HIV Nef-mediated Cellular Phenotypes Are Differentially Expressed as a Function of Intracellular NEF Concentrations*

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Nef is a regulatory protein encoded by the genome of both human and simian immunodeficiency virus. Its expression in T cells leads to CD4 and major histocompatibility complex class I modulation and either enhancement or suppression of T cell activation. How this viral protein achieves multiple and at times opposing activities has been unclear. Through direct measurements of Nef and the Nef-GFP fusion protein, we find that these events are mediated by different Nef concentrations. Relative to the intracellular concentration that down-modulates surface CD4, an order of magnitude increase in Nef-GFP expression is required for a comparable modulation of major histocompatibility complex class I, and a further 3-fold increase is necessary to suppress T cell activation.

The role of the viral nef gene in HIV1 and simian immunodeficiency virus infection has been defined by the Nef-mediated increase in in vivo viral titer and the development of pathogenesis (1). Some reported Nef activities, such as enhancement of infectivity (2–4), could contribute to this in vivo picture. However, other findings, such as molecular associations, receptor modulations, and effects on cell activity (5–7), are less definitive and at times contradictory. For example, whereas some (8–11) have concluded that Nef increases T cell activity, others (12–14) have demonstrated that Nef expression leads to suppression of T cell activation. In part, these differences may be due to either the use of stable cellular clones, which may not be representative of the entire cell population, or the use of promoters with varied activity. One study that used non-clonal cells examined T cell receptor stimulation-induced expression of the activation marker CD69 and convincingly found that all Nef-expressing cells (defined by CD4 down-modulation) also suppressed activation-induced CD69 expression (15). CD69 is one of several surface markers that identify activation of the biochemical pathways leading from the T cell receptor (16). Other Nef-mediated cellular phenotypes include down-modulation of CD4 and MHC I from the cell surface (17–19).

Our own studies have made use of T cell populations, continuous lines as well as primary CD4 T cells, that have been transduced with a retroviral vector. Nef expressed in these non-clonal populations resulted in enhanced T cell activation, as defined by IL-2 secretion, and down-modulation of surface CD4 (8, 10, 20). A Nef-mediated suppression (or enhancement) of CD69 induction or down-modulation of MHC I in transduced cells had not been noted in our experimentation with Nef. In this report, we have chosen to study the effect of one variable, the concentration of intracellular Nef, on the biological activity of this HIV protein. The development of a quantitative chemiluminescent Western assay for Nef permitted an estimation of Nef concentrations in cell lysates, either transduced with the nef gene or infected with HIV. In order to define Nef concentrations at the cellular level, we have made use of a Nef-green fluorescent protein (GFP) fusion protein. By flow cytometry, we correlated the level of cellular Nef-GFP directly to measurements of Nef-modulated cell surface markers of cell function. We found that T cell activation enhancement and CD4 modulation, decreased MHC I surface expression, and suppression of CD69 induction were dependent, respectively, on increasing concentrations of Nef. These findings imply that the numerous Nef-mediated cellular phenotypes are possible within the same cell but are differentially expressed as a function of Nef concentrations.

EXPERIMENTAL PROCEDURES

Antibodies, Plasmids, and Retrovirus—For flow cytometry, (R)-phycoerythrin (RPE)-conjugated antibodies to CD4 and CD69 were purchased from Caltag (South San Francisco, CA). The RPE conjugate antibody to HLA-ABC antigen (clone W6/32) was purchased from Dako A/S (Denmark). Standard beads with known molecular equivalents of soluble fluorochromes (MESF) of fluorescein were from Sigma. pQBI25 (green fluorescence protein (GFP) under CMV promoter) was purchased from Quantum (Canada), and pNA7GFP (NefGFP under CMV promoter) was kindly provided by Drs. Michael E. Greenberg and Jacek Skowronski (21). The pNA7GFP plasmid encodes a fusion product of HIV Nef NA7 and genetically modified green fluorescent protein GFPSg25 (22). To construct the retroviral expression plasmids pLGFPSN and pLNefGFPSN, primers were first synthesized (Midland Certified Reagent Co.) as follows: g5, 5’-ATATGAAATTCATGCTACGACAAAGGAGAAGAAGCTCTTCACTGG-3’; g3, 5’-TTAAGGATCCTCAATCGATGTTGTACAGTTCATCCATG-3’. By using g5 and g3 as primers and pQBI25 as the template, a polymerase chain reaction fragment was digested with EcoRI and BamHI and then inserted into the EcoRI-BamHI site of pLXSN. The recombinant is the retroviral expression vector pLGFPSN. Plasmid pNA7GFP was digested with XbaI and blunted with T4 DNA polymerase and then digested with BamHI. The small fragment was inserted into the HpaI and BamHI site of pLXSN, resulting in pLNefGFPSN. Recombinant retrovirus containing LGFPSN and LNefGFPSN were derived from the packaging line GP293 (CLONTECH) according to the

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1 The abbreviations used are: HIV, human immunodeficiency virus; MHC I, major histocompatibility complex class I; HLA-A, human major histocompatibility complex class I region A; GFP, green fluorescence protein; RPE, (R)-phycoerythrin; CMV, cytomegalovirus; LTR, retroviral long terminal repeat, used here to define promoter; MESF, molecular equivalents of soluble fluorochrome; PMA, phorbol 12-myristate 13-acetate; IL-2, interleukin-2.

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protocol of the manufacturer. Briefly, pLGFPNS and pNefGFPNS were cotransfected with pSVS (encoding the vesicular stomatitis virus envelope) into GP293 cells, and virus was collected 3 days later. Retroviral vectors LXSNS, LNL4SNS, and LG2ASN packaged by PA317 and used to transduce T cells were described previously (10).

**T Cell Culture, Transfection, and Transduction—**VB (23) and Jurkat E6-1 (24) T cells were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health. Cells were grown in complete growth medium (RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 g/liter sodium bicarbonate, 1 mM non-essential amino acids, 10 mM sodium pyruvate, 4 mM l-α-carnosine, and 50 μg/ml gentamicin, adjusted to pH 7.4). E6-1 T cells were transfected with pQBI25 or pN7GFP using GenePORTER Transfection Reagent (Gene Therapy Systems) according to the manufacturer’s protocol. VB T cells were transfected with pQBI25 or pNA7GFP by electroporation (T820; BTX Electronic, Genetica), set at 200 V, 65 ms, and 1 pulse. Cells (5 × 10^6/ml) were electroporated in RPMI 1640 (without fetal calf serum) with DNA at 30 μg/ml at room temperature. For transduction, cells were incubated with recombinant retroviruses carrying the expression vectors LXSNS, LG2ASN, LNL4SNS, LGFPNS, or LNefGFPNS and treated the next day with 2 mg/ml genetinin for E6-1 cells and 1 mg/ml for VB T cells. After 7 days in selection, cells were returned to complete medium, and whole populations of genetinin-resistant cells were used in subsequent studies.

**HIV-1 Infection of Primary CD4+ T Cells—**The peripheral lymphocyte fraction from healthy donors was obtained by leukapheresis and countercentrifugal elutriation by the Department of Transfusiology at the National Institutes of Health (25) and purified as described previously (10). Proliferation of purified CD4 T cells was achieved by addition of CD3/CD28 beads (10). CD4 T cells were infected with either wild type HIV-1 strain NL4-3 or a mutant NL4-3 virus containing a double single-point mutation in the nef gene (DS) (26) or mock-infected with complete media. After a 2-h incubation period at 37°C, the cells were washed in 20 ml of RPMI 1640 to remove unad sorbed virions. Cell pellet was resuspended in complete media at 2 × 10^5 cells/ml and returned to 37°C. Cells were then stimulated with anti-CD3/CD28 beads. Viral replication was followed by determination of secreted HIV core antigen p24 by enzyme-linked immunosorbent assay (Coulter, Miami, FL). At peak p24 secretion (1–2 weeks), cells were pelleted, lysed, and examined for Nef protein as described below.

**Western Blot Detection of Nef and GFP—**Analysis of Nef and green fluorescent protein on Western blots was performed essentially as reported previously (10). Prior to lysis, cell count and volume were determined with a Coulter Z2 particle analyzer (Beckman Coulter, Fullerton, CA) and analyzed by flow cytometry. Cells were lysed in 1% Triton X-100 in 20 mM Tris (pH 8.0), 150 mM NaCl, 2 mM EDTA and supplemented with 1.0 μg/ml leupeptin, 1.0 μg/ml aprotinin, 1 μg/ml pepstatin A, and 250 μg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride, followed by protein determination by BCA microassay (Pierce). Nef was then precipitated by rabbit polyclonal anti-Nef (27) (obtained through the AIDS Research and Reference Reagent Program, NIAID, National Institutes of Health) and run on SDS-gel electrophoresis and blotted onto a cellulose nitrate membrane. This was probed with anti-Nef antibody with a chemiluminescence system. Recombinant Nef at various levels is shown in panels A, B, and C. Cells infected with recombinant Nef at 10 ng of recombinant Nef, respectively. DS HIV were similarly processed and placed in lanes 1, 2, 3, 4, 5, and 6. Recombinant Nef at various levels is shown in panels A, B, and C. Cells infected with recombinant Nef at 10 ng of recombinant Nef (lane 1), 5 ng of recombinant Nef (lane 2), 2.5 ng of recombinant Nef (lane 3), and 1.25 ng of recombinant Nef (lane 4). Nef detection in 107-transduced VB cells (NL4-3 Nef, lane 7) with recombinant Nef at 10 ng (lane 5), 2.5 ng (lane 6), and 1.25 ng (lane 7). Nef expression in NL4-3 HIV-infected primary CD4 T cells. Infected T cells were harvested on day 16. At this time ~50% of cells displayed cytopathology, and p24 secretion was at peak levels. The equivalent of 10^7 cells was applied to lanes 1, 2, 3, and 4, and 10^8 cells per lane for lanes 5, 6, and 7. The wild type Nef (Fig. 1A, lane 1) and the first point in the log of the fluorescence (Fig. 1A, lane 2) was considered to be an independent distribution. The means of these distributions were plotted against the x scale value of fluorescence of GFP or Nef-GFP. The 95% confidence bands were also calculated. These bands broadened at x ≥ 3 running average, and viewed as a contour plot with GFP or Nef-GFP displayed along the y axis and the T cell antigens along the x axis. Because the individual cell measurements were independent, each of the 90 columns in the unsmoothed histograms was considered to be an independent distribution. The means of these distributions were plotted against the x scale value of fluorescence of GFP or Nef-GFP. The 95% confidence bands were also calculated. These bands broadened at higher GFP levels because the distributions broaden and the numbers of cells diminish. For comparison purposes, the y values for all bins were adjusted so that the mean value of the left-hand no/low GFP region was identical for both plots.

**RESULTS**

**Determination of Cellular Nef Concentration—**Jurkat E6-1 cells were transduced with both Nef from HIV NL4-3 or a mutant, non-myristoylated NL4-3 Nef, generated by a glycinato alanine switch at residue position 2 (G2A). This mutant Nef is known to lack the numerous bioactivities of the native Nef protein (10, 15, 28–30). Cells were transduced with these nef genes in the Moloney LTR-based LXSNS vector (18). The cellular concentration of Nef was determined by a chemiluminescence-based Western blot, where quantitation of emitted light was achieved with a cooled charge-coupled device camera system (see “Experimental Procedures”). This analysis of cell lysates demonstrated the specific expression of the Nef proteins in cells transduced with either the wild type Nef or the G2A mutant Nef (Fig. 1A). Recombinant Nef at various levels is shown in lanes 1–4 of Fig. 1A. The band intensities in this particular Western blot indicated that the NL4-3 (Fig. 1A, lane 1) and the G2A (Fig. 1A, lane 7) Nef transduction of the Jurkat cell resulted in an expressed 2.0 ng of Nef per 7 × 10^6 cells for both transductions. Thus, each transductant contained ~0.3 fg of Nef per cell.

The VB T cell line used in this report is characterized in Fig. 1B, with recombinant Nef in lanes 1–4. The wild type Nef (Fig. 1B, lane 5) is expressed at 0.3 fg per cell, and the G2A mutant
Nef (Fig. 1B, lane 6) is at 0.4 fg per cell. These results are representative of multiple T cell transductions using this retroviral expression system. Typically, geneticin-selected T cell populations expressed between 0.3 and 1.0 fg per cell.

We also applied this Nef assay system to HIV-infected primary CD4 T cells. Purified and activated CD4 T cells were infected with either wild type NL4-3 HIV or a Nef-negative HIV mutant NL4-3 DS (26) as described under “Experimental Procedures.” The HIV-infected cells were harvested following the peak of HIV core antigen p24 secretion for maximal Nef generation (31). At this time a minimum of 50% of the cells displayed signs of infection by microscopic examination. The lysate was applied to Western analysis for Nef content. HIV infection of CD4 T cells yielded 1 ng per 10^6 cells or 1 fg Nef per cell (Fig. 1C, lane 3). Although all cells stained positive for intracellular p24 (data not shown), this measured level of Nef has to be taken as a conservative estimate with a minimum of 50% of the cells displaying infection by microscopic examination. Lysate from cells infected with the Nef-negative DS mutant HIV lacked detectable protein (lane 4). These findings suggest that retroviral transduction as performed here results in cellular Nef concentrations that approximate those seen in HIV infection.

Effect of Transduced Nef on T Cell Activation and Surface CD4 and MHC I—Retroviral transduction of T cells with Nef has been demonstrated previously by this laboratory (10, 20) to enhance T cell activation and to modulate surface CD4. Accordingly, activation of the Nef-expressing Jurkat T cell resulted in an enhanced secretion of IL-2 (Fig. 2). Stimulation of the Jurkat cell can also be evaluated through measurement of CD69, which occurs with standard beads with known MESF of fluorescein. As shown in Fig. 4, A and B, the expression of GFP did not affect expressed levels either of CD4 or MHC I (defined by fluorescence on y axis). Cells positive for Nef-GFP expression displayed a 77% down-modulation of surface CD4 (Fig. 4C), whereas with the identical cells stained for MHC I, there was no measurable modulation (Fig. 4D). The mean fluorescence of the Nef-GFP-positive population in Fig. 4 was 67 relative fluorescent units (x axis), which in this run was equivalent to 4.4 × 10^4 MESF (fluorescein equivalents). With the VB cell we have seen that transduction with either native Nef (Fig. 3C) or Nef-GFP (Fig. 4C) has resulted in CD4 modulation. However, in order to permit a direct comparison of the two transductants, we need to compare only Nef-expressing cells. Although a mechanism has not been defined, the lack of full expression of Nef from an integrated LnefSN (LTR-Nef-SV40-Neo) retroviral vector has been documented previously (18). That is, cells that have been selected for geneticin resistance (Neo; neomycin phosphotransferase) do not always coexpress Nef. The Nef-GFP-positive cells in the transduced population displayed in Fig. 4C are 38% of the total geneticin-resistant population, and our measurement of CD4 modulation only included the Nef-GFP-expressing cells. Estimation of CD4 modulation in cells transduced by the native Nef, for example in Fig. 3, is not so straightforward. Indeed, our calculation for percent modulation, which comes from mean fluorescence, included all geneticin-resistant cells and thus included cells that could be negative for Nef expression. Corrections can be achieved, however, by only including cells that functionally display the presence of Nef. The modulation of CD4 in the Nef-transduced VB
population of Fig. 3 occurred to 65% of the selected cells (histogram subtraction, see “Experimental Procedures”). A comparison of the mode fluorescence between the CD4-modulated population (peak of solid line plot of Fig. 3C; 179 relative fluorescent units on x axis) and the mode of the control CD4 levels (dashed line; 991 relative fluorescent units) yielded an 82% decrease in surface CD4. Additionally, if Nef expression is limited to 65% of the cells, one can deduce that the native Nef-transduced cells possessed a Nef concentration of 0.5 fg per cell (up from 0.3 fg in the total population). Thus, transduction of VB cells with either native Nef or Nef-GFP resulted in 80% modulation of surface CD4 but with no measurable change in MHC I. Also, transduction of the Jurkat E6-1 cell did not lead to MHC I modulation or loss of CD69 induction. Previous works have demonstrated that Nef can affect these latter functions, but there remains the need to define the mechanism by which the varied outcomes are possible. With the ability to measure Nef-GFP at the cellular level with flow cytometry, we then characterized T cell surface CD4, MHC I, and CD69 as a function of Nef-GFP concentration.

Correlation of Cellular GFP Protein Concentration and Cellular Fluorescence—In order to correlate MESH values from flow cytometry with protein concentration of cellular GFP, we first established a stable Jurkat clone expressing a high uniform level of GFP (Fig. 5A). Attempts to generate a stable highly fluorescent Nef-GFP clone were non-productive. The GFP clone yielded a mean fluorescence intensity of 95.1 relative units, which from standard beads yielded 1.22 \times 10^5 \text{MESF}. To correlate these values with cellular GFP, the lysate equivalent of 10^5 cells was applied to an SDS gel along with varied levels of recombinant GFP (Fig. 5B). This permitted an estimate of 1.7 ng of GFP from the cell aliquot or 17 fg per cell. An additional two independent determinations yielded similar estimates (data not shown).

We can use the relationship of 17 fg of GFP per cell yielding a fluorescence intensity equal to 1.22 \times 10^5 \text{MESF} units per cell to estimate expressed GFP levels in cells from the cytometry-derived MESF values. The GFP VB population of Fig. 4B, the cells of which possess a fluorescence intensity of 1.45 \times 10^5 \text{MESF} units, would thus be predicted to contain 20 fg of GFP per cell. If the GFP moiety of the Nef-GFP fusion product
possessed fluorescence equivalent to the non-fusion GFP molecule, then the 4.4 x 10^5 MESF units of Nef-GFP population of VB cells (Fig. 4) multiplied by the same factor (17 fg of GFP/1.22 x 10^5 MESF) yields 6.1 fg of GFP (12.2 fg of Nef-GFP; Nef and GFP are of comparable molecular weight).

As a means to examine the utility of the derived factor for estimating Nef-GFP (and GFP) concentration, we then performed a similar Western analysis on the two VB populations (GFP- and Nef-GFP-positive cells) defined in Fig. 4. This analysis differs from the analysis of the GFP clone in that neither population is homogeneously positive and that one of the populations expresses the fusion protein Nef-GFP in place of GFP. The lysate equivalent of 10^5 cells of either the GFP clone (lane 5) or the control Jurkat cell (lane 4) was probed with anti-GFP sera. Recombinant GFP was also applied to the gel (2.5 ng, lane 1; 1.25 ng, lane 2; 0.63 ng, lane 3). C, Western analysis of Nef-GFP and GFP transduced VB cells described in Fig. 4. Following application of the equivalent of 6 x 10^5 cells to the SDS-gel electrophoresis, the blot was probed as with the anti-GFP sera. Lane 6 contains the lysate from Nef-GFP VB cells; lane 7 contains lysate from GFP cells. Recombinant GFP was also applied (10 ng, lane 1; 5 ng, lane 2; 2.5 ng, lane 3; 1.25 ng, lane 4; 0.63 ng, lane 5).

**FIG. 5.** Measurement of GFP and Nef-GFP expression in cells. A, GFP-positive Jurkat E6-1 cell clone (solid line) compared with control E6-1 cell (dashed line). The clone yielded a mean fluorescence intensity equivalent to 1.22 x 10^5 MESF. B, Western analysis of GFP clone. The lysate equivalent of 10^5 cells of either the GFP clone (lane 5) or the control Jurkat cell (lane 4) was probed with anti-GFP sera. Recombinant GFP was also applied to the gel (2.5 ng, lane 1; 1.25 ng, lane 2; 0.63 ng, lane 3). C, Western analysis of Nef-GFP and GFP transduced VB cells described in Fig. 4. Following application of the equivalent of 6 x 10^5 cells to the SDS-gel electrophoresis, the blot was probed as with the anti-GFP sera. Lane 6 contains the lysate from Nef-GFP VB cells; lane 7 contains lysate from GFP cells. Recombinant GFP was also applied (10 ng, lane 1; 5 ng, lane 2; 2.5 ng, lane 3; 1.25 ng, lane 4; 0.63 ng, lane 5).

mode fluorescence (214 versus 67 relative fluorescence units for GFP- and Nef-GFP-positive cells, respectively, in Fig. 4, B and D). The differences in steady state concentration may be related to differences in the half-lives of the two moieties of the Nef-GFP fusion (see “Discussion”). We then compared, at the cellular level, Nef-GFP concentration to cell surface marker modulation.

**FIG. 6.** Suppression of CD69 induction by Nef-GFP in Jurkat cells. Jurkat cells were transfected with a CMV-based expression plasmid for either GFP (A) or Nef-GFP (B) and on day 2 were activated by anti-CD3 antibody. Surface CD69 was measured by flow cytometry on day 3. The mean value of CD69-mediated fluorescence as a function of green fluorescence is plotted as the thick solid line banded by 95% confidence levels (thin lines). C, mean CD69 values from A and B. D, mean values for CD69 induction by PMA treatment for Nef-GFP (solid line) and GFP (dashed line) Jurkat cells.
within the transfected population became positive. The x axis fluorescent intensity of 1000 relative fluorescence units in Fig. 6 corresponded to $1 \times 10^6$ MESF, a fluorescence intensity that is 20-fold higher than the mode fluorescence of the Nef-GFP retroviral transduced population of Fig. 4. These cell populations expressing varying levels of GFP or Nef-GFP were activated by either T cell receptor perturbation with anti-CD3 antibody or by addition of the phorbol ester PMA. Cells were then stained for CD69 (y axis, Fig. 6). Induction of CD69 on the GFP cells appeared to be unaffected by GFP levels (Fig. 6A); however, there was significant suppression of CD69 induction at higher concentrations of the Nef-GFP fusion protein (Fig. 6B). Averaging of these CD69 (y axis) data yielded the plots shown in Fig. 6, A and B (thick line), bordered by the 95% confidence bands. These mean value plots from A and B are replotted in Fig. 6C, with the induced CD69 levels for the Nef-GFP cell represented by the solid line (dashed line is the GFP control). Fig. 6C demonstrates the ability of Nef-GFP to inhibit CD3-induced CD69 expression in a concentration-dependent fashion. At the lower levels of Nef-GFP, there is a shallow decline in CD69 with increasing Nef-GFP, and at 1000 relative fluorescent units ($1 \times 10^6$ MESF) a steeper decline is initiated. Coincidentally, this level of Nef-GFP corresponds to a 50% drop (from the median CD69 value in activated non-GFP cells) in CD3-induced CD69 expression. This MESF value corresponds to 280 fg of Nef-GFP per cell. A 1-log drop in CD69 is approached at Nef-GFP levels corresponding to $8 \times 10^6$ MESF (2.2 pg of Nef-GFP per cell). PMA-induced CD69 expression for the GFP and Nef-GFP cells was examined by identical methods and plotted in Fig. 6D. For the PMA induction of CD69, Nef-GFP has no discernible suppression at levels below $10^6$ MESF (1000 relative fluorescence units, x axis Fig. 6D). This corroborates the previous demonstration that Nef does not effectively suppress CD69 induction by PMA (15). At the highest level of Nef-GFP, however, we do see slight suppression.

We then wished to apply this system to Nef-mediated modulation of constitutively expressed cell surface receptors. The Jurkat E6-1 cells were transfected with the GFP or Nef-GFP plasmids. The measurement of surface levels of MHC I as a function of GFP or Nef-GFP fluorescence, resulted in the curves displayed in Fig. 7. GFP-expressing cells are represented by the dashed lines and Nef-GFP by solid lines (as in Fig. 6, the lines represent the mean value bordered by 95% confidence bands). Nef-GFP mediated a 50% drop in HLA-A at fluorescence levels corresponding to $5 \times 10^5$ MESF, with a log drop at $2 \times 10^6$ MESF (140 and 560 fg per cell, respectively). As with CD69 suppression, GFP expression was without effect.

To establish the quantitative differences in Nef-mediated down-modulation of CD4 and MHC I, we then electroporated VB cells with the GFP and Nef-GFP CMV-based DNA expression vectors previously used in the Jurkat line. As shown in Fig. 8, we see a range of expressed GFP and Nef-GFP levels. Expression of GFP appeared to play no role in the surface density of either MHC I or CD4 (Fig. 8, A or B, respectively). Modulation of these receptors in the Nef-GFP cells was apparent (Fig. 8, C and D); however, CD4 modulation occurred at a lower Nef-GFP concentration. The average fluorescence of stained CD4 and MHC I surface receptors was plotted against GFP or Nef-GFP fluorescence in Fig. 8E, along with the 95% confidence bands. Compared with cells negative for Nef-GFP, 50% of surface CD4 is modulated at a Nef-GFP fluorescence of 20 relative fluorescence units (x axis), equal to $2 \times 10^4$ MESF, and a 1-log drop occurred at $3 \times 10^5$ MESF (5.6 and 84 fg per cell). By comparison, one-half of surface MHC I molecules are removed at a Nef-GFP level equivalent to $4 \times 10^5$ MESF, and 1 log of surface receptor was modulated at $3 \times 10^6$ MESF (110 and 840 fg per cell). This finding demonstrates that the mechanisms for CD4 and MHC I modulation are different and that modulation of MHC I requires higher levels of Nef, corroborating previously described (34–37) mechanistic differences. The potency by which Nef-GFP similarly alters the two receptors differs by an order of magnitude.

**DISCUSSION**

The ability of Nef to mediate numerous and at times opposite effects has been difficult to ascribe to a definitive mechanism. This work defines effector functions of Nef in terms of expressed cellular protein levels. The results suggest that these functions are realized at different Nef concentrations. This work also describes the measurement of intracellular concentrations of Nef protein.

The use of GFP fusion proteins to characterize in vivo function is widespread (38–40), and in particular Nef-GFP has been successfully used to characterize the intracellular localization and function of Nef (21). We have used this fusion protein to compare relative Nef concentrations required to mediate particular activities attributed to Nef. In our early exploration of this expression plasmid, we found that sub-optimal transfection led to a minor population of cells that uniformly expressed a large range of fusion protein, and thus permitted the establishment of GFP dose curves for various T cell functions. By correlating relative Nef-GFP levels with fluorescence intensity of cells stained for surface markers, we have been able to compare the relative concentrations of Nef-GFP needed to achieve defined outcomes. Relative to the concentration of Nef-GFP required to decrease CD4 by 50%, similar reductions in MHC I and in CD69 induction require 20- and 60-fold increases in intracellular concentration, respectively.

The generated curves for Nef-GFP-mediated suppression of CD3 and PMA-induced CD69 expression (Fig. 6) suggest that there may be two sites for Nef interaction within this pathway. At lower concentrations Nef appears to affect only cells induced with CD3, whereas CD69 induction with PMA is affected only at the highest concentrations. This suggests that there are molecular targets above (and potentially independent of PMA) and at or below the PMA site of interaction within this path-
A of Nef-GFP concentration. dashed line by Fig. 6; Nef-GFP modulation of CD4 is depicted by solid line. Gall CD4, when compared with the modulation of HLA antigen. Le modulation would appear to be more restricted than in CD4 mod-
ulation. This work is consistent with previous efforts that have
the C terminus of Nef (attachment site for GFP) are critical for
unclear; for example, mutational studies of the residues within
the C-terminal alteration in these differential dosage effects is
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the C-terminal alteration in these differential dosage effects is
dosage differences with Nef-GFP may be affected through
mediated effects clearly describe different mechanisms, the
CD69 induction pathway. Moreover, since the previous
demonstrations that Nef retroviral transduction of primary
cells (10, 11) or HIV infection (43) leads to T cell activation
enhancement, it seems likely that Nef suppression of T cell
activity would not be functionally prominent in HIV infection.

The utility of a GFP fusion protein can be limited by loss of
function of one of the two moieties, in this case the biological
activity of the Nef or the fluorescence of the GFP. Our data
suggest that the GFP moiety in the Nef fusion protein main-
tains its fluorescent function. However, the fusion of Nef to
GFP appears to alter the steady state concentration of the
GFP moiety. Stable transduction (with the same expression
vector) results in a 3.5-fold increase of GFP content when not
coupled to Nef (compare Fig. 4, B and D). The decrease in
steady state concentration of Nef-GFP may be related to the
shorter half-life of Nef, which is ~12 h (8), compared with the
more stable GFP (44).

Although there appears to be no loss of the fluorescence
function of the Nef-GFP fusion, the bioactivity of Nef does
appear to be affected by the fusion with GFP. The transduction
of VB cells by a retroviral vector expressing either native Nef or
the Nef-GFP fusion protein resulted in similar down-modula-
tion in surface CD4 (see Fig. 3C and 4C), and yet the steady
state concentration of Nef-GFP in the same cell line is an order
of magnitude higher than native Nef. The decrease in efficacy
for Nef-GFP to modulate CD4 (relative to native Nef) suggests
that the fusion of the GFP moiety to Nef may alter the ability
of the viral protein to associate with cellular targets. As
mentioned above, this fusion also increases the cellular half-life
of the viral protein. It is not known whether the relative short
half-life of Nef is related to its capacity to target surface mol-
ecules such as CD4 and MHC I to cellular degradative pro-
cesses; however, when expressed as a transmembrane fusion
protein with CD8, the cytosolic Nef moiety is found responsible
for targeted degradation (45).

Although we use the term “dosage” in our descriptions of how
a range of expressed Nef-GFP alters the level of constitutive
and induced surface antigens, we do not suggest that the gen-
erated data are representative of single binding site dose-
response curves. While such analyses permit a meaningful com-
parison of the numerous effector functions of Nef, these are not
ture dose-response curves. They are population analyses. There
is no assurance that any one cell would show this functional
relationship were it possible to do the experiment. An
obvious future direction will be to define the biochemical
mechanisms of dose dependence for the various functions. In
part this will involve identification of molecular targets and a
demonstration of relevant dose effects. Furthermore, HIV in-
fects a variety of other cells, which will possess an array of
different molecular targets.

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