Phorbol Ester–induced Downregulation of CD4 is a Multistep Process Involving Dissociation from p56^{lck}, 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 p56^{lck}, 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 p56^{lck}-expressing transfected cells, these effects were preceded by the PMA-induced dissociation of CD4 and p56^{lck}, 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, p56^{lck} (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 $p56^{lck}$ (30). In the present study, we have examined the mechanisms of phorbol ester–induced CD4 modulation. Since $p56^{lck}$ clearly has a significant influence on the endocytic trafficking of CD4 (30), we have used CD4-transfected non-lymphoid 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 not dependent on the presence of $p56^{lck}$. 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 $p56^{lck}$-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 $p56^{lck}$.

Materials and Methods

Materials. Horseradish peroxidase (HRP)[1] 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 radioiodinated as described (27, 28). Q4120 was labeled with tetramethyl-rhodamine isothiocyanate (TRITC; Cambridge Bio-Science, Cambridge, UK) according to the manufacturer's instructions, whereas the fluorescein-conjugated mAb to the transferrin receptor (TfR), L01.1, 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 $p56^{lck}$, anti-$p56^{lck}$ (KERP), raised against a peptide covering residues 478–509 of murine $p56^{lck}$, has been described (30). A second antiserum, anti-$p56^{lck}$ (RNGS), was raised against a peptide covering residues 39–64 of murine $p56^{lck}$ starting with the sequence N'-RNGS (prepared by Dr. Torben Saermark, 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 Cell Culture. Adherent HeLa and NIH-3T3 cell lines transfected with the cDNAs of human CD4 or mutant CD4^{cyt-}, as well as M22, an NIH-3T3-CD4 cell line which has been super-transfected with the murine *lck* cDNA, were cultured as described (28, 30) and used 3 d after subculture unless otherwise indicated. CD4^{cyt-} 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 ^{125}I-labeled anti-CD4 mAb (Leu3a or Q4120) or 0.5 nM ^{125}I-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 16-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 ^{125}I-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 succinic 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 and 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 were 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-

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[1] *Abbreviations used in this paper:* BM, binding medium; CD4^{cyt-}, CD4 molecules from which the cytoplasmic domain has been deleted; CI-MPR, cation independent mannose 6-phosphate receptor; HRP, horseradish peroxidase; MES, morpholinoethanesulfonic acid; TfR, transferrin receptor; TRITC, tetramethylrhodamine isothiocyanate.

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. 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 Ca^{2+}/Mg^{2+}-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/p56^*lck* 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× 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-p56^*lck* (RNGS) and anti-p56^*lck* (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 Abs 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 radioiodinated 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
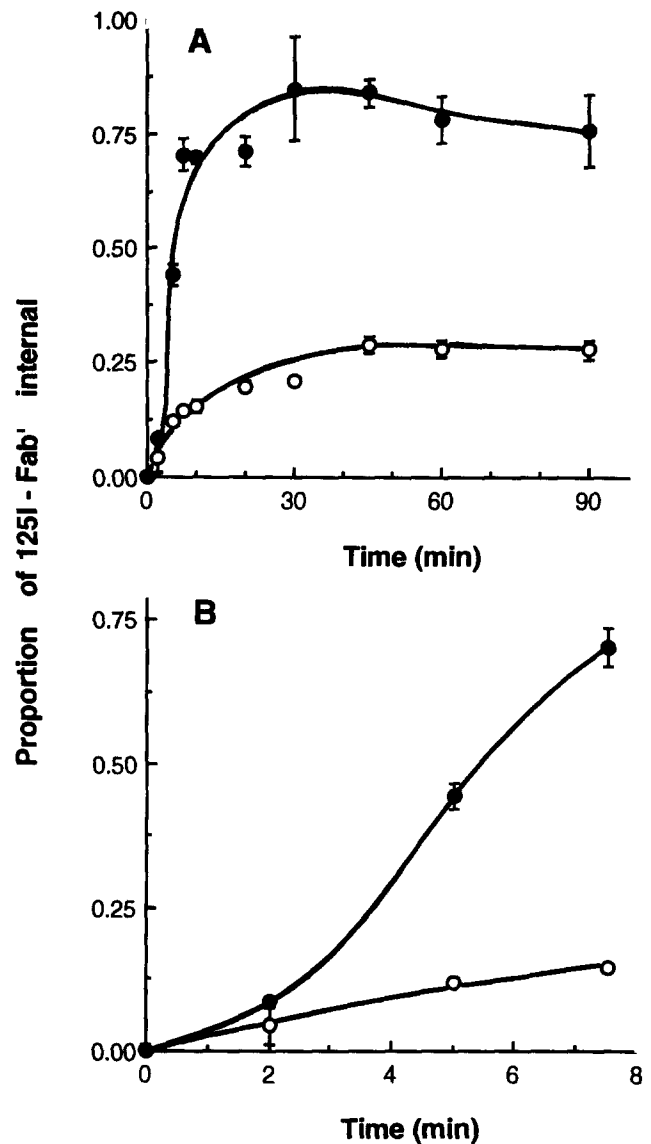

Figure 1. Effect of PMA on CD4 endocytosis in HeLa-CD4 cells. (*A*) Time course of internalization of CD4 on 3-d-old HeLa-CD4 cells in the presence (●) or absence (○) of 100 ng/ml PMA. CD4 endocytosis was traced with ¹²⁵I-labeled Fab' fragments of Leu3a. The plot shows the ratios of acid-resistant ¹²⁵I-Fab' to the total cell-associated label after various times at 37°C. The initial portion of the graph (0–8 min) is expanded (*B*).

Table 1. *Effect of PMA on CD4 Endocytosis*

Cell line	Tracer used	Control		Plus PMA		No. of experiments
		Endocytosis rate	Percent internal at 60 min	Endocytosis rate	Percent internal at 60 min	
		%/min		%/min		
HeLa-CD4 (3-d-old)	^{125}I-Fab'	2.0	28	11.3	78	1*
	^{125}I-Leu3a	1.8 ± 0.9	37 ± 1	8.4 ± 3.3	62 ± 14	7
HeLa-CD4 (2-d-old)	^{125}I-Q4120	2.6 ± 0.2	46 ± 7	6.4 ± 0.2	79 ± 11	2
NIH-3T3-CD4	^{125}I-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^{cyt-}; 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^{lck}. As CD4 downregulation is believed to occur by endocytosis, and CD4 endocytosis is influenced by p56^{lck} expression, we first sought to understand the mechanisms of downregulation in the p56^{lck}-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 ^{125}I-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 ^{125}I 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 [125]I (representing degraded Ab ligand), when calculated as a proportion of the amount of ^{125}I-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 ^{125}I 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
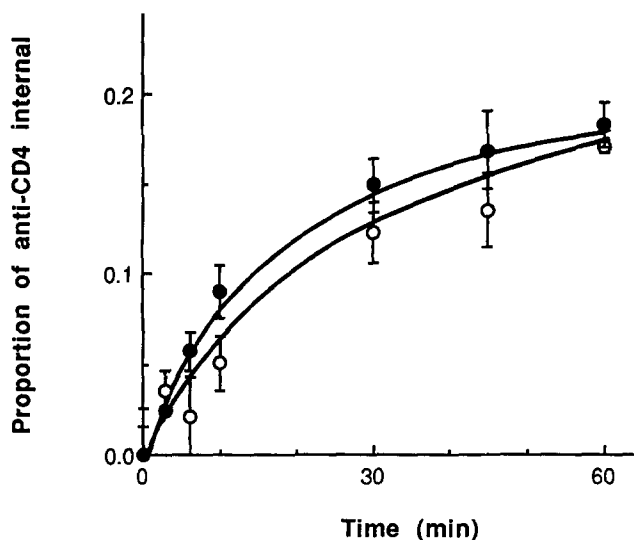
Figure 2. Time course of internalization of ^{125}I-labeled Leu3a in HeLa-CD4^{cyt-} cells that express CD4 molecules from which the cytoplasmic domain has been deleted. The plot shows the ratios of acid-resistant ^{125}I-Leu3a to the total cell-associated label on cells warmed in the presence (●) or absence (O) of 100 ng/ml of PMA.

occurs by this pathway, we used hypertonic media (45) to inhibit the formation of clathrin-coated vesicles (46). In our hands, incubation in media 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
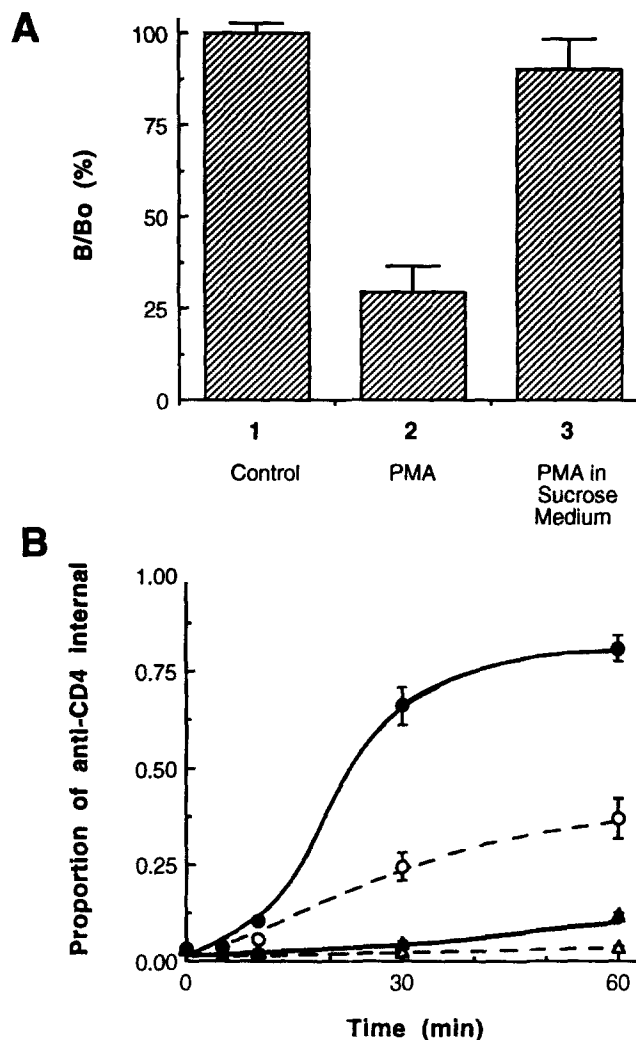

presence of the cytoplasmic domain (33, 36, 42). We have shown that CD4^{cyt-} molecules can be internalized by bulk flow transport through coated pits in both HeLa and lymphoid cell lines (28). To determine whether phorbol ester treatment also affected the internalization of CD4^{cyt-}, we measured endocytosis on HeLa-CD4^{cyt-} 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 6×10^{-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

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 ^{125}I-labeled Q4120. The plot shows binding of ^{125}I-Q4120 compared to the untreated cells (B/B$_0$) 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 ^{125}I-Q4120. The plot shows the ratios of acid-resistant ^{125}I-Q4120 to the total cell-associated label after various times at 37°C.

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)	3
2 min	346	314	(90.8)	18	(5.2)	10	(2.9)	4
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

Distribution of Leu3a/protein A–gold particles were analyzed as detailed in the text and in reference 28.
* Only particles observed immediately juxtaposed to the clathrin coat were counted in this category.

almost completely inhibited CD4 downregulation (Fig. 3 *A*). When CD4 internalization was assayed directly, the hypertonic medium inhibited CD4 endocytosis by more than 90%. The increased CD4 endocytosis observed in the presence of PMA was also inhibited to a similar extent (Fig. 3 *B*), suggesting that both constitutive and PMA-induced uptake of CD4 occurred through coated pits and vesicles.

The findings that PMA does not stimulate vesicular traffic from the surface of HeLa-CD4 cells and that inhibition of coated vesicle–mediated endocytosis inhibits phorbol ester–induced downregulation suggested that the increased 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 ^{125}I-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 ^{125}I-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 ^{125}I-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 ^{125}I-labeled Ab. Furthermore, there was very little recycling of internalized tracer when cells were loaded with the ^{125}I-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.
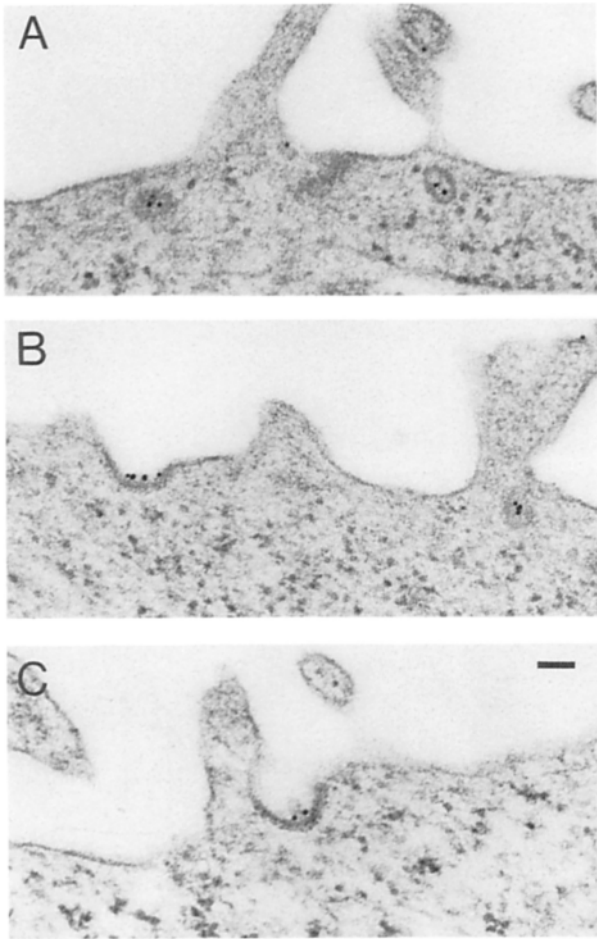
Figure 4. Electron microscopic localization of CD4 in PMA-treated HeLa-CD4 cells. 2-d-old HeLa-CD4 cells were labeled on ice with 8 nM Leu3a followed by protein A–gold, washed, and warmed to 37°C in the presence of 100 ng/ml PMA for 2 min (*A* and *B*) or 3 min (*C*). Scale bar, 100 nm.

Modeling of CD4 Endocytosis.

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
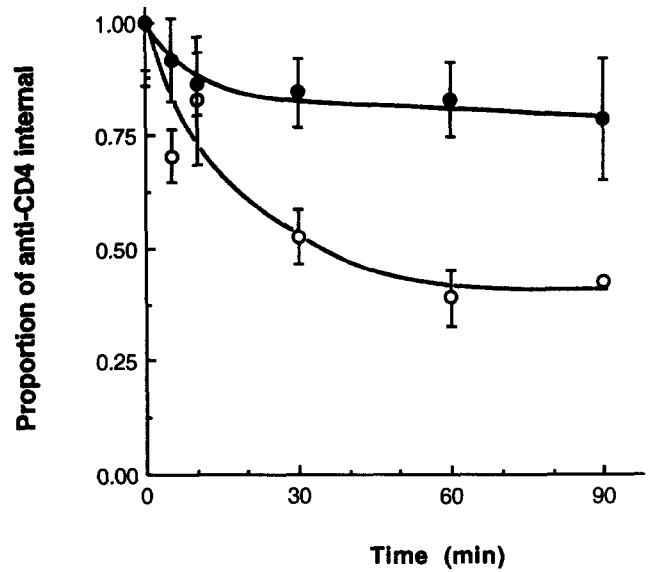

Figure 5. Effect of PMA on recycling of CD4. 3-d-old cultures of HeLa-CD4 cells were surface labeled with ¹²⁵I-labeled Fab' fragments of Leu3a, and then warmed for 30 min to 37°C in the presence (●) or absence (○) of 100 ng/ml PMA to allow the tracer to be internalized. Cells were then cooled and all ¹²⁵I-Fab' removed from the cell surface by acid washing. The time course of ¹²⁵I-Fab' recycling was then determined by rewarming the cells to 37°C for the times indicated. Cells were returned to ice and the acid-resistant activity determined after a second acid wash. The plot shows the ratios of acid-resistant ¹²⁵I-Fab' to the total cell–associated label.
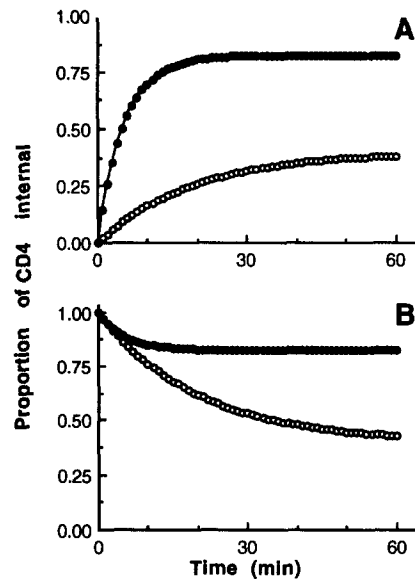

Figure 6. Mathematical modeling of CD4 endocytosis (*A*) and recycling (*B*) during endocytosis at 2% per min (○) or 14% per min (●). For details, see text.

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
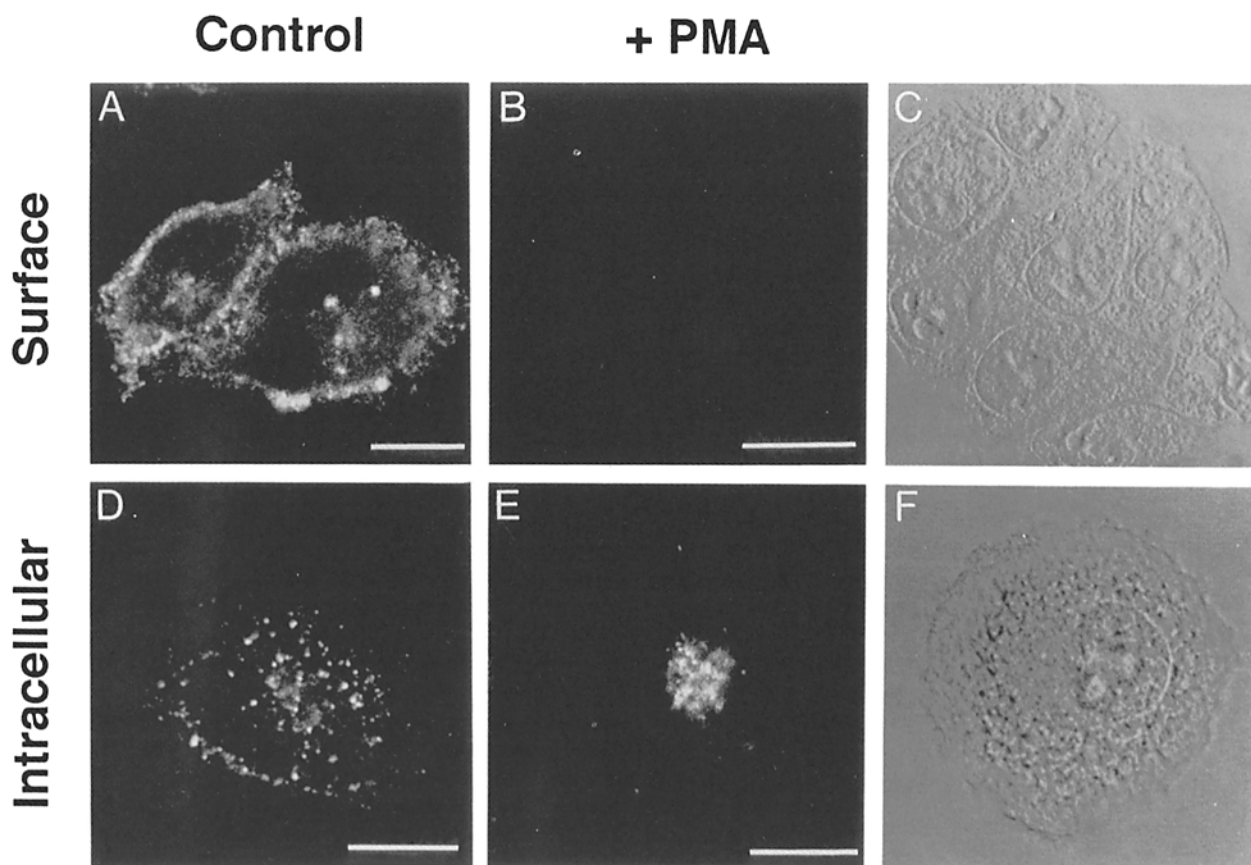
1215   Pelchen-Matthews et al.

Control   + PMA

Figure 7. The distribution of internalized CD4 in the presence of PMA. 2-d-old HeLa-CD4 cells were labeled with Leu3a at 4°C and then incubated at 37°C for 1 h in the absence (*A* and *D*) or presence (*B, C, E,* and *F*) of 100 ng/ml PMA. Cells were then fixed and stained with anti–mouse rhodamine second Ab to reveal CD4 at the cell surface (*A–C*). Alternatively, cell surface mAb was removed by acid washing before the cells were fixed, permeabilized, and stained with anti–mouse rhodamine to reveal internalized Ab (*D–F*). (*C* and *F*) Phase contrast views of *B* and *E*, respectively. All images are of 2-μm thick optical sections. Scale bars, 10 μm.

the cell surface decreased from 60 to about 20%, effectively giving downregulation. Endocytosis (Fig. 6 *A*) and recycling plots (Fig. 6 *B*) 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 ^{125}I-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 in the presence or absence of PMA, before processing to reveal cell surface or internalized mAb (Fig. 7). In unstimulated cells, this revealed CD4 at the cell surface (Fig. 7 *A*), and in intracellular vesicular structures located throughout the cytoplasm (Fig. 7 *D*). 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. 7 *B*), whereas the internalized anti-CD4 mAb was located in clusters of vesicles in the juxta-nuclear region of the cells (Fig. 7 *E*). 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-
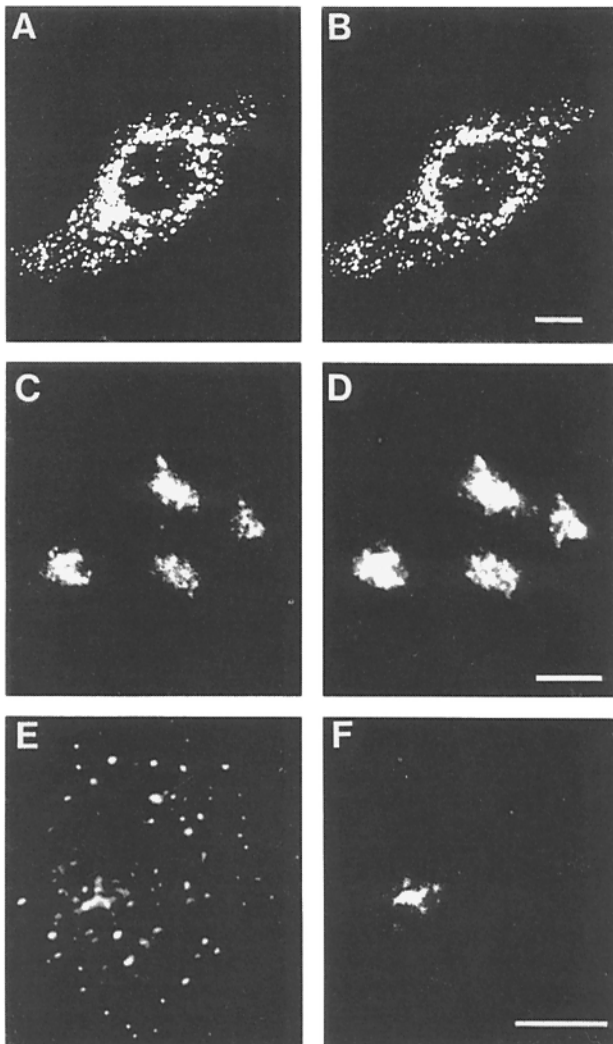
Figure 8. Colocalization of internalized CD4 with the TfR or CI-MPR. 2-d-old HeLa-CD4 cells were labeled with TRITC-Q4120 (*A* and *B*) or Leu3a (*C–F*) at 4°C and then incubated at 37°C for 1 h in the presence (*C* and *D*) or absence (*A*, *B*, *E*, and *F*) of 100 ng/ml PMA, and internalized anti-CD4 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.

ized for 1 h in the presence of PMA accumulated in the juxta-nuclear region in structures that could be costained with Abs to the CI-MPR (Fig. 8, *C* and *D*). No significant colocalization with the CI-MPR was observed in cells that had internalized CD4 in the absence of PMA (Fig. 8, *E* and *F*). Together these data indicate that CD4 internalized into early endosomes is, in the presence of PMA, diverted from the recycling pathway to a CI-MPR–containing late endosome compartment. Thus, in addition to its effect on CD4 endocytosis, PMA also altered the trafficking of internalized CD4 inside the cells.
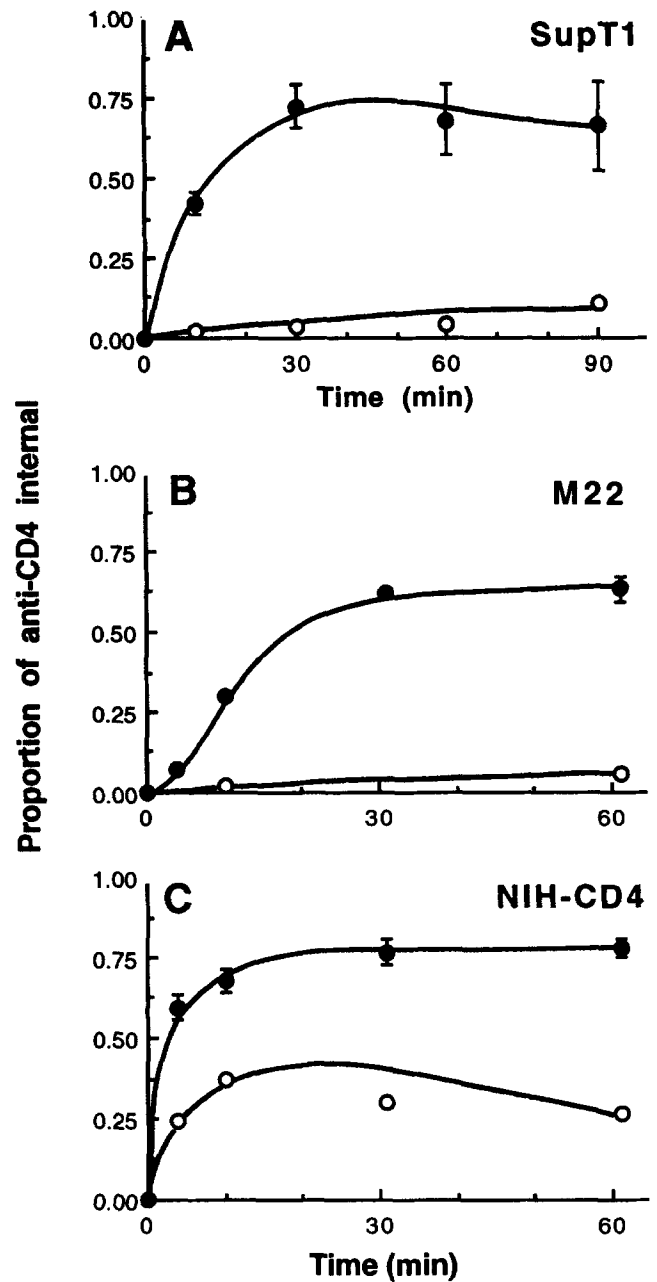
Figure 9. Effect of PMA on CD4 endocytosis in p56^{lck}-expressing cells. Time courses of internalization of CD4 on SupT1 (*A*), p56^{lck} transfected M22 cells (*B*), and the p56^{lck}-negative parental NIH-3T3-CD4 cells (*C*) in the presence (●) or absence (○) of 100 ng/ml PMA. CD4 endocytosis was traced with ^{125}I-labeled anti-CD4 mAb Q4120. The plots show the ratios of acid-resistant ^{125}I-mAb to the total cell–associated label after various times at 37°C.

The Role of p56^{lck} in Phorbol Ester–Induced CD4 Downregulation. As demonstrated above, in p56^{lck}-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^{lck} has been shown to regulate CD4 endocytosis (30), and since the CD4 expressed on T cells, thymocytes, and lym-
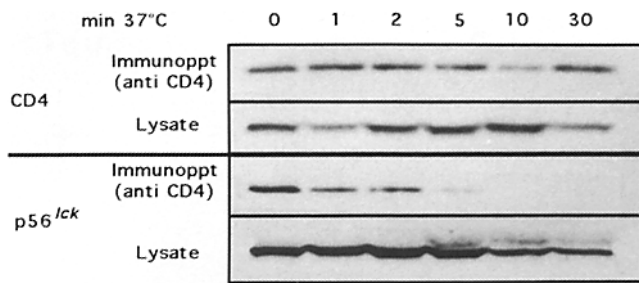
Figure 10. PMA treatment causes dissociation of CD4 and p56^{lck} in M22 cells. M22 cells were incubated in medium containing 100 ng/ml PMA at 37°C. At various times, cells were cooled, washed, and lysed. Cell lysates were directly analyzed by SDS-PAGE, or CD4 was immunoprecipitated from the lysates before SDS-PAGE and blotting for CD4 (*top*) or p56^{lck} (*bottom*).

phoid cell lines can also be downregulated (9, 22), we investigated the effect of PMA on CD4 endocytosis in p56^{lck}-expressing cells. In the lymphoid cell line SupT1, very little uptake of CD4 was observed in the absence of phorbol ester (0.2% per min; 28), and PMA stimulated CD4 endocytosis more than 20-fold, to 4.2% per min (Fig. 9 *A*). The p56^{lck}-transfected NIH-3T3-CD4 cell line M22 (30) responded similarly to PMA: CD4 endocytosis was increased 16-fold, from 0.24% per min to 3.8% per min (Fig. 9 *B*). Significantly, in M22 cells a lag could be observed before the increased CD4 endocytosis, and PMA-stimulated CD4 endocytosis was not as rapid, nor as extensive as that observed on NIH-3T3-CD4 cells lacking p56^{lck} (Fig. 9 *C*).

The association of CD4 and p56^{lck} was studied by analyzing lysates of PMA-treated M22 cells by Western blotting before or after immunoprecipitation of CD4. This demonstrated that PMA induced a very rapid dissociation of CD4 and p56^{lck} (Fig. 10). Quantitative densitometry of the blots indicated that more than 50% of p56^{lck} had dissociated from CD4 within 2 min of the addition of PMA, and CD4 precipitates from lysates of cells that had been treated with PMA for 5 min contained less than 10% of the amount p56^{lck} associated with CD4 in untreated cells. The rapid dissociation of CD4 and p56^{lck} therefore preceded the increase in CD4 endocytosis (Fig. 9 *B*), and the time taken for p56^{lck} and CD4 to dissociate may explain the lag in the increase of CD4 endocytosis observed in these cells. The level of CD4 or p56^{lck} detectable in the lysates did not vary significantly over the time course examined. Hence, in p56^{lck}-expressing cells, the increase in CD4 endocytosis is preceded by the dissociation of CD4 from p56^{lck}, thereby allowing CD4 to interact with coated pits.

Discussion

The observation that CD4 is downmodulated when T cells are stimulated by APCs or through cross-linking with Abs suggests that the control of plasma membrane CD4 levels is important in T cell physiology and function. Antigen-induced CD4 downregulation can be mimicked by treatment

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 p56^{lck} and CD4 (54), and recent data indicates that p56^{lck} dissociates before CD4 downregulation (55, 56). Internalized CD4 is believed to be directed to lysosomes and there 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 p56^{lck}-containing cells involves an initial dissociation of the CD4–p56^{lck} 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 CD4^{cyt-} 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 β turns, and the substitution of amino acids which would disrupt the β 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 γ and δ 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 downregulation 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 β turn structures (61), the residues surrounding Ser408 show a strong tendency to form an α 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 downregulation 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 costained with Abs to the TfR 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 costained 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 downregulation 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 phos-

phorylation 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, $p56^{lck}$ prevents CD4 entry into coated pits. To understand whether phorbol ester induces a relocation of $p56^{lck}$ together with CD4, or whether other mechanisms might account for CD4 downregulation in these cells, we analyzed the interaction of $p56^{lck}$ and CD4 in the NIH-3T3-CD4/$p56^{lck}$ 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 $p56^{lck}$ 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 $p56^{lck}$ is known to involve two cysteine residues at positions 420 and 422, a region that is not required for downregulation (65, 66). Nevertheless, it is conceivable that conformational changes in the cytoplasmic domain of CD4 induced by serine phosphorylation may disrupt the interaction with $p56^{lck}$.

The dissociation of CD4 from $p56^{lck}$ 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 downregulation induced by PMA in peripheral T cells and lymphocytic cells is much more rapid and extensive (21, 22). Thus, dissociation of CD4 and $p56^{lck}$ cannot alone account for the level of downregulation observed (55, 56) and downregulation 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 $p56^{lck}$ and CD4 will cause an underestimate in the observed rate of PMA-stimulated CD4 endocytosis in $p56^{lck}$-expressing cells. Thus PMA must also significantly increase CD4 internalization in lymphocytic cells.

In conclusion, we have demonstrated that phorbol ester–induced CD4 downregulation involves a series of sequential changes in the trafficking properties of CD4. First, in $p56^{lck}$-containing CD4$^+$ cells, phorbol ester stimulates dissociation of CD4 and $p56^{lck}$. 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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