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Human immunodeficiency virus type 1 Nef and p56^{lck} protein-tyrosine kinase interact with a common element in CD4 cytoplasmic tail

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ABSTRACT **The human immunodeficiency virus type 1 *nef* gene induces endocytosis of CD4 antigen and disrupts the association between CD4 and p56^{lck} protein-tyrosine kinase (EC 2.7.1.112). We demonstrate that in T cells these effects of the viral protein require a cluster of hydrophobic amino acids in a membrane-proximal region of the CD4 cytoplasmic tail; other amino acids in the C-terminal segment of CD4 cytoplasmic tail also contribute to the interaction. Mutations in CD4 that prevent down-modulation by Nef also decrease CD4 association with p56^{lck} and prevent Nef-induced disruption of CD4–p56^{lck} complexes. Together, the overlap in CD4 sequences required for interaction with Nef and p56^{lck} and the tight correlation between Nef-induced CD4 down-modulation and disruption of CD4–p56^{lck} association suggest that Nef, or cellular factors recruited by Nef, interact with this segment of CD4 to displace p56^{lck} from the complex and induce CD4 endocytosis.**

nef genes of human and simian immunodeficiency viruses (HIV, SIV) encode related N-terminally myristoylated cytoplasmic proteins (1, 2). *nef* is essential for high viral load and pathogenesis *in vivo* (3) but is not required for viral replication under commonly used *in vitro* conditions (3–5). The mechanism by which Nef stimulates viral replication *in vivo* is unknown but may involve down-modulation of CD4 antigen expression on the cell surface, as has been observed with a large fraction of *nef* alleles derived from laboratory HIV and SIV isolates and from peripheral blood leukocytes of HIV-infected patients (6–11). Nef-induced down-modulation of CD4 on the cell surface reflects accelerated CD4 endocytosis and its degradation in the lysosomal compartment (12, 13).

CD4 is a transmembrane glycoprotein expressed at high levels on helper T cells and is essential for their ontogeny and antigen-specific responses (14–17). In T cells, the short cytoplasmic domain of CD4 is involved in at least two interactions. One is with the p56^{lck} protein-tyrosine kinase (EC 2.7.1.112) (18, 19) and this association is required for antigen-specific signaling (15). The CD4–p56^{lck} association requires cysteine motifs in the CD4 cytoplasmic tail and the N terminus of p56^{lck} and may involve direct binding of the two proteins (20, 21). The cysteine motif in CD4 is not sufficient for binding p56^{lck}, as a deletion of the membrane-proximal segment of the CD4 cytoplasmic tail prevents association of the two proteins (13, 21).

The other interactions involve CD4 endocytosis induced by Nef and/or phorbol ester [phorbol 12-myristate 13-acetate (PMA)]. PMA-induced (22) and Nef-induced CD4 endocytosis (12) and CD4 targeting to the lysosomal compartment require a di-leucine motif in the membrane-proximal region of CD4 cytoplasmic tail. CD4 endocytosis induced by PMA is initiated by phosphorylation of serines located in a close proximity to the di-leucine motif, an event mediated by protein kinase C (22, 23). In contrast, the effect of Nef on CD4 does not involve serine phosphorylation, or formation of stable complexes between the two proteins (7, 12, 13), and the events involved in Nef interaction with CD4 are not known. Neither the effect of Nef nor the effect of PMA requires p56^{lck} or the cysteine motif in CD4 cytoplasmic tail, which is important for association with p56^{lck}; yet CD4 endocytosis induced by either of these agents involves disruption of CD4 association with p56^{lck} (12, 13, 23–27). Mechanisms mediating this process are not clear. To address the mechanism of Nef interaction with CD4–p56^{lck} complexes, we have studied the sequence requirements within the CD4 cytoplasmic tail for down-modulation by Nef and for interaction with p56^{lck} in T cells.

MATERIALS AND METHODS

Construction of CD4 Mutants and Expression in 171.22 T Cells. Mutant CD4 cDNAs were constructed by oligonucleotide-directed site-specific mutagenesis (8) and subcloned into the *Bam*HI site of pBABE(neo) retroviral expression vector (28); the resulting constructs were transfected into the GPE-86 packaging cell line (29). 171.22 cells, a clonal line isolated from the previously described 171 T-cell hybridoma (ref. 15; S.S. and J.S., unpublished data), were infected by coculture with producer GPE-86 cells. CD4$^+$ 171.22 T cells were isolated by cell sorting. For analysis of CD4 expression on the cell surface, 2×10^5 cells were incubated with saturating amounts of phycoerythrin-conjugated Leu3A monoclonal antibody (mAb) (Becton Dickinson) and analyzed on the EpicsC flow cytometer as described (8, 9).

Expression of HIV-1 Nef in CD4$^+$ 171.22 Cells. pBABE-(puro) vector containing NA13 *nef* (8) was introduced into GPE-86 cells and supernatants were used to infect 171.22 cells expressing various forms of CD4 proteins. Transduced cells were selected for 4 days with puromycin (0.8 μg/ml).

Immunoprecipitation and Immunoblot Analysis. Cytoplasmic lysates from a total of 2×10^7 to 4×10^7 cells were prepared and immunoblot analysis of Nef expression was performed as described (9). For immunoprecipitation, aliquots of extracts were precleared for 1 hr with 30 μl of protein G-agarose beads (GIBCO) and then incubated for 4 hr with 10 μl of protein G beads that had been allowed to react with 4 μg of OKT4 mAb (Ortho Diagnostics). Immune complexes were washed as described (9). For immunoblotting, immune complexes were denatured for 15 min at 70°C in nonreducing sample buffer, resolved on 12% polyacrylamide gels, and transferred to poly(vinylidene difluoride) membrane (9). Immunoblot analysis with rabbit serum reacting with p56^{lck} (Santa Cruz Biotechnology, Santa Cruz, CA) or with human CD4 (Cambridge Biotech) was performed as described (9) and developed using the ECL detection system (Amersham).

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Abbreviations: HIV-1, human immunodeficiency virus type 1; PMA, phorbol 12-myristate 13-acetate; mAb, monoclonal antibody.

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RESULTS

In T Cells Nef-Induced CD4 Endocytosis Involves a Cluster of Hydrophobic Amino Acid Residues in the CD4 Cytoplasmic Tail. To examine the CD4 sequences required for down-modulation by Nef, CD4 proteins bearing various mutations in the cytoplasmic tail were expressed in the CD4$^-$ 171.22 T-cell hybridoma. The derived cell lines were subsequently transduced with a retroviral vector containing either an active allele of HIV-1 *nef* (NA13) (8) or an empty control vector. Expression of CD4 on the cell surface of the resultant populations was then analyzed by flow cytometry (Fig. 1*A*). Representative results from such an experiment are shown in Fig. 1*B*. Expression of the strong (NA13) HIV-1 *nef* allele in the context of wild-type CD4 resulted in complete loss of CD4 antigen from the surface of 171.22 cells (compare panels 1A and 1B in Fig. 1*B*). In agreement with previous observations (12, 13), deletion of the last 15 amino acids from the cytoplasmic tail of CD4 (d418), including residues C420 and C422, which are essential for CD4–p56^{lck} association, had no detectable effect on CD4 down-modulation. However, more extensive truncations past residue K418, or deletions eliminating residues M407 to R412 or R412 to K417, severely attenuated the Nef-induced CD4 down-modulation (Fig. 1*A*, mutants d415, d409, d402, or d407–412, d412–417, respectively). This indicated a critical role for one or more residues located between positions M407 and K412, in addition to leucine 413/414. Notably, the residual responsiveness of the latter two mutants was eliminated by combining them with a deletion of the last 10 C-terminal amino acids of the CD4 cytoplasmic tail (see d407–412/d423 and d412–417/d423, Fig. 1*A*). Thus, although the region distal to residue C422 is not essential for down-modulation by Nef in the context of wild-type CD4, it does contribute in the context of partially unresponsive CD4 proteins.

To assess which amino acids in the CD4 cytoplasmic tail are required for down-modulation by Nef, mutants bearing double or triple amino acid substitutions in this region were analyzed. As shown in Fig. 1*A*, substitutions at positions proximal to M407 and distal to K418 had no detectable effect on Nef-induced CD4 down-modulation (Q403A, E405G, R406A; T419A, Q421A; C420A, C422A). In contrast, two sets of substitutions in the region between M407 and S416 resulted in CD4 proteins that were unresponsive to Nef. Replacement of

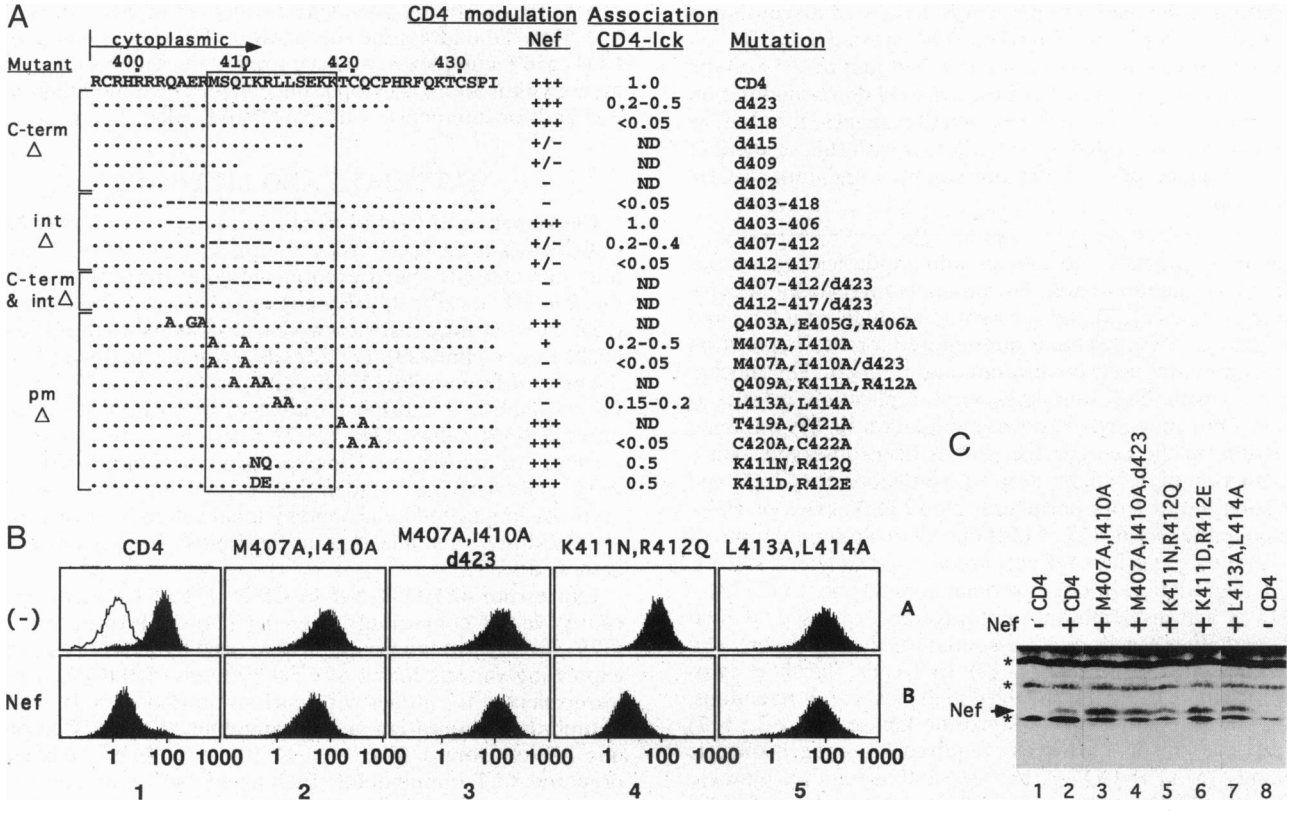

FIG. 1. Analysis of HIV-1 Nef-induced down-modulation of CD4 in 171.22 T cells. (*A*) Effect of mutations in CD4 cytoplasmic domain on down-modulation by Nef and on association with p56^{lck}. Amino acid sequences of the cytoplasmic tails of mutant CD4 proteins are aligned on the left with that of the wild-type human CD4. Dots (.) indicate amino acid identities with the wild-type protein, dashes (–) indicate the extent of internal deletions, and letters identify amino acid substitutions in the single-letter code. The 12-amino acid membrane-proximal region in CD4 cytoplasmic tail required for down-modulation by Nef is boxed. Mutant CD4 proteins were expressed in 171.22 cells and analyzed as described in the text. Following staining with Leu3A anti-CD4 mAb, fluorescence of cells expressing various CD4 proteins was 50–60 channels (on the 256-channel logarithmic scale) higher than that of the parental CD4$^-$ 171.22 cells. The extent of change in CD4 expression following transduction with HIV-1 NA13 *nef* expression vector (Nef) is indicated by +++, ++, +, +/– and –, which reflect a decrease in the fluorescence intensity of 35–55, 20–35, 10–20, 5–10, and <5 channels, respectively. Quantitation of CD4–p56^{lck} association was performed by Western blot analysis, as shown in the Fig. 2, and is based on two independent experiments. The degree of p56^{lck} association with mutant CD4 proteins was normalized to that observed with wild-type CD4 (1.0). ND, not determined. (*B*) Down-modulation of selected mutant CD4 proteins by HIV-1 NA13 Nef in 171.22 T cells. 171.22 cells expressing human CD4 (panel 1) or selected CD4 mutants (panels 2–6) and transduced with a control empty vector (row A) or with HIV-1 *nef* (row B) were stained with phycoerythrin-Leu3A anti-CD4 antibody and analyzed on an EPICS C flow cytometer (row A). The abscissa gives the fluorescence intensity in a logarithmic scale. The ordinate gives a relative cell number. (*C*) Immunoblot analysis of Nef expression. Detergent extracts prepared from cells expressing wild-type CD4 or mutant proteins and transduced with HIV-1 *nef* expression vector were analyzed by immunoblotting with HIV-1 *nef* antiserum (lanes 2–7). Extracts prepared from the parental cells expressing CD4 only were used as a negative control (lanes 1 and 8). Asterisks (*) indicate cross-reacting background bands.

leucines 413 and 414 by alanines abrogated down-modulation by Nef (L413A, L414A, panels 5A and 5B in Fig. 1*B*), while the double alanine substitution for methionine 407 and isoleucine 410 (M407A, I410A) still responded to Nef, but at much reduced levels (panels 2A and 2B in Fig. 1*B*). Deleting 10 amino acids from the C-terminal end of the latter mutant CD4 resulted in unresponsiveness to Nef (M407A, I410A/d423, panels 3A and 3B in Fig. 1*B*). Together these data demonstrate that CD4 down-modulation by Nef requires a cluster of hydrophobic amino acids in the membrane-proximal region of CD4 cytoplasmic tail and provide further evidence that the C-terminal region of the tail contributes to the interaction of CD4 with Nef. Immunoblot analysis demonstrated steady-state levels of Nef protein in mutant CD4 cell lines (M407A, I410A; M407A, I410A/d423; and L413A, L414A) at levels greater than, or equal to, that in the wild-type CD4 cell line (Fig. 1*C*).

Efficient CD4-p56^{lck} Association Requires the Nef-Responsive Element. In T cells, the cytoplasmic tail of CD4 is associated with p56^{lck} protein-tyrosine kinase (18, 19). As the hydrophobic element required for Nef-induced CD4 endocytosis and the double cysteine motif (C420, C422) involved in CD4 association with p56^{lck} (20, 21) are located in close proximity in the CD4 cytoplasmic tail, we tested whether the region that is involved with CD4 endocytosis also interacts with p56^{lck}.

Association of mutant CD4 proteins with endogeneous p56^{lck} was analyzed by immunoblot analysis of anti-CD4 immune complexes prepared from detergent lysates of 171.22 cells expressing selected CD4 proteins. Immunoblotting with CD4-specific serum revealed that similar amounts of the wild-type and mutant CD4 proteins were expressed in these cells (compare lanes 8–19 with lanes 2–6, Fig. 2, lower panel). Several of the mutations affecting residues M407 through K418, the region required for CD4 endocytosis, resulted in a decrease in CD4-associated p56^{lck} (see upper panel in Fig. 2 and compilation in Fig. 1*A*). A large deletion spanning residues Q403 through K418 resulted in a >20-fold reduction of CD4–p56^{lck} association (d403–418, compare lanes 8 and 10, Fig. 2) as did a smaller deletion spanning residues R412 through K417 (d412–417, lane 13). In contrast, deletion of residues M407 through R412 had a less dramatic effect, resulting in an ≈4- to ≈8-fold decrease in coimmunoprecipitated p56^{lck} (d407–412, compare lanes 8 and 12), whereas deletion of more membrane-proximal residues Q403 through R406 had little, if any, effect on CD4–p56^{lck} association (d403–406, lane 11). Interestingly, deletion of the C-terminal 10 amino acids in the cytoplasmic tail also showed a 2- to 4-fold decrease in CD4-associated p56^{lck} (d423, compare lanes 9 and 8). Thus, the results of deletional analysis indicate that CD4 sequences required for down-modulation by Nef also contribute to interaction with p56^{lck}.

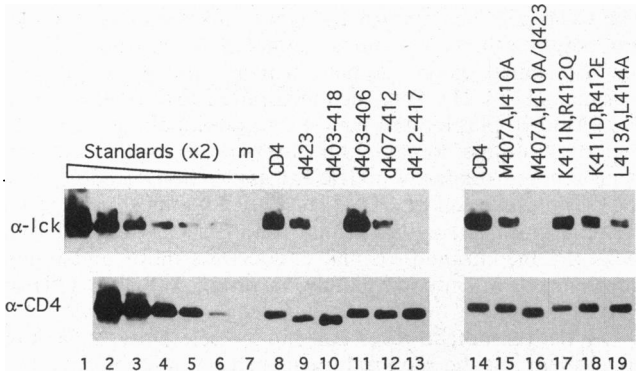

FIG. 2. Effect of mutations in CD4 cytoplasmic tail on association with p56^{lck}. Lysates were prepared from 2×10^7 cells expressing wild-type human CD4 (CD4) or selected mutant CD4 proteins and were immunoprecipitated with OKT4 anti-CD4 mAb. Western blots of immune complexes were probed with antiserum specific for p56^{lck} (α-lck, upper panel) or human CD4 (α-CD4, lower panel). Two-fold serial dilutions of protein extracts prepared from COS7 cells transiently transfected with p56^{lck}, or human CD4, expression vectors were used as standards for quantitation (lanes 1–6 in the upper and lower panels, respectively). Aliquots of an extract prepared from untransfected COS7 cells were used as a negative control (m, lanes 7).

This is more clearly seen with analysis of alanine substitution mutants. For example, substitution of alanines for M407 and I410, which dramatically reduces CD4 down-modulation by Nef, resulted in an ≈2- to ≈4-fold reduction in CD4-associated p56^{lck} (M407A, I410A, compare lanes 14 and 15, Fig. 2, upper panel). When the same mutations were combined with deletion d423 (which lacks 10 C-terminal amino acids but retains residues C420 and C422 and is completely unresponsive to Nef), no detectable p56^{lck} was coimmunoprecipitated (M407A, I410A/d423, lane 16). Moreover, when alanines were substituted for L413 and L414, which abolishes CD4 internalization induced by Nef, the amount of coimmunoprecipitated p56^{lck} was only one-eighth to one-fourth of that observed with wild-type CD4 (L413A, L414A, compare lanes 14 and 19). In contrast, substitutions at K411 and R412, which had no effect on Nef-induced CD4 endocytosis (Fig. 1*A*), resulted in only a modest decrease in association with p56^{lck} (compare lanes 17 and 18 with lane 19). These results indicate that the hydrophobic amino acid residues in the membrane-proximal region in the CD4 cytoplasmic tail that are required for down-modulation by Nef are also involved with efficient recruitment of p56^{lck} into a complex with CD4 and/or with stabilization of the complex.

Different Sequence Requirements for Disruption of CD4–p56^{lck} Association by Nef and PMA. PMA-induced CD4 endocytosis involves dissociation of CD4–p56^{lck} complexes prior to internalization of the CD4 molecule (25–27). To assess the relationship between Nef-induced disruption of CD4–p56^{lck} association and CD4 internalization, we asked whether these two functions can be separated genetically by mutations in the CD4 cytoplasmic tail.

As shown in Fig. 3*A*, coexpression of wild-type CD4 and NA13 Nef resulted in an ≈2- to ≈3-fold lower steady-state level of CD4 (compare lanes 1 and 3, lower panel). This decrease is consistent with an accelerated CD4 turnover and lysosomal degradation induced by Nef proteins (12, 13). The residual CD4 detected in cells expressing Nef reflects the intracellular pool, as no detectable staining of these cells with anti-CD4 antibody was observed by flow cytometry analysis (see Fig. 1*B*, panels 1A and 1B). Similar reduction of the steady-state CD4 was also detected following PMA treatment, and the effects of Nef and PMA were not additive (lanes 2 and 4). In contrast, immunoblot analysis with p56^{lck} antiserum revealed an ≈20-fold decrease in CD4-associated p56^{lck} in Nef-expressing or PMA-treated cells (compare lanes 2, 3, and 1, upper panel), indicating that the majority of the intracellular pool of CD4 was not complexed with p56^{lck}. The higher molecular forms of p56^{lck} observed with PMA-treated cells, but not Nef-expressing cells, may reflect its serine phosphorylation known to be associated with activation of protein kinase C (see lanes 2 and 3 in upper panel; refs. 27 and 30).

Fig. 3 *B* and *C* show that two sets of amino acid substitutions that compromise Nef-induced CD4 down-modulation and reduce CD4–p56^{lck} association also prevented disruption of CD4–p56^{lck} association by Nef. Alanine substitutions at residues M407 and I410 (M407A, I410A), which compromise CD4 internalization induced by Nef (see Fig. 1*B*, panels 2A and 2B), prevented a decrease in the steady-state level of CD4 (M407A, I410A, compare lanes 1 and 3, Fig. 3*B*, lower panel). Surprisingly, this mutation resulted in a 2- to 3-fold increase in the amount of associated p56^{lck} in the presence of Nef (M407A,

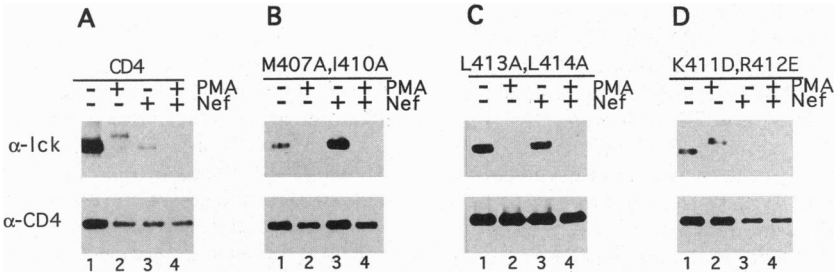

FIG. 3. Effect of HIV-1 Nef or PMA on the association of $p56^{lck}$ with mutant CD4 proteins in 171.22 T cells. (*A*) Lysates were prepared from parental cells expressing human CD4 (lanes 1 and 2) and from a derivative population transduced with HIV-1 *nef* (lanes 3 and 4), which were cultured for 1 hr in the presence (lanes 2 and 4) or absence (lanes 1 and 3), of PMA (50 ng/ml). A similar analysis was performed with M407A, I410A (*B*), L413A, L414A (*C*), and K411D, R412E (*D*) CD4 mutants. Immune complexes were isolated and analyzed as described in the legend to Fig. 2.

I410A, compare lanes 1 and 3, Fig. 3*B*, upper panel). This increase may result from altered affinity of the CD4 cytoplasmic domain for $p56^{lck}$ in Nef-expressing cells or may reflect differences in the turnover of free CD4 and CD4–$p56^{lck}$ complexes. Alanine substitution for residues L413 and L414 (L413A, L414A, Fig. 3*C*) also prevented the effect of Nef on the steady-state level of CD4 protein and on CD4–$p56^{lck}$ association (compare lanes 1 and 3 in the lower and upper panels, respectively). Remarkably, for both of these mutations (M407A, I410A and L413A, L414A), the mutant CD4–$p56^{lck}$ complexes were still sensitive to PMA (compare lanes 1, 2, and 4, upper panels).

In contrast, substitution of K411 and R412 with negatively charged amino acids (K411D, R412E), which had no detectable effect on Nef-induced CD4 internalization (see Fig. 1*B*, panels 4A and 4B), also had no detectable effect on the disruption of CD4–$p56^{lck}$ association by the viral protein (K411D, R412E, compare lanes 1 and 3, Fig. 3*D*, upper panel). However, this mutation prevented CD4 internalization (data not shown) and disruption of $p56^{lck}$ complexes by PMA (Fig. 3*D*, lanes 1 and 2). These results indicate that disruption of CD4–$p56^{lck}$ association by Nef correlates tightly with CD4 down-modulation, suggesting that both processes are tightly coupled.

DISCUSSION

We have studied amino acid sequence requirements within the cytoplasmic tail of CD4 for down-modulation by HIV-1 Nef and for association with $p56^{lck}$ protein-tyrosine kinase. Our observations indicate that in T cells the CD4 down-modulation by Nef is critically dependent on a membrane-proximal cluster of hydrophobic amino acids, including methionine 407 and/or isoleucine 410 and a di-leucine motif (leucines 413 and 414). In addition, the C-terminal segment of the CD4 cytoplasmic tail also contributes to the effect of Nef.

How might Nef interaction with these residues trigger CD4 down-modulation on the cell surface? The di-leucine motif, required for internalization and lysosomal targeting of CD4 is thought to mediate CD4 interaction with clathrin-coated pits and is required for Nef- and PMA-induced CD4 endocytosis (12, 22, 23, 26). Similar di-leucine motifs present in cytoplasmic tails of the γ and δ chains of the CD3 complex are involved in endocytosis and degradation of these molecules (31). In the assembled multisubunit CD3 complexes at the cell surface, the di-leucine motifs of the γ and δ chains are inaccessible for interaction with the endocytotic machinery allowing stable residence of the CD3 complex at the plasma membrane; they become activated (unmasked) by phosphorylation of adjacent serine residues, an event initiated by stimuli that mimic antigenic stimulation and trigger endocytosis of the CD3 complex (31, 32).

The mechanism that occludes leucines 413 and 414 in the CD4 cytoplasmic tail is not clear. Previous studies indicated that $p56^{lck}$ binding to CD4 prevents its association with coated pits and inhibits constitutive CD4 endocytosis via this route (25, 26). However, preventing $p56^{lck}$ association with CD4 by mutations in either of the two proteins, or expression of wild-type CD4 in nonlymphoid cells, which do not contain $p56^{lck}$, does not result in acceleration of CD4 endocytosis comparable to that observed in Nef-expressing cells (12, 25). Thus, even in the absence of association with $p56^{lck}$, leucines 413 and 414 are apparently rendered inaccessible by a particular conformation of the tail or are occluded by an interaction with a protein other than $p56^{lck}$.

PMA-dependent CD4 internalization requires phosphorylation of serines 408 and/or 415 (22, 24), which is thought to promote the interaction of the di-leucine motif with clathrin-coated pits. The effect of PMA is not affected by alanine substitutions at methionine 407 or isoleucine 410 (data not shown). In contrast, CD4 down-modulation by Nef does not involve serine phosphorylation (7, 12) but requires methionine 407 and/or isoleucine 410. We suggest that these residues may be required for an unmasking of the di-leucine motif induced by Nef, by a mechanism independent of serine phosphorylation.

Our results indicate that, in addition to the double cysteine motif, the membrane-proximal region in the cytoplasmic tail that is involved in CD4 endocytosis is also essential for efficient formation or stabilization of CD4–$p56^{lck}$ association. The CD4–$p56^{lck}$ complex is thought to involve direct interaction between the two proteins, mediated by cysteine motifs in the N-terminal unique domain in $p56^{lck}$ and the CD4 cytoplasmic tail (20, 21). Thus the membrane-proximal segment in the CD4 cytoplasmic tail may form additional contacts with the $p56^{lck}$ unique domain. Our observations explain the previously noted relatively inefficient association of $p56^{lck}$ with the cytoplasmic tail of CD8 (21, 33). CD8 binds $p56^{lck}$ via a cysteine motif analogous to that found in CD4 (20, 21) but lacks the membrane-proximal endocytosis motif and is not internalized, or only marginally, in response to Nef (34) or PMA (35, 36).

Our data do not, however, rule out the possibility that a third component may be required for formation or stabilization of CD4–$p56^{lck}$ complexes. Indeed, the relatively tight correlation between the Nef-induced CD4 down-modulation and formation of CD4–$p56^{lck}$ observed with mutant CD4 proteins suggests a model where an additional factor(s) could interact with the membrane-proximal region in CD4 cytoplasmic tail masking the endocytotic motif and stabilizing CD4–$p56^{lck}$ association. Possible candidates for such factors are suggested by a recent observation that CD4 also associates with phosphatidylinositol (PI) 3-kinase and PI 4-kinase in T cells (37). Although the functional significance of PI-3 and PI-4 kinase association with CD4 has not been demonstrated, a role in

endocytosis is implied by a recent observation that PI-3 kinase binding site is required for endocytosis of platelet-derived growth factor receptor and by the involvement of both kinases in protein sorting to lysosome-like vacuolar compartment in *Saccharomyces cerevisiae* (38, 39).

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