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Human Immunodeficiency Virus Type 1 Nef-Induced Down-Modulation of CD4 Is Due to Rapid Internalization and Degradation of Surface CD4

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Human immunodeficiency virus type 1 (HIV-1) Nef is a myristylated protein with a relative molecular mass of 27 kDa, is localized to the cytoplasmic surfaces of cellular membranes, and has been reported to down-modulate CD4 in human T cells. To understand the mechanism of HIV-1 Nef-mediated down-modulation of cell surface CD4, we expressed Nef protein in human T-cell line VB. Expression of HIV-1 Nef protein down-modulated surface CD4 molecules. In pulse-chase experiments, CD4 molecules in Nef-expressing cells were synthesized at normal levels. However, the bulk of newly synthesized CD4 protein was degraded with a half-life of approximately 6 h, compared with the 24-h half-life in control cells. This Nef-induced acceleration of CD4 turnover was inhibited by lysosomotropic agents NH_4Cl and chloroquine as well as by the protease inhibitor leupeptin. Surface CD4 biotinylation experiments demonstrated that CD4 molecules in Nef-expressing T cells are transported to the plasma membrane with normal kinetics but are then rapidly internalized. Therefore, HIV-1 Nef-induced down-modulation of CD4 is due to rapid internalization of surface CD4 and subsequent degradation by an acid-dependent process, potentially lysosomal. Additionally, in a Nef-expressing cell, we find accelerated dissociation of the T-cell tyrosine kinase $p56^{lck}$ and CD4 but only after the complex reaches the plasma membrane. This implies that HIV-1 Nef protein might play a role in triggering a series of T-cell activation-like events, which contribute to $p56^{lck}$ dissociation and internalization of surface CD4 molecules.

Human immunodeficiency virus type 1 (HIV-1), the etiologic agent of AIDS, possesses at least six genes coding for regulatory proteins in addition to the typical retroviral genes (*gag*, *pol*, and *env*). In the past decade, these gene products have been intensively studied to understand their roles during HIV-1 infection. However, the precise function of these regulatory proteins in HIV-1 replication and pathogenesis in virus-infected individuals remains unclear, even though our understanding has greatly increased.

One of the regulatory genes, the *nef* gene, was first identified as an open reading frame of 648 bp near the 3′ end of the genome, partially overlapping the U3 region (49, 64). It encodes an approximately 27-kDa protein covalently modified with the amino-terminal myristic acid from multiply spliced mRNA transcripts (4, 18). After viral infection, Nef proteins are expressed from the most abundant, multiply spliced mRNA, up to 80% of the total early viral transcripts, and are found predominantly in the cytoplasm associated with the plasma membrane (52). In vivo expression of Nef protein was supported by detection of antibodies specific to this protein in HIV-1- and -2-infected individuals and simian immunodeficiency virus (SIV)-infected monkeys (5, 48).

The open reading frame of the *nef* gene is well retained among all primate lentiviruses (HIV-1, HIV-2, and SIV) with highly conserved amino acid sequences, although amino acid variations with an in-frame termination codon can be as high as 17% in laboratory isolates of HIV-1 (4, 14, 50). Blumberg et al. (9), using targeted PCR amplification and DNA sequencing, showed that the *nef* gene is normally open in clones derived directly from pathologic brain and spleen tissues of HIV-1-infected children and adults. This selective pressure for the functional *nef* gene suggested that HIV-1 Nef protein may play an important role during in vivo infection, as has been noted with the SIV system by Kestler et al. (24). In that study, it was suggested that the *nef* gene product of SIVmac functions as a positive effector that is required for a high viral load and disease progression in infected macaques. Furthermore, it has also been demonstrated that HIV-1 Nef protein in a cell model with primary lymphocytes and macrophages plays a positive role in promoting viral infection and replication (15, 41, 59). In contrast, in transformed human T-cell lines, Nef protein did not have a positive effect on viral replication (19, 25).

In addition to the in vivo biological function, many biochemical activities have been ascribed to Nef protein. The earliest studies suggested that HIV-1 Nef proteins function as a negative regulatory factor by repressing long terminal repeat-driven transcription (2, 32, 34, 43). However, subsequent studies by Kim et al. (25) and Hammes et al. (19) have failed to support these findings. Additionally, Nef protein was described as binding GTP and possessing GTPase activity (18), which was also subsequently refuted (23, 37, 42). Reproducible, somewhat less conflicting in vitro studies have shown that HIV-1 and SIV Nef proteins down-regulate the levels of cell surface CD4 in human T-cell lines, such as CEM, HPB-All, and HUT78, as well as in the monocytic cell line U937 (7, 17, 35, 60). Furthermore, murine and simian CD4 molecules on the cell surfaces of murine T-cell lines (16) as well as murine CD4 of T cells from transgenic mice (57) were shown to be down-modulated by HIV-1 Nef expression. These results suggested that Nef proteins utilize a mechanism of action commonly shared among different species, although the precise mechanism remains undefined.

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The CD4 molecule is a glycoprotein of approximately 55 kDa that consists of extracellular domains homologous to immunoglobulin variable and joining regions, a hydrophobic transmembrane domain, and a highly charged cytoplasmic domain. The cytoplasmic domain of human CD4, consisting of 38 amino acids, is highly conserved among human, murine, and rat CD4 molecules. To better understand the mechanism of HIV-1 Nef-mediated down-modulation of this molecule, human $CD4^+$ T-cell line VB was stably transduced with amphotropic retroviral vectors designed to express HIV-1 Nef. In this report, we demonstrate that the kinetics of CD4 synthesis and transport to the plasma membrane are unaltered by the presence of Nef. Nef-mediated CD4 down-modulation is found to be due to rapid internalization of surface CD4 and subsequent degradation in an intracellular lysosomal compartment. Furthermore, through surface labeling of CD4 molecules and assessment of $p56^{lck}$ association, we show that the Nef-mediated process leading to CD4 down-modulation includes accelerated dissociation of the CD4-$p56^{lck}$ complex and that this dissociation is initiated after the complex reaches the plasma membrane.

MATERIALS AND METHODS

DNAs and cells. To express HIV-1 Nef protein in human T cells, a 1.0-kbp *nef* gene fragment of HIV-1 SF2 clone (kindly provided by Jay A. Levy, University of California, San Francisco) was cloned into the murine retrovirus expression vector pLXSN (40). The resulting plasmid was designated pNEF-SF2. The control plasmid pFEN-SF2 was obtained by inserting the fragment in an antisense orientation.

Both plasmid DNAs were linearized and transfected into the amphotropic retrovirus packaging cell line PA317 (ATCC CRL 9078) by the modified calcium phosphate precipitation method described by Chen and Okayama (11). Vector-expressing PA317 cell lines were selected in medium containing 0.75 mg of the antibiotic G418 per ml, and infectious amphotropic vector-containing culture media were used to infect human T-cell lymphoma VB (the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health) in the presence of 4.0 μg of polybrene per ml. Vector-expressing VB cell lines were grown in medium containing 1.0 mg of G418 per ml and were designated VB_{CON} and VB_{NEF}.

Analysis of surface CD4 expression. Flow cytometric analysis (fluorescence-activated cell sorter [FACS] analysis) was performed on a Becton Dickinson FACScan with Lysis II software to assess CD4 expression on cell surfaces. Half a million exponentially growing cells were labeled with fluorescein-conjugated anti-CD4 antibody (Olympus Immunochemicals, Lake Success, N.Y.). Debris and nonviable cells were gated out by forward scatter and the addition of propodium iodide, respectively.

Radiolabeling, immunoprecipitation, and gel electrophoresis analysis. Exponentially growing cells were starved for 30 min in cysteine- and methionine-free RPMI 1640 (ICN Biomedicals, Costa Mesa, Calif.) and then pulse-labeled for 30 min in labeling medium containing ^{35}S-labeled methionine and cysteine (0.1 mCi/ml, 1,000 Ci/mmol) (Tran^{35}S-label; ICN Biomedicals). Pulse-labeled cells were washed in complete growth medium and aliquoted for the chase. Immediately after labeling or the chase, individual cell aliquots were washed in cold phosphate-buffered saline (PBS) (pH 7.4) and then lysed in PBS containing 0.1% sodium dodecyl sulfate (SDS), 0.5% deoxycholate, 1.0% Triton X-100, 1 μg of leupeptin per ml, 1 μg of aprotinin per ml, and 1 mM phenylmethylsulfonyl fluoride (RIPA buffer).

Radiolabeled CD4, Nef protein, and $p56^{lck}$ were immunoprecipitated with rabbit anti-CD4 antibody (American Biotechnology, Cambridge, Mass.), rabbit anti-Nef antibody (the AIDS Research and Reference Reagent Program 331, Division of AIDS, National Institute of Allergy and Infectious Diseases), and rabbit anti-$p56^{lck}$ antibody (a gift from Mark Egerton, National Institutes of Health, Bethesda, Md.), respectively. The immune complex was precipitated with fixed *Staphylococcus aureus* cells and then separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (51).

Radioactivity incorporated into protein was quantified by exposing the dried gel to a storage phosphor screen and scanning the screen with a Fuji Bas2000.

Cell surface CD4 biotinylation. Biotinylation of cell surface CD4 was carried out with sulfo-NHS-biotin (Pierce Chemical Co., Rockford, Ill.) as described previously with some modifications (31). Briefly, VB_{CON} and VB_{NEF} cells were pulse-labeled for 30 min and chased for 1, 2, and 4 h in complete growth medium as described above. Cells were pelleted, washed once in ice-cold PBS supplemented with 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$ (PBS-plus), and resuspended in freshly prepared 1 mg of sulfo-NHS-biotin per ml in PBS-plus at 10^7 cells per ml. After binding for 1 h at 4°C with constant gentle agitation, cells were collected by centrifugation and washed once in serum-free RPMI 1640 and twice in PBS-plus. Total radiolabeled CD4 molecules were precipitated with rabbit anti-CD4 antiserum, and then biotinylated surface CD4 proteins were reprecipitated with streptavidin-linked magnetic beads (Dynabeads; Bioproducts for Science, Indianapolis, Ind.).

Coimmunoprecipitation of $p56^{lck}$ with surface CD4. To detect $p56^{lck}$ associated with cell surface CD4, cells were pulse-chased and incubated in serum-free RPMI 1640 with anti-CD4 monoclonal antibody (HT3.10-28, kindly provided by Peter Rieber, University of Munich, Munich, Germany) for 1 h at 4°C with constant gentle agitation as described above. Cells were lysed in NTNE buffer (1% Nonidet P-40, 20 mM Tris hydrochloride [pH 8.4], 0.15 M NaCl, 2.0 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) on ice, and then excess unlabeled cell lysates were added to the lysates. CD4 complexes (CD4 and CD4-associated proteins) coupled with antibody were precipitated with protein G-agarose (Genex Corp., Gaithersburg, Md.). The immune precipitates were divided in half and reimmunoprecipitated with fixed *S. aureus* cells coupled with either anti-CD4 antibody or anti-$p56^{lck}$ antibody.

RESULTS

Expression of HIV-1 Nef protein in human T cells. To understand the mechanism by which HIV-1 Nef proteins down-regulate the expression of cell surface CD4, the *nef* gene fragment was obtained from HIV-1 isolate SF2 and stably transduced into human T-cell lymphoma VB with an amphotropic retroviral vector as described in Materials and Methods. Two G418-resistant cell populations, expressing either the *nef* gene-containing vector pNEF-SF2 or the control vector pFEN-SF2, were designated VB_{NEF} and VB_{CON}, respectively.

In order to determine whether Nef protein is synthesized in VB_{NEF} cells, cellular proteins were radiolabeled with [^{35}S]methionine-cysteine for 30 min and Nef protein was immunoprecipitated with rabbit anti-Nef antibody (Fig. 1A). A band of approximately 27 kDa, corresponding to Nef protein, could be seen only in the lysates of VB_{NEF} cells, confirming expression

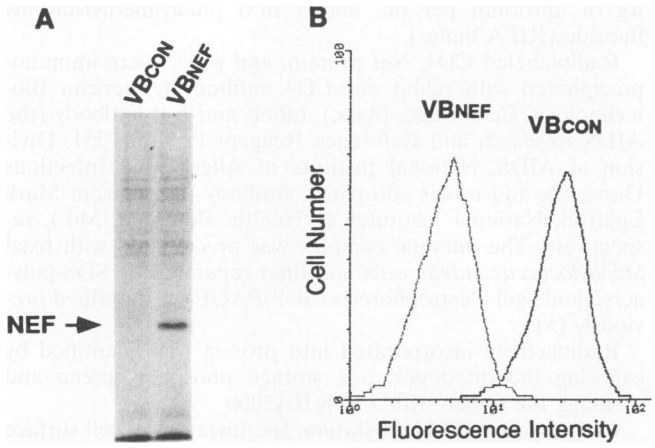

FIG. 1. Nef expression and surface CD4 down-modulation in Nef-expressing VB cells. To express HIV-1 Nef protein in human T-cell line VB, cells were stably transduced with the HIV-1 SF2 *nef* gene by using the murine retrovirus expression vector pLXSN. (A) In order to determine whether Nef protein was synthesized, cell lines VB_{CON} and VB_{NEF}, expressing only the *neo* gene or both the *nef* and *neo* genes, respectively, were pulse-labeled for 30 min with [^{35}S]methionine-cysteine (100 μCi/ml). Nef protein was immunoprecipitated with rabbit anti-Nef antiserum and then separated by SDS-PAGE (10% polyacrylamide). In VB_{NEF} cells, a band corresponding to Nef protein with a relative molecular mass of 27 kDa is visible (indicated by an arrow) while no band is detected in VB_{CON} cells. (B) Surface CD4 down-modulation in VB_{NEF} cells was determined by FACS analysis. Cells were directly stained with fluorescein-conjugated anti-CD4 antibody and then analyzed with a FACScan. A significant reduction in fluorescence intensity in VB_{NEF} cells with 20 to 30% of surface CD4 labeling is observed, compared with that in VB_{CON} cells.

of the 27-kDa HIV-1 Nef protein in VB cells. Pulse-labeled cells were further chased for 2 to 24 h (data not shown). In VB cells, Nef protein is efficiently synthesized and very stable, with a half-life of approximately 24 h.

Nef protein has been shown to down-modulate surface expression of CD4 in human T-cell lines. The human T-cell line VB expresses high levels of CD4 on cell surfaces and has been reported to be susceptible to HIV-1 infection in a manner that approximates infection in nontransformed human T helper/inducer cells (29). It was, therefore, of interest to determine whether Nef protein is functional in VB cells. Cells were directly stained with fluorescein-conjugated anti-CD4 monoclonal antibody and analyzed with a FACScan (Fig. 1B) as described in Materials and Methods. In VB_{NEF} cells, CD4 surface expression is also down-modulated; there is a 70 to 80% drop in fluorescence intensity compared with that of VB_{CON} cells. The effect of Nef on CD4 in VB cells is specific since no significant reduction in surface expression of CD3 and HLA-ABC was observed (data not shown).

In Nef-expressing cells, CD4 protein is synthesized at normal levels but degraded rapidly. Previously, Nef protein has been reported to have no effect on the steady-state levels of CD4 mRNA and CD4 protein (7, 17). However, it is not clear whether CD4 proteins are synthesized and turned over under normal kinetics in Nef-expressing cells. Therefore, we analyzed the fates of newly synthesized CD4 molecules by a pulse-chase experiment. Cells were pulse-labeled with [^{35}S]methionine-cysteine for 30 min and chased for 2, 4, 6, and 24 h. After each time point, cells were lysed and then divided in half to precipitate CD4 and $p56^{lck}$ with either anti-CD4 or anti-$p56^{lck}$ antisera, respectively. The results of this experiment are shown in Fig. 2. Since $p56^{lck}$ is stable with a half-life of 32 h and is not modulated in Nef-expressing cells (Fig. 2B), $p56^{lck}$ was used as an internal control in this experiment. Quantifying and comparing the amounts of CD4 and $p56^{lck}$ at each time point by phosphor imaging demonstrated that at the end of the pulse both cell populations had synthesized equivalent levels of CD4 protein (Fig. 2A, lanes 0).

In control (VB_{CON}) cells, no significant proportion of pulse-labeled CD4 proteins were lost during the 6-h chase (Fig. 2A, lanes 0 to 6) and after the 24-h chase, approximately 50% remained (lane 24). Thus, CD4 protein has a half-life of approximately 1 day. In contrast, in VB_{NEF} cells, CD4 degradation became evident following a 2-h lag after the pulse (Fig. 2A, lanes 0 and 2) and after that the loss of protein is well described by a single exponential function with an approximate 6-h half-life (lanes 4 to 24).

These results suggest that Nef expression in VB cells triggers a rapid degradation of CD4 molecules, which results in down-modulation of CD4 surface expression on Nef-expressing T cells. However, it is important to rule out the possibility that this observed loss of CD4 was due to the secretion of CD4 into the medium or to a failure to solubilize CD4. In order to demonstrate that this loss can be attributed mostly to lysosomal degradation, pulse-chase experiments were carried out in the absence (Fig. 3, lanes 1 and 2) and presence of inhibitors of lysosomal function: a protease inhibitor to inhibit lysosomal proteolysis, leupeptin (100 μg/ml, lanes 3), and weak-base amines to neutralize lysosomal acidity, 50 mM NH_4Cl (lanes 4) and 100 μM chloroquine (lanes 5). As shown in Fig. 3, after the 6-h chase, the loss of radiolabeled CD4 in VB_{NEF} cells (approximately 50%) (lane 2) was suppressed by the addition of individual inhibitors to the chase medium (lanes 3 to 5). Thus, CD4 protein in Nef-expressing cells is routed to a low pH vesicular compartment, presumably the lysosomes, and subsequently degraded.

Newly synthesized CD4 glycoproteins are transported to the plasma membrane with normal kinetics but internalized rapidly. Previous studies by Jabbar and Nayak (22), and Crise et al. (12) showed that CD4 and HIV-1 envelope glycoprotein can interact in the endoplasmic reticulum (ER) and that this process can lead to the retention of both proteins within the ER. Thus, the loss of surface CD4 in cells infected with HIV-1 has been proposed to be a consequence of altered intracellular localization of this protein. Therefore, we examined whether CD4 was retained in the ER of Nef-expressing cells.

Since CD4 is a glycoprotein with two potential N-linked oligosaccharide side chains, the transport of this protein from the ER to the Golgi apparatus was monitored by following the acquisition of resistance to digestion by endoglycosidase H (endo H). Both VB_{NEF} and VB_{CON} cells were pulse-labeled for 30 min and chased for 2 h, and cell lysates were treated with endo H as described previously (51). A majority of pulse-labeled CD4 molecules from both cells were susceptible to endo H digestion but became resistant within 2 h (data not shown), implying that CD4 transport from the ER to the Golgi apparatus is not blocked by the presence of Nef. Therefore, in VB_{NEF} cells, the loss of CD4 is not due to retention and degradation of the molecule within the ER.

We examined whether CD4 glycoproteins in a Nef-expressing cell are first transported to the plasma membrane and then internalized and delivered to the lysosome, as has been noted for several lysosomal membrane proteins (10, 30). Because surface expression of CD4 at any instant in Nef-expressing cells is substantially reduced, we performed a combined experiment of pulse-chase labeling and surface protein labeling with biotin (Fig. 4), as described in Materials and Methods.

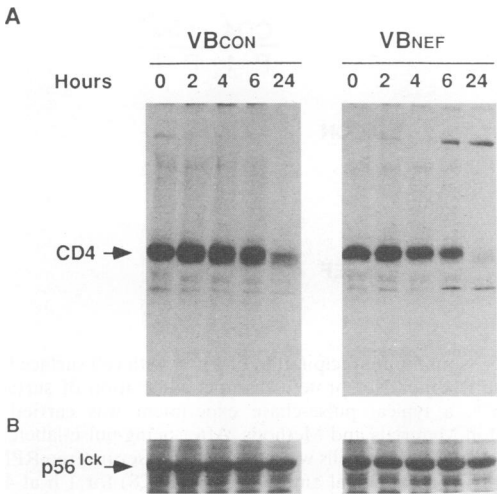

FIG. 2. Kinetics of CD4 protein turnover in control and Nef-expressing human T cells. To determine the rate of CD4 synthesis and its turnover, VB_{CON} and VB_{NEF} cells were pulse-labeled for 30 min with [^{35}S]methionine-cysteine (100 μCi/ml) and chased for 2, 4, 6, and 24 h in complete growth medium. Radiolabeled CD4 and $p56^{lck}$ were immunoprecipitated with either rabbit anti-CD4 or anti-$p56^{lck}$ antiserum, respectively, and then separated by SDS-PAGE (10% polyacrylamide). (A) In control (VB_{CON}) cells, pulse-labeled CD4 (indicated by an arrow) (lane 0) is detected without significant loss during 2-, 4-, and 6-h chases (lanes 2 to 6), and following the 24-h chase, 50% is still detectable. In Nef-expressing (VB_{NEF}) cells, the amount of pulse-labeled CD4 is measured to be equivalent to that in VB_{CON} cells (lane 0) and remains without any loss during the 2-h chase (lane 2). However, following a 2-h lag, a gradual decrease in the intensity of the CD4 band can be observed after 4- and 6-h chases (lanes 4 and 6), with one-half of the pulse-labeled CD4 found after the 6-h chase (lane 6). Following the 24-h chase (lane 24), pulse-labeled CD4 is hardly detectable. (B) In both cell lines, $p56^{lck}$ (indicated by an arrow) is stably expressed and turned over with similar kinetics: the half-life of the protein is >32 h. Lane designations are the same as those for panel A.

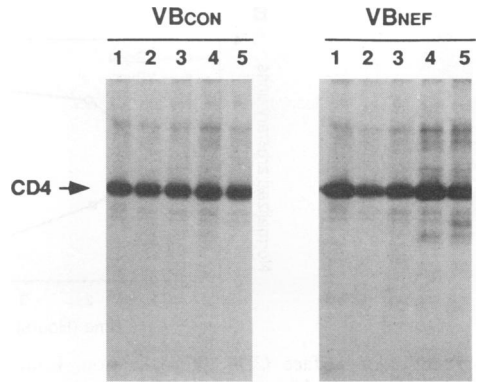

FIG. 3. Effects of leupeptin, NH_4Cl, and chloroquine on CD4 degradation. Pulse-chase experiments were performed to determine whether the loss of CD4 during the chase in VB_{NEF} cells, shown in Fig. 2, resulted from lysosomal degradation of the protein. VB_{CON} and VB_{NEF} cells were pulse-labeled for 30 min with [^{35}S]methionine-cysteine (100 μCi/ml) (lanes 1) and chased for 6 h in the absence (lanes 2) and presence of 100 μg of leupeptin per ml (lanes 3), 50 mM NH_4Cl (lanes 4), or 100 μM chloroquine (lanes 5). CD4 was immunoprecipitated with rabbit anti-CD4 antiserum and separated by SDS-PAGE (10% polyacrylamide). In VB_{NEF} cells, following the 6-h chase (lane 2), the amount of radiolabeled CD4 (indicated by an arrow) is reduced to 50% of the level after the pulse (lane 1). However, this drastic reduction could not be detected in the presence of 100 μg of leupeptin per ml (80 to 85% of pulse-labeled CD4) (lane 3), 50 mM NH_4Cl (nearly 100%) (lane 4), or 100 μM chloroquine (>90%) (lane 5). Signals for CD4 in VB_{CON} cells can be seen at equivalent levels in the lanes after a pulse (lane 1) and 6-h chases without (lane 2) or with individual inhibitors (lanes 3 to 5).

In non-Nef-expressing cells (VB_{CON}), about 20% of newly synthesized CD4 proteins were already delivered to cell surfaces during the 30-min pulse-labeling (Fig. 4A, lane 0) and the percentage increased over the chases (Fig. 4B): 40 to 45% had reached the cell surface 1 h after synthesis (Fig. 4A, lane 1) and 60 to 70% after the 2-h chase (lane 2). Following the 4-h chase, no significant increase in surface CD4 was observed and the levels of radiolabeled CD4 were maintained at 65 to 75% (Fig. 4A, lane 4). Similar levels of pulse-labeled CD4 were found on the surface of VB_{NEF} cells after the 30-min pulse (about 20%) (Fig. 4A, lane 0) and after the 1-h chase (35 to 40%) (lane 1). However, a significant loss in labeled CD4 was observed following the 2-h chase (about 25% of pulse-labeled CD4) (Fig. 4A, lane 2). After the 4-h chase, pulse-labeled CD4 was hardly detected on the surface (less than 10%) (Fig. 4A, lane 4). Thus, Nef expression resulted in a 60% drop (relative to that of control cells) in surface residency after the 2-h chase, and surface residency decreased further, a nearly 90% drop from that of the controls, by the 4-h chase.

The implications of the above results are twofold. First, shortly after synthesis, CD4 molecules in VB_{NEF} cells were delivered to cell surfaces and accumulated there with kinetics similar to those observed in the absence of Nef protein. Thus, we can conclude that Nef expression per se has no effect on CD4 transport from the ER to the plasma membrane. Secondly, the turnover of newly synthesized cell surface CD4 in a Nef-expressing cell is even more dramatic than the decrease in half-life for total cellular CD4. HIV-1 Nef protein appears to induce rapid internalization of cell surface CD4, but this surface phenomenon appears to involve a 1- to 2-h lag, consistent with the observation described in the legend to Fig. 2, in which newly synthesized CD4 was not subject to degradation within the 2-h chase in VB_{NEF} cells.

Prior to internalization in a Nef-expressing cell, surface CD4 molecules dissociate from $p56^{lck}$. In T lymphocytes, a CD4 molecule is noncovalently associated with the protein tyrosine kinase $p56^{lck}$ via the cytoplasmic tail of CD4 and the amino terminus of $p56^{lck}$ (55, 61, 63). Formation of the CD4-$p56^{lck}$ complex has been reported to occur in the ER (13). After exposure of $CD4^+$ T cells to either phorbol esters or appropriate antigen-presenting cells, surface CD4 molecules are rapidly internalized (1, 8, 20, 56). Furthermore, it has recently been shown that disruption of the CD4-$p56^{lck}$ complex precedes internalization of CD4 (21, 58), leading to the proposal that association with $p56^{lck}$ may function to keep CD4 molecules on cell surfaces and that perturbation of this association may provide a trigger to initiate internalization of CD4. However, the mechanism of dissociation of $p56^{lck}$ from the CD4 molecule remains unclear. Therefore, it was of interest to determine whether Nef protein disrupts association of surface CD4 with $p56^{lck}$.

Lack of CD4 accumulation on the surfaces of Nef-expressing cells led us to design an experiment in which we could trace newly surface-delivered CD4 and its association with $p56^{lck}$ before endocytosis occurred. Our previous results, both with VB_{CON} cells and with VB_{NEF} cells, demonstrated that 1 h after synthesis 40 to 45% of total CD4 molecules are transported to

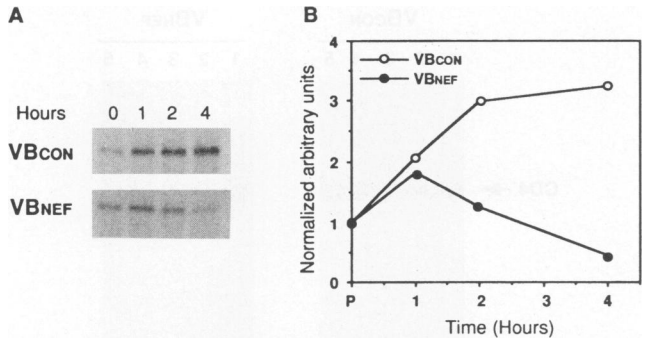

FIG. 4. Kinetics of surface CD4 internalization. Endocytosis of surface CD4 was determined by a combined experiment of pulse-chase labeling and surface protein labeling with biotin as described in Materials and Methods. Cells were pulse-labeled for 30 min with [^{35}S]methionine-cysteine (100 μCi/ml), chased for 1, 2, and 4 h in complete growth medium, and then incubated with freshly prepared 1 mg of sulfo-NHS-biotin per ml in PBS-plus at 10^7 cells per ml for 1 h at 4°C with constant gentle agitation. Cells were lysed, and total CD4 molecules were immunoprecipitated with rabbit anti-CD4 antiserum. CD4 was recovered from the immune precipitates by boiling in sample buffer, and biotinylated CD4 proteins were reprecipitated with streptavidin-linked magnetic beads and separated by SDS-PAGE (10% polyacrylamide) (A). The amount of radiolabeled CD4 was measured by phosphor imaging and normalized by the amount of total pulse-labeled CD4. To simplify the presentation of data, the initial fraction of CD4 on the surface at time zero (pulse period, P) was assigned an arbitrary unit of 1. The change in the amount of surface CD4 was then plotted against time (B). (A and B) After pulse-labeling, radiolabeled CD4 can be detected on cell surfaces in both cell lines, which contain approximately 20% of the total radiolabeled CD4 (lanes 0). During the chases (panel A, lanes 1 to 4), increasing amounts of CD4 can be seen in VB_{CON} cells: 40 to 45% after the 1-h chase (lane 1) and 60 to 70% after the 2-h chase (lane 2). Following the 4-h chase, no significant increase in surface CD4 is observed, but the levels of radiolabeled CD4 remain at 65 to 75% (lane 4). The levels of pulse-labeled CD4 detected on the surfaces of VB_{NEF} cells after the 30-min pulse are similar (about 20%) (lane 0); furthermore, an increasing amount of surface CD4 can be observed after the 1-h chase (35 to 40%) (lane 1). However, significant losses of labeled CD4 are observed following 2- and 4-h chases: about 25% after the 2-h chase (lane 2) and less than 10% after the 4-h chase (lane 4).

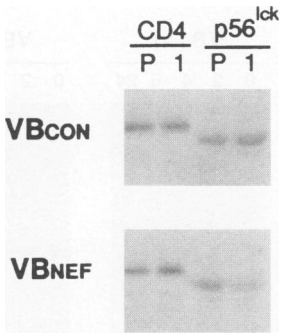

FIG. 5. Coimmunoprecipitation of $p56^{lck}$ with cell surface CD4. To determine whether Nef protein disrupts association of surface CD4 with $p56^{lck}$, a typical pulse-chase experiment was carried out as described in Materials and Methods. After being pulse-labeled for 30 min and chased for 1 h, cells were incubated in serum-free RPMI 1640 with anti-CD4 monoclonal antibody (HT3.10-28) for 1 h at 4°C with constant gentle agitation and then solubilized in NTNE buffer (1% Nonidet P-40, 20 mM Tris hydrochloride [pH 8.4], 0.15 M NaCl, 2.0 mM EDTA, 1 mM phenylmethylsulfonyl fluoride). Surface CD4 complexes (CD4 and CD4-associated proteins) coupled with antibody were precipitated with protein G-agarose and recovered by boiling in sample buffer. Samples were divided in half, and CD4 and $p56^{lck}$ were reimmunoprecipitated with anti-CD4 antibody and anti-$p56^{lck}$ antibody, respectively. In VB_{CON} cells during the pulse, pulse-labeled cell surface CD4 molecules are coprecipitated with pulse-labeled $p56^{lck}$ proteins at a ratio of almost 1:0.8 to 0.9 of CD4 to $p56^{lck}$ for radioactivity incorporated into the proteins (lanes P). After the 1-h chase, more CD4 and $p56^{lck}$ can be detected but the ratio remains unchanged (lanes 1). In VB_{NEF} cells following the pulse, CD4 and $p56^{lck}$ can be observed at a ratio similar to that in VB_{CON} cells (lanes P). However, following the 1-h chase (lanes 1), an increasing amount of cell surface CD4 can be seen with a decreasing amount of $p56^{lck}$, generating a drop in the CD4/$p56^{lck}$ ratio to 1:0.4.

the plasma membrane and retained on the cell surface. No significant endocytosis of surface CD4 was observed during the first 1-h chase in a Nef-expressing cell, even though most was internalized within the next 1-h chase. After being pulse-labeled for 30 min and chased for 1 h, cells were incubated with anti-CD4 monoclonal antibody for 1 h at 4°C, as described in Materials and Methods. Surface CD4 complexes (CD4 and CD4-associated proteins) coupled with antibody were precipitated and resuspended, and then CD4 and $p56^{lck}$ were separately reimmunoprecipitated with anti-CD4 and anti-$p56^{lck}$ antibody, respectively.

The results of this experiment are shown in Fig. 5. In VB_{CON} cells, cell surface CD4 molecules synthesized during the pulse were coprecipitated with pulse-labeled $p56^{lck}$ proteins at a ratio of almost 1:0.8 to 0.9 of CD4 to $p56^{lck}$ for radioactivity incorporated into proteins (Fig. 5, lanes P). After the 1-h chase, more CD4 and $p56^{lck}$ were detected but the ratio remained unchanged (Fig. 5, lanes 1). Intracellular CD4 was separately coimmunoprecipitated with $p56^{lck}$ to demonstrate that association of CD4 with $p56^{lck}$ was observed at the same ratio as that seen with surface CD4 (data not shown). This is well corroborated by the results reported by Crise and Rose (13) that CD4 molecules associate with $p56^{lck}$ in the ER and are transferred to and presented on the plasma membrane as the CD4-$p56^{lck}$ complex forms.

Shortly after synthesis, CD4 in the presence of Nef protein interacted with $p56^{lck}$ and was delivered to the membrane at the normal molar ratio (VB_{NEF}; Fig. 5, lanes P). Also, internal CD4 was detected with $p56^{lck}$ at the same ratio (data not shown). These results imply that expression of HIV-1 Nef protein does not interfere with complex formation of CD4-$p56^{lck}$ in the ER or presentation of the complex on the plasma membrane. However, following the 1-h chase, a 50% reduction in the CD4/$p56^{lck}$ ratio was observed (VB_{NEF}; Fig. 5, lanes 1). During the chase, increased amounts of CD4 were detected on the surface, whereas much less $p56^{lck}$ was coprecipitated, generating a drop in the CD4/$p56^{lck}$ ratio to 1:0.4. It appears that Nef protein triggers surface CD4 molecules to be rapidly internalized and to dissociate from $p56^{lck}$ prior to internalization.

DISCUSSION

In this article, we have described the mechanism by which HIV-1 Nef protein down-modulates the levels of cell surface CD4 in human T cells. This Nef-mediated down-regulation has been observed in many $CD4^+$ cells, including human and murine T-cell lines (7, 17, 35, 60). Initial efforts to define the mechanism demonstrated that expression of HIV-1 Nef protein does not alter the steady-state levels of CD4 mRNA or CD4 protein (7, 17). More recently, Garcia et al. (16) showed that this posttranslational process requires the cytoplasmic

domain of human CD4 molecules and that Nef functions in a cell-type- and species-independent manner. Nevertheless, the precise mechanism of this Nef activity has not been defined. Therefore, we designed experiments to determine the fate of newly synthesized CD4 proteins in Nef protein-expressing T cells.

Human $CD4^+$ T-cell line VB, stably transduced with the HIV-1 SF2 *nef* gene in an amphotropic retroviral vector, experienced a significant reduction in surface expression of CD4 as evidenced by a 70 to 80% drop in fluorescence intensity compared with that of control cells. This reduction is not due to inhibition of CD4 synthesis in Nef-expressing cells, since normal levels of CD4 were synthesized during pulse-labeling. This result supports early reports that Nef induces CD4 down-modulation at the posttranslational level (7, 17). Unlike those in control cells, newly synthesized CD4 molecules are rapidly degraded with a half-life of approximately 6 h in Nef-expressing cells. From data shown in Fig. 1 and 3, the loss of pulse-labeled CD4, seen over the chases in Nef-expressing cells, is largely due to degradation by a lysosomal process since this loss can be suppressed in the presence of drugs (leupeptin, NH_4Cl, and chloroquine) that inactivate lysosomal enzymes with or without raising intravesicular pH (28, 54). It is of interest that CD4 degradation was observed after a 2-h lag after synthesis. During the 2-h lag, CD4 became resistant to endo H digestion, implying that proteins were transported to the Golgi apparatus without being retained in the ER. Furthermore, surface CD4 labeling with biotin showed that newly synthesized CD4 was transported to the plasma membrane in Nef-expressing cells with kinetics similar to those of control cells. Newly delivered CD4 in Nef-expressing cells was then internalized with a short half-life on the plasma membrane, following an approximately 1-h lag after synthesis. Therefore, we conclude that HIV-1 Nef protein triggers rapid endocytosis of cell surface CD4 followed by degradation in the lysosomal compartment, resulting in reduced levels of surface CD4. These results are corroborated well by the recent work of Aiken et al. (3). However, the mechanism described here for Nef-induced CD4 down-modulation is different from those observed with HIV-1 envelope glycoprotein gp160 (12, 13, 22) and Vpu protein in conjunction with gp160 (65). These studies demonstrated that the reduced surface expression of CD4 was achieved by trapping the molecule in the ER. Envelope glycoprotein gp160 appears to form stable complexes of CD4-gp160 in the ER which block transport of CD4 from the ER. Furthermore, ER-retained CD4 is subject to rapid degradation in the presence of Vpu proteins.

The human CD4 molecule is a glycoprotein of approximately 55 kDa with two potential N-linked glycosylation sites. It is expressed primarily on a subset of T lymphocytes and appears to function in the development of immature T lymphocytes in the thymus and in the activation of mature T cells in the periphery (39, 44, 66). In addition, CD4 serves as the primary receptor for HIV (26, 33, 38), and as such, it has been proposed that Nef-mediated CD4 down-modulation may prevent superinfection (7). Soon after HIV-1 infection, Nef expression can achieve this process most effectively and rapidly by internalizing surface CD4 molecules. Later in viral infection, this can be reinforced by expression of an envelope glycoprotein and Vpu, which prevents newly synthesized CD4 from leaving the ER. It has been reported (6, 53) that the interaction of $CD4^+$ T cells with gp120-expressing cells or crossed-linked gp120, followed by T-cell receptor stimulation, leads to programmed cell death (apoptosis). Nef-mediated down-modulation of CD4 in newly infected cells would inhibit potentially lethal interaction with gp120. Of course, even in light of in vitro activities, the in vivo role of Nef remains entirely unknown.

The normal role of CD4 in T-cell activation is highly dependent on its interaction with $p56^{lck}$. In T cells, most of the $p56^{lck}$ is associated with CD4 and its enzymatic activity is regulated by CD4 association (62). Thus, Nef-mediated CD4 down-modulation would increase the amount of free $p56^{lck}$, which could lead to alterations in intracellular signaling. Recent studies with transgenic mice expressing Nef in T cells have demonstrated that Nef appears to enhance the T-cell receptor response (57), suggesting that in the presence of Nef these T cells become sensitized. Such a positive process would be important for HIV infectivity in vivo since viral replication requires the activation of host lymphocytes. The relevance of this positive effect of Nef is supported by ex vivo studies of HIV-1 in which Nef protein has enhanced the ability of primary, quiescent human lymphocytes to replicate HIV-1 (15, 41, 59).

During T-cell activation or activation of the protein kinase C pathway by phorbol esters such as phorbol-12-myristate-13-acetate, CD4 and CD8 are rapidly phosphorylated on cytoplasmic serine residues (1, 8, 20, 56). Subsequently, CD4 molecules are rapidly internalized from the surfaces of cells, whereas CD8 surface expression is not affected. Both CD4 and CD8 physically associate with lymphocyte-specific protein tyrosine kinase, $p56^{lck}$, an essential participant in antigen-specific signal transduction and thymic maturation of T cells. Studies of phorbol-12-myristate-13-acetate-induced internalization of CD4 (21, 58) demonstrated that dissociation of CD4 from $p56^{lck}$ occurred prior to rapid internalization of CD4. In contrast, no disruption of the CD8-$p56^{lck}$ complex was observed. In resting lymphocytes, endocytosis of surface CD4 is remarkably slow, whereas in nonlymphoid cells, which do not express $p56^{lck}$, it occurs much faster, at rates of 2 to 3% of the cell surface pool per minute (36, 45, 46). Therefore, it was proposed that association with $p56^{lck}$ may prevent CD4 molecules on cell surfaces from being internalized. This was supported by expression of $p56^{lck}$ in nonlymphoid cells, which slows the rate of endocytosis of CD4 (47). Furthermore, disruption of this association may initiate the endocytic pathway of CD4. However, the mechanism of the dissociation of $p56^{lck}$ from a CD4 molecule is unknown. Interestingly, dissociation was observed prior to Nef-induced CD4 endocytosis (Fig. 5). Recently, Aiken et al. (3) showed that two adjoining leucine residues in the CD4 cytoplasmic domain play a critical role in Nef-mediated CD4 internalization. This dileucine-based motif was previously identified in the CD3 γ and δ chains as an endocytosis and lysosomal targeting signal (27). Therefore, one can hypothesize that Nef-induced $p56^{lck}$ dissociation exposes the dileucine-based endocytosis motif in CD4 to the endocytic apparatus, which results in CD4 internalization and lysosomal degradation. It raises the question of how HIV-1 Nef selectively triggers $p56^{lck}$ dissociation from surface CD4. Previously, Garcia and Miller (17) showed that Nef down-regulates surface CD4 in a serine phosphorylation-independent manner. In this system, a mutant CD4, in which three serine residues in the cytoplasmic domain (potential phosphorylation sites by protein kinase C) were mutated to alanine, was not down-modulated by phorbol ester but was down-modulated by Nef expression. Thus, in Nef-expressing cells, dissociation of $p56^{lck}$ from a CD4 molecule occurs by a mechanism different from that for phorbol ester-induced serine phosphorylation.

We report here that HIV-1 Nef induces rapid internalization of surface CD4 followed by degradation in a lysosomal compartment. These processes, together with dissociation of CD4

from $p56^{lck}$ prior to internalization, are similar to those observed during T-cell activation and indicate a potential role for Nef in T-cell activation. However, the biochemical functions of Nef in T cells have not been defined. Recent studies on the biological role of Nef in HIV pathogenesis have indicated that Nef plays an essential role in HIV replication. Given the fact that shortly after viral induction by T-cell activation, receptor molecules for HIV are down-modulated, one can speculate that Nef may sustain stable HIV infection in vivo by protecting virus-producing CD4 cells before they can be superinfected or be led to apoptotic cell death. In addition, as a consequence of CD4 endocytosis, higher levels of $p56^{lck}$ are no longer regulated by CD4 association. This might enhance T-cell receptor-mediated signaling, leading to a maximal virus burst from infected CD4 cells. These speculations should be addressed in future studies.

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REFERENCES

1. **Acres, R. B., P. J. Conlon, 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. **Ahmad, N., and S. Venkatesan.** 1988. Nef protein of HIV-1 is a transcriptional repressor of HIV-1 LTR. Science **241:**1481–1485.
3. **Aiken, C., J. Konner, N. R. Landau, M. E. Lenburg, and D. Trono.** 1994. Nef induces CD4 endocytosis: requirement for critical di-leucine motif in the membrane-proximal CD4 cytoplasmic domain. Cell **76:**853–864.
4. **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.
5. **Ameisen, J.-C., B. Guy, S. Chamaret, M. Loche, Y. Mouton, J.-L. Neyrinck, J. Khalife, C. Leprevost, G. Beaucaire, C. Boutillon, H. Gras-Masse, M. Maniez, M.-P. Kieny, D. Laustriat, A. Berthier, B. Mach, L. Montagnier, J.-P. Lecocq, and A. Capon.** 1989. Antibodies to the nef protein and to nef peptides in HIV-1-infected seronegative individuals. AIDS Res. Hum. Retroviruses **5:**279–291.
6. **Banda, N. K., J. Bernier, D. K. Kurahara, R. Kurrle, N. Haigwood, R. P. Sekaly, and T. H. Finkel.** 1992. Crosslinking CD4 by human immunodeficiency virus gp120 primes T cells for activation-induced apoptosis. J. Exp. Med. **176:**1099–1106.
7. **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 super infection. J. Exp. Med. **177:**1561–1566.
8. **Blue, M.-L., D. A. Hafler, K. A. Craig, H. Levine, and S. F. Schlossman.** 1987. Phosphorylation of CD4 and CD8 molecules following T cell triggering. J. Immunol. **139:**3949–3954.
9. **Blumberg, B. M., L. G. Epstein, Y. Saito, D. Chen, L. R. Sharer, and R. Anand.** 1992. Human immunodeficiency virus type 1 *nef* quasispecies in pathological tissue. J. Virol. **66:**5256–5264.
10. **Braun, M., A. Waheed, and K. von Figura.** 1989. Lysosomal acid phosphatase is transported to lysosomes via the cell surface. EMBO J. **8:**3633–3640.
11. **Chen, C., and H. Okayama.** 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. **7:**2745–2752.
12. **Crise, B., L. Buonocore, and J. K. Rose.** 1990. CD4 is retained in the endoplasmic reticulum by the human immunodeficiency virus type 1 glycoprotein precursor. J. Virol. **64:**5585–5593.
13. **Crise, B., and J. K. Rose.** 1992. Human immunodeficiency virus type 1 glycoprotein precursor retains a CD4-$p56^{lck}$ complex in the endoplasmic reticulum. J. Virol. **66:**2296–2301.
14. **Delassus, S., R. Cheynier, and S. Wain-Hobson.** 1991. Evolution of human immunodeficiency virus type 1 *nef* and long terminal repeat sequences over 4 years in vivo and in vitro. J. Virol. **65:**225–231.
15. **de Ronde, A., B. Klaver, W. Keulen, L. Smit, and J. Goudsmit.** 1992. Natural HIV-1 NEF accelerates virus replication in primary human lymphocytes. Virology **188:**391–395.
16. **Garcia, J. V., J. Alfano, and A. D. Miller.** 1993. The negative effect of human immunodeficiency virus type 1 Nef on cell surface CD4 expression is not species specific and requires the cytoplasmic domain of CD4. J. Virol. **67:**1511–1516.
17. **Garcia, J. V., and A. D. Miller.** 1991. Serine phosphorylation-independent downregulation of cell-surface CD4 by *nef*. Nature (London) **350:**508–511.
18. **Guy, B., M. P. Kieny, Y. Riviere, C. Le 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.
19. **Hammes, S. R., E. P. Dixon, M. H. Malim, B. R. Cullen, and W. C. Greene.** 1989. Nef protein of human immunodeficiency virus type 1: evidence against its role as a transcriptional inhibitor. Proc. Natl. Acad. Sci. USA **86:**9549–9553.
20. **Hoxie, J. A., D. M. Matthews, K. J. Callahan, D. L. Cassel, and R. A. Cooper.** 1986. Transient modulation and internalization of T4 antigen induced by phorbol esters. J. Immunol. **137:**1194–1201.
21. **Hurley, T. R., K. Luo, and B. M. Sefton.** 1989. Activators of protein kinase C induce dissociation of CD4, but not CD8, from $p56^{lck}$. Science **245:**407–409.
22. **Jabbar, M. A., and D. P. Nayak.** 1990. Intracellular interaction of human immunodeficiency virus type 1 (ARV-2) envelope glycoprotein gp160 with CD4 blocks the movement and maturation of CD4 to the plasma membrane. J. Virol. **64:**6297–6304.
23. **Kaminchik, J., N. Bashan, D. Pinchasi, B. Amit, N. Sarver, M. I. Johnston, M. Fischer, Z. Yavin, M. Gorecki, and A. Panet.** 1990. Expression and biochemical characterization of human immunodeficiency virus type 1 *nef* gene product. J. Virol. **64:**3447–3454.
24. **Kestler, H. W., 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.
25. **Kim, S., K. Ikeuchi, R. Bryn, J. Groopman, and D. Baltimore.** 1989. Lack of a negative influence on viral growth by the *nef* gene of human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA **86:**9544–9548.
26. **Klatzmann, 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:**767–770.
27. **Letourneur, F., and R. D. Klausner.** 1992. A novel di-leucine motif and a tyrosine-based motif independently mediate lysosomal targeting and endocytosis of CD3 chains. Cell **69:**1143–1157.
28. **Libby, P., and A. L. Goldberg.** 1978. Leupeptin, a protease inhibitor, decreases protein degradation in normal and diseased muscles. Science **199:**534–536.
29. **Lifson, J. D., G. R. Reyes, M. S. McGrath, B. S. Stein, and E. G. Engleman.** 1986. AIDS retrovirus induced cytopathology: giant cell formation and involvement of CD4 antigen. Science **232:**1123–1127.
30. **Lippincott-Schwartz, J., and D. M. Fambrough.** 1986. Lysosomal membrane dynamics: structure and interorganellar movement of a major lysosomal membrane glycoprotein. J. Cell Biol. **102:**1593–1605.
31. **Lisanti, M. P., A. Le Bivic, M. Sargiacomo, and E. Rodriguez-Boulan.** 1989. Steady-state distribution and biogenesis of endogenous Madin-Darby canine kidney glycoproteins: evidence for intracellular sorting and polarized cell surface delivery. J. Cell Biol. **109:**2117–2127.
32. **Luciw, P. A., C. Cheng-Mayer, and J. A. Levy.** 1987. Mutational analysis of the human immunodeficiency virus: the orf-B region down-regulates virus replication. Proc. Natl. Acad. Sci. USA **84:**1434–1438.

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. **Maitra, R. K., N. Ahmad, S. M. Holland, and S. Venkatesan.** 1991. Human immunodeficiency virus type 1 (HIV-1) provirus expression and LTR transcription are repressed in NEF-expressing cell lines. Virology **182:**522–533.
35. **Mariani, R., and J. Skowronski.** 1993. CD4 down-regulation by *nef* alleles isolated from human immunodeficiency virus type 1-infected individuals. Proc. Natl. Acad. Sci. USA **90:**5549–5553.
36. **Marsh, M., J. E. Armes, and A. Pelchen-Matthews.** 1990. Endocytosis and recycling of CD4. Biochem. Soc. Trans. **18:**139–144.
37. **Matsuura, Y., M. Maekawa, S. Hattori, N. Ikegami, A. Hayashi, S. Yamazaki, C. Morita, and Y. Takabe.** 1991. Purification and characterization of human immunodeficiency virus type 1 *nef* gene product expressed by a recombinant baculovirus. Virology **184:** 580–586.
38. **McDougal, J. S., M. S. Kennedy, J. M. Sligh, S. P. Cort, A. Mawle, and J. K. A. Nicholson.** 1986. Binding of HTLV-III/LAV to $T4^+$ T cells by a complex of the 110K viral protein and the T4 molecule. Science **231:**382–385.
39. **Miceli, M. C., and J. R. Parnes.** 1991. The roles of CD4 and CD8 in T cell activation. Semin. Immunol. **3:**133–141.
40. **Miller, A. D., and G. J. Rosman.** 1989. Improved retroviral vectors for gene transfer and expression. BioTechniques **7:**980–990.
41. **Miller, M. D., M. T. Warmerdam, I. Gaston, W. C. Greene, 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.
42. **Nebreda, A. R., T. Bryan, F. Segade, P. Wingfield, S. Venkatesan, and E. Santos.** 1991. Biochemical and biological comparison of HIV-1 NEF and *ras* gene products. Virology **183:**151–159.
43. **Niederman, T. M. J., B. J. Thielan, and L. Ratner.** 1989. Human immunodeficiency virus type 1 negative factor is a transcriptional silencer. Proc. Natl. Acad. Sci. USA **86:**1128–1132.
44. **Parnes, J. R.** 1989. Molecular biology and function of CD4 and CD8. Adv. Immunol. **44:**265–311.
45. **Pelchen-Matthews, A., J. E. Armes, G. Griffiths, and M. Marsh.** 1991. Differential endocytosis of CD4 in lymphocytic and nonlymphocytic cells. J. Exp. Med. **173:**575–587.
46. **Pelchen-Matthews, A., J. E. Armes, and M. Marsh.** 1989. Internalization and recycling of CD4 transfected into HeLa and NIH3T3 cells. EMBO J. **8:**3641–3649.
47. **Pelchen-Matthews, A., I. Boulet, D. R. Littman, R. Fagard, and M. Marsh.** 1992. The protein tyrosine kinase $p56^{lck}$ inhibits CD4 endocytosis by preventing entry of CD4 into coated pits. J. Cell Biol. **117:**279–290.
48. **Ranki, A., K. Jarvinen, S.-L. Valle, P. Nurmilaakso, and K. Krohn.** 1990. Antibodies to recombinant HIV-1 Nef detected in HIV-1 infection as well in nonrisk individuals. J. Acquired Immune Defic. Syndr. **3:**348–355.
49. **Ratner, L., W. Haseltine, R. Pataraca, K. J. Livak, B. Starcich, S. F. Josephs, E. R. Doran, J. A. Rafalski, E. A. Whitehorn, K. Baumeister, L. Ivanoff, S. R. Petteway, M. L. Pearson, J. A. Lautenberger, T. S. Papas, J. Ghrayeb, N. T. Chang, R. C. Gallo, and F. Wong-Staal.** 1985. Complete nucleotide sequence of the AIDS virus, HTLV-III. Nature (London) **313:**277–284.
50. **Ratner, L., B. Starcich, S. F. Josephs, B. H. Hahn, E. P. Reddy, K. J. Livak, S. R. Petteway, Jr., M. L. Pearson, W. A. Haseltine, S. K. Arya, and F. Wong-Staal.** 1985. Polymorphism of the 3′ open reading frame of the virus associated with the acquired immune deficiency syndrome, human T-lymphotropic virus type III. Nucleic Acids Res. **13:**8219–8229.
51. **Rhee, S. S., H. Hui, and E. Hunter.** 1990. Preassembled capsids of type D retroviruses contain a signal sufficient for targeting specifically to the plasma membrane. J. Virol. **64:**3844–3852.
52. **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.
53. **Schwartz, O., M. Alizon, J. M. Heard, and O. Danos.** 1994. Impairment of T cell receptor-dependent stimulation in $CD4^+$ lymphocytes after contact with membrane-bound HIV-1 envelope glycoprotein. Virology **198:**360–365.
54. **Segle, P. O.** 1983. Inhibitors of lysosomal function. Methods Enzymol. **96:**737–764.
55. **Shaw, A. S., K. E. Amrein, C. Hammond, D. F. Stern, B. M. Sefton, and J. K. Rose.** 1989. The lck tyrosine protein kinase interacts with the cytoplasmic tail of the CD4 glycoprotein through its unique amino-terminal domain. Cell **59:**627–636.
56. **Shin, J., C. Doyl, 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.
57. **Skowronski, J., D. Parks, and R. Mariani.** 1993. Altered T cell activation and development in transgenic mice expressing the HIV-1 *nef* gene. EMBO J. **12:**703–713.
58. **Sleckman, B. P., J. Shin, V. E. Igras, T. L. Collins, J. L. Strominger, and S. J. Burakoff.** 1992. Disruption of the CD4-$p56^{lck}$ complex is required for rapid internalization of CD4. Proc. Natl. Acad. Sci. USA **89:**7566–7570.
59. **Spina, C. A., T. J. Kwoh, M. Y. Chowers, J. C. Guatelli, and D. D. Richman.** 1994. The importance of *nef* in the induction of human immunodeficiency virus type 1 replication from primary quiescent CD4 lymphocytes. J. Exp. Med. **179:**115–123.
60. **Tsunetsugu-Yokota, Y., S. Matsuda, M. Maekawa, T. Saito, T. Takemori, and Y. Takebe.** 1992. Constitutive expression of the *nef* gene suppresses human immunodeficiency virus type 1 (HIV-1) replication in monocytic cell lines. Virology **191:**960–963.
61. **Turner, J. M., M. H. Brodsky, B. A. Irving, S. D. Levin, R. M. Perlmutter, and D. R. Littman.** 1990. Interaction of the unique N-terminal region of tyrosine kinase $p56^{lck}$ with cytoplasmic domains of CD4 and CD8 is mediated by cysteine motifs. Cell **60:**755–765.
62. **Veillette, A., M. A. Bookman, E. M. Horak, L. E. Samelson, and J. B. Bolen.** 1989. Signal transduction through the CD4 receptor involves the activation of the internal membrane protein tyrosine kinase $p56^{lck}$. Nature (London) **338:**257–259.
63. **Veillette, A., M. A. Bookman, E. M. Horak, and J. B. Bolen.** 1988. The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase $p56^{lck}$. Cell **55:**301–308.
64. **Wain-Hobson, S., P. Sonigo, O. Danos, S. Cole, and M. Alizon.** 1985. Nucleotide sequence of the AIDS virus, LAV. Cell **40:**9–17.
65. **Willey, R. L., F. Maldarelli, M. A. Martin, and K. Strebel.** 1992. Human immunodeficiency virus type 1 Vpu protein induces rapid degradation of CD4. J. Virol. **66:**7193–7200.
66. **Zuniga-Pflucker, J. C., L. A. Jones, L. T. Chin, and A. M. Kruisbeek.** 1991. CD4 and CD8 act as co-receptors during thymic selection of the T cell repertoire. Semin. Immunol. **3:**167–175.