CD4 Phosphorylation Partially Reverses Nef Down-Regulation of CD4

Yong-Jiu Jin, Xiaoping Zhang, J. Gildade Boursiquot and Steven J. Burakoff

http://www.jimmunol.org/content/173/9/5495

References
This article cites 42 articles, 19 of which you can access for free at:
http://www.jimmunol.org/content/173/9/5495.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
CD4 Phosphorylation Partially Reverses Nef Down-Regulation of CD4

Yong-Jiu Jin, Xiaoping Zhang, J. Gildade Boursiquot, and Steven J. Burakoff

HIV Nef down-regulates CD4 from the cell surface in the absence of CD4 phosphorylation, whereas PMA down-regulates CD4 through a phosphorylation-dependent pathway. In this study we show that the down-regulation of CD4 in human Jurkat T cells expressing Nef was nearly complete (~95%), whereas that induced by PMA was partial (~40%). Unexpectedly, treating T cells expressing Nef with PMA restored the surface CD4 up to 35% of the steady state level. Both mutating the phosphorylation sites in the CD4 cytoplasmic tail (Ser408 and Ser415) and the use of a protein kinase C inhibitor, bisindolylmaleimide I, abolished the restoration of surface CD4, suggesting that the restoration required CD4 phosphorylation. CD4 and Nef could be cross-linked by a chemical cross-linker, 3,3-dithiobis(sulfosuccinimidyl-propionate), in control T cell membranes, but not in PMA-treated T cell membrane, suggesting that CD4 and Nef interacted with each other in T cells, and the phosphorylation disrupted the CD4-Nef interaction. We propose that this dissociation switches CD4 internalization from the Nef-mediated, nearly complete down-regulation to a phosphorylation-dependent, partial down-regulation, resulting in a net gain of CD4 on the T cell surface. The Journal of Immunology, 2004, 173: 5495–5500.

CD4 is a coreceptor of the TCR in TCR-mediated T cell activation and signaling. Cell surface CD4 can be down-regulated by PMA treatment (1, 2). PMA treatment activates protein kinase C (PKC), which, in turn, induces the phosphorylation of Ser408 and Ser415 in the cytoplasmic tail of CD4 (3, 4). As a result, CD4 dissociates from Lck and is recruited into the clathrin-coated pits for endocytosis (5, 6). After endocytosis, some of the internalized CD4 molecules are delivered to lysosomes for degradation, whereas others recycle back to the T cell surface (1, 5). Therefore, PMA-induced down-regulation of CD4 is incomplete. In T cells, which express Lck, PMA treatment removes 40–50% of CD4 molecules from the cell surface; in CD4-transfected HeLa cells, which do not express Lck, PMA removes 75% of the surface-expressed CD4 molecules (4, 6).

CD4 is also the primary receptor for HIV to enter cells. HIV Nef protein, expressed early in viral replication (7), is a major determinant of HIV virulence (8–11). One well-characterized Nef function is the down-regulation of CD4 from the cell surface (12), which correlates with Nef-dependent enhancement of viral pathogenicity (13–20).

The Nef down-regulation of CD4 is independent of the phosphorylation of CD4 (12). The available data indicate that Nef down-regulation of CD4 requires its interaction with CD4 on the T cell plasma membrane (21–24). The CD4 tail is necessary for Nef down-regulation of CD4 (21, 22), as is the membrane association of Nef through its N-terminal myristoylation (25). In Nef, W57 and L58 are the residues critical for the Nef-CD4 interaction (26, 27). Replacing the CD4 tail with Nef results in a chimeric protein that down-regulates CD4 in trans as well as the chimeric protein itself in cis (23). Several studies have shown that CD4 interacts with an AP complex through its C-terminal flexible loop, which is essential for Nef down-regulation of CD4 (30–32). The dileucine motif, including the one in the CD4 tail (2), has the consensus sequence of [DE]XXXL[LI]. The dileucine motifs are known sorting signals, recognizable by adaptor protein complexes, including AP-1, AP-2, AP-3, and AP-4 (33). The prevailing view is that the adaptor protein-2 (AP-2) is involved in endocytosis from plasma membranes, whereas AP-1 and AP-3 are involved in the trans-Golgi network and lysosomal vesicular trafficking, respectively (34). It has been shown that the colocalization of Nef with the AP-2 complex is correlated with Nef down-regulation of CD4 (35). However, yeast two- or three-hybrid studies show that the dileucine motif in HIV Nef interacts mainly with AP-1 and AP-3 and only weakly with AP-2 (36, 37). A GST-tagged HIV Nef binds to AP-1, but not AP-2 (38). Apparently, CD4-Nef interacts with an AP complex, but the specific AP complex involved needs to be further defined.

In an effort to elucidate the differential pathways of CD4 internalization induced by Nef and PMA, we found that Nef down-regulation of CD4 was partially reversed in Jurkat T cells by treatment of PMA. Further analysis suggested that phosphorylation of CD4 induced by PMA disrupted the interaction between Nef and the CD4 tail. A model is proposed in which the Nef down-regulation of CD4 is mediated through a CD4-Nef-AP interaction, whereas the PMA down-regulation of CD4 is through a direct interaction of the phosphorylated CD4 with an AP complex. PMA

*Skirball Institute of Biomedical Research, †Department of Medicine, New York University School of Medicine, New York, NY 10016; and ‡Department of Pharmacy, Rutgers University, Piscataway, NJ 08854

Received for publication June 29, 2004. Accepted for publication August 20, 2004.

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.

1 This work was supported in part by grants from National Institutes of Health, the Association for International Cancer Research (United Kingdom), and a pilot AIDS funding from New York University.

2 Address correspondence and reprint requests to Dr. Yong-Jiu Jin, Skirball Institute of Biomedical Research, 540 1st Avenue, Lab 5-1, New York University School of Medicine, New York, NY 10016. E-mail address: jiny@saturn.med.nyu.edu

3 Abbreviations used in this paper: PKC, protein kinase C; AP, adaptor protein complex; BIM1, bisindolylmaleimide 1; DTSSP, 3,3-dithiobis[sulfosuccinimidyl-propionate]; wt, wild type.
switches the connection of CD4 from the former route to the latter, resulting in a net gain of CD4 on the T cell surface.

Materials and Methods

Cells, Abs, and chemicals
The Jurkat T cell line J77, a variant of clone E6-1 (from American Type Culture Collection, Manassas, VA) was cultured in RPMI 1640 medium containing 10% FBS at 37°C in a 5% CO₂ humidified atmosphere. PE-conjugated anti-CD4 (Leu 3A) was purchased from BD Immunocytometry (San Diego, CA), and polyclonal anti-CD4 Ab was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). mAb OKT4D was prepared from a hybridoma purchased from American Type Culture Collection. Anti-HIV-1 Nef rabbit serum was obtained from the National Institutes of Health AIDS Research and Reference Reagent Repository provided by Dr. R. Swanstrom. The PKC inhibitor bisindolylmaleimide 1 (BIM1) was purchased from CalBiochem (San Diego, CA), and 3,3-dithiobis[ sulfosuccinimidylpropionate] (DTSSP) was obtained from Pierce (Rockford, IL).

Generation of mutant CD4 and HIV Nef
The construction and the stable expression of wild-type (wt) CD4, 2C CD4, and tail-less CD4 in Jurkat T cells have been described previously (39). The serine mutant of CD4 used in this study was similarly constructed. The PCR product containing Ser to Ala substitutions at residues 408 and 415 was digested with Sfi1/BamHI, and the resultant fragment was used to replace the corresponding wt CD4 fragment in wt CD4 cDNA originally cloned in pcDNA3 vector (Invitrogen Life Technologies, Carlsbad, CA). The CD4 Ser mutant plasmid was then stably transfected into Jurkat T cells. Nef (NA7)-GFP plasmid was provided by Dr. J. Skowronski (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY: Ref. 35). The non-GFP-tagged Nef was constructed by PCR subcloning of Nef (NA7) into pcDNA3 vector (Invitrogen Life Technologies). NefΔLck, NefΔNH2, NefΔL, GFP, and NefΔNH2/GFP were generated by PCR mutagenesis using Nef or Nef-GFP as the template following the protocol of the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). All mutations generated in this study were confirmed by DNA sequencing.

PMA treatment and two-color cytometric analysis
For transient expression, 20 × 10⁶ CD4 T cells at 800 µM and 250 V. Sixteen to 18 h after transfection, cells were treated with 100 ng/ml PMA in RPMI 1640 medium containing 1 mg/ml BSA at 37°C for 15 min. For CD4 staining, cells were incubated with PE-conjugated anti-CD4 Ab diluted 1/100 in PBS on ice for 45 min. Cells were then subjected to FACS analysis, vertical and horizontal lines were drawn to demarcate CD4- and Nef-positive and -negative cell populations. The line settings were based on the cell surface staining intensities of CD4 and GFP, which varied somewhat among transfected cell lines. The relative surface CD4 expression (percentage) was the ratio of the medium CD4 staining of PMA-positive cells to that of PMA-untreated cells (−PMA). The 100% level (steady state) was the CD4 expression in mock-transfected control cells.

Subcellular fractionation, chemical cross-linking, and immunoblotting
The subcellular fractionation of Jurkat cells and the immunoblotting were conducted as previously described (39). After washing with PBS, cells were incubated in hypotonic buffer (10 mM Tris (pH 7.5) and 330 mM sucrose) for 10 min on ice. Cells were then passed 10 times through a 30-gauge needle while on ice. The extract was centrifuged at 250 × g for 10 min to remove the nuclei and intact cells. To separate the cytosolic fraction from membrane, the postnuclear supernatant was centrifuged at 50,000 rpm for 1 h using a TL-100 centrifuge and a TLS-55 rotor (Beckman Coulter, Fullerton, CA).

Results

PMA treatment partially restored the surface CD4 staining in T cells expressing Nef-GFP
Because both Nef and PMA can down-regulate CD4, apparently by two independent mechanisms, we started this study by asking the question whether Nef and PMA were synergistic in CD4 down-regulation. We transfected Jurkat T cells with Nef-GFP. The cells were previously stably transfected with wt CD4, 2C CD4 (a non-Lck binding CD4), or tail-less CD4 as previously described (39).

For transient expression, cells were surface-stained with PE-conjugated anti-CD4 for two-color cytometric analysis (Fig. 1a). FL1 shows the CD4 surface staining. Without PMA treatment (−PMA), Nef-GFP down-regulated wt CD4 and 2C CD4 in a dose-dependent manner. In cells expressing high levels of Nef-GFP (FL1, >500), CD4 was almost completely down-regulated. Unexpectedly, with PMA treatment (+PMA), the Nef down-regulated wt CD4 and 2C CD4 were partially restored to the cell surface in Nef-GFP-positive cells (FL1 >10). In contrast, CD4 was down-regulated by PMA in Nef-GFP-negative cells (FL1, <10).

For a better comprehension, Fig. 1b quantitatively compared the effects of PMA on the surface CD4 expression in Nef-positive or Nef-negative cells. The left panel shows the percentage of surface CD4 expression in Nef-negative cells. PMA treatment reduced the surface CD4 of wt CD4 and 2C CD4 by 40 and 75%, respectively (converting the results in Fig. 1a from a log scale to a linear scale). The right panel shows the percentage of surface CD4 in Nef-positive cells. Without PMA treatment, Nef-GFP down-regulated wt CD4 and 2C CD4 to 5 and 3% of the steady state level (medium CD4 staining without PMA treatment). With PMA treatment, the surface wt CD4 and 2C CD4 were restored from 5 to 35% and from 3 to 20%, respectively. The tail-less CD4 was not down-regulated by PMA treatment or by Nef expression (Fig. 1a and b), in agreement with the observations by others that CD4 tail was necessary for the PMA-induced (3) and the Nef-dependent CD4 down-regulation (21, 22). The surface expression of tail-less CD4 in Nef-expressing cells was also not affected by PMA treatment, suggesting that the CD4 tail may be the part in CD4 that participated in the restoration.

The dose of PMA and the time required for the restoration were determined (Fig. 1c). PMA at 10–20 ng/ml was sufficient, and the process started right after the addition of PMA and completed within 10 min, with kinetics similar to those of PMA activation of PKC.

Partial restoration of surface CD4 by PMA may require the phosphorylation of CD4 at Ser408 and Ser415
It is known that the PMA-induced phosphorylation of Ser408 and Ser415 in the CD4 tail is necessary for the down-regulation of CD4 (3, 4). To determine whether the phosphorylation of Ser408 and Ser415 was also involved in the partial restoration of the surface CD4 in Nef-expressing T cells, we generated a serine CD4 mutant (Ser CD4), in which Ser408 and Ser415 were replaced by alanine. T cells stably expressing Ser CD4 were transfected with Nef-GFP. Serine mutation did not affect the Nef-induced down-regulation of CD4 (Fig. 2a, left panel). When treated with PMA (Fig. 2a, right panel), however, unlike wt CD4, the surface staining of Ser CD4 was not restored. Moreover, the restoration of the surface staining of wt CD4 by PMA was abolished when Jurkat T cells were preincubated with a PKC inhibitor, BIM1, at 50–100 mg/ml.
nM (Fig. 2b). The results thus suggested that the PMA-induced phosphorylation of Ser^{408} and Ser^{415} was required for the restoration of the surface CD4 in Nef-GFP-expressing T cells.

PMA triggered a reversal of the Nef down-regulation of CD4

A simple explanation of the partial restoration of CD4 by PMA is the reversal of Nef down-regulation of CD4, possibly at the assembly step of the CD4-Nef-AP complex. Therefore, we examined the effects of PMA on CD4 down-regulation in cells expressing Nef-GFP mutants defective for linking CD4 to the AP complex. In NefWL-GFP mutant, residues W57 and L58 essential for Nef interaction with CD4 were substituted by alanines. In NefLL-GFP mutant, the dileucine motif (L164-L165) essential for Nef interaction with an AP complex was similarly substituted. FACS analysis indicated that, in contrast to wt Nef-GFP (Fig. 3, upper left panel), NefWL-GFP and NefLL-GFP did not down-regulate CD4 (Fig. 3, middle left and lower left panels), excluding a few highly NefWL-GFP-positive cells (FL1, >500) where there was a low level of CD4 down-regulation. Unlike the case with wt Nef-GFP (upper right panel), PMA treatment did not increase surface CD4 that was not down-regulated in cells expressing NefWL-GFP and NefLL-GFP (Fig. 3, middle right and lower right panels), excluding a few highly NefWL-GFP-positive cells where the surface CD4 staining was partially restored. The results indicated that only when CD4 was down-regulated by Nef-GFP was PMA able to partially restore the surface staining of CD4, suggesting that PMA triggered a reversal of the Nef down-regulation of CD4, probably by affecting the CD4-Nef interaction, because PMA-induced phosphorylation of the CD4 tail may alter its conformation (3, 4).
Cell fractionation and chemical cross-linking showed that PMA treatment disrupted the interaction between CD4 and Nef.

To determine whether PMA-induced phosphorylation of the CD4 tail disrupts the CD4-Nef interaction, we set out to show that there was an association between CD4 and Nef in T cells. Before our study, attempts to coimmunoprecipitate wt Nef and CD4 in samples from mammalian cells had failed (24). Therefore, we tried to coimmunoprecipitate CD4 and NefLL, because NefLL interacts with, but does not down-regulate, CD4. To our disappointment, like wt Nef, NefLL too was not coimmunoprecipitated with CD4 (complete data not shown; cf., Fig. 4a, lane 2). We then resorted to cell fractionation and chemical cross-linking. Cells were cotransfected with constructs expressing GFP and each of the three non-GFP-tagged Nef (wt Nef, NefLL, and NefWL). The use of the nontagged Nef was necessary to avoid a possible nonspecific cross-linking between CD4 and the GFP-tag. Approximately 15–20% of the cells expressed Nef according to the expression of the cotransfected GFP (data not shown). The cells were then fractionated into membrane and cytosol fractions. The immunoblotting analysis showed that CD4 and all three Nef were predominantly membrane-associated (Fig. 4a, lane 1, -PMA). Anti-CD4 blotting (Fig. 4a, upper panel) showed that the membrane-associated CD4 was ~20% less in cells transfected with wt Nef (lane 6) than in cells mock-transfected (lane 5), NefLL-transfected (lane 7), or NefWL-transfected (lane 8; analyzed by densitometry). Considering that only 15–20% cells expressed Nef, the results indicated that CD4 was down-regulated by wt Nef, but not by NefLL or NefWL, in agreement with the FACS results with expression of GFP-tagged Nef (Fig. 3). PMA treatment increased the CD4 level in the cytosol fractions (Fig. 4a, lanes 9–12 vs lanes 1–4) and decreased the CD4 level in the membrane fractions (Fig. 4a, lanes 3–8 vs lanes 13–16). Also, PMA treatment attenuated the difference in membrane-associated CD4 amounts between cells transfected with wt Nef and Nef mutants (lanes 13–16). The results suggested a reversal of Nef down-regulation of CD4 by PMA in agreement with the results by FACS (Fig. 1). PMA treatment essentially did not affect the membrane distribution of Nef, including wt Nef, NefLL, and NefWL (Fig. 4a, bottom panel). At least 3–4 times more NefLL (lanes 3, 7, 11, and 15) than wt Nef (lanes 2, 6, 10, and 14) and

**FIGURE 3.** Effects of the expression of NefLL and NefWL mutants on PMA-induced CD4 internalization. Cells expressing wt CD4 were transfected with Nef-GFP, NefLL-GFP, and NefWL-GFP for 18 h; treated with PMA; then stained with CD4-PE for FACS.

**FIGURE 4.** Effects of PMA on membrane distribution and chemical cross-linking of CD4 and Nef mutants. Cells were transfected with nontagged wt Nef (WT), NefLL (LL), or NefWL (WL) or were mock-transfected (−), treated with PMA, and then fractionated into membrane and cytosolic fractions. a, Anti-CD4 and anti-Nef immunoblotting of cytosol and membrane fractions. The amounts of cytosol and membrane fractions represented 1/30th and 1/6th of each treated cells, respectively. Cells were expressing wt CD4. After protein transfer, the polyvinylidene difluoride membrane was cut into two pieces. The top part was immunoblotted with polyclonal anti-CD4 (top panel); the bottom was immunoblotted with anti-Nef rabbit antiserum (bottom panel). b, The membrane fractions were cross-linked by DTSSP, except in lane 2, and then lysed in Nonidet P-40 lysis buffer. After immunoprecipitation with anti-CD4, the samples were immunoblotted with anti-Nef rabbit antiserum (bottom panel). c, Anti-CD4 and anti-Nef immunoblotting of cytosol and membrane fractions. The amounts of cytosol and membrane fractions represented 1/30th and 1/6th of each treated cells, respectively. Cells were expressing wt CD4. After protein transfer, the polyvinylidene difluoride membrane was cut into two pieces. The top part was immunoblotted with polyclonal anti-CD4 (top panel); the bottom was immunoblotted with anti-Nef rabbit antiserum (bottom panel). The membrane fractions were cross-linked by DTSSP, except in lane 2, and then lysed in Nonidet P-40 lysis buffer. After immunoprecipitation with anti-CD4, the samples were immunoblotted with anti-Nef rabbit antiserum (bottom panel).
Nef<sub>WL</sub> (lanes 4, 8, 12, and 16) was detected, even though cells were transfected with equal amounts of different plasmid DNAs. For chemical cross-linking experiments, we used the membrane fraction only because the CD4-Nef interaction occurred on the T cell membrane (25). In addition to the membrane fraction from the wt Nef-transfected cells, we used the membrane fraction from the Nef<sub>LL</sub>-transfected cells, because among wt Nef and two Nef mutants, it was expressed at the highest level, and it was defective in Nef-AP interaction, which would make the interpretation of results less complicated than for the wt Nef. The wt Nef and Nef<sub>LL</sub> were transfected into cells expressing wt CD4, tail-less CD4, or Ser CD4. After fractionation, the membrane fractions were treated with DTSSP, a thiol-cleavable cross-linker. The samples were then immunoprecipitated and analyzed by immunoblotting (Fig. 4b). Anti-CD4 recognized a protein band of ~80–85 kDa on nonreducing SDS gel from the Nef<sub>LL</sub>-transfected samples treated with DTSSP (lanes 3 and 5), but not from the mock-transfected samples (lane 1) or sample not treated with DTSSP (lane 2). Anti-Nef also recognized the 80- to 85-kDa band on nonreducing SDS gel, but recognized a 27-kDa band on reducing SDS gel, which was the cleaved product of the 80- to 85-kDa band by β-ME and corresponded to the size of Nef (lane 7). The results thus indicated that the 80- to 85-kDa band was the chemically cross-linked CD4-Nef product. Tail-less CD4 was not cross-linked (lane 4), indicating that the CD4 tail was required for the interaction.

The effects of PMA on the chemical cross-linking are shown in the right panel of Fig. 4b. From samples transfected with wt CD4, the 80- to 85-kDa cross-linking products (lanes 8 and 9) disappeared upon PMA treatment (lanes 10 and 11). In contrast, from samples transfected with the nonphosphorylatable Ser CD4, the similar 80- to 85-kDa band was detected regardless of PMA treatment (lanes 12–15). The results thus suggested that the PMA-induced serine phosphorylation of CD4 might disrupt the interaction between CD4 and Nef. Note that the 80- to 85-kDa band for Nef<sub>LL</sub> (lanes 8, 12, and 14) was more intense than that for wt Nef (lanes 9, 13, and 15), consistent with the above observation that more Nef<sub>LL</sub> than wt Nef was present on T cell membrane (Fig. 4a).

By anti-Nef immunoblotting, the amount of non tagged Nef<sub>LL</sub> in the membrane fraction was ~3- to 4-fold more than that of wt Nef or Nef<sub>WL</sub> (Fig. 4c), whereas by FACS on GFP fluorescence, similar amounts of wt Nef-GFP, Nef<sub>LL</sub>-GFP, and Nef<sub>WL</sub>-GFP were detected (Fig. 3). To verify that tagging GFP to Nef made the disappearance of the overexpression of Nef<sub>LL</sub> relative to the other two Nef, we side-by-side compared the expression levels of non-tagged and GFP-tagged Nef by anti-Nef immunoblotting (Fig. 4c). Again, no difference was found among Nef-GFP, whereas Nef<sub>LL</sub> was ~3- to 4-fold more than the wt Nef and Nef<sub>WL</sub>. Therefore, the phenomenon proved true. A plausible explanation might be that the dileucine motif in Nef might be involved in Nef endocytosis and/or degradation and that tagging GFP to Nef might slow down Nef degradation to the extent that the difference in the amounts of the three GFP-tagged Nef becomes greatly diminished. This possibility is currently under further investigation.

**Discussion**

In this study we found that HIV Nef-down-regulation of CD4 from the Jurkat T cell surface was reversible by PMA treatment (Fig. 1). The reversal appeared to require the phosphorylation of CD4, because both mutating Ser<sup>408</sup> and Ser<sup>415</sup>, the PMA phosphorylation sites in CD4 (3, 4), and the use of a PKC inhibitor, BIM1, abolished the reversal (Fig. 2). CD4 and Nef could be cross-linked by DTSSP in membranes isolated from T cells not treated with PMA, but not in membranes from PMA-treated cells (Fig. 4). Taken together, the data suggested that CD4 and Nef were associated with each other in T cells, and the Ser phosphorylation of CD4 disrupted the interaction.

It has been shown that the CD4-associated Nef binds to the β-coatomer protein on endosomes, targeting CD4 for lysosomal degradation (40). As a result, Nef prevents down-regulated CD4 molecules from recycling back to the cell surface. In the absence of Nef-β-coatomer protein interaction, ~45% of internalized CD4 molecules recycled back to the cell surface within 10 min (40). This explains why the magnitude of CD4 down-regulation by Nef is more complete than that by PMA. In this study, for instance, CD4 down-regulation by Nef was nearly complete (95%), whereas that by PMA was partial (40%; Fig. 1, a and b).

A postulated mechanism of the reversal is as follows. The Nef down-regulation of CD4 is mediated through the CD4-Nef interaction. PMA induces phosphorylation of the CD4 tail, which disrupts the CD4-Nef interaction. The phosphorylated CD4 tail now bypasses Nef, binding directly to an AP complex, most likely to AP-2 (our preliminary data not shown). In other words, PMA treatment switches CD4 internalization from a Nef-dependent to a Nef-independent pathway, which allows some of the down-regulated CD4 molecules to recycle back to the cell surface, resulting in a net gain in cell surface CD4 in Nef-expressing T cells.

Our hypothesis is consistent with the known facts about CD4 and Nef. The CD4 tail comprises a single α-helix from Arg<sup>402</sup> to Lys<sup>417</sup> (41). Leu<sup>413</sup> and Leu<sup>414</sup> are all part of this α-helix. The dileucine motif (Leu<sup>413</sup> and Leu<sup>414</sup>) involved in the CD4-Nef interaction and CD4 internalization is close to Ser<sup>408</sup> and Ser<sup>415</sup>. Therefore, the phosphorylation of Ser<sup>408</sup> and Ser<sup>415</sup> may directly disrupt the CD4-Nef interaction or may indirectly do so by altering the conformation of the CD4 tail. In this respect, it is known that the phosphorylation of Ser<sup>408</sup> and Ser<sup>415</sup> of CD4 results in the dissociation of Lck from the CD4 tail as a result of a conformational change in the CD4 tail (5). It is conceivable that the same conformational change that breaks the interaction between CD4 and Lck may also break the interaction between CD4 and Nef. Consistent with this is the observation that Lck and Nef appear to complete for binding to CD4 (42).

**Acknowledgments**

We thank J. Skowronski for the Nef-GFP constructs. We also thank John Hirst for FACS analysis.

**References**


20. Stoddart, C. A., R. Geleziunas, S. Ferrell, V. Linquist-Stepps, M. E. Moreno,


17. Glushakova, S., J. Munch, S. Carl, T. C. Greenough, J. L. Sullivan, L. Margolis,


14. Mariani, R., and J. Skowronski. 1993. CD4 down-regulation by nef alleles iso-


5500 REVERSIBILITY OF Nef-DOWN-REGULATION OF CD4

Golgi and at the plasma membrane.

1997. The HIV-1 Nef protein acts as a connector with sorting pathways in the

1996. The CD4 determinant for downregulation by HIV-1 Nef directly binds to Nef: mapping of the Nef binding surface by NMR. Biochemistry 35:10256.

R. C. Desrosiers. 1995. Brief report: absence of intact nef sequences in a long-


Immunologic and virologic status after 14 to 18 years of infection with an

R. C. Desrosiers. 1995. Brief report: absence of intact nef sequences in a long-

for downregulation of CD4 to CD4 binds the AP-1 clathrin adaptor.


0. Virol. 1:1.