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Induction of Phosphorylation of Human Immunodeficiency Virus Type 1 Nef and Enhancement of CD4 Downregulation by Phorbol Myristate Acetate

TIANCI LUO,[1] JAMES R. DOWNING,[2,3] AND J. VICTOR GARCIA[1]*

Departments of Virology and Molecular Biology,[1] Pathology and Laboratory Medicine,[2] and Tumor Cell Biology,[3] St. Jude Children's Research Hospital, Memphis, Tennessee 38101

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The *nef* gene of the human and simian immunodeficiency viruses (HIV and SIV) encodes a 27 to 34 kDa myristoylated protein that induces downregulation of CD4 from the cell surface and enhances virus infectivity. As shown by experiments on SIV-infected adult macaques, Nef is important in pathogenesis and disease progression. In vitro, protein kinase C (PKC) phosphorylates Nef, but the role of phosphorylation in the function and expression of this protein has not yet been determined. Here we show that in HIV type 1-infected cells, phosphorylation of Nef increased 8- to 12-fold after treatment with phorbol myristate acetate and phytohemagglutinin (PMA/PHA). Basal and PMA/PHA-induced phosphorylation occurred on serine residues of Nef and was independent of other HIV proteins. The PMA/PHA-induced phosphorylation of Nef was inhibited by bisindolylmaleimide I, a potent and specific inhibitor of PKC, but was unaffected by H89, an inhibitor of protein kinase A. In contrast, treatment with bisindolylmaleimide I did not affect the basal level of Nef phosphorylation, suggesting two different phosphorylation pathways. A PMA-insensitive CD4 mutant in which three serine residues in the cytoplasmic domain have been replaced by alanines was used to determine whether PMA-induced phosphorylation affects Nef-induced CD4 downregulation. In Nef-expressing cells, treatment with PMA enhanced downregulation of the CD4 serine triple mutant from the cell surface, suggesting that phosphorylation is important for Nef function.

Nef is encoded by an open reading frame that overlaps the 3′ long terminal repeats of the types 1 and 2 human immunodeficiency viruses (HIV-1 and -2) and simian immunodeficiency virus (SIV) (5, 15, 46). Expression of Nef, which occurs early after infection from multiply spliced messages (26, 28, 36, 41), is essential for maintaining high levels of virus replication and for pathogenesis in adult macaques infected with SIV (25). In addition, Nef enhances pathogenesis and replication in HIV-1-infected SCID-hu mice (4, 23). The growth advantage of *nef*-containing viruses over those lacking *nef* is due at least in part to enhanced viral infectivity (3, 19, 35, 45).

Nef downregulates the cell surface expression of CD4 (2, 6, 7, 11, 17, 18, 21, 34), the receptor for both HIV and SIV (16, 24, 27, 30, 33). Nef accelerates the endocytosis of CD4, leading to its subsequent degradation in lysosomes (2, 31, 40). Because a serine triple mutant of CD4 has been shown to be refractory to phorbol myristate acetate (PMA) but sensitive to Nef, downregulation of CD4 by Nef has been deemed to be independent of the CD4 phosphorylation induced by phorbol esters (1, 18, 48).

Nef is myristoylated and phosphorylated (5, 9, 21, 22). Myristoylation is essential to the ability of Nef to enhance virus infectivity and to downregulate CD4 expression at the cell surface (19, 20). These findings suggests that the association of Nef with cell membranes may be critical for its function, perhaps by facilitating interaction with key cellular factors. The amino acid sites at which Nef is phosphorylated, the mechanism underlying this phosphorylation, and its role in the function(s) of Nef remain largely unknown. Although the threonine residue at position 15 (Thr-15) has been thought to influence its phosphorylation, Nef has not been shown to be phosphorylated at this site (9, 21, 22). The significance of the contribution of Thr-15 is questionable because this residue is not conserved among functional Nef isolates (e.g., SF2, NL4-3, and SIVmac239) (37). Although associated with a cellular serine/threonine kinase, Nef does not appear to be phosphorylated by this kinase (43, 44). However, bacterially expressed Nef is efficiently phosphorylated by protein kinase C (PKC) in vitro, suggesting that PKC may modify Nef in vivo.

Here we show that Nef is phosphorylated at a basal level in human T cells that have been either acutely infected with HIV-1_{SF2} or transduced by a retrovirus vector expressing SF2 Nef and that stimulation of these cells with PMA and phytohemagglutinin (PHA) significantly enhanced the phosphorylation of Nef. We also show that both the basal and PMA/PHA-induced phosphorylation reactions occur on serine residues of Nef. Interestingly, enhanced phosphorylation of Nef led to increased downregulation of CD4 from the cell surface.

Treatment with PMA/PHA increased the phosphorylation of Nef. Because PKC phosphorylates Nef in vitro (21, 22), we determined whether Nef is phosphorylated during HIV-1 infection. HIV-1_{SF2}-infected HuT 78 cells were labeled with P_i in the absence or presence of PMA/PHA, and Nef was then immunoprecipitated by using a specific monoclonal antibody. The basal level of phosphorylation (Fig. 1A, lane 2) was significantly enhanced after stimulation with PMA/PHA (Fig. 1A, lane 3). The amount of Nef protein present increased slightly after treatment with PMA/PHA, but Nef-specific phosphorylation rose by more than eightfold (Fig. 1B). These findings suggest that the enhanced level of phosphorylation after treatment with PMA/PHA was not due to the effect of PMA/PHA on Nef expression.

To establish whether phosphorylation of Nef occurs inde-

* Corresponding author. Mailing address: Dept. of Virology and Molecular Biology, St. Jude Children's Research Hospital, Memphis, TN 38101. Phone: (901) 495-2611. Fax: (901) 523-2622. E-mail: victor.garcia@stjude.org.

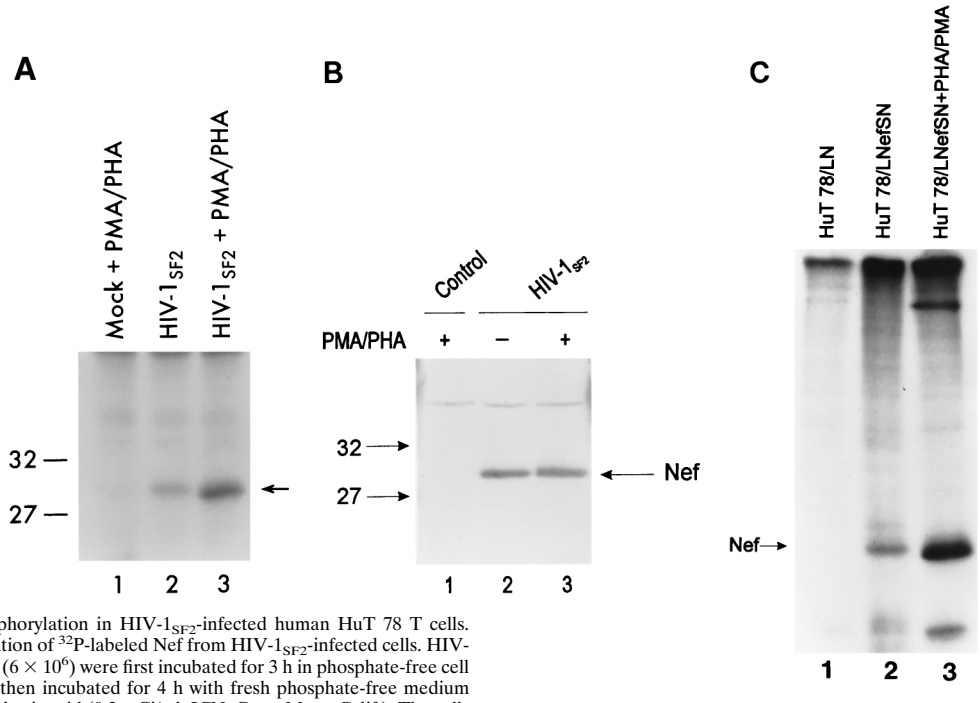


FIG. 1. Nef phosphorylation in HIV-1_{SF2}-infected human HuT 78 T cells. (A) Immunoprecipitation of ^{32}P-labeled Nef from HIV-1_{SF2}-infected cells. HIV-infected HuT 78 cells (6×10^6) were first incubated for 3 h in phosphate-free cell culture medium and then incubated for 4 h with fresh phosphate-free medium containing [^{32}P]phosphoric acid (0.2 mCi/ml; ICN, Costa Mesa, Calif.). The cells were washed with ice-cold phosphate-buffered saline and lysed in lysis buffer (1% Triton X-100, 10 mM Tris, 5 mM Na_2-EDTA, 50 mM NaCl, 30 mM $Na_4P_2O_7$, 50 mM NaF, 100 μM Na_3O_4, 1 μg of aprotinin per ml, 1 μg of leupeptin per ml, 1 mM phenylmethylsulfonyl fluoride). The cell lysates were subjected to immunoprecipitation with a Nef-specific monoclonal antibody. Lane 1, mock-infected cells treated with PMA/PHA; lane 2, HIV-1-infected cells; lane 3, HIV-1-infected cells treated with PMA/PHA. PMA (50 ng/ml) and PHA (5 μg/ml) (both from Sigma, St. Louis, Mo.) were added to the medium during the ^{32}P labeling. Molecular mass markers (in kilodaltons) are on the left. The radioactivity levels of the bands were quantitated with a PhosphorImager (Molecular Dynamics model 425F). (B) Western blot analysis of Nef expression. Equal amounts (100 μg) of total cellular protein were loaded. Lane 1, mock-infected HuT 78 cells; lane 2, HIV-1_{SF2}-infected HuT 78 cells; lane 3, HIV-1_{SF2}-infected HuT 78 cells treated with PMA/PHA. The blot was probed with rabbit anti-Nef antisera and detected with goat anti-rabbit immunoglobulin G conjugated with alkaline phosphatase (Promega, Madison, Wis.) as previously described (6, 7, 31). (C) Nef phosphorylation in the absence of other HIV proteins. Immunoprecipitation of ^{32}P-labeled Nef was done as described in the legend for panel A. Lane 1, HuT 78/LN control cells; lane 2, HuT 78 cells transduced with LNefSN; lane 3, PMA/PHA-treated HuT 78 cells transduced with LNefSN.

pendently of the expression of other HIV-1 proteins, HuT 78 cells were transduced to express HIV-1_{SF2} Nef (7, 31). The transduced cells expressed Nef and showed decreased levels of surface CD4, as expected (7, 31, 32) (data not shown). The basal level at which Nef was phosphorylated in these cells was similar to that seen in the HIV-1-infected cells (Fig. 1C, lane 2). When cells were treated with PMA/PHA, the phosphorylation of Nef increased approximately 12-fold (Fig. 1C, lane 3). Subsequently, we found that PMA alone induced the same level of Nef phosphorylation as the PMA/PHA combination (data not shown). These results suggest that both the basal and the PMA-induced levels of Nef phosphorylation are independent of the expression of other HIV-1 genes.

Phosphorylation of Nef occurred on serine residues. Phosphoamino acid analysis was performed to determine the identity of the amino acids on which Nef is phosphorylated and whether treatment with PMA/PHA changes this pattern. Only serine residues were phosphorylated under both the basal and the PMA/PHA-induced conditions (Fig. 2 and data not shown). These results suggest that phosphorylation of Nef is mediated by a cellular serine kinase(s).

A specific inhibitor of PKC blocked the PMA/PHA-induced but not the basal phosphorylation of Nef. PMA activates PKC in intact cells (8, 10, 12, 29, 38). To determine if PKC is involved in the phosphorylation of Nef in vivo, cells were treated with bisindolylmaleimide I, a potent and specific inhibitor of PKC (49). A parallel sample was similarly treated with H89, an inhibitor of PKA (14). As shown in Fig. 3A, H89 had no significant effect on Nef phosphorylation but bisindolylmaleimide I blocked the PMA/PHA-induced phosphorylation of Nef. These results support a role for PKC in the PMA-induced phosphorylation of Nef. In contrast to its dramatic effect on PMA/PHA-induced phosphorylation, bisindolylmaleimide I did not affect the basal level of Nef phosphorylation (Fig. 3B). This finding suggests that PKC is not responsible for or involved in the basal phosphorylation of Nef. Therefore, the

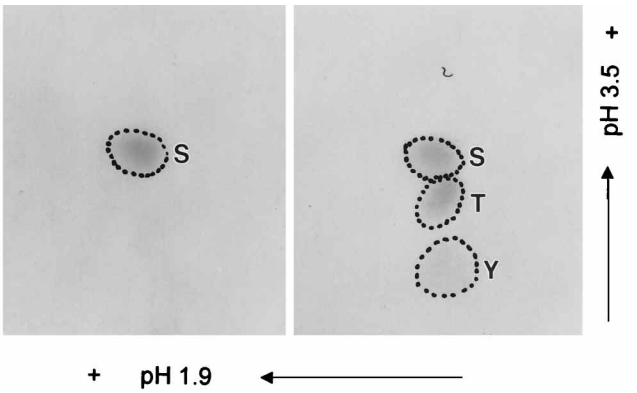
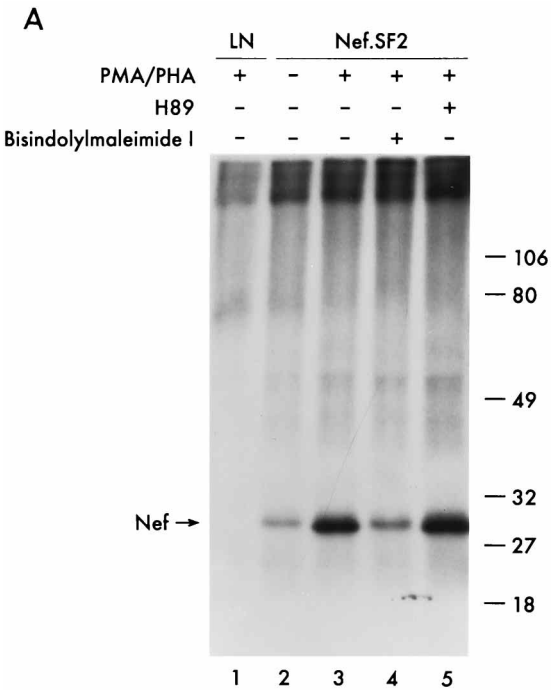


FIG. 2. Nef is phosphorylated on serine. Bands corresponding to phosphorylated Nef from cells after PMA/PHA treatment were eluted from a sodium dodecyl sulfate-polyacrylamide gel and subjected to phosphoamino acid analysis as previously described (13). The right panel shows amino acid standards visualized with ninhydrin. The left panel shows an autoradiogram of phosphorylated amino acids of Nef.



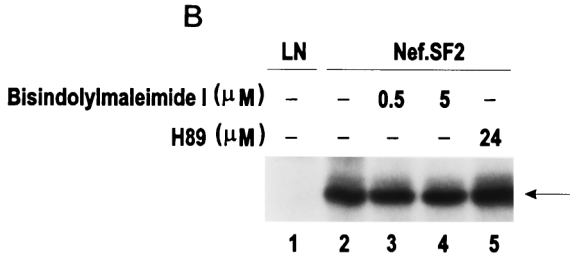


FIG. 3. Effect of a specific PKC inhibitor on Nef phosphorylation. All samples were labeled with ^{32}P and immunoprecipitated in parallel for each experiment as described in the legend for Fig. 1A. (A) Bisindolylmaleimide I, a specific inhibitor of PKC, blocks PMA/PHA-induced phosphorylation of Nef. Lanes: 1, HuT 78/LN (control) cells treated with PMA/PHA; 2, HuT 78 cells transduced with LNefSN (HuT 78/LNefSN cells); 3, HuT 78/LNefSN cells treated with PMA/PHA; 4, HuT 78/LNefSN cells treated with PMA/PHA in the presence of 0.5 μM bisindolylmaleimide I; 5, HuT 78/LNefSN cells treated with PMA/PHA in the presence of 2.4 μM H89, a PKA inhibitor. Both PKC inhibitor bisindolylmaleimide and PKA inhibitor H89 were purchased from Calbiochem-Novabiochem International (San Diego, Calif.). Molecular mass markers (in kilodaltons) are on the right. (B) Bisindolylmaleimide has no effect on the basal level of Nef phosphorylation. Lanes: 1, HuT 78/LN (control) cells; 2, HuT 78/LNefSN cells; 3, HuT 78/LNefSN cells treated with 0.5 μM bisindolylmaleimide I; 4, HuT 78/LNefSN cells treated with 5 μM bisindolylmaleimide I; 5, HuT 78/LNefSN cells treated with 24 μM H89.

kinase pathway that mediates the basal phosphorylation of Nef is different from the pathway that mediates PMA/PHA-induced Nef phosphorylation.

Enhanced phosphorylation by PMA further downregulates a PMA-insensitive CD4 mutant in Nef-expressing cells. To address the possible physiologic relevance of Nef phosphorylation, we examined the effect of PMA-induced Nef phosphorylation on the downregulation of CD4. We used HeLa.H4 cells, which express a PMA-resistant CD4 mutant (18, 47, 48), because treatment with PMA causes a Nef-independent, PKC-mediated downregulation of wild-type CD4 (1, 39, 42). HeLa.H4 cells transduced with LNefSN express SF2 Nef and show a reduction in cell surface CD4 levels (Fig. 4a, graph C, and Fig. 4b). The basal and PMA-induced levels of Nef phosphorylation in the HeLa.H4 cells transduced with LNefSN were similar to those observed in HuT 78 cells (data not shown). There was no significant difference in the levels of Nef in the control and PMA-treated cells (data not shown). These results show that Nef phosphorylation is cell type independent and that the increase in phosphorylation is not due to higher levels of Nef expression. While treatment with PMA has no significant effect on the levels of the CD4 mutant in HeLa.H4 control cells (Fig. 4a, graph B, and Fig. 4b), in Nef-expressing cells there was a further reduction of cell surface CD4 levels (Fig. 4a, graph D, and Fig. 4b). These data suggest that PMA-induced phosphorylation potentiates the Nef-induced cell surface downregulation of this PMA-insensitive CD4 mutant. In addition, these results confirm that CD4 downregulation by Nef does not require serine phosphorylation of CD4 (18).

The data presented in this paper show that HIV-1 Nef is phosphorylated during the infection of HuT 78 human T cells by HIV-1_{SF2} and that phosphorylation of Nef is significantly increased after stimulation with PMA/PHA. We obtained similar results when only HIV-1_{SF2} Nef was expressed, suggesting that no other HIV proteins are important for the phosphorylation of Nef. In previous reports from other laboratories, threonine 15 was implicated in the phosphorylation of HIV-1 Nef because mutation of this site abolished its PMA/PHA-induced phosphorylation (9, 21, 22). However, the actual identities of the phosphorylated amino acids were not explored further. Several functional Nef isolates (including HIV-1_{SF2} Nef) have an alanine at position 15 (37), suggesting that a threonine residue at this site is not essential for function. On the basis of the data presented here, Thr-15 also is not absolutely crucial to the phosphorylation of Nef or for Nef-induced CD4 downregulation.

The phosphoamino acid analysis shown in Fig. 2 demonstrates that the phosphorylation of HIV-1_{SF2} Nef occurs on serine residues. However, depending on the Nef isolate, it is possible that other residues may be phosphorylated. In a previous manuscript, Bandres et al. reported that Nef was not phosphorylated in Jurkat T cells unless they were treated with PMA/PHA (9); our present results show a basal level of phosphorylated Nef in HuT 78 cells. This difference could be due to the fact that different Nef isolates and cell lines were used (HIV-1_{HXB3} versus HIV-1_{SF2}). However, they are more likely a result of improved expression and detection systems.

In vitro studies have shown that Nef purified from a bacterial expression system is phosphorylated by PKC (21, 22). To investigate the possible role of PKC in the phosphorylation of Nef in vivo, we examined the effect of bisindolylmaleimide I, a specific inhibitor of PKC activity. This compound blocked the PMA/PHA-induced phosphorylation of Nef; in contrast, PKA inhibitor H89 had no effect on this process. These results suggest that PKC is involved in PMA/PHA-induced phosphorylation of Nef in vivo. There is a single PKC serine phosphorylation site conserved in all Nef isolates (amino acid 107 for SF2 Nef) (37). Interestingly, a mutation to alanine at this site in SF2 Nef had no effect on the basal or induced levels of Nef phosphorylation (data not shown). In addition, this mutation did not affect CD4 downregulation (data not shown). The fact that in the presence of Nef a PMA-insensitive CD4 mutant was further downregulated from the cell surface suggests that induced Nef phosphorylation potentiates the effect of Nef on CD4 surface expression levels.

Our results highlight the complexity surrounding the regulation of HIV proteins. Nef from HIV-1_{SF2} has 11 serine res-

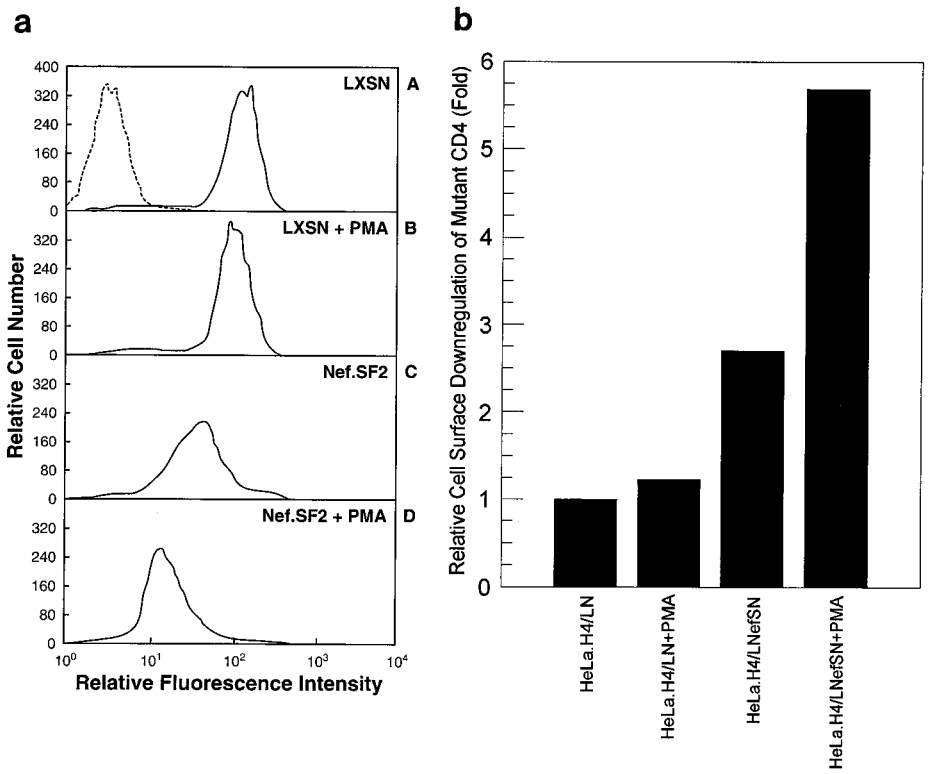


FIG. 4. Enhanced phosphorylation by PMA further downregulates a PMA-insensitive CD4 mutant in Nef-expressing cells. To assess the effect of PMA on cell surface downregulation of the mutant CD4 in HeLa.H4 cells, HeLa.H4/LN (control) or HeLa.H4 cells transduced with LNefSN (HeLa.H4/LNefSN cells) were incubated with PMA (20 ng/ml) for 6 h before immunoprecipitation of Nef or flow cytometry analysis of CD4 expression. (a) Analysis of cell surface CD4 levels. Graph A, HeLa.H4/LN (control) cells stained with isotype-matched phycoerythrin (PE)-conjugated monoclonal negative control antibody (dotted line) or with PE-conjugated anti-CD4 monoclonal antibody (solid line) (both PE-conjugated antibodies were from Exalpha, Boston, Mass.); graphs B, C, and D, cells stained with PE-conjugated anti-CD4 monoclonal antibody (graph B, PMA-treated HeLa.H4/LN cells; graph C, HeLa.H4/LNefSN cells; graph D, PMA-treated HeLa.H4/LNefSN cells). All samples were analyzed in parallel, and the experiment was repeated twice. (b) Relative expression levels of cell surface mutant CD4. The fold reduction of CD4 levels was determined by dividing the mean channel fluorescence for each sample by the mean channel fluorescence of the LN control cells.

idues, most of which are well conserved among various isolates. To address the significance of the basal phosphorylation of Nef in regulating the stability, cellular localization, and function of the protein, extensive mutagenesis and functional analysis will be necessary. Similar experiments will be needed to further explore the role of PMA-induced Nef phosphorylation. However, the enhanced downregulation of a PMA-insensitive CD4 mutant by Nef after treatment with PMA suggests a possible functional role for the phosphorylation of Nef. Further, bisindolylmaleimide I did not block the basal phosphorylation of Nef, leading us to hypothesize that the kinase pathway for this modification differs from the pathway involved in PMA/PHA-induced phosphorylation. Our findings suggest that differential phosphorylation may regulate the various functions of Nef and its diverse interactions with host factors during various stages of the cell and viral life cycles.

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REFERENCES

1. **Acres, R. B., P. J. Canlon, D. Y. Mochizuki, and B. Gallis.** 1986. Rapid phosphorylation and modulation of the T4 antigen on cloned helper T cells induced by phorbol myristate acetate or antigen. J. Biol. Chem. **261:**16210–16214.
2. **Aiken, C., J. Konner, N. R. Landau, M. E. Lenburg, and D. Trono.** 1994. Nef induces CD4 endocytosis: requirement for a critical dileucine motif in the membrane-proximal CD4 cytoplasmic domain. Cell **76:**853–864.
3. **Aiken, C., and D. Trono.** 1995. Nef stimulates human immunodeficiency virus type 1 proviral DNA synthesis. J. Virol. **69:**5048–5056.
4. **Aldrovandi, G. M., and J. A. Zack.** 1996. Replication and pathogenicity of human immunodeficiency virus type 1 accessory gene mutants in SCID-hu mice. J. Virol. **70:**1505–1511.
5. **Allan, J. S., J. E. Coligan, T. H. Lee, M. F. McLane, P. J. Kanki, J. E. Groopman, and M. Essex.** 1985. A new HTLV-III/LAV encoded antigen detected by antibodies from AIDS patients. Science **230:**810–813.
6. **Anderson, S., D. C. Shugars, R. Swanstrom, and J. V. Garcia.** 1993. Nef from primary isolates of human immunodeficiency virus type 1 suppresses surface CD4 expression in human and mouse T cells. J. Virol. **67:**4923–4931.
7. **Anderson, S. J., M. Lenburg, N. R. Landau, and J. V. Garcia.** 1994. The cytoplasmic domain of CD4 is sufficient for its down-regulation from the cell surface by human immunodeficiency virus type 1 Nef. J. Virol. **68:**3092–3101.
8. **Atluru, D., S. Polam, S. Atluru, and G. E. Woloschak.** 1990. Regulation of mitogen-stimulated T-cell proliferation, IL-2 production and IL-2 receptor expression by protein kinase C inhibitor H7. Cell. Immunol. **129:**310–320.
9. **Bandres, J. C., S. Luria, and L. Ratner.** 1994. Regulation of human immunodeficiency virus Nef protein by phosphorylation. Virology **201:**157–161.
10. **Bandres, J. C., and L. Ratner.** 1994. Human immunodeficiency virus type 1 Nef protein down-regulates transcription factors NF-κB and AP-1 in human T cells in vitro after T-cell receptor stimulation. J. Virol. **68:**3243–3249.
11. **Benson, R. E., A. Sanfridson, J. S. Ottinger, C. Doyle, and B. R. Cullen.** 1993. Downregulation of cell-surface CD4 expression by simian immunodeficiency virus Nef prevents viral superinfection. J. Exp. Med. **177:**1561–1566.

12. **Bomsztyk, K., J. W. Rooney, T. Iwaski, N. A. Rachie, S. K. Dower, and C. Hopkins Sibley.** 1991. Evidence of interleukin-1 and phorbol esters activate NF-κB by different pathways: role of protein kinase C. Cell Regul. **2:**329–335.
13. **Boyle, W. J., P. V. D. Geer, and T. Hunter.** 1991. Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. Methods Enzymol. **201:**110–149.
14. **Chijwa, T., A. Mishima, M. Hagiwara, M. Sano, K. Hayashi, T. Inoue, K. Naito, T. Toshioka, and H. Hidaka.** 1990. Inhibition of Forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H89), of PC12D pheochromocytoma cells. J. Biol. Chem. **265:**5267–5272.
15. **Colombini, S., S. K. Arya, M. S. Reitz, L. Jagodzinski, B. Beaver, and F. Wong-Staal.** 1989. Structure of simian immunodeficiency virus regulatory gene. Proc. Natl. Acad. Sci. USA **86:**4813–4817.
16. **Dalgleish, A. G., P. C. L. Beverly, P. R. Clapham, D. H. Crawford, M. F. Greaves, and R. A. Weiss.** 1984. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature (London) **312:**763–767.
17. **Foster, J. L., S. J. Anderson, A. L. B. Frazier, and J. V. Garcia.** 1994. Specific suppression of human CD4 surface expression by Nef from the pathogenic simian immunodeficiency virus SIVmac239^{open}. Virology **201:**373–379.
18. **Garcia, J. V., and A. D. Miller.** 1991. Serine phosphorylation-independent downregulation of cell-surface CD4 by Nef. Nature (London) **350:**508–511.
19. **Goldsmith, M. A., M. T. Warmerdam, R. E. Atchison, M. D. Miller, and W. C. Green.** 1995. Dissociation of the CD4 downregulation and virus infectivity enhancement functions of human immunodeficiency virus type 1 Nef. J. Virol. **69:**4112–4121.
20. **Greenway, A. L., D. A. McPhee, E. Grgacic, D. Hewish, A. Lucantoni, I. Macreadie, and A. Azad.** 1994. Nef 27, but not the Nef 25 isoform of human immunodeficiency virus-type 1 pNL4.3, down-regulates surface CD4 and IL-2R expression in peripheral blood mononuclear cells and transformed T cells. Virology **198:**245–256.
21. **Guy, B., M. P. Kieny, Y. Riviere, C. L. Peuch, K. Dott, M. Girard, L. Montagnier, and J. P. Lecocq.** 1987. HIV F/3′ orf encodes a phosphorylated GTP-binding protein resembling an oncogene product. Nature (London) **330:**266–269.
22. **Guy, B., Y. Riviere, K. Dott, A. Regnault, and M. P. Kieny.** 1990. Mutational analysis of the HIV Nef protein. Virology **176:**413–425.
23. **Jamieson, B. D., G. M. Aldrovandi, V. Planelles, J. B. M. Jowett, L. Gao, L. M. Bloch, I. S. Y. Chen, and J. A. Zack.** 1994. Requirement of human immunodeficiency virus type 1 *nef* for in vivo replication and pathogenicity. J. Virol. **68:**3478–3485.
24. **Kannagi, M., J. M. Yetz, and N. L. Letvin.** 1985. In vitro growth characteristics of simian T-lymphotropic virus type III. Proc. Natl. Acad. Sci. USA **82:**7053–7057.
25. **Kestler, H. W., III, D. J. Ringler, K. Mori, D. L. Panicali, P. K. Sehgal, M. D. Daniel, and R. C. Desrosiers.** 1991. Importance of the nef gene for maintenance of high virus loads and for development of AIDS. Cell **65:**651–662.
26. **Kim, S., R. Byrn, J. Groopman, and D. Baltimore.** 1989. Temporal aspects of DNA and RNA synthesis during human immunodeficiency virus infection: evidence for differential gene expression. J. Virol. **63:**3708–3713.
27. **Klatzman, D., E. Champagne, S. Chamaret, J. Gruest, D. Guetard, T. Hercend, J. C. Gluckman, and L. Montagnier.** 1984. T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. Nature (London) **312:**767–768.
28. **Klotman, M. E., S. Kim, A. Buchbinder, A. Derossi, D. Baltimore, and F. Wong-staal.** 1991. Kinetics of expression of multiply spliced RNA in early human immunodeficiency virus type 1 infection of lymphocytes and monocytes. Proc. Natl. Acad. Sci. USA **88:**5011–5015.
29. **Krasnow, S. W., L. Zhang, K. Leung, L. Osborn, S. Kunkel, and G. J. Nabel.** 1991. Tumor necrosis factor alpha, interleukin-1, and phorbol myristate acetate are independent activators of NF-κB which differentially activate T cells. Cytokine **3:**372–379.
30. **Landau, N. R., M. Warton, and D. R. Littman.** 1988. The envelope glycoprotein of human immunodeficiency virus binds to the immunoglobulin-like domain of CD4. Nature (London) **334:**159–162.
31. **Luo, T., S. J. Anderson, and J. V. Garcia.** 1996. Inhibition of Nef- and phorbol ester-induced CD4 degradation by macrolide antibiotics. J. Virol. **70:**1527–1534.
32. **Luo, T., and J. V. Garcia.** 1996. The association of Nef with a cellular serine/threonine kinase and its enhancement of infectivity are viral isolate dependent. J. Virol. **70:**6493–6496.
33. **Maddon, P. J., A. G. Dalgleish, J. S. Mcdougal, P. R. Clapham, R. A. Weiss, and R. Axel.** 1986. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. Cell **47:**333–348.
34. **Mariani, R., and J. Skowronski.** 1993. CD4 downregulation by nef alleles isolated from human immunodeficiency virus type 1-infected individuals. Proc. Natl. Acad. Sci. USA **90:**5549–5553.
35. **Miller, M. D., M. T. Warmerdam, J. Gaston, W. C. Green, and M. B. Feinberg.** 1994. The human immunodeficiency virus-1 nef gene product: a positive factor for viral infection and replication in primary lymphocytes and macrophages. J. Exp. Med. **179:**101–113.
36. **Munis, J. R., R. S. Kornbluth, J. C. Guatelli, and D. D. Richman.** 1992. Ordered appearance of human immunodeficiency virus type 1 nucleic acids following high multiplicity infection of macrophages. J. Gen. Virol. **73:**1899–1906.
37. **Myers, G., B. Korber, S. Wain-Hobson, R. F. Smith, and G. N. Pavlakis.** 1993. Human retroviruses and AIDS: a compilation and analysis of nucleic acid and amino acid sequences. Los Alamos National Laboratory, Los Alamos, N.Mex.
38. **Nishizuka, Y.** 1986. Studies and perspectives of protein kinase C. Science **233:**305–312.
39. **Petersen, C. M., E. I. Christensen, B. S. Andrensen, and B. K. Moller.** 1992. Internalization, lysosomal degradation and new synthesis of surface membrane CD4 in phorbol ester-activated T-lymphocytes and U-937 cells. Exp. Cell Res. **201:**160–173.
40. **Rhee, S. S., and J. W. Marsh.** 1994. Human immunodeficiency virus type 1 Nef-induced down-modulation of CD4 is due to rapid internalization and degradation of surface CD4. J. Virol. **68:**5156–5163.
41. **Robert-Guroff, M., M. Popovic, S. Gartner, P. Markham, R. C. Gallo, and M. S. Reitz.** 1990. Structure and expression of *tat*-, *rev*-, and *nef*-specific transcripts of human immunodeficiency virus type 1 in infected lymphocytes and macrophages. J. Virol. **64:**3391–3398.
42. **Ruegg, C. L., S. Rajasekar, B. S. Stein, and E. G. Engleman.** 1992. Degradation of CD4 following phorbol ester-induced internalization in human T-lymphocytes. J. Biol. Chem. **267:**18837–18843.
43. **Sawai, E. T., A. S. Baur, H. Strubel, B. M. Peterlin, J. A. Levy, and C. Cheng-Mayer.** 1994. Human immunodeficiency virus type 1 Nef associates with a cellular serine kinase in T lymphocytes. Proc. Natl. Acad. Sci. USA **91:**1539–1543.
44. **Sawai, E. T., A. S. Baur, B. M. Peterlin, J. A. Levy, and C. Cheng-Mayer.** 1995. A conserved domain and membrane targeting of Nef from HIV and SIV are required for association with a cellular serine kinase activity. J. Biol. Chem. **270:**15307–15314.
45. **Schwartz, O., V. Marechal, O. Danos, and J. M. Heard.** 1995. Human immunodeficiency virus type 1 Nef increases the efficiency of reverse transcription in the infected cells. J. Virol. **69:**4053–4059.
46. **Shibata, R., T. Miura, M. Hayami, K. Ogawa, H. Sakai, T. Kiyomasu, A. Ishimoto, and A. Adachi.** 1990. Mutational analysis of the human immunodeficiency virus type 2 (HIV-2) genome in relation to HIV-1 and simian immunodeficiency virus SIV_{AGM}. J. Virol. **64:**742–747.
47. **Shin, J., C. Doyle, Z. Yang, D. Kappes, and J. L. Strominger.** 1990. Structural features of the cytoplasmic region of CD4 required for internalization. EMBO J. **9:**425–434.
48. **Shin, J., R. L. Dunorack, Jr., S. Lee, and J. L. Strominger.** 1991. Phosphorylation-dependent down-modulation of CD4 requires a specific structure within the cytoplasmic domain of CD4. J. Biol. Chem. **266:**10658–10665.
49. **Toullec, D., P. Pianetti, H. Coste, P. Bellevergue, T. Grand-Perret, M. Ajakane, V. Baudet, P. Boissin, E. Boursier, F. Loriolle, L. Duhamel, D. Charon, and J. Kirilovsky.** 1991. The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. J. Biol. Chem. **266:**15771–15781.