Phorbol Ester–induced Downregulation of CD4 is a Multistep Process Involving Dissociation from p56lc, Increased Association with Clathrin-coated Pits, and Altered Endosomal Sorting

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Summary

The phorbol ester phorbol myristate acetate (PMA) induces a rapid downregulation of CD4 from the surface of T cells and lymphocytic cell lines, as well as from CD4-transfected nonlymphoid cells. Here we have studied the mechanisms of this phorbol ester-induced CD4 modulation. Using HeLa-CD4 or NIH-3T3-CD4 cells, in which the endocytosis of CD4 is not influenced by the protein tyrosine kinase p56lc, we show that PMA enhanced the uptake of CD4, increasing the rate of CD4 endocytosis three to five-fold, and doubling the proportion of CD4 found inside the cells. Trafficking of a CD4 mutant lacking the major portion of the cytoplasmic domain, as well as fluid phase endocytosis were not affected by PMA treatment. Studies in which clathrin-coated pits were disrupted through the use of hypertonic media indicated that both the constitutive and PMA-induced CD4 uptake occurred through coated vesicles. Electron microscopy demonstrated directly that PMA increases the association of CD4 with coated pits. Immunofluorescent staining of internalized CD4 showed that PMA also diverted CD4 from the early endosome–plasma membrane recycling pathway to a mannose 6-phosphate receptor–containing late endosomal compartment. In lymphoid or p56lc-expressing transfected cells, these effects were preceded by the PMA-induced dissociation of CD4 and p56lc, which released CD4 and made possible increased endocytosis and altered intracellular trafficking. Together these results indicate that phorbol esters have multiple effects on the normal endocytosis and trafficking of CD4, and suggest that phosphorylation may influence the interaction of CD4 with coated pits.

CD4 is a type I integral cell surface glycoprotein that is expressed primarily on thymocytes and MHC class II-restricted peripheral T cells (1, 2). The molecule is a member of the Ig supergene family and appears to function in T lymphocyte ontogeny (3) and in the activation of mature CD4+ T cells (4). In addition, CD4 acts as the primary receptor for the HIVs (5). The ectodomain of CD4 contains sites that can interact with nonpolymorphic regions of the MHC class II antigens and also bind to the gp120 component of the HIV envelope glycoproteins (6, 7). In addition, the cytoplasmic domain of CD4 interacts with a lymphocyte-specific Src-related protein tyrosine kinase, p56lc (8), and CD4 may therefore also function in signal transduction.

Although the primary site of CD4 function appears to be at the cell surface, it is known that various physiological and experimental stimuli can induce its downregulation. Indeed, loss of cell surface CD4 could be involved in the generation of CD4+CD8+ thymocytes, and the downregulation of CD4 on peripheral T cells may contribute to the induction of anergy and tolerance. Exposure of specific T cells to an appropriate antigen (9–11), or to cross-linking Abs against CD4 (12, 13), the CD3-TCR complex (11), or CD2 (14) can induce a reduction in cell surface CD4 expression. In addition, cell surface CD4 levels can be modulated during HIV infection (15), after treatment with a soluble form of HIV gp120 (16, 17) or after exposure to gangliosides (18, 19). The modulation of CD4 that occurs during antigen encounter can be mimicked by treating cells with phorbol esters (9, 10). These activators of protein kinase C have been shown to cause transient phosphorylation of the CD4 cytoplasmic domain (9, 20, 21), which may then induce CD4 downmodulation by endocytosis (21–23). However, the mechanisms by which CD4 is cleared from the cell surface have not been elucidated in detail, and, although it appears that internalized CD4 is degraded after phorbol ester stimulation (23–26), the exact fate of the downregulated CD4 molecules has not been determined.

We have previously demonstrated that on transfected nonlymphoid (HeLa-CD4 or NIH-3T3-CD4) and monocytic cell lines (HL-60 and U937), CD4 is constitutively internalized
and recycled to the cell surface (27–29). Internalization occurs through coated pits and coated vesicles and, at steady state, ~40% of the CD4 is found inside the cells. In contrast, the CD4 expressed in lymphoid cell lines is not internalized (28), but is restricted to the cell surface through its interaction with p56Δ (30). In the present study, we have examined the mechanisms of phorbol ester–induced CD4 modulation. Since p56Δ has a significant influence on the endocytic trafficking of CD4 (30), we have used CD4-transfected nonlymphoid cells for these experiments. We demonstrate that human CD4 expressed on HeLa–CD4 or NIH–3T3–CD4 cells can be downregulated by phorbol ester, and that this modulation is independent of the presence of p56Δ. The initial effect of phorbol ester is to increase the rate of CD4 endocytosis through coated pits. Furthermore, the level of CD4 recycling observed in the presence of phorbol esters is reduced as CD4 is diverted from the recycling pathway between early endosomes and the plasma membrane to a mannose 6-phosphate receptor–containing late endosomal compartment in the endocytic pathway. In lymphoid or p56Δ-expressing transfected cells, phorbol ester–induced CD4 downregulation appears to occur by a similar mechanism. However, the enhanced CD4 endocytosis is only possible after the rapid dissociation of CD4 from p56Δ.

**Materials and Methods**

**Materials.** Horseradish peroxidase (HRP) type II and PMA were purchased from Sigma Chemical Co. Ltd. (Poole, Dorset, UK). A stock solution of PMA (2 mg/ml in ethanol) was stored at –20°C. The anti-CD4 mAb Leu3a was obtained from Becton Dickinson & Co. (Mountain View, CA), and Fab′/fragments were prepared as described (27). The Q4120 Ab, developed by Dr. Quentin Sattentau (Centre d’Immunologie de Marseille-Luminy, INSERM-CNRS, Marseille, France) (31) was provided by the Medical Research Council AIDS Directed Programme Reagents Programme (South Mimms, Potters Bar, Herts, UK). Abs or Fab′ fragments were radiiodinated as described (27, 28). Q4120 was labeled with tetramethyl-rhodamine isothiocyanate (TRITC; Cambridge Bioscience, Cambridge, UK) according to the manufacturer’s instructions, whereas the fluorescein-conjugated mAb to the transferrin receptor (TR), L011, was purchased from Becton Dickinson & Co. A rabbit polyclonal serum specific for the cation independent mannose 6-phosphate receptor (CI-MPR) was kindly provided by Dr. W. J. Brown (Cornell University, Ithaca, NY) and has been previously characterized (32). The rabbit antiserum to p56Δ, anti-p56Δ (KERP), raised against a peptide covering residues 478–509 of murine p56Δ, has been described (30). A second antiserum, anti-p56Δ (RNGS), was raised against a peptide covering residues 39–64 of murine p56Δ starting with the sequence N′-RNGS (prepared by Dr. Torben Sørensen, University of Copenhagen, Copenhagen, Denmark, for the European Community Concerted Action programme), and was affinity purified using the peptide immobilized on Reactigel (Pierce and Warriner, Chester, UK). Peroxidase-conjugated, and rhodamine- or fluorescein-labeled anti-rabbit and anti-mouse reagents were purchased from Pierce and Warriner.

**Cells and Culture.** Adherent HeLa and NIH–3T3–CD4 cells transfected with the cDNAs of human CD4 or mutant CD4Δ were, as well as M22, an NIH–3T3–CD4 cell line which has been supertransfected with the murine lcκ cDNA, were cultured as described (28, 30) and used 3 d after subculture unless otherwise indicated. CD4Δ is a CD4 mutant from which the major portion of the cytoplasmic domain (comprising amino acids 403–433) has been deleted (33). The lymphocytic cell line SupT1 was grown in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml of penicillin, and 0.1 mg/ml streptomycin and was used when growing exponentially.

**Ab Binding and Endocytosis Assays.** To determine levels of CD4 remaining on the cell surface after phorbol ester treatment, cells were cooled by washing twice in ice-cold binding medium (BM; RPMI 1640 lacking bicarbonate, supplemented with 0.2% BSA, and 10 mM Hepes, pH 7.4) and incubated for 2 h on ice in medium containing 0.3 nM 125I-labeled anti-CD4 mAb (Leu3a or Q4120) or 0.5 nM 125I-Fab′ of Leu3a. Unbound Ab or Fab′ was washed away in three changes of medium and two rinses of PBS, before cells were harvested by dissolving in 0.2 M NaOH for γ-counting.

Internalization of CD4 was measured as described (27). Briefly, adherent cells grown in 1-mm tissue culture wells were labeled with radioiodinated anti-CD4 Abs or Fab′ fragments, as detailed above, and warmed to 37°C to allow endocytosis of the Ab tracer. At various times cells were cooled and either harvested directly, or cell surface 125I-label was removed by washing in cold BM buffered to pH 2 or 3 with 10 mM morpholinoethanesulfonic acid (MES) and HCl for Ab or Fab′ fragments, respectively. For the suspension cell line SupT1, the assay was adapted as described (28). The proportion of acid resistant to total cell counts was calculated for each time point and plotted. For recycling studies, cells were labeled as above, incubated in BM at 37°C for 30 min, cooled, surface stripped in cold medium at pH 3 and returned to BM at 37°C for various times before analysis as described above. To study Ab degradation, all warm media from the endocytosis and recycling studies were collected, and aliquots analyzed by γ-counting before and after precipitation with 20% TCA for 1 h on ice.

To inhibit endocytosis through coated pits, cells were preincubated for 5 min at 0–4°C in hypertonic medium (0.45 M sucrose in RPMI 1640 medium lacking bicarbonate, supplemented with 0.2% BSA, 20 mM MES, and 20 mM sucinic acid, pH 5.7) and the endocytosis assay was performed in the presence of hypertonic medium.

Fluid phase endocytosis of HRP was assayed as described (30).

**Electron Microscopic Localization of CD4.** The distribution of CD4 at the cell surface and during endocytosis was determined as described (28, 30). Briefly, HeLa-CD4 cells grown on 22-mm² glass coverslips were labeled with 8 nM Leu3a or 9 nm protein A gold (provided by Dr. Gareth Griffiths, EMBL, Heidelberg, Germany; 28) and warmed to 37°C for 1–4 min in the presence or absence of 100 ng/ml PMA. Cells were cooled, fixed, and embedded as described (28, 30) and ultrathin sections were examined with an electron microscope (model CM12; Philips Analytical, Cambridge, UK). For quantitative analysis, cells were examined systematically, noting the location of every gold particle encountered.

**Immunofluorescence Endocytosis Assay.** To follow CD4 endocytosis, HeLa–CD4 cells grown on glass coverslips, and labeled at 0–4°C with 8 nM Leu3a or rhodamine-conjugated Q4120 for 2 h. The cells were washed extensively and then warmed to 37°C for various times to allow endocytosis of the Ab-labeled CD4 mol-
ecules. After warming, cells on some of the coverslips were cooled on ice and washed in medium adjusted to pH 2 to remove cell surface mAb. Subsequently, all cells were fixed in 3% paraformaldehyde in PBS for 30 min on ice and quenched with 50 mM NH₄Cl. Some samples were permeabilized with 0.1% Triton X-100 to reveal internalized Ab. Subsequently, all cells were fixed in 3% paraformaldehyde in PBS for 30 min on ice and quenched with 50 mM NH₄Cl. Some samples were permeabilized with 0.1% Triton X-100 to reveal internalized Ab. To detect the Leu3a Ab, cells were stained with rhodamine-labeled goat anti-mouse diluted 1:2,000. Cells on some coverslips were counter stained in rabbit anti-CI-MPR at 1:200 followed by FITC-conjugated goat anti-rabbit diluted 1:1,000, whereas the TfR was detected using FITC-conjugated L01.1 diluted 1:100. Cells were washed and mounted in Moviol, and observed by confocal microscopy (model MRC 600; Bio-Rad Laboratories, Hemel Hempstead, Herts, UK).

Immunoprecipitation and Immunoblotting. M22 cells were washed once in Ca²⁺/Mg²⁺-free PBS and harvested by scraping into PBS. The cells were centrifuged at 1,500 rpm for 5 min at 4°C, and resuspended in 20 mM Tris-HCl lysis buffer, pH 8.0, containing 3% NP-40, 150 mM NaCl, 2 mM EDTA, and protease inhibitors (1 mM PMSF and 10 μg/ml each of chymostatin, leupeptin, antipain, and pepstatin) for 10 min on ice. Detergent-insoluble material was removed by centrifugation at 4°C for 30 min at full speed in a benchtop microfuge. The supernatants were collected and aliquots taken for protein determination using the bicinchoninic acid assay (34), immunoprecipitation, and immunoblotting.

To precipitate CD4 and CD4/p56κκ complexes, aliquots of each lysate containing equal amounts of cell protein were precleared by incubation for 30 min at 4°C with 50 μl packed, prewashed protein A-Sepharose (Sigma Chemical Co.). Q4120 covalently coupled to protein A-Sepharose (20 μl of a 50% slurry) was then added and the lysate incubated for 2 h at 4°C. The beads were collected by centrifugation (1 min at 1,000 rpm) and washed three times with lysis buffer and twice with 25 mM Hepes, pH 7.2, containing 0.1% NP-40, resuspended in 20 μl SDS-PAGE sample buffer without reducing agents, and run on 10% SDS-PAGE minigels. Aliquots of the lysate before immunoprecipitation were mixed 4:1 with a 5 x concentrated sample buffer, and run on identical minigels. After electrophoresis, cellular proteins were transferred to nitrocellulose. The blots were blocked using 10% dried skimmed milk powder (Marvel) in PBS at 4°C overnight, and then incubated in primary Ab. The affinity-purified anti-p56κκ (RNGS) and anti-p56κκ (KERP) were used at dilutions of 1:1,000 and 1:500, respectively, whereas Q4120 was used at 1.6 μg/ml. After incubation with Ab and washing, the blots were probed with peroxidase-conjugated goat anti-rabbit or goat anti-mouse Ab and developed using an enhanced chemiluminescence detection system (Amersham International, Amersham, Bucks, UK) according to the manufacturer's instructions.

Results

Downregulation of CD4 on Transfected Nonlymphocytic Cells. Phorbol esters such as PMA have been shown to cause rapid downmodulation of CD4 from the surface of human PBL and thymocytes, as well as a number of lymphocytic or myeloid cell lines (9, 14, 22, 35). In addition, PMA can downregulate CD4 expressed on nonlymphoid HeLa cells after transfection (24, 33, 36). Using a binding assay with a radiolabeled anti-CD4 mAb, we determined the concentration dependence of CD4 downregulation by PMA on 3-d-old HeLa-CD4 cells. Half-maximal downregulation (ED₅₀) was observed with 0.5 ng/ml PMA (0.8 nM). Thus, the potency of PMA on HeLa-CD4 cells is similar to that reported for PBL (22, 37, 38).

As already described for lymphocytic cells (9, 14, 22), PMA (100 ng/ml) induced rapid CD4 downregulation on HeLa-CD4 cells, with the majority of the CD4 molecules being removed from the cell surface in 1 h (36). Cell surface CD4 expression remained at a low level (20–30% of that on untreated cells) for up to 8–10 h, but subsequently the amount of cell surface CD4 increased again, recovering to near the original levels after 24–48 h of continuous treatment with phorbol ester. This recovery of CD4 expression was not due to inactivation of the phorbol ester, since PMA-containing medium taken from cells after 24 h was still able to induce CD4 downregulation in fresh HeLa-CD4 cells. Studies with

![Graph](image-url)
Table 1. Effect of PMA on CD4 Endocytosis

| Cell line       | Tracer used | Endocytosis rate | Percent internal at 60 min | Endocytosis rate | Percent internal at 60 min | No. of experiments |
|-----------------|-------------|------------------|----------------------------|------------------|-----------------------------|--------------------|
|                 |             |                  |                            |                  |                             |                    |
| HeLa-CD4 (3-d-old) | 125I-Fab'   | 2.0              | 28                         | 11.3             | 78                          | 1*                 |
|                 | 125I-Leu3a  | 1.8 ± 0.9        | 37 ± 1                     | 8.4 ± 3.3        | 62 ± 14                     | 7                  |
| HeLa-CD4 (2-d-old) | 125I-Q4120 | 2.6 ± 0.2        | 46 ± 7                     | 6.4 ± 0.2        | 79 ± 11                     | 2                  |
| NIH-3T3-CD4     | 125I-Leu3a  | 3.9 ± 1.2        | 41 ± 10†                   | 12.9 ± 2.8       | 88                          | 2                  |

* Data from multiple experiments are expressed as mean ± SD.
† Internal at 30-60 min.

cycloheximide indicated that the recovery requires protein synthesis (data not shown), and may involve the PMA-induced downregulation of protein kinase C (39, 40), as well as effects of the phorbol ester on CD4 transcription and translation (41). This has not been examined further in the present study.

Downregulation was dependent on the presence of the CD4 cytoplasmic domain, and was not observed in HeLa cells transfected with a mutant CD4 lacking the cytoplasmic domain (HeLa-CD4<sup>Δcyt</sup>; 33, 36, 42).

Like the HeLa-CD4 cells, NIH-3T3 cells transfected with CD4 (NIH-3T3-CD4) downregulated their cell surface CD4 in response to phorbol ester. The amount of CD4 downregulation on NIH-3T3-CD4 cells was consistently greater than that observed on HeLa-CD4 cells, with >70% of the CD4 being removed from the cell surface during the first 30 min of PMA treatment.

Thus, CD4 can be downregulated from the cell surface of lymphoid and nonlymphoid cells by similar concentrations of phorbol esters. This indicates that CD4 downregulation is not T cell dependent and does not require the presence of p56<sup>Δk</sup>. As CD4 downregulation is believed to occur by endocytosis, and CD4 endocytosis is influenced by p56<sup>Δk</sup> expression, we first sought to understand the mechanisms of downregulation in the p56<sup>Δk</sup>-negative nonlymphoid cells.

Effects of Phorbol Esters on CD4 Endocytosis. The CD4 molecules expressed in HeLa-CD4 and NIH-3T3-CD4 cells are constitutively internalized and recycled (27, 29). To study the effects of phorbol ester on this constitutive CD4 endocytosis, cells were surface labeled at 0-4°C with 125I-labeled anti-CD4 mAb or Fab' fragments. After 2 h, the cells were washed and warmed to 37°C in the presence or absence of 100 ng/ml PMA. At various times, the level of internalized 125I tracer was detected by acid washing as described in Materials and Methods. The result of a typical CD4 endocytosis experiment is shown in Fig. 1. PMA increased the rate of CD4 endocytosis on 3-d-old HeLa-CD4 cells by four to sixfold. This increase was observed after a brief lag of about 2 min (Fig. 1 B). Comparable results were obtained when CD4 endocytosis was measured using Fab' fragments or intact anti-CD4 mAb (Table 1). Similar effects were seen on 2-d-old HeLa-CD4 or on NIH-3T3-CD4 cells, although the increase in CD4 endocytosis was less striking, possibly because these cells had somewhat higher CD4 endocytosis rates in the absence of phorbol ester. In addition to its effect on the rate of CD4 endocytosis, PMA increased the steady state distribution of CD4 in the cells from 40 to about 80% (Table 1). This effect was generally more pronounced on 2-d-old HeLa-CD4 and on the NIH-3T3-CD4 cells.

To investigate whether the internalized Ab ligands were degraded, the media in which the cells had been warmed for endocytosis were precipitated with TCA. The level of TCA-soluble 125I (representing degraded Ab ligand), when calculated as a proportion of the amount of 125I-ligand initially bound to the cells, was proportional to the amount of internalized mAb, regardless of the presence of PMA. When levels of TCA-soluble 125I were calculated as a proportion of the endocytosed plus degraded counts (i.e., as a proportion of all the activity that was or had been inside the cells), significant increases in TCA-soluble counts (30-40%) were only apparent in cells treated with PMA for 2 h or longer. At these long time points, significant dissociation of the mAb from CD4 could have occurred, especially since CD4 and mAb would have entered acidic organelles. The appearance of degraded activity may not, therefore, reflect the fate of CD4.

These data indicated that the one important effect of phorbol ester is to increase the rate of CD4 endocytosis in HeLa-CD4 or NIH-3T3-CD4 cells. This shifts the steady state distribution of CD4 so that more than 80% is intracellular, with a concomitant decrease in cell surface CD4.

The Specificity of the Phorbol Ester Effect on CD4 Endocytosis. Previous experiments have indicated that phorbol ester-induced downregulation of CD4 is dependent on the
Figure 2. Time course of internalization of 12SI-labeled Leu3a in HeLa-CD4-cy- cells that express CD4 molecules from which the cytoplasmic domain has been deleted. The plot shows the ratios of acid-resistant 12SI-Leu3a to the total cell-associated label on cells warmed in the presence (○) or absence (○) of 100 ng/ml of PMA.

presence of the cytoplasmic domain (33, 36, 42). We have shown that CD4cy- molecules can be internalized by bulk flow transport through coated pits in both HeLa and lymphoid cell lines (28). To determine whether phorbol ester treatment also affected the internalization of CD4cy-, we measured endocytosis on HeLa-CD4cy- cells. Virtually identical CD4 endocytosis curves were obtained in the presence or absence of PMA (Fig. 2). This observation suggests that the PMA-induced enhancement of CD4 endocytosis described above was specific for full-length CD4 molecules, and not due to a general stimulation of bulk flow endocytosis by phorbol ester, as has been described for example, for macrophages (43, 44). To demonstrate directly that phorbol esters do not stimulate the basal endocytic capacity in HeLa-CD4 cells, we measured the rates of fluid phase endocytosis using HRP. PMA did not affect the initial rate of fluid uptake measured over the first 10 min in HRP medium (fluid uptake occurred at $6 \times 10^{-4}$ nl/min/µg of cell protein). However, cells incubated with PMA for longer periods (>1 h) accumulated 20-25% more fluid than control cells, suggesting that PMA-treated cells may retain more of the internalized marker than untreated cells.

Together these results indicate that in these HeLa-CD4 cells, phorbol esters do not have a significant effect on the vesicular traffic from the cell surface, and hence that the phorbol ester-induced increase in CD4 uptake is not due to a general stimulation of endocytosis.

Phorbol Esters Increase CD4 Endocytosis Through Coated Pits. In HeLa and NIH-3T3 cells, CD4 is internalized through clathrin-coated pits and vesicles (28, 30), suggesting that it contains sequences in its cytoplasmic domain that allow it to cluster into coated pits. To examine whether the increased uptake of CD4 observed in the presence of phorbol ester also occurs by this pathway, we used hypertonic media (45) to inhibit the formation of clathrin-coated vesicles (46). In our hands, incubation in medium containing 0.45 M sucrose gave a more complete, yet reversible, inhibition of CD4 internalization than methods of acidifying the cytosol (cf. 47, 48), although long-term cell viability was improved when the hypertonic medium was slightly acidified (Pelchen-Matthews, A., and M. Marsh, manuscript in preparation). Treatment of HeLa-CD4 cells for 1 h with PMA in the presence of medium containing 0.45 M sucrose and adjusted to pH 5.7

Figure 3. The effect of hypertonic medium on CD4 downregulation and endocytosis on 3-d-old HeLa-CD4 cells. (A) Cell surface expression of CD4 on HeLa-CD4 cells pretreated for 1 h in binding medium (1), medium containing 100 ng/ml PMA (2), or in hypertonic medium containing 100 ng/ml PMA (3). After incubation, cells were cooled and levels of CD4 remaining on the cell surface determined using 0.3 nM 12SI-Q4120. The plot shows binding of 12SI-Q4120 compared to the untreated cells (B/Bo) after correction for cellular protein content. (B) Time courses of internalization of CD4 in the presence (filled symbols) or absence (open symbols) of 100 ng/ml PMA in normal (circles, solid lines) or hypertonic medium (triangles, dashed lines). CD4 endocytosis was traced with 0.3 nM 12SI-Q4120. The plot shows the ratios of acid-resistant 12SI-Q4120 to the total cell-associated label after various times at 37°C.
The findings that PMA does not stimulate vesicular traffic from the surface of HeLa-CD4 cells and that inhibition of coated vesicle-mediated endocytosis suggests that the increase rate of CD4 endocytosis may be due to increased association of CD4 with clathrin-coated pits. This was demonstrated directly by immunolabeling electron microscopy. 2-d-old HeLa-CD4 cells were labeled on ice with Leu3a and protein A-gold. After washing to remove any free gold conjugate, cells were warmed to 37°C in the presence or absence of PMA, fixed, and prepared for electron microscopy. Control experiments in which the kinetics of 125I-Leu3a uptake were examined after incubation with protein A-gold showed that internalization of the mAb was not affected by the gold probe (28). In the absence of phorbol ester, 4.0–5.5% of the gold particles at the cell surface were found in close apposition to coated plasma membrane (Table 2), in agreement with our previous study (28). In samples treated with PMA, gold particles were also observed in coated pits and vesicles (Fig. 4). When the distribution of labeled CD4 was determined at increasing times after warming in PMA medium, there was a transient increase in the number of gold particles seen in coated pits or vesicles, peaking with more than 10% of all gold particles (or 12% of the gold at the cell surface) adjacent to coated plasma membrane. This represents a threefold increase over the basal coated pit association of CD4, and can account for the increase in the rate of CD4 endocytosis observed (cf. Table 1). Significantly, the increased association of CD4 with coated pits is observed just before the enhanced endocytosis of CD4 as measured biochemically (the lag shown in Fig. 1 B). Since ligand located in coated pits at the cell surface is still accessible to acid washing in the biochemical experiments, and the lifetime of a coated pit at the cell surface is believed to be about 1–2 min (49–51), the increased association of CD4 with coated pits can completely explain the phorbol ester-induced enhancement of CD4 uptake.

**Effects of Phorbol Esters on CD4 Recycling.** To investigate whether PMA also affects the recycling of internalized CD4, 3-d old HeLa-CD4 cells were loaded with 125I-labeled anti-CD4 Fab' fragments by warming in the presence or absence of 100 ng/ml PMA. The cells were then briefly treated with acid medium at 4°C to remove any Fab' remaining on the cell surface, and reincubated at 37°C. Subsequently, the cells were subjected to a second acid wash and recycling detected as a decrease in the level of acid-resistant 125I-Fab' tracer associated with the cells, and a loss of the previously internalized tracer intact (i.e., TCA precipitable) into the medium. In the absence of PMA, the recycling of CD4 observed was similar to our previous results (27). In contrast, very little recycling was observed in cells that had been loaded in the presence of PMA (Fig. 5). Identical results were observed when CD4 recycling was monitored using intact 125I-labeled Ab. Furthermore, there was very little recycling of internalized tracer when cells were loaded with the 125I-mAb in the absence of phorbol ester, and PMA only added to the recycling medium (data not shown). As in the endocytosis experiments, the level of anti-CD4 tracer found inside the cells at steady state was increased from about 40% in the absence of phorbol ester to about 80% with PMA.

### Table 2. Effect of PMA on the Distribution of Gold-labeled CD4 on HeLa-CD4 Cells

| Time at 37°C | Total No. of particles counted | Particles over noncoated plasma membrane | Particles over coated pits and vesicles | Internalized particles | Unclassified |
|-------------|-------------------------------|----------------------------------------|----------------------------------------|------------------------|-------------|
|             |                               | %                                      | %                                      | %                      | %           |
| A: Control  |                               |                                        |                                        |                        |             |
| 0           | 511                           | 475 (93.0)                             | 21* (4.1)                              | 0 (0.0)                | 15          |
| 1 min       | 349                           | 310 (88.8)                             | 18 (5.2)                               | 18 (5.2)               | 4           |
| 2 min       | 346                           | 314 (90.8)                             | 18 (5.2)                               | 10 (2.9)               | 8           |
| 3 min       | 416                           | 330 (79.3)                             | 23 (5.5)                               | 56 (13.5)              | 7           |
| 4 min       | 316                           | 252 (79.7)                             | 8 (2.5)                                | 48 (15.2)              | 8           |
| B: Plus PMA |                               |                                        |                                        |                        |             |
| 1 min       | 378                           | 325 (86.0)                             | 27 (7.1)                               | 25 (6.6)               | 1           |
| 2 min       | 329                           | 253 (76.9)                             | 34 (10.3)                              | 29 (8.8)               | 13          |
| 3 min       | 372                           | 275 (73.9)                             | 33 (8.9)                               | 52 (14.0)              | 12          |
| 4 min       | 289                           | 185 (64.0)                             | 15 (5.2)                               | 82 (28.4)              | 7           |

* Only particles observed immediately juxtaposed to the clathrin coat were counted in this category.
Fig. 4. Electron microscopic localization of CD4 in PMA-treated HeLa-CD4 cells. 2-d-old HeLa-CD4 cells were labeled on ice with 0.5 nM Leu3a followed by protein A-gold, washed, and warmed to 37°C in the presence of 100 ng/ml PMA for 2 min (A and B) or 3 min (C). Scale bar, 100 nm.

Modeling of CD4 Endocytosis. The studies described above have demonstrated that one of the main effects of phorbol ester is to increase CD4 endocytosis through coated pits. To determine if this increase in CD4 internalization is alone sufficient to explain CD4 downregulation, we designed a simple mathematical model of CD4 endocytosis. The experimentally determined CD4 internalization and recycling rates were used to calculate surface and internal CD4 levels and the proportion of internalized molecules at different times using an iterative routine. Thus, for 3-d-old HeLa-CD4 cells, the model was set with 60% of the CD4 molecules at the cell surface, 40% in the endosome compartment, internalization at a rate of 2% per min, and recycling back to the cell surface at 3% of the internal level per min (27, 28), thereby maintaining a steady state. To model our endocytosis experiments, only the pool of molecules initially at the cell surface was considered labeled. This yielded a CD4 uptake curve that corresponded closely to the actual data (open circles in Fig. 6 A). Similarly, when only molecules initially internal were considered labeled, the resulting curve (Fig. 6 B) resembled the CD4 recycling plot determined experimentally (Fig. 5). To imitate the effect of the phorbol ester, the endocytosis rate in the model was increased to 10-14% per min, whereas all other parameters were maintained. This altered the steady state distribution of CD4 so that the proportion of CD4 at
the cell surface decreased from 60 to about 20%, effectively giving downregulation. Endocytosis (Fig. 6A) and recycling plots (Fig. 6B) calculated under these conditions again corresponded closely to the experimental data.

Thus, an increase in CD4 endocytosis and consequent shift in the steady state distribution of the cellular CD4 could explain the CD4 endocytosis and recycling curves observed biochemically. However, in the endocytosis experiments we did observe some degradation of internalized 125I-mAb tracer by TCA precipitation (see above). Furthermore, a number of reports have suggested that, upon phorbol ester stimulation, internalized CD4 is rapidly degraded in lysosomes (23-26).

We previously showed by immunofluorescent staining that in NIH-3T3-CD4 cells CD4 is internalized into vesicles distributed throughout the cytoplasm and resembling early endosomes (52). If phorbol ester treatment simply alters the steady state between CD4 internalization and recycling, then a similar distribution of internalized CD4 should be observed in the presence of PMA, although the quantity of CD4 in the intracellular compartment would be increased. To test this, we followed the fate of internalized CD4 by immunofluorescent staining.

**Phorbol Esters Alter the Distribution of Internalized CD4.** As in the biochemical endocytosis experiments, 2-d-old HeLa-CD4 cells were labeled with anti-CD4 mAb at 4°C and then incubated at 37°C for 1 h in the presence or absence of PMA, before processing to reveal CD4 at the cell surface (Fig. 7). In unstimulated cells, this revealed CD4 at the cell surface (Fig. 7A), and in intracellular vesicular structures located throughout the cytoplasm (Fig. 7D). Early after the addition of phorbol ester (5 and 10 min), the internalized CD4-anti-CD4 complexes were observed in a similar distribution. At later times (30 min-1 h), cell surface CD4 had become largely undetectable (Fig. 7B), whereas the internalized anti-CD4 mAb was located in clusters of vesicles in the juxta-nuclear region of the cells (Fig. 7E). To identify the intracellular compartments further, we performed double-staining studies, using the TfR and the CI-MPR as markers for early and late endosomes, respectively (53). In unstimulated cells which had internalized a TRITC-labeled anti-CD4 mAb for 1 h, counter-staining with a FITC-conjugated anti-TfR mAb, revealed that most of the CD4-containing vesicles were also stained for the TfR (Fig. 8, A and B), and are therefore likely to be early endosomes. In contrast, CD4 internal-
Figure 8. Colocalization of internalized CD4 with the Tfr or CI-MPR.
2-d-old HeLa-CD4 cells were labeled with TRITC-Q4120 (A and B) or Leu3a (C–F) at 4°C and then incubated at 37°C for 1 h in the presence (C and D) or absence (A, B, E, and F) of 100 ng/ml PMA, and internalized anti-CD4 was detected as described in Fig. 7. In the absence of PMA, CD4-containing vesicles (A) could be costained with FITC-labeled anti-Tfr mAb (B), whereas in the presence of PMA, CD4 (C) colocalized with the CI-MPR (D). In the absence of PMA, CD4 (E) remained in vesicles similar to those observed in A and B, which did not costain with the CI-MPR (F). The figure shows optical sections of thickness ~3 μm (A and B) or 1 μm (C–F). Scale bars, 10 μm.

Figure 9. Effect of PMA on CD4 endocytosis in p56^k^k-expressing cells.
Time courses of internalization of CD4 on SupT1 (A), p56^k^k transfected M22 cells (B), and the p56^k^k-negative parental NIH-3T3-CD4 cells (C) in the presence (●) or absence (○) of 100 ng/ml PMA. CD4 endocytosis was traced with 1^25I^-labeled anti-CD4 mAb Q4120. The plots show the ratios of acid-resistant 1^25I^-mAb to the total cell-associated label after various times at 37°C.

The Role of p56^k^k in Phorbol Ester–Induced CD4 Downregulation. As demonstrated above, in p56^k^k-negative cells expressing CD4 after transfection, PMA increases the rate of CD4 internalization and reroutes the internalized CD4 molecules to later compartments of the endocytic pathway. Since p56^k^k has been shown to regulate CD4 endocytosis (30), and since the CD4 expressed on T cells, thymocytes, and lym-
of cells with phorbol esters, allowing biochemical and morphological analysis of the mechanisms involved. Downregulation occurs by endocytosis (21-23) and appears to require phosphorylation of serine residues in the cytoplasmic domain of the molecule, in particular of Ser408 (33, 36). In T cells, phorbol ester treatment leads to the dissociation of p56kk and CD4 (54), and recent data indicates that p56kk dissociates before CD4 downregulation (55, 56). Internalized CD4 is believed to be directed to lysosomes and then degraded (23-26). Here we have examined the cellular mechanisms of CD4 downregulation induced by phorbol ester. Our studies indicate that downregulation is a multistep process, involving both increased CD4 endocytosis and altered endosomal sorting. In addition, the downregulation in p56kk-containing cells involves an initial dissociation of the CD4-p56kk complex.

Phorbol Ester-induced Endocytosis of CD4. Under normal conditions, the CD4 expressed in HeLa-CD4 and NIH-3T3-CD4 cells is constitutively internalized through coated pits and vesicles. Here we demonstrated that within minutes of the addition of phorbol ester, there was a three- to fivefold increase in the rate of CD4 internalization. Control experiments indicated that PMA does not modulate fluid phase endocytosis in HeLa-CD4 cells and does not affect the uptake and cycling properties of CD4low- molecules. Thus, the increased uptake of CD4 induced by phorbol ester is not due to general effects on vesicular traffic from the cell surface, but must be due to an increased association of CD4 with endocytic coated pits. This was confirmed in studies with hypertonic media, which inhibit coated vesicle formation, and by electron microscope observation of the association of CD4 with coated pits and vesicles.

Recently, several motifs have been identified that allow plasma membrane receptors to interact with components of clathrin-coated pits and lead to rapid endocytosis of these molecules. Most of these motifs consist of four to six amino acids with flanking aromatic or large hydrophobic residues (e.g., sequences of the form ar-x-x-ar or ar-x-x-x-x-ar), where one of the aromatic residues is frequently a tyrosine (57, 58). Where information is available, these structures show a strong tendency to form $\beta$ turns, and the substitution of amino acids which would disrupt the $\beta$ turn has been shown to reduce the efficacy of these endocytosis signals. The cytoplasmic domain of CD4 does not contain such a motif. Nonetheless, the fact that we observe endocytosis of CD4 and clustering into coated pits indicates that an alternative signal(s) must exist (59). Recently, Letourneur and Klausner (60) suggested that a pair of leucine residues forms part of the signal responsible for the endocytosis and lysosomal targeting of the $\gamma$ and $\delta$ subunits of CD3. A di-leucine sequence is found in CD4 (Leu413 and Leu414), and Shin et al. (24) have reported that these leucine residues, as well as other hydrophobic amino acids (Met407 and Ile410) are required for CD4 downregulation. Hence the di-leucine in CD4 may be a component of the endocytosis signal.

Previously it has been demonstrated that phorbol esters induce a rapid transient phosphorylation of serine residues in the cytoplasmic domain of CD4 (9, 21, 36), and that mu-
tation of these serines to alanine reduces the efficiency of down-regulation (33, 36, 42). The serine at position 408 is believed to be the most important of the three cytoplasmic serine residues in this respect (36). Although the effects of these residues on phorbol ester--induced down-regulation have been recorded, their role in CD4 endocytosis has not been evaluated, and it is unclear why phosphorylation of Ser408 should increase the efficiency of CD4 endocytosis. In contrast to many serine phosphorylation sites that are located at the tip of B turn structures (61), the residues surrounding Ser408 show a strong tendency to form an a helix (24). Phosphorylation of CD4 might be expected to influence the structure of this helix and thereby alter the disposition of sequences involved in endocytosis. The role of phosphorylation in coated vesicle--mediated endocytosis has been controversial (58). However, our studies indicate that PMA directly increases the association of CD4 with coated pits and hence the rate of CD4 endocytosis, and that this may be one situation in which phosphorylation does create or enhance an endocytosis signal.

Phorbol Ester--induced Endosomal Sorting of CD4. Modeling of CD4 endocytosis suggests that the PMA-induced increase in the rate of CD4 endocytosis and the consequent alteration of the steady state distribution of CD4 between the cell surface and the endosome compartment could account for the observed down-regulation of CD4. However, several previous studies suggested that CD4 internalized in the presence of PMA may be diverted from the endosome compartment and degraded in lysosomes. We used immunofluorescence microscopy to demonstrate directly that PMA affects the distribution of the CD4--anti-CD4 mAb complexes internalized from the cell surface. Thus, in unstimulated cells, internalized mAb was located in vesicular organelles throughout the cytoplasm which contained with Abs to the TIR and correspond to early endosomes. After PMA treatment, the distribution of CD4 was altered, so that the bulk of the internalized anti-CD4 mAb was seen clustered in a perinuclear region of the cell. In contrast to the early endosomes, these perinuclear clusters could be contained with Abs to the CI-MPR and may represent components of the late endosome compartment. Thus CD4 internalized into early endosomes in the presence of phorbol ester is diverted from the constitutive recycling pathway to the late endosome/lysosome pathway, and as a consequence, there is a reduction in the recycling of internalized CD4.

These observations imply that, in addition to the effects on endocytosis, the phorbol ester--induced phosphorylation of CD4 may also generate a signal that targets internalized CD4 to late endosomes and lysosomes. As with the endocytosis signal, the precise nature of this sorting signal is unclear. However, as truncation experiments (24) suggest that down-regulation requires the membrane proximal half of the cytoplasmic domain of CD4, this signal may also involve, or overlap with, the L-L sequence. Significantly, it has recently been reported that phosphorylation of the cytoplasmic domain of the CI-MPR and of the polymeric Ig receptor may be involved in the endosomal sorting or targeting of these molecules (62, 63). For the CI-MPR one of the phosphorylation sites is close to an L-L sequence that has been implicated in sorting to late endosomes (62, 64).

CD4 Downregulation in Lymphoid Cells. Phorbol ester--induced CD4 downregulation in nonlymphoid cells occurs through a combination of increased CD4 endocytosis and sorting of the internalized CD4 molecules to CI-MPR--containing late endosomes and lysosomes. In lymphoid cells, p56^k^ prevents CD4 entry into coated pits. To understand whether phorbol ester induces a relocation of p56^k^ together with CD4, or whether other mechanisms might account for CD4 down-regulation in these cells, we analyzed the interaction of p56^k^ and CD4 in the NIH-3T3-CD4/p56^k^ cell line M22 (30). These cells also downregulate CD4 in response to phorbol ester, although the rate of CD4 internalization is slower than that seen in phorbol ester--treated HeLa-CD4 and NIH-3T3-CD4 cells and is similar to that observed on lymphoid cells. Significantly, compared with NIH-3T3-CD4 cells, there is a lag after the addition of phorbol ester to M22 cells before significant endocytosis of CD4 is observed. When we immunoprecipitated CD4 from phorbol ester--treated M22 cells, we found that p56^k^ was dissociated from CD4 with a t_1/2 of 1-2 min. Thus, the dissociation appears to occur during the lag described above and to precede the onset of CD4 internalization. The region of the CD4 cytoplasmic domain that interacts with p56^k^ is known to involve two cysteine residues at positions 420 and 422, a region that is not required for down-regulation (65, 66). Nevertheless, it is conceivable that conformational changes in the cytoplasmic domain of CD4 induced by serine phosphorylation may disrupt the interaction with p56^k^.

The dissociation of CD4 from p56^k^ alone would release CD4 and result in CD4 uptake with similar kinetics and to a similar extent as the constitutive CD4 endocytosis observed in nonlymphoid cells (i.e., 2-3% per min, and 40% internal at steady state). This would lead to a partial reduction of cell surface CD4 levels. However, CD4 down-regulation induced by PMA in peripheral T cells and lymphocytic cells is much more rapid and extensive (21, 22). Thus, dissociation of CD4 and p56^k^ cannot alone account for the level of down-regulation observed (55, 56) and down-regulation is likely also to involve the changes in endocytosis and trafficking of CD4 described for the HeLa-CD4 cells. Indeed, the rates of CD4 endocytosis observed in PMA-treated lymphocytic cell lines are increased more than 20-fold (e.g., to 4.2% per min in SupT1 cells, see above) and exceed the rates of constitutive CD4 endocytosis in nonlymphocytic cells (cf. Table 1). The effect of PMA on CD4 endocytosis is likely to be greater than this, as asynchronous and/or incomplete dissociation of p56^k^ and CD4 will cause an underestimate in the observed rate of PMA-stimulated CD4 endocytosis in p56^k^--expressing cells. Thus PMA must also significantly increase CD4 internalization in lymphocytic cells.

In conclusion, we have demonstrated that phorbol ester--induced CD4 down-regulation involves a series of sequential changes in the trafficking properties of CD4. First, in p56^k^--containing CD4^+^ cells, phorbol ester stimulates dissociation of CD4 and p56^k^ (64). The released CD4 is then able to interact
with endocytic coated pits and vesicles. Second, phorbol esters increase the clustering of CD4 into coated pits and thereby increase the rate of CD4 endocytosis. Finally, phorbol esters induce the redistribution of internalized CD4 from the constitutive recycling pathway to degradative pathways, leading to delivery of CD4 to CI-MPR-containing late endosomes and lysosomes. Although these events have been observed in this study after stimulation with phorbol ester, it is likely that other stimuli, such as ligation of the TCR by antigen, would lead to CD4 downregulation by similar mechanisms.

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References

1. Parries, J.R. 1989. Molecular biology and function of CD4 and CD8. Adv. Immunol. 44:265.
2. Robey, E., and R. Axel. 1990. CD4: collaborator in immune recognition and HIV infection. Cell. 60:697.
3. Zühlig-Pflücker, J.C., J.A. Jones, L.T. Chin, and A.M. Kruisbeek. 1991. CD4 and CD8 act as co-receptors during thymic selection of the T cell repertoire. Semin. Immunol. 3:167.
4. Miceli, M.C., and J.R. Parries. 1991. The roles of CD4 and CD8 in T cell activation. Semin. Immunol. 3:133.
5. Sattentau, Q.J., and R.A. Weiss. 1988. The CD4 antigen: physiological ligand and HIV receptor. Cell. 52:631.
6. Lamarre, D., D.J. Capon, D.R. Karp, T. Gregory, E.O. Long, and R.-P. Sékaly. 1989. Class II MHC molecules and the HIV gp120 envelope protein interact with functionally distinct regions of the CD4 molecule. EMBO (Eur. Mol. Biol. Organ.) J. 8:3271.
7. Fleury, S., D. Lamarre, S. Meloche, S.-E. Ryu, C. Cantin, W.A. Hendrickson, and R.-P. Sékaly. 1991. Mutational analysis of the interaction between CD4 and class II MHC: class II antigens contact CD4 on a surface opposite the gp120 binding site. Cell. 66:1037.
8. Veillet, A., N. Abraham, L. Caron, and D. Davidson. 1991. The lymphocyte-specific tyrosine protein kinase p56
6. Semin. Immunol. 3:143.
9. Acres, B.R., P.J. Conlon, D.Y. Mochizuki, and B. Gallis. 1986. Rapid phosphorylation and modulation of the T4 antigen on cloned helper T cells induced by phorbol myristate acetate or antigen. J. Biol. Chem. 34:16210.
10. Weyand, C.M., J. Goronzy, and C.G. Fathman. 1987. Modulation of CD4 by antigenic activation. J. Immunol. 138:1351.
11. Rivas, A., S. Takada, J. Koide, G. Sonderstrup-McDevitt, and E.G. Engleman. 1988. CD4 molecules are associated with the antigen receptor complex on activated but not resting T cells. J. Immunol. 140:2912.
12. Cole, J.A., S.A. McCarthy, M.A. Rees, S.O. Sharrow, and A. Singer. 1989. Cell surface comodulation of CD4 and T cell receptor by anti-CD4 monoclonal antibody. J. Immunol. 143:397.
13. Thullier, L., F. Selz, P. Métézeau, and J.-L. Pérignon. 1990. The activation of protein kinase C is not necessary for the monoclonal antibody-induced modulation of CD3 and CD4 antigens. Eur. J. Immunol. 20:1197.
14. Blue, M.-L., J.F. Daley, H. Levine, K.R. Branton, and S.F. Schlossman. 1989. Regulation of CD4 and CD8 surface expression on human thymocyte subpopulations by triggering through CD2 and CD3-T cell receptor. J. Immunol. 142:374.
15. Hoxie, J.A., J.D. Alpers, J.L. Rackowski, K. Hubein, B.S. Haggarty, A.J. Cedarbaum, and J.C. Reed. 1987. Alterations in T4 (CD4) protein and mRNA synthesis in cells infected with HIV. Science (Wash. DC). 234:1123.
16. Juszczak, R.J., H. Turchin, A. Truneh, J. Culp, and S. Kassis. 1991. Effect of human immunodeficiency virus gp120 glycoprotein on the association of the protein tyrosine kinase p56
6 with CD4 in human T lymphocytes. J. Biol. Chem. 266:11176.
17. Ceftai, D., M. Ferrer, N. Serpente, T. Idziorek, and A. Dautry-Varsat, P. Debre, and G. Bismuth. 1992. Internalization of HIV glycoprotein gp120 is associated with down-modulation of membrane CD4 and p56
6 together with impairment of T cell activation. J. Immunol. 149:285.
18. Offner, H., T. Thieme, and A.A. Vandenbark. 1987. Gangliosides induce selective modulation of CD4 from helper T lymphocytes. J. Immunol. 139:3295.
19. Saggioro, D., C. Sorio, F. Calderazzo, L. Callegaro, M. Panozzo, G. Berton, and L. Chieco-Bianchi. 1993. Mechanism of action of the monosialoganglioside GM1 as a modulator of CD4 expression: evidence that GM1-CD4 interaction triggers dissociation of p56
6 from CD4, and CD4 internalization and degradation. J. Biol. Chem. 268:1368.
20. Blue, M.-L., D.A. Hafler, K.A. Craig, H. Levine, and S.F. Schlossman. 1987. Phosphorylation of CD4 and CD8 molecules following T cell triggering. J. Immunol. 139:3949.
21. Hoxie, J.A., J.L. Rackowski, B.S. Haggarty, and G.N. Gaulton.
1988. T4 endocytosis and phosphorylation induced by phorbol esters but not by mitogen or HIV infection. J. Immunol. 140:786.

22. Hoxie, J.A., D.M. Matthews, K.J. Callahan, D.L. Cassel, and R.A. Cooper. 1986. Transient modulation and internalization of T4 antigen induced by phorbol esters. J. Immunol. 137:1194.

23. Petersen, C.M., E.I. Christensen, B.S. Andresen, and B.K. Møller. 1992. Internalization, lysosomal degradation and new synthesis of surface membrane CD4 in phorbol ester-activated T-lymphocytes and U937 cells. Exp. Cell Res. 201:160.

24. Shin, J., R.L. Dunbrack, S. Lee, and J.L. Strominger. 1991. Phosphorylation-dependent down-modulation of CD4 requires a specific structure within the cytoplasmic domain of CD4. J. Biol. Chem. 266:10658.

25. Baenziger, J.W., A. Okamoto, E. Hall, S. Verma, and C.G. Davis. 1991. The tail of CD4 targets chimeric molecules to a degradative pathway. New Biologist. 3:1233.

26. Ruegg, C.L., S. Rajasekar, B.S. Stein, and E.G. Engleman. 1992. Degradation of CD4 following phorbol-induced internalization in human T lymphocytes. J. Biol. Chem. 267:18837.

27. Pelchen-Matthews, A., J.E. Armes, and M. Marsh. 1989. Internalization and recycling of CD4 transfected into HeLa and NIH3T3 cells. EMBO (Eur. Mol. Biol. Organ.) J. 8:6341.

28. Pelchen-Matthews, A., J.E. Armes, G. Griffiths, and M. Marsh. 1991. Differential endocytosis of CD4 in lymphocytic and non-lymphocytic cells. J. Exp. Med. 173:575.

29. Marsh, M., J.E. Armes, and A. Pelchen-Matthews. 1990. Endocytosis and recycling of CD4. Biochem. Soc. Trans. 18:139.

30. Pelchen-Matthews, A., I. Boulet, D.R. Littman, R. Fagard, and M. Marsh. 1992. The protein tyrosine kinase p56^k inhibits CD4 endocytosis by preventing entry of CD4 into coated pits. J. Cell Biol. 117:279.

31. Healey, D., L. Dianda, J.P. Moore, J.S. McDougal, M.J. Moore, P. Estess, D. Buck, P.D. Kwong, E.C.L. Beverley, and Q.J. Sattentau. 1990. Novel anti-CD4 monoclonal antibodies separate human immunodeficiency virus infection and fusion of CD4^+ cells from virus binding. J. Exp. Med. 172:1233.

32. Brown, W.J., and M.G. Farquhar. 1987. The distribution of 215-kilodalton mannose 6-phosphate receptors within cis (heavy) and trans (light) Golgi subfractions varies in different cell types. Proc. Natl. Acad. Sci. USA. 84:9001.

33. Maddon, P.J., J.S. McDougal, P.R. Clapham, A.G. Dalgleish, S. Jamal, R.A. Weiss, and R. Axel. 1988. HIV infection does not require endocytosis of its receptor, CD4. Cell. 54:865.

34. Smith, P.K.R.J., Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, and D.C. Klenk. 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150:76.

35. Clapham, P.R., R.A. Weiss, A.G. Dalgleish, M. Exley, D. Whitby, and N. Hogg. 1987. Human immunodeficiency virus infection of monocytic and T-lymphocytic cells: receptor modulation and differentiation induced by phorbol ester. Virology. 158:44.

36. Shin, J., C. Doyle, Z. Yang, D. Kappes, and J.L. Strominger. 1990. Structural features of the cytoplasmic region of CD4 required for internalization. EMBO (Eur. Mol. Biol. Organ.) J. 9:425.

37. Favero, J., J.F.P. Dixon, P.C. Bishop, R. Larguier, and J.W. Parker. 1990. Lymphocyte mitogenesis and CD4 modulation induced by different phorbol esters: comparative studies. Int. J. Immunopharmacol. 12:769.

38. Møller, B.K., B.S. Andresen, E.I. Christensen, and C.M. Petersen. 1990. Surface membrane CD4 turnover in phorbol ester stimulated T-lymphocytes. FEBS (Fed. Eur. Biochem. Soc.) Lett. 276:59.

39. Collins, M.K.L., and E. Rozengurt. 1982. Binding of phorbol esters to high affinity sites on murine fibroblastic cells elicits a mitogenic response. J. Cell. Physiol. 112:42.

40. Young, S., P.J. Parker, A. Ullrich, and S. Stabel. 1987. Down-regulation of protein kinase C is due to an increased rate of degradation. Biochem. J. 244:775.

41. Neudorf, S., M. Jones, S. Parker, R. Papes, and D. Lattier. 1991. Phorbol esters down-regulate transcription and translation of the CD4 gene. J. Immunol. 146:2836.

42. Bedinger, P., A. Moriarty, R.C. von Borstel II, N.J. Donovan, K.S. Steimer, and D.R. Littman. 1988. Internalization of the human immunodeficiency virus does not require the cytoplasmic domain of CD4. Nature (Lond.) 334:162.

43. Swanson, J.A., B.D. Yirinec, and S.C. Silverstein. 1985. Phorbol esters and horseradish peroxidase stimulate pinocytosis and redirect the flow of pinocytosed fluid in macrophages. J. Cell Biol. 100:851.

44. Phaire-Washington, I., E. Wang, and S.C. Silverstein. 1980. Phorbol myristate acetate stimulates pinocytosis and membrane spreading in mouse peritoneal macrophages. J. Cell Biol. 86:634.

45. Daukas, G., and S.H. Zigmond. 1985. Inhibition of receptor-mediated but not fluid phase endocytosis in polymorphonuclear leukocytes. J. Cell Biol. 101:1673.

46. Heuser, J.E., and R.G.W. Anderson. 1989. Hypertonic media inhibit receptor-mediated endocytosis by blocking clathrin-coated pit formation. J. Cell Biol. 108:389.

47. Davoust, J., J. Gruenberg, and K.E. Howell. 1987. Two threshold values of low pH block endocytosis at different stages. EMBO (Eur. Mol. Biol. Organ.) J. 6:3601.

48. Sandvig, K., S. Olsnes, O.W. Petersen, and B. van Deurs. 1987. Acidification of the cytosol inhibits endocytosis from coated pits. J. Cell Biol. 105:679.

49. Anderson, R.G.W., M.S. Brown, and J.L. Goldstein. 1977. Role of the coated endocytic vesicle in the uptake of receptor-bound low density lipoprotein in human fibroblasts. Cell. 10:351.

50. Marsh, M., and A. Helenius. 1980. Adsorptive endocytosis of Semliki Forest virus. J. Mol. Biol. 142:439.

51. Griffiths, G., R. Back, and M. Marsh. 1989. A quantitative analysis of the endocytic pathway in baby hamster kidney cells. J. Cell Biol. 109:2703.

52. Marsh, M., P. Reid, I. Parsons, C. Hermon, and A. Pelchen-Matthews. 1992. Morphological analysis of the regulation of CD4 endocytosis by p56^k. Biochem. Soc. Trans. 20:719.

53. Griffiths, G., B. Hoflack, K. Simons, I. Mellman, and S. Kornfeld. 1988. The mannose 6-phosphate receptor and the biogenesis of lysosomes. Cell. 52:329.

54. Hurley, T.R., K. Luo, and B.M. Sefton. 1989. Activators of protein kinase C induce dissociation of CD4, but not CD8, from p56^k. Science (Wash. DC). 245:407.

55. Sleckman, B.P., J. Shin, V.E. Igras, T.L. Collins, and J.L. Strominger. 1992. Disruption of the CD4-p56^k complex is required for rapid internalization of CD4. Proc. Natl. Acad. Sci. USA. 89:7566.

56. Yoshida, H., Y. Koga, K. Nakamura, G. Kimura, and K. Nomoto. 1992. A lymphocyte-specific protein tyrosine kinase, p56^k, regulates the PMA-induced internalization of CD4. Biochem. Biophys. Acta. 1137:321.

57. Vaux, D. 1992. The structure of an endocytosis signal. Trends Cell Biol. 2:189.

58. Trowbridge, I.S., J.F. Collawn, and C.R. Hopkins. 1993.
Signal-dependent membrane protein trafficking in the endocytic pathway. *Annu. Rev. Cell Biol.* In press.

59. Miettinen, H.M., K. Matter, W. Hunziker, J.K. Rose, and I. Mellman. 1992. Fc receptor endocytosis is controlled by a cytoplasmic domain determinant that actively prevents coated pit localization. *J. Cell Biol.* 116:875.

60. Letourneau, F., and R.D. Klausner. 1992. A novel di-leucine motif and a tyrosine-based motif independently mediate lysosomal targeting and endocytosis of CD3 chains. *Cell.* 69:1143.

61. Kennelly, P.J., and E.G. Krebs. 1991. Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. *J. Biol. Chem.* 266:15555.

62. Méresse, S., and B. Hoflack. 1993. Phosphorylation of the cation-independent mannose 6-phosphate receptor is closely associated with its exit from the trans-Golgi network. *J. Cell Biol.* 120:67.

63. Casanova, J.E., P.P. Breitfeld, S.A. Ross, and K.E. Mostov. 1990. Phosphorylation of the polymeric immunoglobulin receptor required for its efficient transcytosis. *Science (Wash. DC).* 248:742.

64. Johnson, K.F., and S. Kornfeld. 1992. The cytoplasmic tail of the mannose 6-phosphate/insulin-like growth factor-II receptor has two signals for lysosomal enzyme sorting in the Golgi. *J. Cell Biol.* 119:249.

65. Turner, J.M., M.H. Brodsky, B.A. Irving, S.D. Levin, R.M. Perlmutter, and D.R. Littman. 1990. Interaction of the unique N-terminal region of tyrosine kinase p56	extsuperscript{1k} with cytoplasmic domains of CD4 and CD8 is mediated by cysteine motifs. *Cell.* 60:755.

66. Shaw, A.S., J. Chalupny, J.A. Whitney, C. Hammond, K.E. Amrein, P. Kavathas, B.M. Sefton, and J.K. Rose. 1990. Short related sequences in the cytoplasmic domains of CD4 and CD8 mediate binding to the amino-terminal domain of the p56	extsuperscript{1k} tyrosine protein kinase. *Mol. Cell. Biol.* 10:1853.