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Second-Messenger Regulation of Receptor Association with Clathrin-coated Pits: A Novel and Selective Mechanism in the Control of CD4 Endocytosis

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CD4, a member of the immunoglobulin superfamily, is not only expressed in T4 helper lymphocytes but also in myeloid cells. Receptor-mediated endocytosis plays a crucial role in the regulation of surface expression of adhesion molecules such as CD4. In T lymphocytes $p56^{lck}$, a CD4-associated tyrosine kinase, prevents CD4 internalization, but in myeloid cells $p56^{lck}$ is not expressed and CD4 is constitutively internalized. In this study, we have investigated the role of cyclic AMP (cAMP) in the regulation of CD4 endocytosis in the myeloid cell line HL-60. Elevations of cellular cAMP were elicited by 1) cholera toxin, 2) pertussis toxin, 3) forskolin and IBMX, 4) NaF, or 5) the physiological receptor agonist prostaglandin E_1. All five interventions led to an inhibition of CD4 internalization. Increased cAMP levels did not inhibit endocytosis per se, because internalization of insulin receptors and transferrin receptors and fluid phase endocytosis were either unchanged or slightly enhanced. The mechanism of cAMP inhibition was further analyzed at the ultrastructural level. CD4 internalization, followed either by quantitative electron microscopy autoradiography or by immunogold labeling, showed a rapid and temperature-dependent association of CD4 with clathrin-coated pits in control cells. This association was markedly inhibited in cells with elevated cAMP levels. Thus these findings suggest a second-messenger regulation of CD4 internalization through an inhibition of CD4 association with clathrin-coated pits in $p56^{lck}$-negative cells.

INTRODUCTION

CD4 is a 55-kDa integral cell surface glycoprotein of the immunoglobulin superfamily (Maddon *et al.*, 1985). It is expressed on thymocytes, helper T lymphocytes, cells of the macrophage/monocytes lineage, and hematopoietic progenitor cells (Reinherz *et al.*, 1979; Wood *et al.*, 1983; Stewart *et al.*, 1986; Maddon *et al.*, 1987; Frederickson and Basch, 1989; Filion *et al.*, 1990; Hanna *et al.*, 1994; Louache *et al.*, 1994; Sotzik *et al.*, 1994; Zauli *et al.*, 1994). In T helper lymphocytes, CD4 acts 1) as an adhesion molecule by tightening the interaction between the T cell receptor and major histocompatibility complex II (MHCII;[1] Doyle and Strominger, 1987) and 2) as a signal-transducing receptor by triggering the activation of the lymphocyte-specific CD4-associated protein tyrosine kinase $p56^{lck}$ (Veillette *et al.*, 1988; Rudd, 1990). The physiological role of CD4 in $p56^{lck}$-negative nonlymphocytic cells is not known; however, CD4-dependent signaling in such cells has been described (Foti *et al.*, 1995).

Endocytosis is an important mechanism regulating the cell surface expression of most receptors. Internalization does not only modulate the cell surface density of receptors; it also allows the termination of the re-

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[1] Abbreviations used: IBMX, 3-isobutyl-1-methylxanthine; MHCII, major histocompatibility complex type II.

ceptor–ligand interaction, serial receptor occupation (Valitutti *et al.*, 1995), and endocytosis of bound ligands (van Deurs *et al.*, 1989). Receptor internalization is therefore a crucial determinant of many diverse biological processes, such as establishment of neuronal connection (Hu *et al.*, 1993), interaction of lymphocyte T cell receptors with MHCII (Doyle and Strominger, 1987; Parnes, 1989), supply of cell nutrients (Féger *et al.*, 1994), and determination of cell sensitivity to ligand binding (Carpentier, 1993, 1994).

For many receptors, internalization is regulated by intracellular signals. Autologous and heterologous regulation of receptors may be distinguished. Autologous regulation occurs in many instances through the activation of intrinsic or associated tyrosine kinases, e.g., insulin receptor (Carpentier, 1989). Heterologous regulation (i.e., receptor cross-talk) occurs when one receptor generates a second messenger that regulates the internalization of another receptor. Examples for the latter include the increased internalization of type 1 complement receptor after stimulation of the *N*-formylmethionylleucylphenylalamine (f-MLP) receptor (presumably via protein kinase C; Carpentier *et al.*, 1991) and cyclic AMP (cAMP) enhanced internalization of adherence receptors in neurons (through an increase in the number of clathrin-coated pits; Hu *et al.*, 1993). cAMP has also been implicated in the regulation of CD4 capping and endocytosis after receptor multimerization in human T lymphocytes (Kammer *et al.*, 1988).

In this study, we have addressed the role of cAMP in the regulation of CD4 internalization in the $p56^{lck}$-negative myeloid cell line HL-60. Our results demonstrate a cAMP-dependent inhibition of CD4 internalization through a decrease in CD4 association with clathrin-coated pits.

MATERIALS AND METHODS

Reagents

Goat anti-mouse IgG (whole molecule), goat anti-mouse IgG (whole molecule, gold conjugate, 10 nm), 3-isobutyl-1-methylxanthine (IBMX), forskolin, holo-transferrin (iron-saturated), Lucifer yellow, cAMP, dimethyl sulfoxide (DMSO), γ-globulins, prostaglandins E_1 (PGE_1), and HEPES were purchased from Sigma (St. Louis, MO). RPMI 1640 culture medium and fetal calf serum were purchased from Life Technologies (Paisley, Scotland, United Kingdom). Cholera toxin (CTX) and pertussis toxin (PTX; islet-activating protein) were purchased from List Biological Laboratories (Campbell, CA). Purified mouse anti-human CD4 IgG1 RPA-T4 was obtained from PharMingen (San Diego, CA), and anti-human T cells CD4 IgG1 Leu3a were obtained from Becton Dickinson (Erembodegen, Belgium). Iodo-Beads as iodination reagent and ImmunoPure Fab preparation kit were purchased from Pierce (Rockford, IL), and ^{125}I was obtained from Amersham (Buckinghamshire, United Kingdom). [^{125}I]Insulin (specific activity, 350 μCi/μg of protein) was a generous gift from Hoechst (Frankfurt, Germany). Anti-human insulin receptor was a generous gift from K. Siddle (Cambridge, United Kingdom). [^{3}H]Adenine (NET Q63) and [^{14}C]cAMP (NEC-463) were purchased from Dupont de Nemours (Dreieich, Germany). Other chemicals were of analytical grade and were obtained from Fluka (Buchs, Switzerland) or Sigma. When drugs were added as DMSO solutions, the final concentration of DMSO in the medium did not exceed 1% and control conditions were equally treated with DMSO. Experiments were performed in phosphate-buffered saline (PBS) or a medium containing 138 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 20 mM glucose, and 20 mM HEPES, pH 7.4 (referred as medium 1).

Cell Lines and Cultures

HL-60 cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum. Cells were induced to differentiate to the granulocytes phenotype by addition of 1.3% (vol/vol) DMSO for the first 72 h and next with 0.65% for the last 48 h to the culture medium, as described previously (Demaurex *et al.*, 1992). All experiments shown in this article were performed with DMSO-differentiated HL-60 cells. The inhibition of CD4 endocytosis by cAMP-elevating agents was, however, similarly observed in undifferentiated HL-60 cells (our unpublished results).

Iodination of Intact Antibodies, Fab Fragments, and Holo-Transferrin

Iodination of purified mouse anti-human CD4 RPA-T4, Leu3a-Fab fragments, and iron-saturated holo-transferrin was performed with Iodo-Beads, according to the manufacturer's instructions (Markwell, 1982). Briefly, Iodo-Beads were washed and preloaded with 0.5–1 mCi of Na^{125}I in Dulbecco's PBS for 5 min at room temperature. Free-carrier RPA-T4 (50 μg, 330 pmol), Leu3a Fab fragments (35–45 μg, 700–900 pmol), or holo-transferrin (100 μg, 1.23 nmol) was added and incubation was continued for 15 min at room temperature. Iodination was stopped by removing the beads from the mixture reaction and free ^{125}I was discarded by passing the solution through a gel filtration Sephadex G-25 column (Pharmacia Biotech, Uppsala, Sweden). Iodinated proteins were stored at 4°C in PBS containing 1% bovine serum albumin (BSA). Specific activity was between 2 and 3 μCi/μg of protein for ^{125}I-labeled RPA-T4, 4.5 and 6.5 μCi/μg of protein for ^{125}I-labeled Leu3a Fab fragments, and 3 and 4 μCi/μg of protein for ^{125}I-labeled transferrin.

Generation of Fab Fragments

Fab fragments were generated with the kit from Pierce using enzymatic digestion by papain-coated Sepharose beads according to the manufacturer's instructions. No specific binding could be detected with Fab fragments from the anti-CD4 monoclonal antibody RPA-T4. We have therefore generated Fab fragments from the Leu3a monoclonal antibody; Fab fragments from this antibody have already been previously used for CD4 endocytosis assays (Pelchen Matthews *et al.*, 1989).

Acid Wash Assay

Internalization was assayed using the acid-wash technique as described previously (Iacopetta *et al.*, 1986; Pelchen Matthews *et al.*, 1989, 1991; Aiken *et al.*, 1994). Briefly, $10–20 \times 10^6$ cells were washed and incubated for 2 h at 4°C with ^{125}I-labeled RPA-T4 (0.02–0.04 μCi/10^6 cells, i.e., 7–20 ng/10^6 cells), ^{125}I-labeled Leu3a Fab fragments (0.1–0.2 μCi/10^6 cells, i.e., 15–44 ng/10^6 cells), ^{125}I-labeled insulin (0.02–0.04 μCi/10^6 cells, i.e., 57–115 pg/10^6 cells), or ^{125}I-labeled transferrin (0.01–0.02 μCi/10^6 cells, i.e., 3–7 ng/10^6 cells) in 0.5 ml of ice-cold PBS/BSA (1%). Cells were next washed twice by centrifugation ($200 \times g$, 5 min) to remove unbound radiolabeled molecules and then incubated at 37°C to allow endocytosis. Cells were washed twice in their appropriated buffer at low pH (pH 2) to remove surface-bound radiolabeled antibodies or ligands. Percentages of ^{125}I-labeled anti-CD4, ^{125}I-labeled anti-CD4 Fab fragments, ^{125}I-labeled transferrin, and ^{125}I-labeled insulin internalization were

expressed as the ratio of acid-wash–resistant radioactivity to total radioactivity associated to cells at neutral pH. Counting was performed in a Beckman 5500 gamma counter. Experimental conditions for the acid-wash assay were determined with the iodinated RPA-T4 antibody. Antibody binding at 4°C was maximal after 90 min of incubation. The nonspecific binding was between 3 and 5% of the total recovered radioactivity. The sequential washes with buffer at pH 2 detached more than 95% of the surface bound antibodies (our unpublished results). To study the endocytosis of cross-linked CD4, cells were first incubated for 90 min at 4°C with the iodinated antibody, washed, and reincubated for 45 min at 4°C with a goat anti-mouse IgG (0.2 $\mu g/10^6$ cells).

Fluid Phase Endocytosis Assay

Duplicate of 15×10^6 cells were incubated for 5 min at 37°C or at 4°C in the presence of Lucifer yellow (1.5 mg/ml in medium 1 free of Ca^{2+} and Mg^{2+}). At the end of each incubation period, the endocytotic process was stopped by cooling the cells at 4°C. Cells were next washed four times in medium 1 free of Ca^{2+} and Mg^{2+} at 4°C and lysed in medium 1 containing 0.1% Triton X-100 (Krischer *et al.*, 1993). Cell-associated fluorescence was measured in a Perkin Elmer-Cetus LS-3 fluorimeter (excitation, 415 nm; emission, 535 nm). Finally, fluorescence values were reported to protein content of each samples. Protein determination was performed with the Pierce kit using bicinchonic acid.

cAMP Measurements

cAMP levels were determined by sequential chromatography (Johnson *et al.*, 1994). Briefly, HL-60 cells (10^6 cells/ml) were incubated for 3 h in medium 1 containing 1 mM $CaCl_2$ and 1.5 mM [^{3}H]adenine at 37°C. Next, cells were washed and stimulated by either PGE_1 (20 μM, 30 min, 37°C), CTX (1 μg/ml, 1 h, 37°C), PTX (250 ng/ml, 1 h, 37°C), IBMX (0.5 mM, 30 min, 37°C)/forskolin (100 μM, 5 min, 37°C), or NaF (20 mM, 10 min, 37°C). Cells were lysed using 30% trichloroacetic acid, and cAMP degradation was stopped by addition of 1 mM IBMX and 100 μM cAMP. Cell lysates were sequentially chromatographed on Dowex and alumina ion-exchange columns to separate [^{3}H]cAMP from other ^{3}H-labeled nucleotides. Column recovery were corrected by adding [^{14}C]cAMP in samples before the chromatographic steps. Controls were obtained by measuring simultaneously in parallel cAMP production in nontreated cells in the conditions used for treated cell.

Electron Microscopic Autoradiography

HL-60 granulocytes were incubated with buffer only or stimulated with CTX (1 μg/ml, 1 h, 37°C), washed in PBS, and incubated for 2 h at 4°C with ^{125}I-labeled anti-CD4 (RPA-T4; 100 ng/10^6 cells) in ice-cold PBS/BSA (1%). After antibody binding, cells were washed twice by centrifugation ($200 \times g$, 5 min) in ice-cold PBS to remove antibody excess and warmed for various times at 37°C to allow endocytosis. Cells were then fixed in glutaraldehyde, postfixed in osmium tetroxide, and processed for quantitative electron microscopic autoradiography as described previously (Carpentier *et al.*, 1978, 1981, 1982, 1992; Fan *et al.*, 1982). Thin sections were examined in a Philips EM 300 electron microscope (Philips, Eindhoven, the Netherlands), and autoradiographic grains were quantitatively analyzed at a magnification of 16,600× on cells that were judged to be well preserved. Two experiments were performed. For each incubation time in each experiment, two Epon blocks were prepared, and sections were cut from each block. For each time point studied, a total of 800–900 grains was analyzed. Autoradiographic grains within a distance of ±250 nm from the plasma membrane were considered associated with the cell surface, whereas grains overlying the cytoplasm and >250 nm from the plasma membrane were considered internalized (Carpentier *et al.*, 1981, 1982, 1993). Grains associated with the plasma membrane were divided into the following classes: 1) microvilli, 2) clathrin-coated pits, and 3) nonvillous nonclathrin-coated segments. Grains were considered associated with microvilli or clathrin-coated pits if their center was <250 nm from these surface domains. This approach has been extensively used and validated in previous studies (Carpentier *et al.*, 1978, 1981, 1992; Fan *et al.*, 1982).

Immunogold Labeling of CD4

HL-60 granulocytes were stimulated with CTX (1 μg/ml, 1 h, 37°C), washed in PBS, and incubated for 2 h at 4°C with Leu3a (40 ng/10^6 cells) in ice-cold PBS/BSA (1%). After antibody binding, cells were washed twice by centrifugation ($200 \times g$, 5 min) in ice-cold PBS to remove antibody excess and incubated a second time with an anti-mouse IgG coupled to 10-nm colloidal gold particles for 2 h at 4°C [antibodies used at a dilution of 1:15 for a cell suspension of 5×10^7 cells/ml of PBS/BSA (1%)]. Unbound secondary antibody was removed by washing twice with ice-cold PBS, and cells were warmed for 5 min at 37°C to allow endocytosis. Cells were finally fixed 30 min at room temperature with 2.5% glutaraldehyde in phosphate buffer, dehydrated, and processed for electron microscopy as described previously (Carpentier *et al.*, 1991). Thin sections were examined in a Philips EM 300 (Philips), and gold particles were quantitatively analyzed on cells considered well preserved. Gold particles associated with the plasma membrane were divided in the following classes: 1) particles over microvilli, 2) particles over nonvillous noncoated plasma membrane segments, and 3) particles over clathrin-coated pits/vesicles. Data are means ± range of two experiments totaling together: 1) 180 cells/1462 gold particles and 180 cells/1158 gold particles counted after incubations at 4°C and after 5 min warming at 37°C, respectively, for the control conditions and 2) 184 cells/1631 gold particles and 188 cells/1289 gold particles counted after incubations at 4°C and after 5 min warming at 37°C, respectively, for cells treated with CTX.

To rule out the involvement of Fc receptors in the observed gold labeling, cells were preincubated with 100 μg/ml nonspecific human immunoglobulin. This preincubation did not change the number of gold particles per cell (for 20 cells counted, 201 gold particles were found in the absence of nonspecific human immunoglobulin and 205 were found in the presence).

Quantitative Electron Microscopic Analysis

Thin sections of HL-60 cells were randomly photographed and analyzed on a Leica Quantimet 500+. The ratio of nonvillous to villous plasma membrane was calculated for each condition (cells incubated with buffer only or treated 1 h with CTX at 1 μg/ml) on a sampling of 51–53 electron micrographs of cells considered to be well conserved (Carpentier *et al.*, 1993). Percentage of the plasma membrane occupied by clathrin-coated structures (clathrin-coated pits or vesicles) was also estimated using the same procedure. For each condition, thin sections (n = 50–52 electron micrographs of cells considered to be well conserved) used to quantify radiolabeled CD4 surface distribution were randomly photographed and analyzed on a Leica Quantimet 500+.

RESULTS

CD4 Internalization in HL-60 Cells

In addition to its expression in T helper lymphocytes, it is now generally accepted that CD4 is present in cells of myeloid lineage, such as the HL-60 cells. However, the control of CD4 surface expression may be substantially different in myeloid cells and in lymphocytes. Indeed, myeloid cells lack the protein tyrosine kinase $p56^{lck}$, which in lymphocytes is associated with CD4 and plays a crucial role in the regulation of CD4

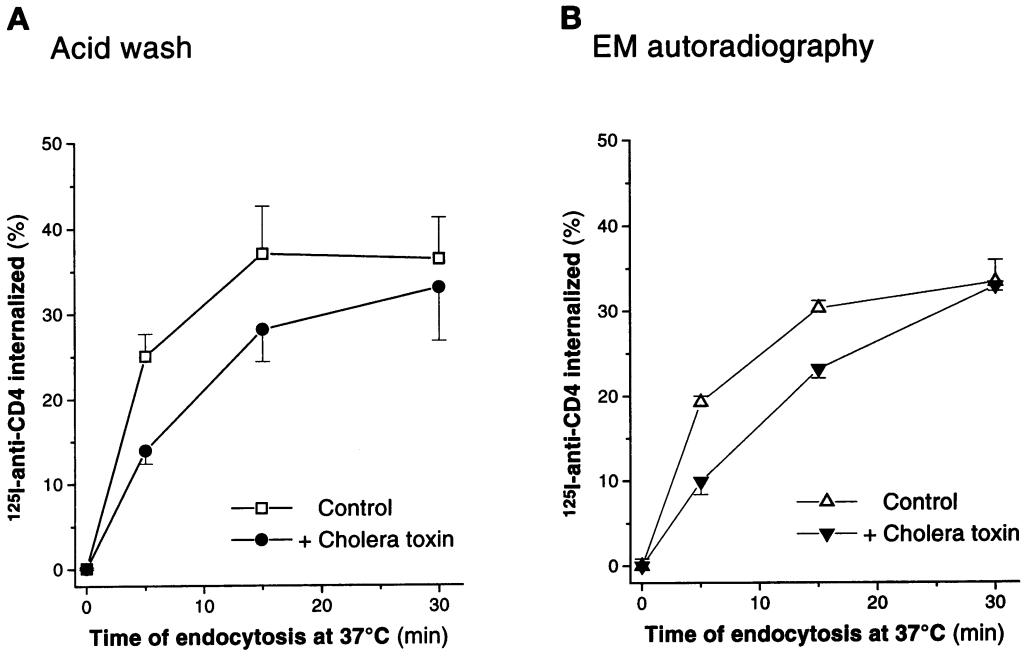

Figure 1. Kinetics of CD4 endocytosis in control and CTX-treated HL-60 cells. HL-60 were preincubated with 1 μg/ml CTX or buffer only (1 h, 37°C). ^{125}I-labeled anti-CD4 antibody was allowed to bind to cell surface CD4 at 4°C for 2 h. After removal of unbound antibody, endocytosis of the ^{125}I-labeled antibody–CD4 complex was allowed to occur by raising the temperature to 37°C. (A) An acid-wash assay was used to measure the CD4 internalization as described in MATERIALS AND METHODS. CD4 internalization was calculated as the ratio of acid-wash–resistant radioactivity to total cell-associated radioactivity at neutral pH. Data are means ± SE of three experiments, each performed in duplicate. (B) CD4 internalization was morphologically quantitated on cells processed for EM autoradiography as described in MATERIALS AND METHODS. Data are means ± range of two experiments. The CTX concentration used in this study (1 μg/ml) was not cytotoxic, as assessed by trypan blue exclusion.

endocytosis. The aim of this study was, therefore, to investigate possible mechanisms that might regulate CD4 endocytosis in HL-60 cells.

Internalization of surface CD4 was assessed by analyzing the cellular uptake of radiolabeled antibodies directed against CD4 in pulse experiments, i.e., after a 2-h preincubation in the presence of ^{125}I-labeled antibody, cells were washed extensively to remove unbound antibodies and internalization was allowed by raising the temperature to 37°C. Under these conditions, the initial rate of CD4 internalization was 4–5%/min (Figure 1), similar to results obtained in another study carried out in HL-60 cells (Pelchen Matthews *et al.*, 1991). The intracellular CD4 accumulation reached a plateau after approximately 15 min (Figure 1A). As an alternative approach to quantify CD4 internalization, we have tracked ^{125}I-labeled anti-CD4 antibody by quantitative electron microscope (EM) autoradiography. The kinetics of CD4 internalization observed using this method were very similar to those obtained in the course of the acid-wash assay (Figure 1B).

cAMP Inhibition of CD4 Internalization in HL-60 Cells

cAMP is a universal second messenger, which has been previously implicated in the regulation of endocytotic processes (Bradbury *et al.*, 1992; Hu *et al.*, 1993; Eker *et al.*, 1994). In a first attempt to assess the role of cAMP in CD4 internalization, HL-60 cells were treated with CTX, an agent known to stimulate adenylate cyclase (Gilman, 1987). The rate of intracellular CD4 accumulation was decreased by the CTX, as measured by the acid-wash assay (Figure 1A) or EM autoradiography (Figure 1B). The effect was most obvious at early time points (which predominantly reflect receptor internalization). The plateau reached after 15–30 min (which include the effects of receptor recycling) was not markedly influenced by the toxin. We have therefore restricted the quantitative analysis to the 5-min time point.

To verify that the above-mentioned inhibition of CD4 internalization correlated with increased cAMP levels, cells were incubated in the presence of various compounds known to pharmacologically increase cAMP, PTX (Gilman, 1987), IBMX/forskolin (Seamon *et al.*, 1981), and NaF (Blackmore *et al.*, 1985). All of them 1) significantly elevated cellular cAMP levels[2]* (Figure 2B) and 2) inhibited CD4 internalization (Figure 2A). Already relatively small

[2] The method used does not allow us to obtain absolute values for cellular cAMP levels. However, previous studies in HL-60 granulocytes have demonstrated basal cAMP levels of 1–3 pmol/10^6 cells (Prytz *et al.*, 1991; Bang *et al.*, 1993). Our results show that PGE_1, PTX, CTX, forskolin/IBMX, and NaF raise cellular cAMP levels by factors of 4.0 ± 0.5, 7.7 ± 2.8, 38.5 ± 4.3, 19.0 ± 8.8, 141.8 ± 12.5, respectively.

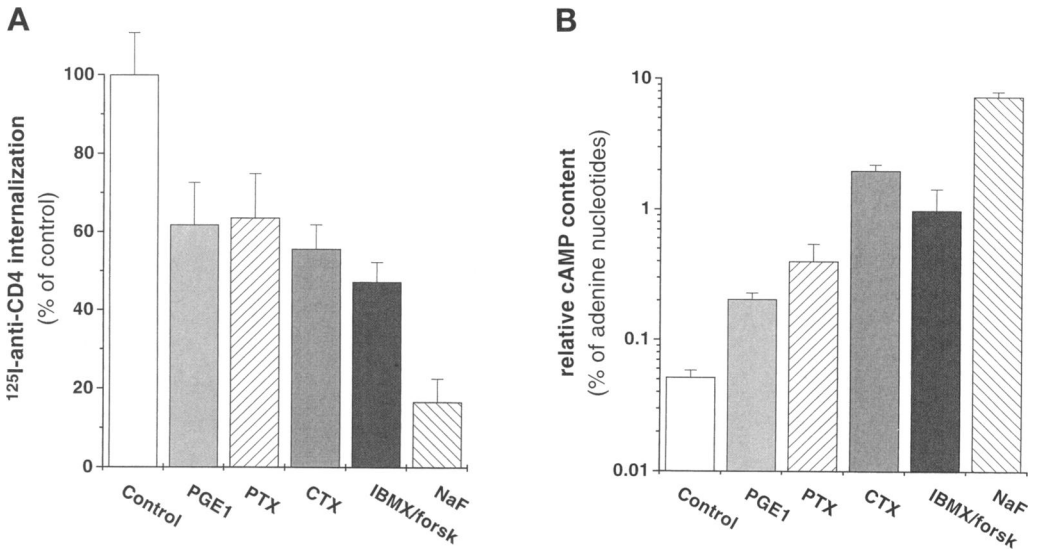

Figure 2. CD4 internalization is decreased by compounds that induce cAMP elevations. HL-60 cells were pretreated with incubation buffer only (1 h at 37°C, control), PGE_1 (20 μM, 30 min at 37°C), PTX (250 ng/ml, 1 h at 37°C), CTX (1 μg/ml, 1 h at 37°C), IBMX (0.5 mM, 30 min at 37°C)/Forskolin (forsk.; 100 μM, 5 min at 37°C), or NaF (20 mM, 10 min at 37°C). (A) Effect of various compounds on CD4 internalization. An acid-wash assay was used to measure CD4 internalization. ^{125}I-labeled anti-CD4 antibody (^{125}I-labeled RPA-T4) was allowed to bind to cell surface CD4 at 4°C for 2 h. Endocytosis of the ^{125}I-labeled antibody–CD4 complex was allowed to occur during 5 min by raising the temperature to 37°C. CD4 internalization was calculated as the ratio of acid-wash–resistant radioactivity to total cell-associated radioactivity at neutral pH. Data are expressed as percentage of control (i.e., pretreatment with incubation buffer only). CD4 internalization in control cells was 25.1 ± 2.6% per 5 min. Data are the means ± SE of three experiments, each performed in duplicate. (B) Effect of various compounds on cAMP levels. Cells were loaded with [^{3}H]adenine and pretreated with cAMP-elevating compounds as described above. Cells were next lysed by addition of trichloroacetic acid and cAMP was separated from other ^{3}H-labeled adenosine nucleotides by sequential ion-exchange chromatography. [^{3}H]cAMP levels are expressed as percentage of total cellular ^{3}H-labeled adenosine nucleotides. Cells pretreated with cAMP-elevating compounds incorporated 110.2 ± 19.2% of ^{3}H-labeled nucleotides as compared with control cells. Data are means ± range of two experiments, each performed in triplicate.

elevations of cAMP induced by the PTX (eightfold as compared with levels in control cells) led to a marked inhibition of CD4 internalization. Approximately 50% inhibition was obtained with intermediate cAMP levels, such as those induced by the CTX and IBMX/forskolin (20- to 40-fold as compared with levels in control cells) and 80% inhibition was reached with fluoride, which increased cAMP ~150-fold. Thus, our data demonstrate that cAMP blocks CD4 endocytosis, as 1) all compounds that raised cAMP blocked CD4 endocytosis and 2) there was an all-over relationship between the cAMP levels and the inhibition of CD4 endocytosis. We next investigated whether the physiological receptor agonist PGE_1, which is known to raise cAMP myeloid cells (Scott, 1970; Bourne *et al.*, 1971), also had effects on CD4 endocytosis. PGE_1 increased cellular cAMP levels approximately fourfold (Figure 2B) and inhibited CD4 internalization by ~40% (Figure 2A). Thus, receptor-mediated cAMP elevations lead to an inhibition of CD4 internalization, suggesting a physiological relevance of the observed cAMP effect.

cAMP Effect on Transferrin, Insulin, and Fluid Phase Endocytosis

The cAMP effect might 1) be receptor-specific, 2) apply to endocytosis of other receptors internalized by clathrin-coated pits, or 3) affect various form of endocytosis including fluid phase endocytosis. To distinguish between these possibilities, the effect of cAMP-elevating compounds on internalization of insulin and transferrin receptors and on fluid phase endocytosis of Lucifer yellow were investigated. The insulin receptor is a signaling receptor found outside clathrin-coated pits (on microvilli) in its unoccupied state. In response to insulin binding, it associates with clathrin-coated pits to be internalized (Carpentier *et al.*, 1992, 1993). In contrast, the transferrin receptor, a carrier of cargo molecules, is concentrated in its unoccupied state within clathrin-coated pits and is constitutively internalized (Watts, 1985). Eventually, fluid phase endocytosis represents the receptor-independent endocytotic activity of the cell; at least part of fluid phase endocytosis is thought to occur via clathrin-coated pits (van Deurs *et al.*, 1989). As shown in Figure 3, neither internalization of insulin and transferrin receptors tagged with their respective ^{125}I-labeled ligands nor fluid phase endocytosis were inhibited by elevated cAMP levels: the internalization of transferrin receptors and fluid phase endocytosis was even slightly enhanced. Thus, high intracellular cAMP levels inhibit neither receptor-mediated endocytosis in general nor the nonspecific fluid phase uptake, which suggests that the cAMP-dependent inhibitory effect on receptor internalization was specific to CD4.

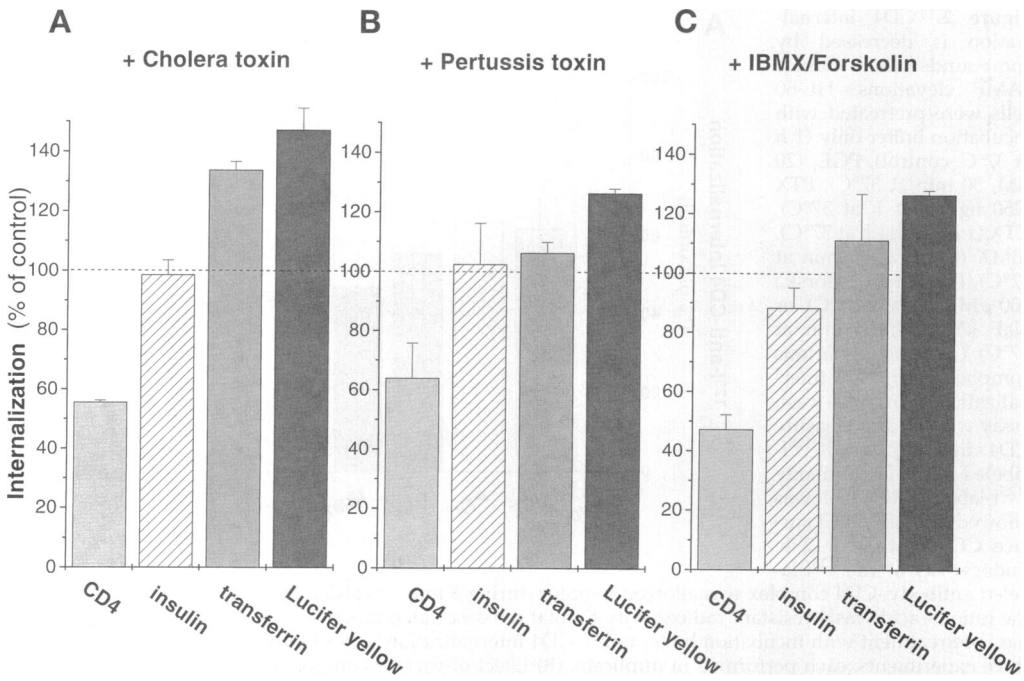

Figure 3. Increases in cellular cAMP levels selectively decrease CD4 internalization. An acid-wash assay was used to measure the CD4, insulin, and holotransferrin internalization. Iodinated antibody/ligands were allowed to bind to cell surface-specific receptors at 4°C for 2 h. Endocytosis of the ^{125}I-labeled molecules was allowed to occur by raising the temperature to 37°C for 5 min. ^{125}I-labeled molecules internalization was calculated as the ratio of acid-wash–resistant radioactivity to total radioactivity associated with cells at neutral pH. Data are expressed as percentage of control (i.e., pretreatment with incubation buffer only). Internalization in control cells were 25.1 ± 2.6% per 5 min, 16.9 ± 4.6% per 5 min, and 43.2 ± 5.0% per 5 min for CD4, insulin, and holo-transferrin, respectively. Data are the means ± SE of three experiments, each performed in duplicate. Fluid phase endocytosis was assessed by incubating cells with Lucifer yellow at 4°C and endocytosis was allowed to occur by raising the temperature to 37°C for 5 min. Fluid phase uptake was estimated by calculating the ratio of cell-associated fluorescence over protein content of each sample. Results are expressed as percentage of the control (no raise of the temperature). Data are means ± SE of three experiments, each performed in duplicate. (A) Cells treated with 1 μg/ml CTX for 1 h at 37°C. (B) Cells treated with 250 ng/ml PTX for 1 h at 37°C. (C) Cells treated with 100 μM forskolin for 5 min at 37°C and 0.5 mM IBMX for 30 min at 37°C.

cAMP Inhibition of CD4 Internalization Does Not Critically Depend on Receptor Cross-Linking

Receptor internalization may be sensitive to the aggregation state induced by ligands or antibodies (Kammer *et al.*, 1983; Heldin, 1995). To assess the potential role of receptor cross-linking induced by antibody binding (Sakihama *et al.*, 1995), CD4 internalization in the presence of divalent or monovalent (anti-CD4 Fab fragments) were compared. In agreement with results observed in other nonlymphocytic cells (Pelchen Matthews *et al.*, 1989), no difference was observed between these two experimental conditions (Figure 4A). Similarly, the effect of cAMP on CD4 internalization was unchanged when a monovalent instead of a divalent antibody was used (Figure 4B). In contrast, after cross-linking with a secondary antibody, an approximately 2.5-fold increase in CD4 internalization occurred (Figure 4A). The cAMP inhibition of CD4 internalization was also present after CD4 cross-linking by a secondary antibody; however, the relative decrease was smaller under these conditions (Figure 4B). For the insulin receptor, the absence of a cAMP effect on internalization was observed in the presence and the absence of a cross-linking antibody (Figure 4C). Thus, the cAMP effect on CD4 internalization does not critically depend on receptor cross-linking by anti-CD4 antibodies.

CD4 Surface Localization and Association with Clathrin-coated Membranes

CD4 has been proposed to be internalized through clathrin-coated pits, both in lymphocytes and in myeloid cells (Pelchen Matthews *et al.*, 1991). To understand how cAMP affected CD4 internalization in HL-60 myeloid cells, we studied at the EM ultrastructural level the surface localization of CD4 and in particular its association with clathrin-coated structures in the first minutes of endocytosis. As CD4 endocytosis is increased by multivalent ligands (e.g., cross-linking by a secondary antibody; Figure 4), we used in parallel two approaches: 1) CD4 was tagged with ^{125}I-labeled anti-CD4 antibodies and localized by quantitative EM autoradiography (primary cross-link, Figure 5), and 2) CD4 was labeled by a primary anti-CD4 antibody followed by a secondary gold-conjugated antibody (secondary cross-link, Figure 6).

After a 2-h incubation with the radiolabeled antibody at 4°C, approximately 20% of the plasma membrane labeling was found on microvilli. The bulk of CD4 was located on nonvillous non-clathrin-coated membranes, and only a small fraction of plasma membrane labeling (~5%) was recovered in clathrin-coated pits (Figure 7A). When cells were incubated for 5 min at 37°C to initiate endocytosis, CD4 was recruited into clathrin-coated sur-

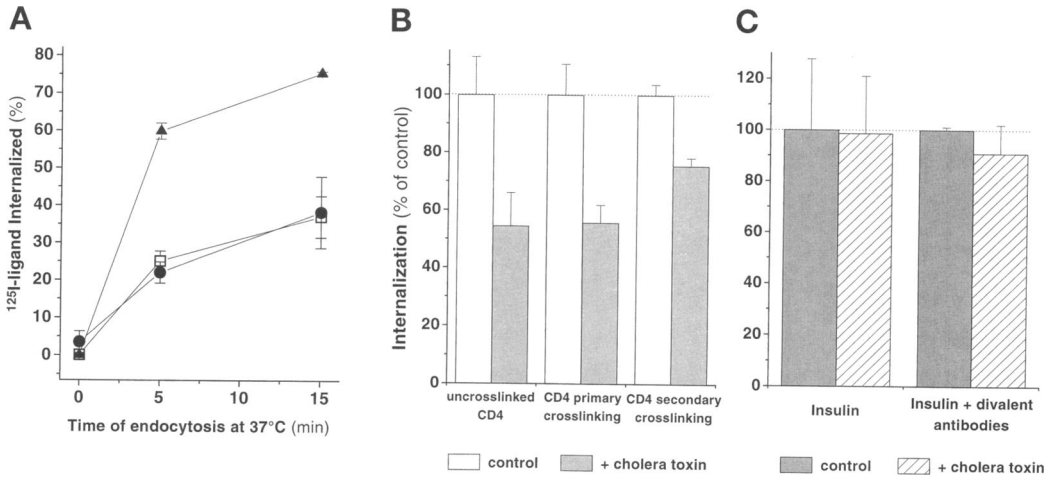

Figure 4. cAMP effect does not critically depend on the receptor cross-linking. An acid-wash assay was used to follow receptor internalization. ^{125}I-labeled ligands were allowed to bind to cells at 4°C for 2 h, and their internalization was allowed to occur by raising the temperature to 37°C. The internalization of iodinated ligands was calculated as the ratio of acid-wash–resistant radioactivity to total radioactivity associated with cells at neutral pH. (A) Time course of CD4 internalization assessed by ^{125}I-labeled divalent anti-CD4 antibodies (□), ^{125}I-labeled monovalent Fab fragments (●), and ^{125}I-labeled divalent anti-CD4 antibodies cross-linked by a secondary antibody (▲). (B) Effect of CTX on CD4 internalization (5 min at 37°C), assessed by ^{125}I-labeled monovalent Fab fragments (uncross-linked CD4), ^{125}I-labeled divalent anti-CD4 antibodies (CD4 primary cross-linking), and ^{125}I-labeled divalent anti-CD4 antibodies cross-linked by a secondary antibody (CD4 secondary cross-linking). (C) Effect of CTX on insulin receptor internalization (5 min at 37°C) assessed by ^{125}I-labeled insulin in the absence (insulin) or presence of a divalent anti-insulin receptor antibody (insulin + divalent antibodies). Divalent antibodies against the insulin receptors were added 1 h before initiation of internalization. In the absence of CTX, 16.9 ± 4.6% and 15.1 ± 0.2% of total cell-associated ^{125}I-labeled insulin was internalized in the absence and presence of the divalent antibody, respectively. Data are means ± range or SE of two or three experiments, each performed in duplicate.

faces leading to a twofold increase in the fraction of cell surface CD4 present in these structures (Figure 7, A and B). By contrast in the presence of CTX after 5 min of endocytosis at 37°C, only a small increase (~1.1-fold), as compared with untreated cells, of the CD4 association with clathrin-coated structures was observed (Figure 7B). Thus, CTX treatment (i.e., high intracellular cAMP levels) decreases significantly the temperature-dependent association of CD4 with clathrin-coated structures. This decreased association of CD4 with clathrin-coated structures was consistent with the observed inhibition of the internalization (Figure 1).

Qualitatively, the data obtained by immunogold labeling of CD4 yielded the same results as data obtained by EM autoradiography. At 4°C, CD4 was mainly located on nonvillous non-clathrin-coated membranes, and only a small fraction (~6%) was found associated with clathrin-coated structures (Figure 7C). After 5 min at 37°C, an increased association of CD4 with clathrin-coated surfaces was detected. This increased temperature-dependent association of CD4 with clathrin-coated surfaces was also reduced by CTX treatment of the cells (Figure 7D).

There were, however, marked quantitative differences between the results obtained with the EM autoradiographic detection of ^{125}I-radiolabeled CD4 and the EM detection of gold-labeled CD4. The major difference between these two detection methods is the extent of the CD4 cross-linking prior to endocytosis. In the EM autoradiographic detection, CD4 is weakly cross-linked by the primary radiolabeled antibody (by virtue of the antibody divalence); however, this primary cross-link does not influence the efficiency of CD4 internalization as compared with non-cross-linking conditions (as shown by the acid-wash assay in Figure 4A). By contrast, when CD4 is tagged with gold particles, the receptor is cross-linked to a higher extent through a primary and a secondary gold-conjugated antibody (secondary cross-link). Under these conditions the temperature-dependent association with clathrin-coated structures was more pronounced, and the relative inhibition by CTX was less efficient (Figure 7, compare B and D). These results are consistent with those obtained through the acid-wash assay in which a secondary cross-linking of CD4 enhances its internalization but attenuates the inhibitory effect of CTX (see Figure 4).

cAMP Effects on Cell Geometry and Quantities of Clathrin-coated Pits

In some cell types, cAMP has been shown to affect the cytoskeleton organization (Kammer *et al.*, 1983; Morton and Tchao, 1994; Lang *et al.*, 1996) or the quantities of clathrin-coated pits on the cell surface (Hu *et al.*, 1993). We have therefore analyzed the effect of cAMP on the general cell geometry and on the number and

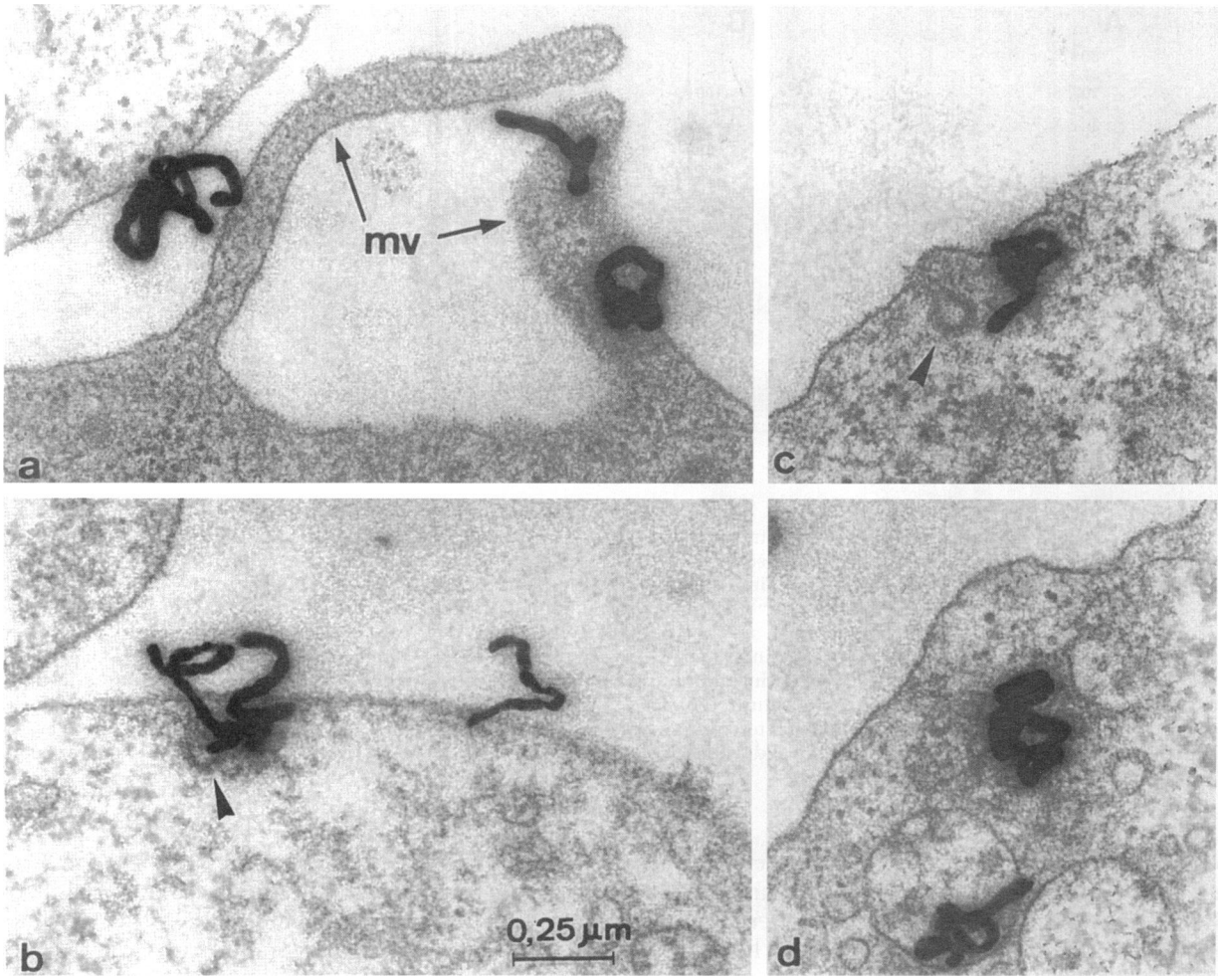

Figure 5. Representative electron micrographs of CD4 radiolabeling in HL-60 cells. Cells were incubated for 2 h at 4°C with an iodinated anti-CD4 antibody (^{125}I-labeled RPA-T4) and endocytosis of the ^{125}I-labeled antibody–CD4 complex was allowed to occur by raising the temperature to 37°C. Cells were then processed for EM detection, and autoradiographic grains were quantified for their association with microvilli (a, mv), clathrin-coated pits (b and c, arrowheads), nonvillous membrane (c), and the inner of cells (d). Autoradiographic grains within a distance of ±250 nm from the plasma membrane were considered associated with the cell surface and grains overlying the cytoplasm and >250 nm from the plasma membrane were considered internalized. Grains were considered associated with microvilli or clathrin-coated pits if their center was less than 250 nm from these surface domains.

size of clathrin coated pits (Table 1). Neither the percentage of plasma membrane constituted by microvilli nor the size and density of clathrin-coated pits were significantly affected by cAMP (i.e., CTX treatment of cells). Thus, the CTX inhibition of CD4 association with clathrin-coated pits was not due to a change in cell geometry or clathrin-coated pits/vesicles density.

DISCUSSION

Our study is the first demonstration of a regulatory mechanism that decreases CD4 internalization in p56^{lck}-negative cells. Similar to the action of p56^{lck} in lymphocytes (Pelchen Matthews *et al.*, 1992), cAMP elevations in HL-60 cells led to a decreased CD4 internalization through a decrease of association with clathrin-coated pit components. cAMP-dependent inhibition of receptor-association with clathrin-coated pits represents a novel mechanism regulating receptor-mediated endocytosis.

Basically all receptors that are internalized through clathrin-coated structures have specific sequences in their cytosolic tail, such as exposed hydrophobic residues (e.g., transferrin receptor and insulin receptor; Trowbridge, 1991; Paccaud *et al.*, 1993), that allow association with clathrin-coated pits. Thus, in the absence of mechanisms that inhibit association with clathrin-coated pits, these receptors segregate by default into clathrin-coated pits and are, therefore, con-

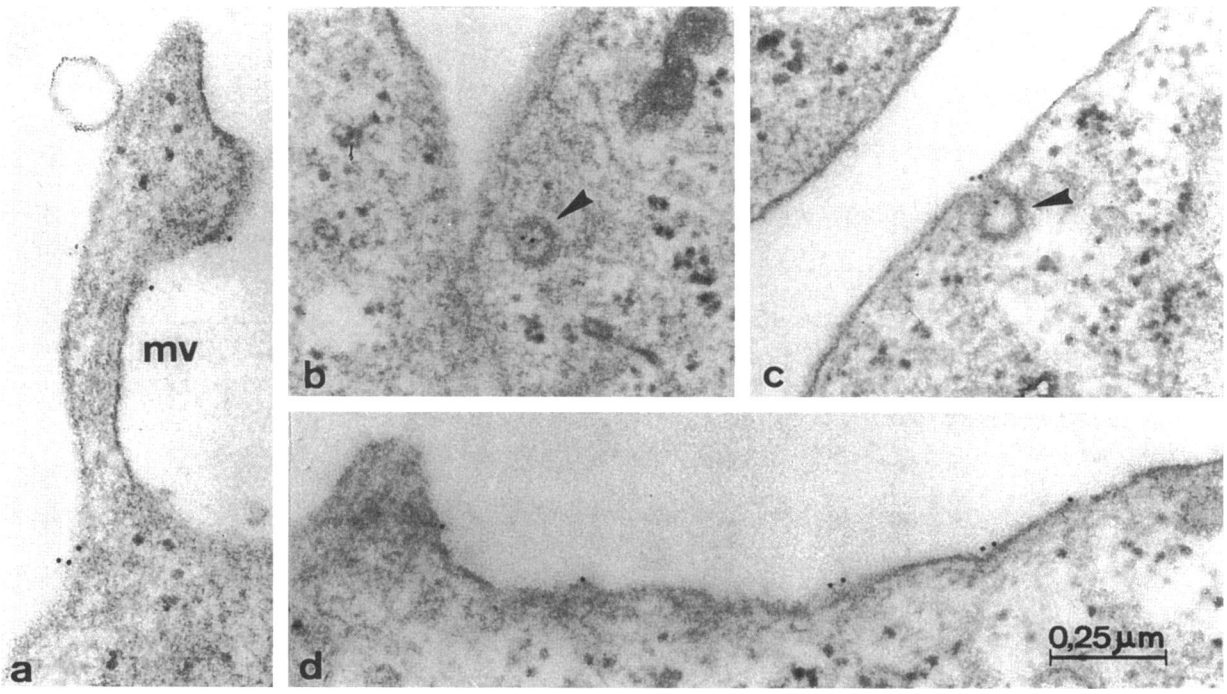

Figure 6. Representative electron micrographs of CD4 immunogold labeling of HL-60 cells. Cells were incubated for 2 h at 4°C with an anti-CD4 antibody (Leu3a) and thereafter with a secondary antibody coupled to colloidal gold particles (10 nm) to allow the detection of the antigen. Endocytosis of the gold-conjugated-CD4 complex was allowed to occur by raising the temperature for 5 min at 37°C. Cells were then processed for EM, and gold particles were quantified for their association with microvilli (a, mv), clathrin-coated pits (b and c, arrowhead), or nonvillous membrane (d). Gold particles were considered associated with clathrin-coated structures when they were observed immediately adjacent (at a distance of <20 nm) to the clathrin coat or totally enclosed in clathrin-coated pits/vesicles. Gold particles close to the entrance of deep invaginations but not adjacent to the clathrin lattice (i.e., the two gold particles near the mouth of the pit in c) were counted as noncoated plasma membrane.

stitutively internalized. A prototype for this behavior is the transferrin receptor. To allow a regulation of its internalization, a receptor needs mechanisms that actively exclude it from clathrin-coated pits. For the insulin receptor, the activation of its endogenous tyrosine kinase by insulin is necessary to counteract mechanisms of this exclusion (Carpentier, 1994; Carpentier and McClain, 1995). For CD4 in lymphocytes, the CD4-associated tyrosine kinase $p56^{lck}$ is instrumental in preventing the association with clathrin-coated pits (Pelchen Matthews *et al.*, 1992).

CD4 in non-lymphocytic $p56^{lck}$-negative cells has been suggested to lack mechanisms of exclusion from clathrin-coated pits and, therefore, to behave as a constitutively internalized receptor (Pelchen Matthews *et al.*, 1992). Our results demonstrate for the first time that cAMP can specifically decrease the constitutive association of a receptor with clathrin-coated pits.

The absence of $p56^{lck}$ in myeloid cells has prompted the research for other CD4-associated tyrosine kinases. However, studies in nonlymphocytic cells did not reveal CD4-associated tyrosine kinase activity (Pelchen Matthews *et al.*, 1991). The cAMP effect in HL-60 cells resembles the effect of $p56^{lck}$ on CD4 endocytosis in lymphocytes: a decrease in CD4 internalization by preventing its association with clathrin-coated pits. The physiological relevance of the cAMP effect is most likely heterologous regulation of CD4 internalization, i.e., a receptor cross-talk. This is demonstrated by the inhibition of CD4 internalization by PGE_1 (Figure 2). Our results therefore predict that the in vivo rate of CD4 internalization in myeloid cells is not invariably high but a function of the cellular environment, i.e., the concentration of hormones and inflammatory mediators that increase cellular cAMP levels.

At this point, we have not investigated how cAMP diminishes the association of CD4 with clathrin-coated pits. An involvement of protein kinase A appears likely, although a direct cAMP effect cannot be excluded. A direct phosphorylation of CD4 is probably not involved, because CD4 does not contain consensus sequences for phosphorylation by protein kinase A. Thus, the cAMP effect presumably occurs at the level of associated proteins that are functionally relevant for the regulation of CD4 internalization. In this respect, the recent description of activation of rap1 through cAMP (Altschuler *et al.*, 1995) is of interest. In neurons, cAMP increases internalization of adhesion receptors

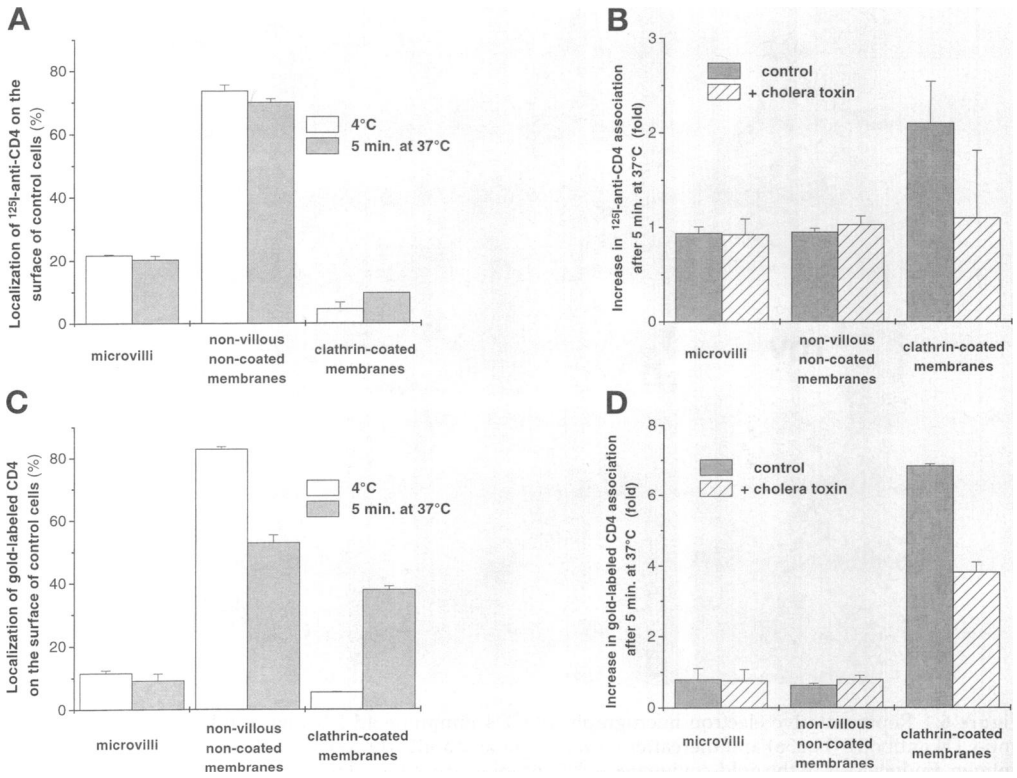

Figure 7. CTX decreases the association of CD4 with clathrin-coated structures. CD4 cell surface localization in HL-60 granulocytes was followed with a radiolabeled antibody (CD4 primary cross-linking; A and B) or with immunogold complexes (CD4 secondary cross-linking; C and D). Cells treated with CTX (1 μg/ml, 1 h at 37°C) or buffer only were labeled for 2 h at 4°C, and then endocytosis of CD4 was allowed to occur by raising the temperature to 37°C for 5 min. Cells were next processed for EM, and the association of labeled CD4 with various cell membrane structures (microvilli, nonvillous noncoated membranes, and clathrin-coated membranes) was quantitatively analyzed. (A) CD4-associated radiolabeling of cell membrane structures in cells pretreated with incubation buffer only. (B) Fold increase in CD4-associated radiolabeling of cell membrane structures after incubation for 5 min at 37°C in control and CTX-treated cells. (C) CD4-associated gold labeling of cell membrane structures in cells pretreated with incubation buffer only. (D) Fold increase in CD4-associated gold labeling of cell membrane structures after incubation for 5 min at 37°C in control and CTX-treated cells. Data are means ± range or SE of two or three experiments.

by increasing the density of clathrin-coated pits through stimulation of clathrin light chain expression (Hu *et al.*, 1993). Our morphometric measurements show that this mechanism of cAMP action does not apply to HL-60 cells. Two fundamentally different mechanisms might account for the cAMP-dependent decrease of CD4 association with clathrin-coated pits: 1) the affinity of CD4 for clathrin coat components could be decreased or 2) the mechanism releasing the constraint that maintains CD4 outside clathrin-coated pits is inhibited. In the case of the insulin receptor, for example, it is thought that the receptor is excluded from clathrin-coated pits by an active constraint, presumably through binding to some structures outside

Table 1. Percentage of plasma membrane constituted by microvilli and clathrin-coated pits/vesicles in control and CTX-treated HL-60

	% of plasma membrane constituted by microvilli	% of plasma membrane constituted by clathrin-coated pits/vesicles		Mean diameter of clatrhin-coated pits/vesicles (nm)	
		4°C	37°C (5 min)	4°C	37°C (5 min)
Control cells	29.6 ± 2.1	2.0 ± 0.4	1.8 ± 0.4	80 ± 3	78.2 ± 3
CTX-treated cells	26.6 ± 2.0	2.3 ± 0.4	2.4 ± 0.5	79 ± 2	82 ± 2

EM thin sections of HL-60 cells considered to be well preserved were randomly photographed and analyzed on a Leica Quantimet 500+. Percentage of plasma membrane constituted of microvilli was determined in each condition as described in MATERIALS AND METHODS from 52 ± 1 EM thin sections representing 2116.4 ± 94.5 μm of plasma membrane. Percentage of plasma membrane occupied by clathrin-coated pits/vesicles and mean diameter of clathrin-coated pits/vesicles were determined in each condition as described in MATERIALS AND METHODS from 51 ± 1 EM thin sections representing 333.9 ± 18.3 μm of plasma membrane. Sections used for this analysis were from the autoradiography experiments (Figure 5).

clathrin-coated pits, and that ligand-binding releases this constraint (Carpentier, 1993; Carpentier and McClain, 1995). Thus, it is conceivable that cAMP increases the affinity of CD4 for non-clathrin-coated membrane areas and—by that mechanism—decreases its association with clathrin-coated pits.

A puzzling aspect of our study is the observation that after incubation for 2 h at 4°C, most (>95%) of cell surface CD4 is found outside clathrin-coated pits; 5 min of incubation at 37°C, however, leads to an increase in the percentage of CD4 present in clathrin-coated pits/vesicles (10% of total surface-labeled CD4). This redistribution might reflect the restoration of a permissive temperature. However, the transferrin receptor remains associated with clathrin-coated pits even after prolonged incubation at 4°C (Watts, 1985). Thus, at this point, we cannot positively affirm that CD4 is a constitutively internalized receptor in HL-60 cells. It is possible that, similar to the situation in lymphocytes, CD4 internalization in HL-60 cells is a ligand-driven process and that antibody binding can substitute for the physiological ligand. Cross-linking experiments, where CD4 internalization is assessed after receptor cross-linking, would be compatible with this view. Indeed, CD4 cross-linking, which is likely to occur in physiological surface stimulation of CD4 internalization (Sakihama *et al.*, 1995), leads to a massive increase of CD4 association with clathrin-coated pits and enhanced CD4 internalization (compare Figures 4 and 7).

The precise physiological role of CD4 in myeloid cells remains uncertain. It is, however, likely that, in the broadest sense of the term, it acts as an adhesion molecule, possibly through the interaction with MHCII or other surface-bound ligands (Foti *et al.*, 1995). Thus, CD4 internalization is, similar to the situation in lymphocytes, presumably relevant for the termination of CD4 interaction with its ligand. Thus, in a simplest scenario, inhibition of CD4 internalization by cAMP constitutes a mechanism that allows a prolonged interaction of CD4 with its ligand. Recent results on the interaction of T lymphocytes with antigen-presenting cells raise another possibility. Serial engagement of a large number of T cell receptors with a small number of specific MHCII–peptide complexes allows continuous signaling (Valitutti *et al.*, 1995). If such a serial engagement also occurs with CD4, then the decrease in CD4 internalization would lead to a decreased CD4-dependent cellular signaling.

Thus, our results give rise to a novel concept in the regulation of receptor-mediated endocytosis through association with clathrin-coated pits. Classical concepts include 1) constitutive association with clathrin-coated pits and 2) constitutive exclusion from and receptor-mediated association with clathrin-coated pits. Here, we show that receptors may be subject to second-messenger–mediated exclusion from clathrin-coated pits. This mechanism is selective for CD4 (when compared with insulin receptor and transferrin receptor) in HL-60 cells. Future studies should establish whether this mechanism is more widespread and also applies to other receptors and other cell types.

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