HIV NEF-MEDIATED CELLULAR PHENOTYPES ARE DIFFERENTIALLY EXPRESSED AS A FUNCTION OF INTRACELLULAR NEF CONCENTRATIONS*

Running title: CONCENTRATION DEPENDENCY OF HIV NEF-MEDIATED EFFECTS

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Nef is a regulatory protein encoded by the genome of both HIV and SIV. Its expression in T cells leads to CD4 and MHC 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 MHC I, and a further three-fold increase is necessary to suppress T cell activation.
The role of the viral nef gene in HIV and SIV 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, while some have concluded that Nef increases T cell activity (8-11), others have demonstrated that Nef expression leads to suppression of T cell activation (12-14). 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 (St. Louis, MO). 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 Skowronska (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 Company, TX) as follows:
gf5:5’ATATGAATTCATGGCTAGCAAAGGAGAAGAACTCTTCACTGG-3’;
gf3:5’TTAGGATCCTCAATCGATGTTGTACAGTTCATCCATG-3’. Using gf5 and gf3 as primers and pQBI25 as the template, a PCR 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 Hpal and BamHI site of pLXSN, resulting in pLNefGFPSN.

Recombinant retrovirus containing LGFPSN and LNefGFPSN were derived from
the packaging line GP293 (Clontech, CA) according to the protocol of the manufacturer. Briefly, pLGFPSN and pLNefGFPSN were cotransfected with pVSV (encoding the VSV envelope) into GP293 cells, and virus was collected three days later. Retroviral vectors LXSN, LNL43SN, 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, NIH. Cells were grown in complete growth medium (RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 g/L sodium bicarbonate, 1 mM non-essential amino acids, 10 mM sodium pyruvate, 4 ul/L β-mercaptoethanol, and 50 μg/ml gentamicin, adjusted to pH 7.4). E6-1 T Cells were transfected with pQBI25 or pNA7GFP using GenePORTER Transfection Reagent (Gene Therapy Systems, CA) according to the manufacturer’s protocol. VB T cells were transfected with pQBI25 or pNA7GFP by electroporation (T820; BTX Electronic Genetics, CA) set at 200V, 65 msec, and 1 pulse. Cells (5 x 10^6/ml) were electroporated in RPMI 1640 (without fetal calf serum) with DNA at 30 μg/ml, room temperature. For transduction, cells were incubated with recombinant retroviruses carrying the expression vectors LXSN, LG2ASN, LNL43SN, LGFPSN, or LNefGFPSN, and treated the next day with 2 mg/ml of geneticin for E6-1 cells and 1mg/ml for VB T cells. After 7 days in selection, cells were returned to complete medium and whole populations of geneticin-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 countercurrent centrifugal elutriation by the Department of Transfusion Medicine at the National Institutes of Health (25), and purified as previously described (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-hour incubation period at 37°C the cells were washed in 20 ml of RPMI 1640 to remove unadsorbed virions. Cell pellets were resuspended in complete media at 2 x 10^6 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 ELISA (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 previously reported (10). Prior to lysis, cell count and volume were determined on a Coulter Z2 particle analyser. Defined numbers of cells were lysed in 1% Triton X-100 in 20 mM Tris (pH 8.0), 150 mM NaCl, 2.0 mM EDTA; supplemented with 1.0 ug/ml leupeptin, 1.0 ug/ml aprotinin, 1 ug/ml pepstatin A, and 250 ug/ml AEBSF, followed by protein determination by BCA microassay (Pierce Chemical, Rockford, IL). Nef was then precipitated by rabbit polyclonal anti-Nef (27) (obtained through the AIDS Research and Reference
Reagent Program, NAID, NIH) and run on SDS gel electrophoresis, blotted onto a cellulose nitrate membrane. This was probed with an anti-C-terminal Nef monoclonal antibody (Advanced Biotechnologies, Inc., Columbia, MD, catalog #13-160-100), followed by a secondary HRP-conjugated goat anti-mouse IgG (Kirkegaard & Perry Labs, Gaithersburg, MD), and then developed with a chemiluminescence substrate (SuperSignal West, Pierce Chemical, Rockford, IL). Quantitation of chemiluminescence was achieved with a cooled CCD camera system (ChemilImager, Alpha Innotech, San Leandro, CA). For detection of GFP and Nef-GFP the blots were probed with rabbit anti-sera against GFP (Santa Cruz Biotechnology, Santa Cruz, CA). To make each experimental Western blot of unknowns quantitative, recombinant protein standards were included.

*T Cell Activation*--T cells were activated and IL-2 was assayed, as previously described (10). For CD69 induction, cells (10^6/ml) were incubated with 3 ug/ml anti-CD3 mAb (clone HIT3a, PharMingen, CA) for 18 hours.

*Analysis of Flow Cytometric Data*--Data were acquired on a Becton Dickinson FACScan with CELLquest software. Histogram subtraction was accomplished with the same software on two populations of cells of equivalent cell number, and involved subtraction of cellular events of one histogram from events of another at each increment of fluorescence intensity. Only positive values are plotted in the resultant differential histogram. Because some of the analyses that we required were not available in the proprietary software, files were converted to Microsoft Excel format with FCS Assistant shareware (www.fscpress.com) and then to text suitable for input
to programs written in the Mathematica programming language and executed under
the Mathematica system (Wolfram Research, Inc., Champaign, IL). The data
consisted of pairs of numbers for each of the cells (n=300,000-700,000). The first of
these was proportional to the log of the fluorescence due to GFP or Nef-GFP and the
second was proportional to the log of the fluorescence of a surface marker for T cell
activation CD69, CD4, or HLA-A. A two dimensional 80 x 80 bin histogram was
formed, smoothed with a 3 x 3 running average and viewed as a contour plot with
GFP or Nef-GFP displayed along the x axis and the T cell antigens along the y axis.
Because the individual cell measurements were independent, each of the 80 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 either Nef from HIV NL4-3 or a mutant, non-myristylated NL4-3 Nef, generated by a glycine to 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 LXSN 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 CCD 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 indicated that the NL4-3 (FIG. 1A, lane 6) and the G2A (FIG. 1A, lane 7) Nef transduction of the Jurkat cell resulted in an expressed 2.0 ng Nef per 7 x 10^6 cells for both transductants. Thus, each transductant contained approximately 0.3 fg 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 0.3-1.0 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 in the Experimental Procedures section. 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 result 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 previously been demonstrated by this laboratory to enhance T cell activation and to modulate surface CD4 (10,20). 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 induction, which occurs to a majority of stimulated cells and is easily measured by flow cytometry. In contrast, IL-2 synthesis in these cells following CD3/CD28 stimulation is confined to only a few percent of the cell
population, and thus, a signal generated by intracellular staining of IL-2 is difficult to discriminate from the noise levels of flow cytometry. The CD69 induction pathway from the T cell receptor is essential for IL-2 production, but is not sufficient for T cell activation and IL-2 secretion (32,33). As shown in FIG. 3A, T cell receptor-mediated CD69 induction was unaffected by Nef expression from the retroviral vector. This suggests that the T cell receptor mediated activation pathway involved in the Nef-mediated enhancement of IL-2 may be distinguishable from the pathway leading to CD69 expression. This is reasonable, since there are numerous pathways leading to IL-2 expression (33). In all panels of FIG. 3 antigen measurements (CD4 or MHC I) in Nef-expressing cells are depicted by a solid line, whereas staining of non-transduced control cells is represented by the dashed line. The E6-1 Jurkat cells express high levels of HLA-A (MHC I); down-modulation of MHC I in Nef-transduced Jurkat cells (FIG. 3B) was absent. Thus, Nef expression from the retroviral vector did not significantly alter either surface MHC I or CD69.

We also examined Nef transduction of VB T cells, which like the E6-1 line, possess high surface expression of MHC I (in the form of HLA-A). Additionally, VB cells express a high level of CD4. The cells were stained for either CD4 (FIG. 3C) or MHC I (FIG. 3D). Nef expression from the retroviral vector resulted in CD4 down-modulation (FIG. 3C), but in the identical population there was no modulation of MHC I (FIG. 3D). CD4 modulation does not occur to all cells in the transduced population (see FIG. 3C and discussion below), but there is a 70% reduction in the mean fluorescence intensity for CD4 by an estimated 0.3 fg native Nef per VB cell (FIG.
Transduction with GFP and Nef-GFP expression vector—To permit a simultaneous measurement of surface antigen modulation with Nef concentration, we then constructed a Moloney LTR-based (LNefGFPSN) retrovirus for transduction of Nef-GFP. The Nef-GFP fusion protein had previously been shown to possess a biologically active Nef, the expressed levels of which correlated proportionally with CD4 modulation (21). Transduction of VB cells with either the Nef-GFP or a control (non-Nef) GFP vector yielded a bimodal distribution of cells either negative or positive for GFP (or for Nef-GFP) expression (FIG. 4). Each of the positive populations represented approximately 40% of the total population (see FIG. 4 legend). Instrumental measurements of fluorescence are in relative units and can fluctuate from day-to-day. To quantitate the generated fluorescence and permit comparisons of data, all flow cytometry experiments that measured GFP and the Nef-GFP fusion protein were co-run with standard beads with known Molecular Equivalents of Soluble Fluorochromes (MESF) of fluorescein. As shown in FIG. 4A 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 mode fluorescence of the Nef-GFP positive population in FIG. 4 was 67 relative fluorescent units (x-axis), which in this run was equivalent to $4.4 \times 10^4$ 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 previously been documented (18). That is, cells that have been selected for geneticin resistance (Neo; neomycin phosphotransferase) do not always co-express 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 which functionally display the presence of Nef. The modulation of CD4 in the Nef-transduced VB population of FIG. 3C 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 approximately 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 $1.22 \times 10^5$ 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 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 GFP per cell yielding a fluorescence intensity equal to $1.22 \times 10^5$ 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 $1.45 \times 10^5$ MESF units, would thus be
predicted to contain 20 fg 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 10^4 MESF units of Nef-GFP population of VB cells (FIG. 4) multiplied by the same factor (17 fg GFP/1.22 x 10^5 MESF) yields 6.1 fg GFP (12.2 fg 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. As shown in FIG. 5C and described in greater detail in the legend, we find that the GFP-positive VB cells express 23 fg GFP per cell (compared to the fluorescence-derived value of 20 fg), whereas the Nef-GFP positive cells express 13 fg Nef-GFP per cell (or the equivalent of 6.5 fg GFP per cell; compared to 6.1 fg as derived from fluorescence readings). The correlation of these estimates suggests that the cytometry determined fluorescence measurements closely predict the protein determination made by quantitative Westerns for both the GFP and Nef-GFP proteins, and that the molecular fluorescence of the Nef-GFP approximates that of GFP.

Another conclusion is that the difference in fluorescence intensity of the GFP and Nef-GFP transduced cells (compare x-axis values of GFP-positive cells in Fig. 4B to Nef-GFP-positive cells in 4D) corresponds to the difference in steady state concentrations of GFP and Nef-GFP. The 3.5-fold increase in GFP protein (23 fg in the
GFP cell vs 6.5 fg of GFP in the Nef-GFP cell) is comparable to the 3.2-fold increase in GFP mode fluorescence (214 vs 67 relative fluorescence units for GFP- and Nef-GFP-positive cells, respectively, in FIG. 4B 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 modulation of cell surface markers by flow cytometry.

Nef dosage effects on CD69, CD4, and MHC I expression--To examine the activity of higher Nef levels on the modulation of CD69 and MHC I, we transfected the E6-1 Jurkat line with a CMV promoter-based Nef-GFP-expression vector. The level of green fluorescence for both the Nef-GFP and the GFP (non-Nef) transfectant populations easily spanned 3 logs (FIG. 6A, B). The conditions used for transfection or electroporation resulted in a greater range of cellular expression levels of GFP and Nef-GFP, when compared to stable transductions with the retroviral vector (see FIG. 4). However, relative to the entire population, the number of GFP and Nef-GFP positive cells was greatly diminished. We found that with the conditions that resulted in uniform expression of GFP or Nef-GFP over a large range (see FIG. 6), fewer than 5% of cells within the transfected population became positive. The x-axis fluorescent intensity of 1000 relative fluorescence units in FIG. 6 corresponded to 1 x 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. 6A 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 x 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 Nef-GFP per cell. A one-log drop in CD69 is approached at Nef-GFP levels corresponding to 8 x 10^6 MESF (2.2 pg Nef-GFP per cell). PMA-induced CD69 expression for the GFP and Nef-GFP cells were examined by identical methods and plotted in FIG. 6D. For the PMA-induction of CD69, Nef-GFP has no discernable 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. 8A or B, respectively). Modulation of these receptors in the Nef-GFP cells was apparent (FIG. 8C 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 to 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 one-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 one 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 mechanistic differences (34-37). 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-fold 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, while 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 pathway. Moreover, since the multi-fold increase in IL-2 with Nef expression (10) is not reflected in the CD69 induction (compare FIG. 2 and 3), we also deduce that the Nef-dependent mechanism for enhancing T cell activation may not be part of the CD69 induction pathway.

While the concentration differences required for the Nef-mediated effects clearly describe different mechanisms, the dosage differences with Nef-GFP may be affected through steric inhibition by the attached GFP functionality. The role of the C-terminal alteration in these differential dosage effects is unclear; for example, mutational studies of the residues within the C-terminus of Nef (attachment site for GFP) are critical for both CD4 modulation and CD69 suppression (15). From the dose response curves in FIG. 8 the role of Nef in MHC I modulation would appear to be more restricted than in CD4 modulation. This work is consistent with previous efforts that have suggested that Nef possesses a higher efficacy in modulating CD4, when compared to the modulation of HLA antigen. Le Gall et al (35) studied the effect of a CMV-based Nef expression vector on CD4 and MHC I modulation. They found that an MHC I-transfected HeLa CD4 line required at least an order of magnitude less plasmid DNA to modulate surface CD4 compared to MHC I modulation. More recently, Walk et al (41) developed a cellular system in which the bioactivity of a pre-expressed Nef-fusion protein can be induced by exogenous
addition of a drug. They found that the required dosage of the specific drug, which uncovered the Nef activity responsible for CD4 modulation, was nearly two orders of magnitude less than that required for MHC I modulation. Our direct demonstration that higher levels of Nef-GFP are required to modulate MHC I than to modulate CD4, corroborates these previous studies. While these results seem to suggest that super-physiological levels of Nef are required for MHC I removal, this does not appear to be the case. It has been demonstrated that the efficient removal of MHC I from T cells by HIV is adversely affected by deletion of either the HIV nef (19) or the HIV vpu gene (42). That is, the Nef and Vpu proteins play critical co-factor roles in this process. If there is a concern, it is that the mechanism (by which high concentrations of Nef modulate MHC I) may be different than the biochemical role Nef plays in concert with Vpu during HIV infection. Moreover, in light of the previous demonstrations that Nef retroviral transduction of primary cells (10,11) or HIV infection (43) lead 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 suggests that the GFP moiety in the Nef fusion protein maintains 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. 4B and D). The decrease in steady-state concentration of Nef-
GFP may be related to the shorter half-life of Nef, which is approximately 12 hours (8), compared to the more stable GFP (44).

While 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-modulation 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 viral protein’s ability to association 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 molecules such as CD4 and MHC I to cellular degradative processes; 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 generated data are representative of single binding site dose response curves. While such analyses permit a meaningful comparison of the numerous effector functions of Nef, these are not true 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-dependency for the various functions. In part this will involve identification of molecular targets and a demonstration of relevant dose effects. Furthermore, HIV infects a variety of other cells, which will possess an array of different molecular targets.
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FOOTNOTES

1 Abbreviations: HIV, human immunodeficiency virus; SIV, simian 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; VSV, vesicular stomatitis virus; CCD, charge coupled device; LTR, retroviral long terminal repeat, used here to define promoter; MESF, molecular equivalents of soluble fluorochrome; HRP, horse radish peroxidase.
FIGURE LEGENDS

FIG. 1. **Western analysis of cellular Nef in transduced and HIV-infected cells.**  
A. The equivalent of $7 \times 10^6$ Jurkat T cells transduced with either NL4-3 Nef (lane 6), G2A NL4-3 Nef (lane 7) or non-transduced Jurkat cells (lane 5) were applied to SDS gel electrophoresis and blotted onto nitrocellulose. Nef was detected by monoclonal anti-Nef antibody with a chemiluminescence system. Recombinant NL4-3 Nef was applied at 10 ng (lane 1), 5 ng (lane 2), and 2.5 ng (lane 3) and 1.25 ng (lane 4).  
B. Nef detection in $10^7$ transduced VB cells (NL4-3 Nef, lane 5; G2A NL4-3, lane 6) or non-transduced cells (lane 7) with recombinant Nef at 10 ng (lane 1), 5 ng (lane 2), 2.5 ng (lane 3), and 1.25 ng (lane 4).  
C. Nef expression in NL4-3 HIV-infected primary CD4 T cells. Infected T cells were harvested on day 16. At this time approximately 50% of cells displayed cytopathology and p24 secretion was at peak levels. The equivalent of $10^6$ cells was applied to lane 3. Cells infected with Nef-negative NL4-3 DS HIV were similarly processed and placed in lane 4. Lanes 1 and 2 are 1 and 10 ng recombinant Nef, respectively.

FIG. 2. **T cell activation enhancement by Nef expression.** Nef expressing (Nef) E6-1 Jurkat T cells and control Jurkat cells (E6-1) were activated by CD3-CD28 beads for 18 hours and IL-2 secretion was measured by IL-2 ELISA. Data represent the mean of 4 determinations plus standard deviation. Control Jurkat
E6-1 cells and G2A Nef transduced cells secreted similar IL-2 levels (data not shown).

FIG. 3. **Surface expression of CD69, MHC I, and CD4 in transduced Nef-expressing cells.** Flow cytometry of Jurkat E6-1 cells (A and B) and VB cells (C and D) transduced with NL43 Nef retroviral expression vector. A. Nef-expressing Jurkat cells were stained with anti-CD69 antibody following T cell receptor stimulation with anti-CD3 antibody (solid line). Activated non-Nef control cells are represented by the dashed line, whereas CD69 levels for the non-activated cells are represented by the dotted line. B. Nef-expressing Jurkat cells were stained with anti-MHC I antibody (solid line) and are compared to non-Nef controls (dashed line). Dotted line represents the isotype control for Nef-expressing cells. C. Flow cytometry of VB cells transduced with NL4-3 Nef and stained for either CD4 (solid line) or isotype 2A (dotted line). CD4 staining for the non-Nef VB control cell is represented by the dashed line. D. Nef-expressing VB cells were stained with either the anti-MHC I antibody (solid line) or the isotype 2A control antibody (dotted line). Control cell staining for MHC I is represented by the dashed line.

FIG. 4. **CD4 and MHC I in VB cells transduced with GFP and Nef-GFP.** VB cells were transduced with either a GFP (A and B) or Nef-GFP (C and D) retroviral expression vector and then stained for either CD4 (A and C) or MHC I
(B and D). In these contour plots cell populations were either positive or negative (+ or -) for expression of GFP or the GFP Nef-GFP fusion product. For the GFP transductant 44% of the cellular population is positive; the Nef-GFP population is 38% positive.

FIG. 5. **Measurement of GFP and Nef-GFP expression in cells.** A. GFP-positive Jurkat E6-1 cell clone (solid line) compared to control E6-1 cell (dashed line). The clone yielded a mean fluorescence intensity equivalent to $1.22 \times 10^5$ 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 $6 \times 10^5$ 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. 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.

FIG. 7. **Modulation of MHC I in Jurkat cells as a function of Nef-GFP concentration.** Jurkat cells were transfected with the GFP (dashed lines) or Nef-GFP (solid lines) vectors as in FIG. 6, and stained for MHC I expression. Mean MHC I values are plotted and banded by 95% confidence levels as in FIG. 6.

FIG. 8. **Modulation of MHC I and CD4 in VB cells as a function of Nef-GFP concentration.** VB cells were electroporated with the GFP (A and B) or Nef-GFP (C and D) vectors as in FIG. 6, and stained for MHC I (A and C) and CD4 (B and D) expression. At least 500,000 events were processed. Cell data from panels C and D (Nef-GFP) were averaged as in FIG. 6; Nef-GFP modulation of CD4 is depicted by solid line and MHC I by dashed line (mean with 95% confidence levels).
Figure 3/ Liu et al

**Jurkat E6-1**

- **A**: Cell Number vs. CD69
- **B**: Cell Number vs. MHC I

**VB**

- **C**: Cell Number vs. CD4
- **D**: Cell Number vs. MHC I
Figure 6/ Liu et al

CD69 (T Cell Activation)

GFP

Nef-GFP

CD3-Stimulated

PMA-Stimulated

Green Fluorescence
Figure 7/ Liu et al

Gene Fluorescence

MHC I

Green Fluorescence

GFP

Nef-GFP
Figure 8/ Liu et al

Nef-GFP

CD4

MHC I

Surface Antigen

Green Fluorescence

MHC I (HLA-A)

CD4

GFP

Nef-GFP
HIV NEF-mediated cellular phenotypes are differentially expressed as a function of intracellular NEF concentrations
Xunxian Liu, Jeffrey A. Schrager, G. David Lange and John W. Marsh

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