Abstract. The sequence of events leading to clathrin-coated pit (CCP) nucleation on the cell surface and to the incorporation of receptors into these endocytic structures is still imperfectly understood. In particular, the question remains as to whether receptor tails initiate the assembly of the coat proteins or whether receptors migrate into preformed CCP. This question was approached through a dissection of the mechanisms implemented by Nef, an early protein of human and simian immunodeficiency virus (HIV and SIV, respectively), to accelerate the endocytosis of cluster of differentiation antigen type 4 (CD4), the major receptor for these viruses. Results collected showed that: (a) Nef promotes CD4 internalization via an increased association of CD4 with CCP; (b) the Nef-mediated increase of CD4 association with CCP is related to a doubling of the plasma membrane area occupied by clathrin-coated structures; (c) this increased CCP number at the plasma membrane has functional consequences preferentially on CD4 uptake and does not significantly affect transferrin receptor internalization or fluid-phase endocytosis; (d) the presence of a CD4 cytoplasmic tail including a critical dileucine motif is required to induce CCP formation via Nef; and (e) when directly anchored to the cytoplasmic side of the plasma membrane, Nef itself can promote CCP formation. Taken together, these observations lead us to propose that CD4 can promote CCP generation via the connector molecule Nef. In this model, Nef interacts on one side with CD4 through a dileucine-based motif present on CD4 cytoplasmic tail and on the other side with components of clathrin-coated surface domain (i.e., adaptins). These Nef-generated complexes would then initiate the nucleation of CCP.

Receptor-mediated endocytosis provides a mechanism through which cells selectively capture nutrients and building blocks from the extracellular medium, transduce extracellular signals, and remove signaling receptors from their surface, thereby modulating their sensitivity to external stimuli (Cohen and Fava, 1985; van Deurs et al., 1989; Rodman et al., 1990; Watts and Marsh, 1992; Carpentier, 1994; Feger et al., 1994). Elucidating the molecular mechanisms governing receptor-mediated endocytosis is, therefore, important to unravel how cell growth and maintenance are controlled. Endocytosis of signaling receptors (i.e., the insulin receptor, the EGF receptor, etc.) occurs in response to ligand binding while endocytosis of transport protein receptors (i.e., the transferrin receptor, the LDL receptor, etc.) is constitutive (Cohen and Fava, 1985; Hanover et al., 1985; Hopkins et al., 1985; Watts, 1985; Carpentier et al., 1992; Sorkin and Carpenter, 1993). In both cases the entry of the receptors inside the cell is mediated by plasma membrane invaginated dom-
advances contrast with the lack of information regarding another key event in receptor-mediated endocytosis, namely the triggering and control of CCP formation. In this respect the question remains open as to whether receptors designed to be internalized induce CCP formation or whether receptors are segregated in preformed CCP. According to one model, CCP components first assemble at the plasma membrane and, in a second step, receptors migrate into these structures to be internalized (Fire et al., 1991; Katzir et al., 1994; Sako and Kusumi, 1994; Lazarovits et al., 1996). This concept is supported by the absence of increase in CCP density in the proximity of stimulated Fc receptors (FelRI) (Santini and Keen, 1996). By contrast, the second model postulates that the assembly of CCP components is initiated by receptors themselves. Along this line, ligand-induced clustering of IgM receptors was shown to lead to clathrin recruitment at the plasma membrane (Salisbury et al., 1980), and overexpression of transferrin receptors caused an increase in clathrin coating at the plasma membrane (Iacopetta et al., 1988; Miller et al., 1991). Likewise, clathrin-coated vesicle formation at the Golgi membranes was demonstrated to be modulated by expression levels of mannose 6-phosphate receptor as long as the cytoplasmic tail is unaltered (Le Borgne and Hoflack, 1997).

The present study was aimed at exploring the process of CCP formation through the study of Nef, an early protein of primate lentiviruses which downregulates the surface expression of CD4 (Garcia and Miller, 1991; Anderson et al., 1993; Mariani and Skowronski, 1993) by inducing its accelerated internalization followed by lysosomal degradation (Aiken et al., 1994; Rhee and Marsh, 1994). Previous work indicated that 20 membrane-proximal residues of the CD4 cytoplasmic domain are sufficient to confer Nef responsiveness to a heterologous molecule, and that within this region a dileucine-based motif plays a critical role (Aiken et al., 1994; Salghetti et al., 1995). Furthermore, the study of chimeric integral membrane proteins containing Nef as their cytoplasmic domain revealed that, in this context, Nef can act in cis to induce the rapid endocytosis of these fusion molecules via clathrin-coated pits (Mangasarian et al., 1997). Taken together, these data support a model where Nef downregulates CD4 by acting as a connector between this receptor and the endocytic apparatus. Here, the mechanisms of Nef-induced CD4 endocytosis were further investigated. This led to the observation that the viral protein triggers the formation of CD4-enriched CCP. This effect required that Nef be tethered to the plasma membrane either by coexpression with CD4, or as part of a chimeric integral membrane protein. These results strongly suggest that receptors can trigger the formation of CCP at the cell plasma membrane.

**Materials and Methods**

**Reagents**

Lucifer yellow, holotransferrin iron-saturated, γ globulins, goat anti-mouse IgG (whole molecule) gold conjugate (10 nm), histidinol, and Heps were purchased from Sigma Chemical Co. (St Louis, MO). RPMI 1640 culture medium and fetal calf serum (FCS) were purchased from GIBCO BRL (Paisley, Scotland). Purified mouse anti-human CD4 (RPA-T4) was obtained from PharMingen (San Diego, CA); purified mouse anti-human CD4 (Leu3a) and purified mouse anti-human CD71 (anti-human transferrin receptor, clone B3/25) were obtained from Boehringer Mannheim (Mannheim, Germany); and monomolecular mouse anti-clathrin (directed against the 180-kD heavy chain) was a gift of R.G.W. Anderson. Iodo-Beads as iodonation reagent were purchased from Pierce Chemical Co. (Rockford, IL) and 125I from Amersham International (Little Chalfont, England). Other chemicals were of analytical grade and were obtained from Fluka AG (Buchs, Switzerland) or Sigma Chemical Co. Experiments were performed in PBS or a medium containing (millimolars) 138 NaCl, 6 KCl, 1 MgCl₂, 20 glucose, and 2 Hepes, pH 7.4 (medium 1).

**DNA Constructions**

The HIV1-Nef allele and the retroviral vectors LNeF and LNefSN, used in these experiments, were described previously (Aiken et al., 1994). CD4 mutants and CD4-Nef chimera (44Nef) were created by ligating DNA fragments generated by PCR (Mangasarian et al., 1997). The constructs were verified by DNA sequence analysis. CD4 mutants and 44Nef chimera were expressed from the cytomegalovirus (CMV) immediate early promoter, in the pCMX plasmid vector (Umesono et al., 1991).

**Cell Lines, Cultures, and Transfections**

CEM and Namalwa cell lines expressing Nef were created using the retroviral vectors LNeF and LNefSN as previously described (Aiken et al., 1994). Stably transfected cells were next cultured in RPMI 1640 medium supplemented with 10% FCS and selected in the presence of 1 mg/ml G418. The Epstein-Barr virus (EBV)-transformed Namalwa human B cell line was maintained in RPMI 1640 medium supplemented with 10% FCS. For the establishment of cell populations stably producing CD4 and derivatives, Namalwa cells were coelectroporated with a mixture comprising a CMV-based plasmid expressing one of these proteins and pSV2-His, at a 1:10 ratio, using a total of 50 μg of DNA for 5 × 10⁶ cells at 250 V and a capacitance of 960 μF. Cells were then selected in histidine-deficient medium containing 1 mM histidinol (Sigma Chemical Co.) (Mangasarian et al., 1997).

**Antibody and Holotransferrin Iodination**

Iodination of purified mouse anti-human CD4 antibodies (RPA-T4) and iron-saturated holotransferrin was performed using Iodo-Beads according to the manufacturer’s instructions (Markwell, 1982). Briefly, Iodo-Beads were washed and preloaded with ~0.5–1 mCi of Na125I in PBS for 5 min at room temperature. Free-carrier mouse anti-human CD4 antibodies (50 μg; 230 pmol) or holotransferrin (100 μg; 1.23 nmol) were added to the beads and incubation was continued for 15 min at room temperature. Iodination was stopped by removing the beads from the mixture reaction and free 125I was discarded by passing the solution through a gel filtration sephadex G-25 column (Pharmacia Biotech, Inc., Piscatway, NJ). Iodinated protein was stored at 4°C in PBS + BSA 1%. Specific activity was comprised between 2–3 μCi/μg of protein and 3–4 μCi/μg of protein for 125I-anti-CD4 and 125I-holo-transferrin, respectively.

**Acid Wash Assay**

Internalization was assayed using the acid wash technique as previously described (Iacopetta et al., 1986; Pelchen Matthews et al., 1989; Pelchen Matthews et al., 1991; Aiken and Trono, 1995). Briefly, cells (10–20 × 10⁶) were washed and incubated for 2 h at 4°C with 125I-RPA-T4 (0.02–0.04 μCi/10⁶ cells, i.e., 7–20 ng/10⁶ cells) or 125I-holotransferrin (0.01–0.02 μCi/10⁶ cells, i.e., 3–7 ng/10⁶ cells) in 0.5 ml cold PBS/BSA 1%. Cells were next washed twice by centrifugation (200 g, 5 min) to remove unbound radiolabeled molecules and then incubated at 37°C to allow endocytosis. Cells were washed twice in their corresponding buffer at low pH (pH 2) to remove surface-bound radiolabeled antibodies or ligands. Percentage of 125I-anti-CD4/125I-holotransferrin internalization was expressed as the ratio of acid-washed-resistant radioactivity to total radioactivity associated with cells at neutral pH. Counting was performed in a Beckman 5,500 γ counter (Fullerton, CA). The iodinated anti-CD4 antibody binding at 4°C was maximal after 90 min of incubation. The nonspecific binding ranged between 3–5% of the total recovered radioactivity. The sequential washes with buffer at pH 2 detached more than 95% of the surface-bound antibodies after a 2-h incubation at 4°C.
Fluid-Phase Endocytosis Assay

Duplicates of 15 × 10^4 cells were incubated for different periods of time at 37°C in the presence of Lucifer yellow (1.5 mg/ml in Ca^2+^- and Mg^2+^-free medium 1). At the end of each incubation period, the endocytic process was stopped by cooling the cells to 4°C. Cells were next washed four times in ice-cold Ca^2+^- and Mg^2+^-free medium and lysed with 0.1% Triton X-100 (Krischer et al., 1995). Cell-associated fluorescence was measured in a Perkin-Elmer LS-3 fluorimeter (excitation 415 nm, emission 535 nm; Perkin-Elmer Corp., Norwalk, CT). Finally, fluorescence value was reported to protein content of each sample. Protein determination was performed with the Pierce kit using bicinchoninic acid.

 Autoradiography

CEM T cells were incubated for 2 h at 4°C with ^125I-anti-CD4 (0.2–0.3 µCi/10^6 cells, i.e., 100 ng/10^6 cells) in PBS (pH 7.4). After antibody binding, cells were washed twice by centrifugation (200 g, 5 min) to remove antibody excess and, and then were washed with buffer for incubation at 4°C to allow endocytosis. Cells were then fixed, dehydrated, and processed for electron microscope autoradiography; labeling was quantitated as previously described (Carpentier et al., 1978, 1981, 1992; Fan et al., 1982). Three experiments were performed. For each incubation time analyzed, three Epon blocks were prepared, and sections were cut from each block. For each time point studied and for each cell line, ~950–1,150 grains were analyzed from cells judged to be morphologically well preserved. Autoradiographic grains within a distance of ~250 nm from the plasma membrane were considered associated with the cell surface; grains overlying the cytoplasm and >250 nm from the plasma membrane were considered internalized. Grains associated with the plasma membrane were considered associated with CCP if their centers were <250 nm from these surface domains. Indeed, assuming a half distance of 80–100 nm (Salpeter et al., 1977), the radioactive source responsible for each autoradiographic grain has a >80% chance of being contained within this distance of 250 nm around each grain. The center of the grains was determined by overlaying them with a circle of 250-nm radius. This approach has been extensively used and validated in previous studies (Salpeter et al., 1977; Carpentier et al., 1978, 1981, 1992; Fan et al., 1982).

 CD4 Immunogold Labeling on EM Thin Sections and Isolated Plasma Membranes

CEM T cells were incubated 2 h at 4°C with anti-CD4 antibodies (Leu3a, 40 ng/10^6 cells) in cold PBS (pH 7.4). After antibody binding, cells were washed twice by centrifugation (200 g, 5 min) in cold PBS to remove antibody excess and incubated a second time with an anti–mouse IgG coupled with 10-nm colloidal gold particles (antibodies diluted 1:15 in 20% FCS, 5 min) to allow the recruitment of tagged receptors into CCP and their visualization. Cells were then fixed for 30 min at room temperature with 2.5% glutaraldehyde in phosphate buffer, dehydrated, and processed for EM as described previously (Carpentier et al., 1991). Thin sections were examined in an electron microscope (model EM 301; Philips Electron Optics). Autoradiographic grains located on the plasma membrane were considered associated with CCP if their centers were <250 nm from these surface domains. Gold particles were considered associated with CCP when they were observed immediately adjacent.

 Results

Nef Increases CD4 Association with CCP

To understand the mechanisms governing Nef-induced CD4 downregulation, CD4 tagged with ^125I-anti-CD4 was tracked morphologically by a quantitative EM autoradiographic analysis in CEM T lymphocytes stably expressing the viral protein (Fig. 1). Confirming previous biochemical observations (Aiken et al., 1994; Rhee and Marsh, 1994), the EM analysis showed that at the permissive temperature of 37°C, endocytosis of ^125I-anti-CD4 bound to plasma membrane of Nef-expressing CEM T cells was significantly increased as compared to control cells (stably expressing an empty vector) (Fig. 2 A). After a 2-h incubation at 4°C in the presence of ^125I-anti-CD4, ~3% of the plasma membrane–bound CD4 was found associated with CCP in control cells. This value slightly increased with time and temperature to reach 5% after 30 min of incubation at 37°C. By contrast, in cells expressing Nef, the association of ^125I-anti-CD4 with CCP peaked after 5 min of incubation at 37°C and plateaued at ~8% (Fig. 2 B). CD4 association with CCP was alternatively determined by EM

Quantitative Determination of Clathrin-coated Plasma Membrane

Quantitative determination of clathrin-coated plasma membrane CCP and clathrin lattices on the inner face of the plasma membrane were visualized as described by Sanan and Anderson (1991) with minor modifications. Briefly, formvar-coated EM nickel grids were treated with 1 mg/ml of poly-l-lysine for 1 h at 4°C. Cells (10^6/ml) in cold PBS were then allowed to sediment on the air-dried grids for 1 h at 4°C. Cells adherent to poly-l-lysine–coated grids were then washed with cold PBS, incubated 30 min with cold PBS/BSA 1%, and washed twice again with cold PBS. Adherent isolated plasma membranes were obtained by incubating the grids with the adherent cells in hypotonic (0.65×) PBS for 30 s and then by sonicating the cells at a weak power. This procedure disrupts the cells but allows a large portion of plasma membranes, with conserved internal structures such as clathrin-coated membranes and cytoskeleton elements, to stay adherent to the poly-l-lysine–coated grids. Adherent membranes were next washed with cold PBS and fixed for 15 min at 4°C followed by 15 min at room temperature with 4% glutaraldehyde in PBS. Adherent membranes were subsequently fixed in 2% osmium tetroxide in PBS for 8 min, 30 s at room temperature, washed three times for 5 min with PBS buffer, incubated with 1% aqueous tannic acid for 10 min, washed twice for 5 min in distilled water, incubated with 1% uranyl acetate for 10 min, and finally washed twice for 1 min with distilled water before air-drying. Membranes considered well preserved were then randomly photographed on an electron microscope (model EM 301; Philips Electron Optics). Eventually, the extent of membrane surfaces (%) decorated by clathrin was quantitated on electron micrographs using a Leica Quantimet 500 Plus, Vienna, Austria.

Concomitant Labeling of CD4 and Transferrin Receptors on EM Thin Sections

Cells in ice-cold PBS/BSA 1% (10^6/ml) were successively (a) incubated with anti-transferrin receptor antibodies (0.5 µg/10^6 cells, 90 min at 4°C), (b) washed and incubated a second time with an anti–mouse IgG coupled with 10-nm colloidal gold particles (antibodies diluted 1:15 in 20% FCS, 90 min at 4°C), (c) washed and saturated with nonspecific globulins (100 µg/10^6 cells, 30 min at 4°C), and (d) washed and incubated a third time with ^125I-anti-CD4 antibodies (100 ng/10^6 cells, 90 min at 4°C). Eventually, cells were washed twice with ice-cold PBS and warmed for 5 min at 37°C to allow the recruitment of tagged receptors into CCP and their endocytosis. Cells were then fixed for 30 min at room temperature with 2.5% glutaraldehyde in phosphate buffer, dehydrated, and processed for EM as described previously (Carpentier et al., 1991). Thin sections were examined in an electron microscope (model EM 301; Philips Electron Optics) using an electron microscope (model EM 301; Philips Electron Optics). Autoradiographic grains located on the plasma membrane were considered associated with CCP if their centers were <250 nm from these surface domains. Gold particles were considered associated with CCP when they were observed immediately adjacent.
immunogold labeling visualized on ultrathin sections. This method allows a more precise localization of anti-CD4 on the cell surface and a better definition of its association with specialized membrane domains such as CCP (Fig. 1 C). Similar conclusions to the one reached using $^{125}$I-anti-CD4 as a ligand were collected through immunogold labeling: in the presence of Nef at 37°C, anti-CD4 association with CCP increased at each time point studied by a factor between two and three (Fig. 2 C). The amount of cell surface $^{125}$I-anti-CD4 internalized, in both control and Nef-expressing cells, was closely related to the propensity of CD4 to associate with CCP, as shown by the linear relationship ($R = 0.76$) connecting the two events (Fig. 2 D).

Thus, in the presence of Nef, the stimulated rate of CD4 internalization was related, at least in part, to an increased association of the receptor with CCP.

**Nef Triggers the Recruitment and Assembly of CCP Components at the Plasma Membrane**

Surprisingly, the Nef-triggered CD4 association with CCP correlated with an increase in the plasma membrane surface coated with clathrin in CEM T cells. As determined on conventional EM thin sections, the plasma membrane surface...
decorated by a clathrin coat (2.0 \pm 0.4\% of the plasma membrane in control CEM T lymphocytes, 102 cell sections analyzed) increased by 63\% in cells transfected with Nef (data not shown). These initial observations were verified on isolated plasma membranes adherent to EM grids and negatively stained (Sanan and Anderson, 1991). This technique allows: (a) an en face view of large surfaces of the inside of the membrane; (b) a distinction between clathrin-coated flat lattices and clathrin-coated invaginations, both easily identified by their typical honeycomb organization; and (c) an accurate quantification of these respective clathrin-coated structures (Fig. 3). Quantitative analysis confirmed that, in the presence of Nef, surfaces occupied by CCP and flat clathrin lattices on the inner leaflet of the plasma membrane increased by 95.4 \pm 37.3\% (Fig. 4). Breaking these structures into their two components showed that CCP and flat clathrin lattices were increased 2.2- and 1.7-fold, respectively, by Nef expression (representing two out of three and one out of three, of the total clathrin-coated membrane increase, respectively) indicating that the increase in clathrin-coated plasma membrane was related primarily to an augmentation of invaginated clathrin-coated struc-

Figure 3. Representative electron micrographs of CCP and clathrin flat lattices on the inner face of CEM plasma membranes. CCP (arrowheads) and flat clathrin lattices (b, arrows) were visualized on adherent plasma membranes as described by Sanan and Anderson (1991) with minor modifications. Briefly, cells were allowed to sediment on poly-l-lysine-coated grids for 1 h at 4\°C and adherent plasma membranes were then obtained by incubating the grids in hypotonic medium followed by sonication at weak power. This procedure disrupts the cells but allows a large portion of plasma membranes, with conserved structures such as clathrin-coated membranes and cytoskeleton elements, to stay adherent to the poly-l-lysine-coated grids. Adherent membranes were next fixed and negatively stained for EM. Membranes considered well conserved were then randomly photographed and the extent of membrane surfaces coated by clathrin was quantitated on electron micrographs as described in Materials and Methods. Membranes were considered well conserved were randomly photographed and the extent of membrane surfaces coated by clathrin was quantitated on electron micrographs as described in Materials and Methods. In (c) cells were incubated for 2 h at 4\°C with anti-CD4 antibodies followed by a 2-h incubation at 4\°C in the presence of anti-mouse IgG coupled with 10-nm colloidal gold particles. Cells were next warmed for various periods of time at 37\°C to allow endocytosis. Gold particles (localizing CD4 and seen by transparency) are associated with CCP (arrowheads).

Figure 4. Nef increases the extent of plasma membrane coated by clathrin-lined structures (CCP and clathrin lattices) in CEM lymphoid T cells. Adherent plasma membranes were visualized as described by Sanan and Anderson (1991) with minor modifications. Briefly, cells were allowed to sediment on poly-l-lysine-coated grids for 1 h at 4\°C and adherent plasma membranes were then obtained by incubating the whole cells with hypotonic medium followed by a sonication at a weak power. This procedure disrupts the cells but allows a large portion of plasma membranes, with conserved internal structures such as clathrin-coated membranes and cytoskeleton elements, to stay adherent to the poly-l-lysine-coated grids. Adherent membranes were next fixed and negatively stained for EM. Membranes considered well conserved were then randomly photographed and the extent of membrane surfaces coated by clathrin was quantitated on electron micrographs as described in Materials and Methods. Data are means \pm SEM of quantitative analysis performed on 110 cells/487.8 \mu m^2 of plasma membrane segments, and 104 cells/447 \mu m^2 of plasma membrane segments for CEM control (transduced with a control retroviral vector) and Nef-expressing cells, respectively.

2. These values do not integrate the curvature of CCP which, if taken into account, would have amplified the difference in favor of CCP.
tures. Under these conditions, CCP size remained unchanged: 86.1 ± 0.9 and 90.8 ± 2.5 nm in control and Nef-expressing cells, respectively. Clathrin synthesis was also not significantly affected by Nef as determined by SDS-PAGE and quantitative analysis of clathrin-specific immunoblots (data not shown). Taken together, these data demonstrate that Nef-induced CD4 internalization is associated with a stimulation of the recruitment and assembly of clathrin coat constituents at the plasma membrane, giving rise to the production of a majority of invaginated CCP.

**Nef Specifically Affects CD4 Endocytosis**

Nef downregulates CD4 and, to a lesser extent, MHC class I (Schwartz et al., 1996). In contrast, Nef does not affect the surface expression of a variety of surface receptors including CD8, CD29, CD45RO, intercellular adhesion molecule-1, CD38, CD69, the transferrin receptor (Tf-R), and a CD4–LDL receptor fusion protein (Benson et al., 1993; Schwartz et al., 1993; Aiken et al., 1994), indicating that the Nef effect on endocytosis is at least partly receptor specific. To verify this point in our experimental conditions, 125I–transferrin uptake was analyzed. The transferrin receptor is a carrier of cargo molecules concentrated in CCP and constitutively internalized through these structures (Hanover et al., 1985; Watts, 1985). Nef expression only moderately affected the uptake of radiolabeled-transferrin, thus confirming the lack of a general effect of Nef on receptor-mediated endocytosis (Fig. 5 A). Similarly, the uptake of a fluid-phase marker (Lucifer yellow) remained unaffected by Nef (Fig. 5 B).

**Nef-induced CCP Preferentially Incorporate CD4**

The observation that Nef triggered the formation of cell surface CCP while concomitantly and specifically inducing CD4 endocytosis suggested that the viral protein was mediating the generation of CD4-enriched CCP. This hypothesis was tested by the simultaneous morphological tracking of CD4 and Tf-Rs. Surface CD4 molecules were traced with 125I–anti-CD4 while Tf-Rs were labeled with an anti–Tf-R primary antibody followed by a secondary antibody coupled with colloidal gold (10 nm) (Fig. 6). In the presence of Nef, the per cell number of CCP containing exclusively CD4 tripled, while the corresponding number of CCP incorporating solely the transferrin receptor only increased by ~7% (Table I). The increase in the number of CCP labeled with both markers showed that additional CCP appearing in Nef-expressing cells do not incorporate exclusively CD4 but may trap, to a low extent, other receptors. This low amount of receptors other than CD4 present in additionally formed CCP is possibly responsible for the small increase in transferrin internalization observed in Nef-expressing cells (Fig. 5 A). The number of unlabeled CCP was also significantly increased by Nef expression. Given that the analysis was performed on thin sections, which does not allow visualization of the entire clathrin-coated pits, and that 125I–anti-CD4 binding was not saturating, results collected led to an underestimation of CD4 potentially present in these structures and to an overestimation of unlabeled CCP. This was verified in the course of an immunogold CD4 labeling detected on adherent plasma membranes preparations (Fig. 3 C). After 5 min of incubation at 37°C of CEM lymphocytes surface-labeled with anti-CD4 immunocomplexes, 13% of the total CCP counted on the adherent membranes incorporated the immunogold probe (data not shown) as compared with the 6.6% observed in the case of 125I–anti-CD4 binding (see above). As compared to the increase in the total number of clathrin-coated pits, these values remain low, which leaves the possibility open that some of the newly formed CCP are not functional, although the preferential Nef-induced formation of CCP versus flat clathrin lattices is in favor of the functionality of the generated structures. Moreover, Nef might generate additional CCP in CEM lymphocytes incorporating some unidentified receptors, the endocytosis of which could also be increased by the viral protein.

**Figure 5.** Nef does not significantly affect receptor-mediated endocytosis in general or the nonspecific fluid-phase endocytosis. (A) Kinetics of 125I-transferrin internalization in CEM T lymphoid cells expressing or not expressing Nef. Cells were incubated 2 h at 4°C with 125I-transferrin and internalization of the 125I-ligand–receptor complex was allowed by raising the temperature to 37°C as previously described (Pelchen Matthews et al., 1991; Aiken et al., 1994). Data are means ± SEM range of two experiments. (B) Kinetics of Lucifer yellow uptake in CEM T lymphoid cells expressing or not expressing Nef. The fluid-phase endocytosis was determined by measuring the nonspecific cell uptake of Lucifer yellow as previously described (Krischer et al., 1993). Data are means ± SEM of three experiments.
Of note, in CEM T lymphocytes as well as in Namalwa B lymphocytes, little Nef-induced MHCI downregulation was observed (data not shown). Together, these morphological analyses strongly suggest that at least part of the Nef-induced CCP preferentially contain CD4.

Generation of New CCP Requires Nef and a Dileucine Motif in CD4 Cytoplasmic Tail

Was CD4 concentrated in Nef-induced preformed CCP or was it the primum movens in the generation of these structures? To answer this question, Namalwa B cells, which do not naturally express CD4, were stably transfected with CD4, Nef, a dileucine mutated form of CD4, (CD4\_DL), CD4+Nef, CD4\_DL+Nef, and the chimera 44Nef. As control, cells transfected with an empty plasmid vector were used. CCP on the inner face of the plasma membrane (PM) were visualized as described by Sanan and Anderson (1991) with minor modifications. Briefly, cells were allowed to sediment on polyl-lysine coated grids for 1 h at 4°C and adherent plasma membranes were then obtained by incubating the whole cells with hypotonic medium followed by a sonication at a weak power. This procedure disrupts the cells but allows a large portion of plasma membranes, with conserved internal structures such as clathrin-coated membranes and cytoskeleton elements, to stay adherent to the polyl-lysine-coated grids. Adherent membranes were next fixed and negative stained for EM. Membranes considered well conserved were then randomly photographed and the amount of CCP present on plasma membrane segments was quantitated on electron micrographs as described in Materials and Methods. Data are means ±SEM of quantitative analysis performed on 155 cells/710.6 μm², 229 cells/988.0 μm², 100 cells/459.3 μm², 106 cells/456.4 μm², 112 cells/445.7 μm², 158 cells/656.3 μm², and 104 cells/474.6 μm² of plasma membrane segments from Namalwa transfected with the empty plasmid, CD4, Nef, CD4\_DL, CD4+Nef, CD4\_DL+Nef, and 44Nef, respectively.

### Table I. Nef-induced CCP Incorporate Preferentially CD4

| Cells (Nb of cells) | Total number of CCP | CCP labeled by CD4 solely | CCP labeled by Tf-R solely | CCP labeled by CD4 and Tf-R | Unlabeled CCP |
|---------------------|---------------------|---------------------------|---------------------------|---------------------------|---------------|
| Control (150)       | 813                 | 15                        | 331                       | 39                        | 429           |
|                     | (1.8%)*             | (41.1%)*                   | (4.8%)*                   | (52.3%)*                  |               |
| Nef (150)           | 1,340               | 46                        | 355                       | 58                        | 880           |
|                     | (3.4%)*             | (26.5%)*                   | (4.3%)*                   | (65.7%)*                  |               |

*Percentage of total CCP counted.

---

**Figure 6.** Representative electron micrographs showing the association of CD4 (a and b, autoradiographic grains) and transferrin receptors (b, gold particles) with CCP (arrowheads) in CEM T lymphoid cells. CD4 and transferrin receptor association with CCP were concomitantly detected in CEM T cells. CD4 was traced with 125I-anti-CD4 with the Tf-R was labeled with an anti-Tf-R primary antibody followed by a secondary antibody coupled with colloidal gold. Endocytosis of the radiolabeled antibody–CD4 complex and gold-conjugated transferrin receptor was allowed to occur by raising the temperature to 37°C for 5 min. After cell processing for EM autoradiography, quantification was carried out as described previously (Carpentier et al., 1978, 1981, 1992; Fan et al., 1982). Autoradiographic grains located on the plasma membrane were considered associated with CCP if their centers were <250 nm from these surface domains. Gold particles were considered associated with CCP when they were observed immediately adjacent (at a distance <20 nm) to the clathrin coat or totally enclosed in CCP.

**Figure 7.** Relative amount of CCP decorating the plasma membrane of transfected Namalwa B lymphocytes. Cells were transfected with CD4, Nef, a dileucine mutated form of CD4, (CD4\_DL), CD4+Nef, CD4\_DL+Nef, and the chimera 44Nef. As control, cells transfected with an empty plasmid vector were used. CCP on the inner face of the plasma membrane (PM) were visualized as described by Sanan and Anderson (1991) with minor modifications. Briefly, cells were allowed to sediment on polyl-lysine coated grids for 1 h at 4°C and adherent plasma membranes were then obtained by incubating the whole cells with hypotonic medium followed by a sonication at a weak power. This procedure disrupts the cells but allows a large portion of plasma membranes, with conserved internal structures such as clathrin-coated membranes and cytoskeleton elements, to stay adherent to the polyl-lysine-coated grids. Adherent membranes were next fixed and negative stained for EM. Membranes considered well conserved were then randomly photographed and the amount of CCP present on plasma membrane segments was quantitated on electron micrographs as described in Materials and Methods. Data are means ±SEM of quantitative analysis performed on 155 cells/710.6 μm², 229 cells/988.0 μm², 100 cells/459.3 μm², 106 cells/456.4 μm², 112 cells/445.7 μm², 158 cells/656.3 μm², and 104 cells/474.6 μm² of plasma membrane segments from Namalwa transfected with the empty plasmid, CD4, Nef, CD4\_DL, CD4+Nef, CD4\_DL+Nef, and 44Nef, respectively.
The present work was intended to dissect the mechanisms responsible for the increased CD4 internalization observed in the presence of the HIV Nef protein. We found that Nef promotes CD4 internalization by increasing the association of this receptor with the classical internalization gates, the CCP. Surprisingly, we also noted that the Nef effect correlated with a doubling of the plasma membrane area occupied by clathrin-coated structures. These changes had functional consequences preferentially on CD4 uptake since the internalization of receptors classically internalized via CCP (e.g., transferrin receptors), or the nonspecific uptake of extracellular fluids (e.g., Lucifer yellow), was not significantly affected by Nef expression. This translated into a preferential increase in CD4-containing CCP over ones carrying transferrin receptor. Nef per se could promote CCP formation when artificially anchored to the cytoplasmic leaflet of the plasma membrane as part of a chimeric integral membrane protein. However, under physiological conditions, the Nef effect required the presence of CD4, and was in particular dependent on a dileucine-based motif within the cytoplasmic tail of this receptor. Taken together, these observations lead us to propose that, if properly tethered to the plasma membrane, Nef can induce CCP formation. It is interesting to note, in this regard, that Nef was recently shown to induce the accumulation of endosomes and lysosomes in human T lymphoid cells (Sanfridson et al., 1997).

The CD4 specificity of Nef-induced endocytosis is supported not only by the observation that the uptake of transferrin receptors was only weakly stimulated by Nef, but also by the results of immunocytochemical experiments carried out at the EM level, revealing a clear contrast between the percentage of clathrin-coated pits labeled with CD4 or with transferrin receptor in cells expressing Nef. These observations are in agreement with previously published results showing that the surface expression of receptors including CD8, CD29, CD45RO, interleukin-2 receptor (α chain), ICAM-1, CD38, CD69, HLA-DR, and a CD4-LDL receptor fusion protein are unaffected by Nef (Benson et al., 1993; Schwartz et al., 1993; Aiken et al., 1994). The only known exception is the MHCI molecule, the internalization of which is also stimulated by Nef, although less efficiently than that of CD4 (Schwartz et al., 1996). Nef also did not affect fluid-phase endocytosis in our experimental conditions. This latter observation supports the recent proposal that the total uptake of membrane and fluids is not strictly regulated by the clathrin-dependent endocytic pathway but might involve alternative mechanisms (Hansen et al., 1993; Cupers et al., 1994; Eker et al., 1994; Damke et al., 1995). The receptor-dependent formation of CD4-specific CCP could appear to contradict previous observations showing that CCP are relatively nonspecific structures capable of concentrating receptors of diverse nature (Dickson et al., 1981; Willingham et al., 1981; Carpentier et al., 1982). It is likely, however, that the selectivity of the observed phenomenon reflects a specific interaction between Nef and CD4 (Harris and Neil, 1994; Greenway et al., 1995; Grzesiek et al., 1996; Rossi et al., 1996). As Nef might interact with other receptors such as MHCI, it may appear surprising that the Nef-dependent CCP formation observed here required the presence of CD4, at least if Nef-induced CD4 and MHCI endocytosis proceed through similar mechanisms. However, levels of
MHCI were found to be minimally affected by Nef expression in Namalwa cells (data not shown).

The relative receptor specificity of the clathrin-coated structures induced by Nef could be interpreted as evidence for the initiation by receptors of the de novo formation of CCP, or alternatively could mean that CD4 is preferentially segregated in preformed Nef-induced CCP. The second possibility can be ruled out since, except in nonphysiological conditions where Nef is constitutively attached to the plasma membrane as part of a chimeric integral membrane protein, the viral protein alone is not able to induce CCP formation. Concomitant expression of CD4 is indeed necessary for this process; furthermore, there is a strict requirement for the preservation, within the CD4 cytoplasmic tail, of a dileucine-based motif previously identified as critical for CD4 response to Nef (Aiken et al., 1994).

These observations demonstrate that, at least in some circumstances, receptor tails can initiate coat protein assembly. This conclusion contrasts with that of previous studies in which a series of transport protein receptors showed a surface mobility consistent with random movements and occasional encounters with CCP (Fire et al., 1991; Ghosh and Webb, 1994; Katsir et al., 1994; Sako and Kusumi, 1994; Lazarovits et al., 1996). Along the same line, the relocation and aggregation of FcRIs induced by multivalent antigens was not found to be accompanied by any change in the distribution of adaptor protein 2 (AP2) or clathrin (Santini and Keen, 1996). However, our findings are in agreement with other studies showing: (a) clathrin recruitment at the plasma membrane subsequent to ligand-induced clustering of IgM receptors in lymphoblastoid cells (Salisbury et al., 1980); and (b) a correlation between the number of surface Tf-Rs and CCP in transfected cells (Iacopetta et al., 1988; Miller et al., 1991). The present work adds another dimension to these results, by providing direct evidence for a causal relationship between the presence of receptors and the formation of CCP.

Since both CD4 and Nef are required for the induction of CCP, what are their respective contributions to this phenomenon? Results presented show that both CD4 and Nef are required for the induction of CCP, and that Nef artificially tethered to the membrane as the cytoplasmic tail of a transmembrane fusion protein is capable of producing the same effect. These observations imply that Nef targeting to the plasma membrane, either through a direct physical interaction with CD4 (or the CD4/p56<sup>ck</sup> complex), or alternatively through part of a chimeric integral protein, is the primary requirement to induce CCP generation. This targeting may either permit the interaction of the viral protein with other proteins involved in signal transduction pathways leading to CCP generation, or allow a direct participation of the viral protein in CCP formation. Clathrin coat assembly is a multistep process in which a pivotal role is played by AP (Smythe and Warren, 1991; Schmid, 1992; Robinson, 1994). These heterotetrameric proteins participate in at least three crucial stages of clathrin-coated pit formation where Nef might act: (a) they must be recruited onto the right membrane, probably through binding to specific adaptor receptors (Mahaffey et al., 1990); (b) they next are activated to make them competent (possibly as part of complexes with other molecules) to bind clathrin triskelions (Peeler et al., 1993); and (c) they initiate the nucleation process leading to clathrin assembly into lattices (Keen et al., 1991; Gallusser and Kirchhausen, 1993). Several studies have provided evidence for a direct interaction of Nef with signal-transducing molecules such as protein tyrosine kinases of the src family (p56<sup>ck</sup>, hck) (Saksela et al., 1995; Collette et al., 1996), serine/threonine protein kinases (Sawai et al., 1995), protein kinase C (Banderet al., 1994), or protein phosphatidylinositol 3-kinase (Graziani et al., 1996), all molecules which may participate in the triggering and/or regulation of endocytotic processes. Via these effects on signal transduction pathways Nef might, therefore, interfere with CD4 endocytosis. However, Nef-induced CD4 downregulation is generally dissociated from the effects of the viral protein on signal transduction pathways (Aiken and Trono, 1995; Chowers et al., 1995; Saksela et al., 1995), suggesting that mechanisms implemented by Nef to induce the formation of CCP are not related to perturbation of signal transduction and might rather involve a more direct participation of the viral protein. Several observations support this alternative: (a) Nef is capable of physical interaction with CD4 and this interaction requires the integrity of a dileucine motif present in CD4 cytoplasmic tail; (b) Nef-induced CD4 endocytosis is specific and is not dependent on CD4 alteration; (c) the chimeric molecule CD4–Nef is internalized efficiently through CCP, suggesting that Nef contains the determinants required to associate with CCP components (Mangasarian et al., 1997); and (d) Nef is capable of physical interactions through different domains with both CD4 and adaptins (Piguet, V., M. Foti, A. Mangasarian, D. Lew, K.-H. Krause, D. Trono, and J.-L. Carpentier, manuscript in preparation). Thus, although we cannot exclude that CCP generation is related to Nef-induced signaling cascades, data collected favor a model where Nef plays the role of a connector between specific recognition signals present in receptor tails (e.g., CD4) and adaptins. They also suggest that the CD4–Nef complex participates together with adaptins in the nucleation process, perhaps via the Nef-mediated activation of adaptins. A third model would be that Nef itself behaves as an adaptin. We do not favor this possibility since it would imply that Nef, which has no structural analogy with adaptins, not only plays all the diverse and complex functions of these molecules, but in addition can generate CCP morphologically identical to those requiring adaptins.

Nef might mimic endogenous molecules capable, in physiological conditions, to link various receptors bearing dileucine-based motifs (or other motifs) to components of the endocytic machinery, and sometimes to induce the formation of CCP. Along this line, if a possible direct interaction of dileucine motifs with adaptins has recently been reported, this concept is not accepted by others (Ohno et al., 1995; Heilker et al., 1996). It is noteworthy that several cellular proteins appear to function as connectors between cell surface receptors and CCP, β-arrestin has thus been demonstrated to act as a clathrin adaptor facilitating the endocytosis of the β<sub>2</sub>-adrenergic receptor (Ferguson et al., 1996; Goodman et al., 1996), while the Eps15 and Shc proteins are thought to participate in connecting the EGF receptor tyrosine kinase with CCP (Okabayashi et al., 1996; Van Delft et al., 1997). However, no data is available to suggest that these proteins can induce the formation of CCP.
factor receptor/kinase complexes in A-431 cells. J. Biol. Chem. 260:12351–12358.

Collette, Y. H. Dutartre, A. Benatante, F. Ramos-Morales, R. Benarous, M. Harris, and D. Ollive. 1996. Physical and functional interaction of Nef with Lck. J. Biol. Chem. 271:6533–6541.

Coppe, P., A. Voit, A. Kaczynski, D. Baudhun, and P.J. Courtoy. 1994. Clathrin polymerization is not required for bulk-phase endocytosis in rat fibroblast. J. Biol. Cell. 127:725–735.

Damke, H., T. Baba, A.M. van der Bliek, and S.L. Schmid. 1995. Clathrin-inde-
dependent pinocytosis is induced in cells overexpressing a temperature-sensitive
mutant of dynamin. J. Cell Biol. 131:69–80.

Dickson, R.B., M.C. Willingham, and I. Pastan. 1981. α2-macroglobulin ad-
sorbed to colloidal gold: a new probe in the study of receptor-mediated en-
docytosis. J. Cell Biol. 90:29.

Elker, P., P.K. Holm, B. van Deurs, and K. Sandvig. 1994. Selective regulation of apical endocytosis in polarized Madin-Darby canine kidney cells by mem-
topar and cAMP. J. Cell. Biol. 126:1807–1819.

Fan, J.Y., J.L. Carpenter, E. Van Obberghen, N.M. Blackett, C. Grundfeld, and L. Orci. 1982. Receptor-mediated endocytosis of insulin: role of microvilli, coated pits, and coated vesicles. Proc. Natl. Acad. Sci. USA. 79:7788–7791.

Feger, J., S. Gil-Falgon, and C. Lamaze. 1994. Cell receptors: definition, mecha-

nisms and regulation of receptor-mediated endocytosis. Cell. Mol. Biol. (Oxf.). 40:1039–1061.

Ferguson, S.S., W.E. Downey, A.M. Colapietro, L.S. Barak, L. Menard, and
M.G. Caron. 1994. Role of β-arrestin in mediating agonist-promoted G pro-
tein-coupled receptor internalization. Science (Wash. DC). 271:363–366.

Fire, E., D.E. Zwart, M.G. Roth, and Y.I. Henis. 1991. Evidence from lateral
mobility studies for dynamic interactions of a mutant influenza hemagglu-
tinin with coated pits. J. Cell. Biol. 115:1585–1594.

Gallusser, A., and T. Kirchhausen. 1993. β1 and β2 subunits of the AP
complexes are the clathrin coat assembly components. EMBO (Eur. Mol. Bio-
logical Org.) J. 12:5237–5244.

Garcia, J.V., and A.D. Miller. 1991. Serine phosphorylation-independent down-
regulation of cell-surface CD4 by nef. Nature (Lond.). 350:508–511.

Ghosh, R.N., and W.W. Webb. 1994. Automated detection and tracking of indi-
vidual and clustered cell surface low density lipoprotein receptor molecules.
Biophys. J. 66:1301–1319.

Goldstein, J.L., M.S. Brown, R.G. Anderson, D.W. Russell, and W.J. Schneider. 1985. Receptor-mediated endocytosis: concepts emerging from the LDL receptor system. Annu. Rev. Cell Biol. 1:1–39.

Greenman, O.B., Jr., J. Krupnick, F. Santini, V.V. Gurevich, R.B. Penn, A.W. Gaggen, J.H. Keen, and J.L. Benovic. 1996. β-Arrestin acts as a clathrin adaptor in endocytosis of the β2-adrenergic receptor. Nature (Lond.). 383:447–450.

Graziani, A., F. Galimi, E. Medico, E. Cottone, D. Gramaglia, C. Boccaccio, and P.M. Comoglio. 1996. The HIV-1 nef protein interferes with phosphatidylinositol 3-kinase activation 1. J. Biol. Chem. 271:6590–6593.

Greenway, A., A. Azad, and D. McPhee. 1995. Human immunodeficiency virus type 1 Nef protein inhibits activation pathways in peripheral blood mononu-
uclear cells and T cell lines. J. Virol. 69:1842–1850.

Grzesiek, S., S.J. Stuhl, P.T. Wingfield, and A. Bax. 1996. The CD4 determinant for downregulation by HIV-1 nef directly binds to nef. Mapping of the nef binding domain by NMR. 55:1585–1625.

Hanover, J.A., L. Beguinot, M.C. Willingham, and I.H. Pastan. 1985. Transit of recepters for epidermal growth factor and transferrin through clathrin-
coated pits. Analysis of the kinetics of receptor entry. J. Biol. Chem. 260: 15938–15945.

Hansen, S.H., K. Sandvig, and B. van Deurs. 1993. Molecules internalized by clathrin-independent endocytosis are delivered to endosomes containing transferrin receptors. J. Cell Biol. 123:89–97.

Harris, M.P., and J.C. Neill. 1994. Myristolation-dependent binding of HIV-1 Nef to CD4. J. Mol. Biol. 241:136–142.

Heilker, R., U. Manning Krieg, J.F. Zuber, and M. Spiess. 1996. In vitro binding of clathrin adaptors to sorting signals correlates with endocytosis and base-

lateral sorting. EMBO (Eur. Mol. Biol. Organ.) J. 15:2893–2899.

Hopkins, C.R., K. Miller, and J.M. Beadmore. 1986. Receptor-mediated en-
docytosis of transferrin and epidermal growth factor receptors: a comparison of constitutive and ligand-induced uptake. J. Cell Sci. (Suppl. 3):173–186.

Iacopetta, B.J., J.L. Carpenter, T. Pozzan, D.P. Lew, P. Gorden, and L. Orci. 1994. Role of intracellular calcium and protein kinase C in the endocytosis of transferrin and insulin by HL60 cells. J. Cell. Biol. 103:851–856.

Iacopetta, B.J., S. Rothenberg, and L. Kahn. 1991. A role for the clathrin-

domain in transferrin receptor sorting and coated pit formation during endocytosis. Cell. 54:485–489.

Katir, Z., N. Nardi, I. Geffen, C. Fuhrer, and Y.I. Henis. 1994. Dynamic inter-
actions of the asialoglycoprotein receptor subunits with coated pits. en-
hanced interactions of H2 following association with H1. J. Biol. Chem. 269: 25186–25187.

Ketten, J.H., K.A. Beck, T. Kirchhausen, and T. Jarrett. 1991. Clathrin domains in recognition by assembly protein AP2. J. Biol. Chem. 266:7950–7956.

Krisher, J., A. Gilbert, P. Gorden, and J.L. Carpenter. 1993. Endocytosis is in-
hibited in hepatocytes from diabetic rats. Diabetes. 42:1303–1309.

Lazarovits, J., H.Y. Nain, A.C. Rodriguez, R.H. Wang, E. Fire, C. Bird, Y.I.
Henis, and M.G. Roth. 1996. Endocytosis of chimeric influenza virus hemagglutinin proteins that lack a cytoplasmic recognition feature for coated pits. J. Cell Biol. 134:339–348.

Le Borgne, R., and B. Hoflack. 1997. Mannose 6-phosphate receptors regulate the formation of clathrin-coated vesicles in the TGN. J. Cell Biol. 137:335–345.

Lin, H.C., M.S. Moore, D.A. Sanan, and R.G. Anderson. 1991. Reconstitution of clathrin-coated pit budding from plasma membranes. J. Cell Biol. 114: 881–891.

Mahaffey, D.T., J.S. Peeler, F.M. Brousky, and R.G. Anderson. 1990. Clathrin-coated pits contain an integral membrane protein that binds the AP-2 subunit with high affinity. J. Biol. Chem. 265:16514–16520.

Mangasarian, A., M. Foti, C. Aiken, D. Chin, J.-L. Carpentier, and D. Trono. 1997. The HIV-1 Nef protein acts as a connector with sorting pathways in the Golgi and at the plasma membrane. Immunity. 6:677–78.

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.

Markwell, M.A.K. 1982. A new solid-state reagent to iodinate proteins. Anal. Biochem. 125:427–432.

Maxfield, F.R., J. Schlessinger, Y. Shechter, I. Pastan, and M. C. Willingham. 1978. Collection of insulin, EGF and m2-macroglobulin in the same patches on the surface of cultured fibroblasts and common internalization. Cell. 14: 805–810.

Miller, K., M. Shipman, I.S. Trowbridge, and C.R. Hopkins. 1991. Transferrin receptors promote the formation of clathrin lattices. Cell. 65:621–632.

Ohno, H., J. Stewart, M.C. Fournier, H. Bosshart, I. Rhee, S. Miyatake, T. Saito, A. Gallusser, T. Kirchhausen, and J.S. Bonifacino. 1995. Interaction of tyrosine-based sorting signals with clathrin-associated proteins. Science (Wash. DC). 269:1872–1875.

Okabayashi, Y., Y. Sugimoto, N.F. Totty, J. Hsuan, Y. Kido, K. Sakaguchi, I. Gout, M.D. Waterfield, and M. Kasuga. 1996. Interaction of Src with adaptor protein adaptins. J. Biol. Chem. 271:5265–5269.

Pelchen Matthews, A., J.E. Armes, T. Serafin, and J.E. Rothman. 1989. Dissection of a single round of vesicular transport: sequential intermediates for intercisternal movement in the Golgi stack. Cell. 56:357–368.

Paccard, J.P., W. Reith, B. Johansson, K.E. Magnusson, B. Mach, and J.L. Carpenter. 1993. Clathrin-coated pit-mediated receptor internalization. Role of internalization signals and receptor mobility. J. Biol. Chem. 268:23191–23196.

Peeler, J.S., W.C. Donzell, and R.G. Anderson. 1993. The appendage domain of the AP-2 subunit is not required for assembly or invagination of clathrin-coated pits. J. Cell Biol. 120:47–54.

Pelchen Matthews, A., J.E. Armes, and M. Marsh. 1989. Internalization and recycling of CD4 translocated into HeLa and NIH3T3 cells. EMBO (Eur. Mol. Biol. Organ.) J. 8:3641–3649.

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.

Rhee, S.S., and J.W. Marsh. 1994. Human immunodeficiency virus type 1 Nef-induced down-modulation of CD4 is due to rapid internalization and degradation of surface CD4. J. Virol. 68:5156–5163.

Robinson, M.S. 1994. The role of clathrin, adaptors and dynamin in endocytosis. Curr. Opin. Cell Biol. 6:530–544.

Rodman, J.S., R.W. Mercer, and P.D. Stahl. 1990. Endocytosis and transcytosis. Curr. Opin. Cell Biol. 2:664–672.

Rossi, F., A. Gallina, and G. Milanesi. 1996. Nef-CD4 physical interaction sensed with the yeast two-hybrid system. Virology. 217:397–403.

Rothman, J.E., and L. Orci. 1992. Molecular dissection of the secretory pathway. Nature (Lond.). 355:409–415.

Sako, Y., and A. Kusumi. 1994. Compartmentalization structure of the plasma membrane for receptor movements as revealed by a nanometer-level motion analysis. J. Cell Biol. 125:1251–1264.

Sakselka, K., G. Cheng, and D. Baltimore. 1995. Proline-rich (PxxP) motifs in HIV-1 Nef bind to SH3 domains of a subset of Src kinases and are required for the enhanced growth of Nef+ viruses but not for down-regulation of CD4. EMBO (Eur. Mol. Biol. Organ.) J. 14:484–491.

Salgheiti, S., R. Mariani, and J. Skowronski. 1995. Human immunodeficiency virus type 1 Nef and p56lck protein-tyrosine kinase interact with a common element in CD4 cytoplasmic tail. Proc. Natl. Acad. Sci. USA. 92:349–353.

Salisbury, J.L., J.S. Condeccis, and P. Satir. 1980. Role of coated vesicles, microfilaments, and calmodulin in receptor-mediated endocytosis by cultured β lymphoblastoid cells. J. Cell Biol. 87:132–141.

Salpeter, M.M., H.C. Fertuck, and E.E. Salpeter. 1977. Resolution in electron microscope autoradiography II. Iodine-125, the effect of heavy metal staining and reassessment of critical parameters. J. Cell Biol. 72:161–173.

Sanan, D.A., and R.G. Anderson. 1991. Simultaneous visualization of LDL receptor distribution and clathrin lattices on membranes torn from the upper surface of cultured cells. J. Histochem. Cytochem. 39:1017–1024.

Sanfrison, A., S. Hester, and C. Doyle. 1997. Nef proteins encoded by human and simian immunodeficiency viruses induce the accumulation of endosomes and lysosomes in human T cells. Proc. Natl. Acad. Sci. USA. 94:873–878.

Santis, F., and J.H. Keen. 1996. Endocytosis of activated receptors and clathrin-coated pit formation: deciphering the chicken or egg relationship. J. Cell Biol. 132:1025–1036.

Sawai, E.T., A.S. Baur, B.M. Peterlin, J.A. Levy, and C. Cheng-Mayer. 1995. A conserved domain and membrane targeting of nef from HIV and SIV are required for association with cellular serine kinase activity. J. Biol. Chem. 270: 15307–15314.

Schmid, S.L. 1992. The mechanism of receptor-mediated endocytosis: more questions than answers. BioEssays. 14:589–596.

Schmid, S.L. 1993. Biochemical requirements for the formation of clathrin- and COP-coated transport vesicles. Curr. Opin. Cell Biol. 5:621–627.

Schmidt, S.L., and E. Smythe. 1991. Stage-specific assays for coated pit formation and coated vesicle budding in vitro. J. Cell Biol. 114:869–880.

Schwartz, O., Y. Riviere, J.M. Heard, and O. Danos. 1993. Reduced cell surface expression of processed human immunodeficiency virus type 1 envelope glycoprotein in the presence of N. J. Virol. 67:3274–3280.

Schwartz, O., V. Marechal, S. Le Gall, F. Lemonnier, and J.M. Heard. 1996. Endocytosis of major histocompatibility complex I molecules is induced by the HIV-1 Nef protein. Nat. Med. J. India 2:338–342.

Smythe, E., and G. Warren. 1991. The mechanism of receptor-mediated endocytosis. Curr. Opin. Cell Biol. 3:634–641.

Sorkin, A., and G. Carpenter. 1993. Interaction of activated EGF receptors with coated pit adaptins. Science (Wash. DC). 261:612–615.

Trowbridge, I.S. 1991. Endocytosis and signals for internalization. Curr. Opin. Cell Biol. 5:621–627.

Umesono, K., K.K. Murakami, C.C. Thompson, and R.M. Evans. 1991. Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D3 receptors. Cell. 65:1255–1266.

Van Deurs, B., O.W. Petersen, S. Olsnes, and K. Sandvig. 1989. The ways of endocytosis. J. Cell Biol. 100:633–637.

Watts, C. 1985. Rapid endocytosis of the transferrin receptor in the absence of bound transferrin. J. Cell Biol. 100:633–637.

Watts, C., and M. Marsh. 1992. Endocytosis: what goes in and how? J. Cell Sci. 103:1–8.

Willingham, M.C., I.H. Pastan, G.G. Sahagian, G.W. Jourdian, and E.F. Neufeld. 1981. Morphologic study of the internalization of a lysosomal enzyme by the mannose 6-phosphate receptor in cultured Chinese hamster ovary cells. Proc. Natl. Acad. Sci. USA. 78:4967–4971.