIV_{mac239} could induce only the endocytosis of the former. Here, we confirm the results of this earlier report and use mutagenesis of the intracytoplasmic domain of human CD4 to demonstrate that the Nef proteins encoded by SIV_{mac239} and by the 7312A isolate of HIV-2 are each dependent upon a significantly more extensive intracytoplasmic target for downregulation of CD4 than is required by HIV-1 Nef. These data strongly suggest that Nef directly participates in the specific recognition of the target sequence for CD4 internalization.

MATERIALS AND METHODS

Molecular clones. All Nef and CD4 proteins were expressed in mammalian cells with the pBC12/CMV plasmid (9). Expression plasmids carrying genes

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encoding human CD4 (pcCD4), the interleukin 2 receptor α chain (IL-2R α) (pIL-2R α), and the Nef protein from HIV-1 isolate NL-43 (pcNef/NL-43) have been described previously (19). The full-length *nef* genes present in HIV-1_{SF2} (24), SIV_{mac239} (21), and HIV-2_{7312A} (14) were amplified by PCR and then inserted into the expression plasmid pBC12/CMV to generate pcNef/SF2, pcNef/mac239, and pcNef/7312A, respectively. Expression plasmids carrying genes encoding carboxy-terminally epitope-tagged NL-43 Nef (pcNef/NL-43/FLAG) or mac239 Nef (pcNef/mac239/FLAG) were generated by PCR with a reverse primer encoding the FLAG epitope DYKDDDDK.

Plasmids carrying genes encoding mutated forms of human CD4 were generated by PCR mutagenesis, as previously described (19), with *Taq* plus DNA polymerase (Stratagene). The names of the resultant CD4 mutants indicate both the residue(s) that is mutated and the identity of the substituted residue. Thus, the Q409A CD4 mutant bears a substitution of alanine for the glutamine normally seen at residue 409. The identities of the resultant CD4 mutants were confirmed by DNA sequencing with Sequenase version 2.0 (Amersham).

CD4 downregulation assay. 293T cell cultures (diameter, 35 mm) were transfected by the calcium phosphate method. To determine the dose response for Nef-mediated downregulation of cell surface CD4, transfection mixtures containing 0.5 μ g of the IL-2R α expression plasmid pIL-2R α , 0.5 μ g of the wild-type human CD4 expression plasmid pcCD4, and increasing amounts of one of the Nef expression plasmids, pcNef/NL-43 or pcNef/mac239, were prepared. The parental pBC12/CMV plasmid served as a negative control. Other CD4 downregulation assays were performed with transfection cocktails containing 0.5 μ g of pIL-2R α , 0.5 μ g of wild-type or mutated forms of the CD4 expression plasmid pcCD4, and 1.5 μ g of pcNef/NL-43 or pcNef/SF2 or 3 μ g of pcNef/mac239 or pcNef/7312A. The matched negative control plasmid pBC12/CMV was added where necessary such that the total amount of DNA in each transfection mixture remained at 4 μ g. At 65 h after transfection, the cells were harvested as a single-cell suspension and stained with fluorescence-labeled antibodies as described previously (19). Cell surface CD4 and IL-2R α expression were then quantified by two-color flow cytometric analysis with a FACScan apparatus (Becton Dickinson).

Western blot analysis. Human 293T cells (35-mm-diameter cultures) were transfected by the calcium phosphate method with 2 μ g of the expression plasmids pcNef/NL-43/FLAG and pcNef/mac239/FLAG, which encode Nef protein tagged with FLAG at their carboxy termini. The parental pBC12/CMV vector served as a negative control. At 65 h after transfection, cells were harvested and proteins were extracted and subjected to electrophoresis on a sodium dodecyl sulfate–12% polyacrylamide gel. The resolved proteins were transferred onto a nitrocellulose membrane (Amersham) and then incubated with a 1:1,000 dilution of the anti-FLAG M2 monoclonal antibody (Eastman Kodak) in phosphate-buffered saline containing 0.1% Tween 20 and 1% nonfat dry milk. After extensive washing of the membrane, bound antibodies were detected with a horseradish peroxidase-conjugated goat-anti-mouse antiserum and by enhanced chemiluminescence (Amersham).

RESULTS

The assay used to measure the efficiency of CD4 downregulation is based on transient expression in the human cell line 293T and has been described previously (16, 19). In brief, 293T cells were transfected with a plasmid carrying genes expressing wild-type or mutant CD4, a second plasmid (pIL-2R α) carrying a gene expressing cell surface IL-2R α , and a third plasmid carrying the *nef* gene. The IL-2R α receptor is not subject to downregulation by Nef (16, 19) and therefore serves as an important control for transfection efficiency. At 65 h after transfection, the transfected cells were stained with a fluorescein isothiocyanate-conjugated monoclonal antibody specific for IL-2R α and a phycoerythrin-conjugated monoclonal antibody specific for the human CD4 receptor. Percent CD4 positive cells was calculated as the number of cells positive for both CD4 and IL-2R α divided by the total number of cells positive for cell surface IL-2R α , as quantified by fluorescence-activated cell sorter (FACS) analysis.

Data from a representative experiment are presented in Fig. 1, where intensity of staining for IL-2R α is quantitated on the *x* axis and CD4 expression is quantitated on the *y* axis. The number in the upper right quadrant of each panel is therefore the percentage of cells that are positive for both IL-2R α and CD4, while the number in the lower right quadrant is the percentage of cells that are positive for IL-2R α only. (The number in the lower right quadrant gives the total percentage of IL-2R α -positive cells.)

In the absence of Nef (Fig. 1A), 41% of the transfected cells were found to be positive for both CD4 and IL-2R α while 66% were positive for IL-2R α . While neither SIV_{mac239} Nef nor HIV-1_{NL-43} Nef expression had any significant effect on the total number of IL-2R α -positive cells, both proteins dramatically reduced the percentage of cells that were double positive (Fig. 1D and G). As discussed in more detail below, some mutations of the CD4 intracytoplasmic domain dramatically inhibited downregulation of CD4 by SIV Nef (Fig. 1E) without affecting downregulation by HIV-1 Nef (Fig. 1H), while other mutations blocked internalization induced by either Nef protein (Fig. 1F and I). None of the CD4 mutations discussed in this work had a significant effect on the level of cell surface CD4 expression in the absence of Nef (Fig. 1 and data not shown).

To compare the specificities of CD4 downregulation by HIV-1 and SIV Nef, it was important to confirm that these proteins were expressed at equivalent levels in transfected cells. We therefore attached a FLAG epitope tag to the carboxy termini of both the HIV-1_{NL-43} and the SIV_{mac239} Nef coding sequence and then measured steady-state levels of expression in transfected 293T cells by Western blot analysis. As shown in Fig. 2A, this experiment demonstrated very similar levels of expression of HIV-1 Nef and of the larger SIV Nef protein. Similar data were also obtained with untagged forms of HIV-1 and SIV Nef by using specific polyclonal antisera (data not shown).

We next determined whether HIV-1_{NL-43} and SIV_{mac239} Nef display significant differences in the efficiency of CD4 downregulation by comparing their dose responses for each viral gene product. As shown in Fig. 2B, at low ratios of CD4 expression plasmid to Nef expression plasmid, it appeared that HIV-1_{NL-43} Nef was somewhat more effective than SIV_{mac239} Nef at inducing CD4 endocytosis. However, at higher ratios of CD4 to Nef (1:5 and above), little difference was observed. To compensate for the apparently greater effectiveness of HIV-1 Nef in this CD4 downregulation assay, subsequent experiments were performed with a 1:3 ratio of CD4 to HIV-1 Nef and a 1:6 ratio of CD4 to SIV Nef.

HIV-1 and SIV Nef use distinct but overlapping CD4 target sites. Previous efforts to mutationally define sequences in the intracytoplasmic domain of CD4 that are important for downregulation have demonstrated that the first 23 aa (residues 396 to 418) of this 38-aa sequence are both necessary and sufficient for wild-type levels of Nef-induced endocytosis (1, 32). Within this short sequence, a dileucine motif located at residues 413 and 414 (Fig. 3A) has been shown to be critical for downregulation by HIV-1 Nef.

To determine whether SIV_{mac239} Nef and HIV-1_{NL-43} Nef show differences in their ability to downregulate specific CD4 mutants, we constructed the set of 10 missense and 3 deletion mutants of CD4 whose sequences are given in Fig. 3A and then determined whether these mutant proteins were responsive to HIV-1 or SIV Nef. As shown in Fig. 3B, both HIV-1_{NL-43} and SIV_{mac239} Nef gradually lost the ability to effectively downregulate CD4, as each was carboxy-terminally truncated, with SIV_{mac239} Nef showing a perhaps slightly lower level of activity than HIV-1_{NL-43} Nef when tested with the Δ 415 CD4 deletion mutant. Of note, all four mutations that affected leucine residues 413 or 414 resulted in a complete loss of responsiveness to both Nef proteins. In contrast, several other mutations (e.g., S408A, Q409A, R412A, and EK416/417AA) had no effect on downregulation by either Nef protein. One mutation, IK410/411AA, markedly inhibited downregulation by both HIV-1 and SIV Nef yet did not affect the dileucine motif (Fig. 3B). However, the RM406/407AA mutation was of particular interest in

FIG. 1. Representative results of FACS analysis of cell surface CD4 and IL-2R α expression. Cultures of 293T cells were transfected with plasmids carrying genes expressing wild-type or mutant human CD4 in the absence or presence of a plasmid carrying a gene expressing a wild-type Nef protein. At ~65 h after transfection, the cells were stained with a phycoerythrin (PE)-conjugated antibody (Ab) specific for human CD4 and with a fluorescein isothiocyanate (FITC)-conjugated antibody specific for IL-2R α prior to FACS analysis. In each panel, the number in the upper right quadrant is the percentage of cells positive for both CD4 and IL-2R α . The number given in the lower right quadrant is the total percentage of cells positive for cell surface IL-2R α , regardless of their CD4 status. Both SIV and HIV-1 Nef efficiently downregulated the wild-type CD4 protein (CD4_{wt}) (A, D, and G), while only HIV-1 Nef proved able to downregulate the E405A mutant of CD4 (B, E, and H). Neither Nef protein affected the cell surface expression of the LS414/415AA mutant of CD4 (C, F, and I). As previously shown (19), no CD4- or IL-2R α -positive cells are detected when mock-transfected 293T cells are used.

FIG. 2. (A) Western blot analysis of Nef protein expression. Steady-state levels of expression of carboxy-terminally epitope-tagged forms of HIV-1_{NL-43} and SIV_{mac239} Nef were measured in transfected 293T cells with an anti-epitope (FLAG-specific) monoclonal antibody. (B) Analysis of the efficiency of cell surface CD4 downregulation in transfected 293T cells in response to increasing levels of HIV-1_{NL-43} or SIV_{mac239} Nef. The parental pBC12/CMV plasmid was utilized as a negative control.

that this CD4 mutant remained fully responsive to HIV-1 Nef yet responded only poorly to the SIV_{mac239} Nef protein. Overall, these data suggested that the target motifs required for downregulation of cell surface CD4 by HIV-1_{NL-43} and SIV_{mac239} Nef are very similar but not identical.

An alanine at residue 405 in murine CD4 specifically blocks downregulation by SIV Nef. Foster et al. (13) have previously reported that the full-length murine CD4 protein is refractory to downregulation by SIV_{mac239} Nef but is efficiently downregulated by HIV-1 Nef. Comparison of the protein sequences of the intracellular domains of human and mouse CD4 reveals eight amino acid differences. However, five of these are located carboxy terminal to residue 418, i.e., outside of the sequences found to be sufficient for downregulation of human CD4 by both HIV-1 and SIV Nef (Fig. 3). The remaining three differences involve substitution of glutamine for the arginine residues found in human CD4 at positions 401 and 402 and sub-

stitution of alanine for the glutamic acid found at position 405 (Fig. 4A). We therefore mutated these three residues in human CD4 to the correct mouse sequence, to give the RRE 401/402/405QQA mutant and determined whether this CD4 protein is internalized in response to HIV-1 and SIV Nef expression. As shown in Fig. 4B, this mutant proved as responsive as wild-type CD4 to the HIV-1_{NL-43} Nef protein but was entirely refractory to downregulation by SIV_{mac239} Nef. A series of additional CD4 mutants were next prepared to determine which of these three residues were critical to this inhibitory effect. This experiment demonstrated that the E405A mutation was both necessary and sufficient to render human CD4 selectively nonresponsive to SIV Nef but that mutation of

FIG. 3. Response of human CD4 mutants to HIV-1_{NL-43} and SIV_{mac239} Nef. (A) Wild-type (WT) sequence of the 38-aa intracytoplasmic tail of human CD4 and primary sequences of a set of CD4 missense and deletion mutants. (B) The efficiencies of Nef-mediated downregulation of human CD4 mutants were measured as described for Fig. 1 and are indicated. The level of CD4 expression seen in the absence of Nef was set at 100% and was not significantly affected by the CD4 mutations examined (Fig. 1 and data not shown). These data represent the average of results from three independent FACS experiments, with the standard deviations indicated. Open bars, HIV-1_{NL-43} Nef; filled bars, SIV_{mac239} Nef.

FIG. 4. Effect of murine CD4 sequences on Nef downregulation. (A) Murine CD4 differs from human CD4 at eight positions, only three of which are located within the minimal target sequence for CD4 downregulation. These three differences were introduced into human CD4 to give the RRE401/402/405QQA triple mutant. The other indicated mutants reflect the introduction of a subset of these three differences into human CD4, as indicated. WT, wild type. (B) The efficiencies of downregulation of these human CD4 derivatives by HIV-1_{NL-43} Nef (open bars) or SIV_{mac239} Nef (filled bars) were measured as described for Fig. 1 and 3. Standard deviations are indicated.

the arginines at positions 401 and 402, either together or separately, had no effect (Fig. 4).

HIV-2 Nef also requires an extended CD4 target sequence. We next wished to determine whether the inability of the SIV_{mac239} Nef protein to effectively downregulate the E405A and RM406/407AA mutants of human CD4 was a unique attribute or whether this inability instead represented a general property of Nef proteins derived from the HIV-2-SIV_{smm}-SIV_{mac} group of primate lentiviruses. For this purpose, we selected the Nef protein encoded by the 7312A isolate of HIV-2 (14). HIV-2_{7312A} and SIV_{mac239} belong to different subgroups within the HIV-2-SIV_{smm}-SIV_{mac} virus family (14). The Nef protein encoded by HIV-2_{7312A} is only 61% identical to SIV_{mac239} Nef yet displays 45% identity to HIV-1_{NL-43} Nef. In addition to HIV-2_{7312A} Nef, we also analyzed a second HIV-1 Nef protein encoded by the SF2 isolate of HIV-1 (24).

As shown in Fig. 5, the HIV-1_{SF2} Nef protein behaved identically to HIV-1_{NL-43} Nef while the HIV-2_{7312A} Nef protein behaved similarly to SIV_{mac239} Nef. In particular, HIV-2_{7312A} Nef proved unable to downregulate the RRE401/402/405QQA and E405A mutants of CD4 and, like SIV Nef, proved ineffective with the RM406/407AA mutant. In contrast, all three of these CD4 mutants were very effectively downregulated by HIV-1_{SF2} Nef. While both these Nef proteins failed to downregulate either the IK410/411AA or the LS414/415AA CD4 mutant, both proteins proved fully able to downregulate cell

surface expression of wild-type CD4 and of the EK416/417AA mutant (Fig. 5).

HIV-2 and SIV_{mac} differ in their CD4 target sequences. The data presented in Fig. 3 to 5 demonstrate that the RM406/407AA double mutant of CD4 is inefficiently downregulated by SIV_{mac239} and HIV-2_{7312A} Nef and that the IK410/411AA double mutant is refractory to downregulation by all Nef proteins tested. To more fully define which of these four residues are important for CD4 target recognition by these different Nef proteins, we generated strains with the four single-residue CD4 mutations R406A, R407A, I410A, and K411A. As shown in Fig. 6, the K411A mutation had little effect on the efficiency of CD4 downregulation by HIV-1_{NL-43}, SIV_{mac239}, or HIV-2_{7312A} Nef. In contrast, the I410A CD4 mutant was as poorly downregulated by these three Nef proteins as was the IK410/411AA double mutant. We therefore conclude that it is the isoleucine residue located at residue 410 in CD4 that is critical for downregulation by HIV-1, HIV-2, and SIV Nef.

A more interesting, and surprising, result was obtained with the R406A and M407A CD4 mutants. Both these single-residue-mutation CD4 mutants were efficiently downregulated by HIV-1_{NL-43} Nef, as was predicted from the finding that the RM406/407AA double mutant also remained fully responsive to HIV-1 Nef (Fig. 3 and 5). In contrast, both the R406A and the M407A CD4 mutant were only very inefficiently downregulated by SIV_{mac239} Nef, thus demonstrating that both these residues are important for CD4 target recognition by this Nef protein. Surprisingly, however, the HIV-2_{7312A} Nef protein gave yet a third response pattern. In particular, HIV-2_{7312A} Nef proved able to efficiently downregulate the R406A CD4 mutant but was unable to downregulate cell surface expression of the M407A CD4 mutant. Therefore, on the R406A mutant of CD4, the HIV-2_{7312A} Nef protein behaved like HIV-1_{NL-43} Nef and unlike SIV_{mac239} Nef. In contrast, on the M407A mutant, HIV-2_{7312A} Nef behaved like SIV_{mac239} Nef and unlike HIV-1_{NL-43} Nef.

FIG. 5. A subset of the CD4 mutants described in the legends to Fig. 3 and 4 were tested for downregulation by Nef proteins derived from the SF2 isolate of HIV-1 (open bars) and the 7312A isolate of HIV-2 (shaded bars). Standard deviations are indicated. Wt, wild type.

FIG. 6. CD4 mutants, bearing the indicated single amino acid changes, were tested for downregulation by HIV-1_{NL-43} Nef (open bars), SIV_{mac239} Nef (filled bars), and HIV-2_{7312A} Nef (shaded bars). Standard deviations are indicated. Wt, wild type.

DISCUSSION

Although the biological activities of the HIV-1 Nef protein have been the subject of intense analysis, these studies have so far failed to provide a complete explanation for the critical importance of Nef for viral replication and pathogenesis *in vivo*. While the finding that Nef expression can enhance virion infectivity (2, 8, 28, 31, 36) is likely to provide an important piece of this puzzle, it remains unclear how internalization and degradation of CD4 leads to enhanced viral replication. This concern has led to the proposal that CD4 internalization, rather than being a critical aspect of Nef function, might instead simply represent a secondary effect of a Nef-induced modulation of cellular activation pathways (25).

Clearly, if CD4 internalization is instead the result of a direct interaction of CD4 with Nef, this argues that removal of CD4 from the infected cell surface is probably an important component of the *in vivo* Nef phenotype. Two lines of evidence suggest that such an interaction is likely to occur. First, although direct binding of CD4 by Nef has been difficult to demonstrate, evidence consistent with such an interaction has been obtained by the yeast two-hybrid system (29), by overexpression in insect cells (18), and by nuclear magnetic resonance spectroscopy (17). However, while these studies do provide evidence for the specificity of the observed CD4-Nef interaction, the observed *in vitro* binding displayed an affinity (K_d , ~1 mM) that is significantly lower than is normally seen for physiologically relevant interactions (17). A second line of evidence suggesting a direct interaction between CD4 and Nef is pro-

FIG. 7. The first 23 aa of the intracytoplasmic domain of human CD4 are fully sufficient for Nef-mediated internalization (1, 32). This figure identifies individual amino acids within this sequence that are critical for downregulation by HIV-1, HIV-2, and SIV Nef. See the text for a detailed discussion.

vided by the observation that fusion of Nef to the extracellular and transmembrane regions of CD4 leads to endocytosis and degradation of the resultant chimeric molecule (26). This induced degradation appeared mechanistically very similar to that observed with native CD4 in the presence of Nef. These data suggest that Nef, acting either in *cis* or in *trans*, may induce CD4 internalization by directly targeting it to a specific cellular degradative pathway (26).

The demonstration that different Nef proteins recognize distinct target sites within the intracellular domain of CD4 would provide a third line of evidence supporting a direct interaction between CD4 and Nef, rather than between CD4 and a Nef-induced cellular cofactor. Evidence suggesting that such a difference is likely to exist was first provided by the report of Foster et al. (13) showing that while HIV-1_{SF2} Nef could efficiently downregulate the full-length murine CD4 protein, SIV_{mac239} Nef was ineffective. In this paper, we have extended the results of this earlier report by showing that both SIV_{mac239} and HIV-2_{7312A} Nef require a glutamic acid residue located at position 405 in human CD4, as well as a methionine residue at position 407, for efficient downregulation of cell surface CD4 (Fig. 3 to 6). In addition, SIV_{mac239} Nef, but not HIV-2_{7312A} Nef, also requires an arginine residue located at position 406 (Fig. 6). In contrast, Nef proteins derived from the NL-43 and SF2 isolates of HIV-1 each proved able to very efficiently downregulate CD4 proteins bearing a mutation at position 405, 406, or 407. Finally, all four of the Nef proteins examined here required both the leucine residues located at positions 413 and 414 in CD4 and the isoleucine residue located at position 410. Considered together, these data therefore suggest that these different Nef proteins have distinct but overlapping target sites for downregulation of cell surface CD4, as summarized in Fig. 7. This finding provides strong genetic support for the hypothesis that there is a direct interaction between cell surface CD4 and Nef in infected cells *in vivo* and emphasizes the remarkable specificity of this receptor downregulation event. In addition, this study describes reagents that will prove useful in the future *in vitro* analysis of this presumably critical protein-protein interaction.

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