

Cluster of Differentiation Antigen 4 (CD4) Endocytosis and Adaptor Complex Binding Require Activation of the CD4 Endocytosis Signal by Serine Phosphorylation

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Cluster of differentiation antigen 4 (CD4), the T lymphocyte antigen receptor component and human immunodeficiency virus coreceptor, is down-modulated when cells are activated by antigen or phorbol esters. During down-modulation CD4 dissociates from p56^{lck}, undergoes endocytosis through clathrin-coated pits, and is then sorted in early endosomes to late endocytic organelles where it is degraded. Previous studies have suggested that phosphorylation and a dileucine sequence are required for down-modulation. Using transfected HeLa cells, in which CD4 endocytosis can be studied in the absence of p56^{lck}, we show that the dileucine sequence in the cytoplasmic domain is essential for clathrin-mediated CD4 endocytosis. However, this sequence is only functional as an endocytosis signal when neighboring serine residues are phosphorylated. Phosphoserine is required for rapid endocytosis because CD4 molecules in which the cytoplasmic domain serine residues are substituted with glutamic acid residues are not internalized efficiently. Using surface plasmon resonance, we show that CD4 peptides containing the dileucine sequence bind weakly to clathrin adaptor protein complexes 2 and 1. The affinity of this interaction is increased 350- to 700-fold when the peptides also contain phosphoserine residues.

INTRODUCTION

Efficient endocytosis of cell surface glycoproteins through clathrin-coated vesicles requires the presence of endocytosis signals (Pelchen-Matthews *et al.*, 1991; Kirchhausen *et al.*, 1997). These signals interact with adaptor proteins that couple the glycoproteins to clathrin lattices. Two types of endocytic adaptor have been identified to date; 1) adaptor protein complex 2 (AP-2)¹ complexes, which have been implicated in the endocytosis of cell surface proteins carrying either YXXØ (where X is any amino acid, and Ø is a large

hydrophobic amino acid) or dileucine-containing endocytosis signals (Trowbridge *et al.*, 1993; Kirchhausen *et al.*, 1997); and 2) the nonvisual arrestins, which facilitate the internalization of certain seven-transmembrane-domain G-protein-coupled receptors (Ferguson *et al.*, 1996a,b; Goodman *et al.*, 1996). The mechanisms through which these proteins recognize and bind sorting signals are not well understood. Here we describe our studies of the dileucine-containing endocytosis signal in cluster of differentiation antigen 4 (CD4) and its interaction with the AP-2 and AP-1 adaptor complexes.

CD4 is a type I integral membrane glycoprotein expressed primarily on the surfaces of major histocompatibility complex (MHC) class II restricted T lymphocytes, thymocytes, and cells of the macrophage-monocyte lineage. This protein undergoes endocytosis

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¹ Abbreviations used: AP, adaptor protein complex; CD4, cluster of differentiation antigen 4; MHC, major histocompatibility complex; PE, phorbol ester; PMA, phorbol 12-myristate 13-acetate; SPR, surface plasmon resonance; wt, wild-type.

in clathrin-coated vesicles; however, its internalization is tightly regulated (reviewed in Marsh and Pelchen-Matthews, 1996). In T cells, association of CD4 with the Src family protein tyrosine kinase p56^{lck} prevents CD4 association with coated pits and endocytosis (Pelchen-Matthews *et al.*, 1992). After T cell activation by antigen, antibody-mediated cross-linking of cell surface proteins, or treatment of cells with phorbol esters (PEs), CD4 dissociates from p56^{lck} and undergoes endocytosis (Pelchen-Matthews *et al.*, 1992, 1993). In CD4^{+ve} cells, which lack p56^{lck} (e.g., monocytes and macrophages), CD4 is constitutively internalized, although the rates of internalization are increased when the cells are treated with PEs (Pelchen-Matthews *et al.*, 1991). Significantly, the endocytic properties of CD4 observed on tissue culture cells are also seen with primary cells *ex vivo* (Pelchen-Matthews *et al.*, 1998).

The endocytic properties of CD4 in the absence of p56^{lck} have been studied in most detail in HeLa and NIH 3T3 cells stably transfected with human CD4 (Pelchen-Matthews *et al.*, 1989). In these cells CD4 undergoes constitutive endocytosis at rates of ~4% of the cell surface pool per minute. This endocytosis is dependent on the presence of the cytoplasmic domain of the molecule and is signal mediated. Removal of the cytoplasmic domain reduces the uptake rate to 1%/min. The truncated molecule is neither included in nor excluded from coated pits and is internalized through bulk flow endocytosis (Pelchen-Matthews *et al.*, 1991), which we subsequently referred to as basal endocytosis. After PE treatment, CD4 undergoes rapid internalization at rates up to 10–12%/min. This increased internalization is due to a three- to fourfold increase in CD4 association with coated pits (Pelchen-Matthews *et al.*, 1991). The cytoplasmic domain of CD4 has no tyrosines, but it does contain a pair of leucine residues (L413 and L414 in human CD4) that are required for PE-induced down-modulation (Shin *et al.*, 1990). In addition, the phosphorylation of adjacent serine residues (S408 and S415) has been implicated in down-modulation (Shin *et al.*, 1991). These serines are phosphorylated in PE-treated cells, and substitution of the serines reduces PE-induced down-modulation (Shin *et al.*, 1990, 1991). Together these data suggest that phosphorylation may be required for rapid endocytosis of CD4 and may regulate the interaction of CD4 with clathrin adaptors.

CD4 down-modulation is a multistep process that involves endocytosis, endosomal sorting, and degradation (Pelchen-Matthews *et al.*, 1993). Here we have used a series of CD4 mutants to investigate whether phosphorylation of the serine residues adjacent to the dileucine sequence is required for clathrin adaptor complex binding and endocytosis. The results indicate that the dileucine sequence is essential for endocytosis, but that the endocytosis signal is only active when S408 and/or S415 are phosphorylated. The motifs for

constitutive and PE-induced endocytosis differ, with phosphorylation of S408 being essential for constitutive endocytosis, whereas phosphorylation of S408 and/or S415 allows PE-induced internalization. Surface plasmon resonance (SPR) analysis demonstrated that peptides encoding a portion of the CD4 cytoplasmic domain including L413, L414, S408, and S415 show weak binding to purified AP-2 adaptors. However, peptides in which S408 and/or S415 are phosphorylated show up to 700-fold increase in binding affinity. Significantly, constitutive endocytosis and adaptor binding are not observed when the serine residues are substituted with glutamic acid, indicating that negative charges cannot substitute for phosphoserine in this signal. Together the data indicate that 1) the endocytosis signal in CD4 involves a dileucine sequence that is only active when adjacent serine residues are phosphorylated, and 2) this phosphoserine- and dileucine-containing signal is recognized by clathrin adaptor complexes. We also observed that some CD4 constructs lacking endocytosis signals were internalized relatively efficiently in PE-treated cells. This suggested that PE treatment may also modify adaptor complexes, or other components of the endocytic machinery, to enhance CD4 recruitment into coated pits.

MATERIALS AND METHODS

Reagents and Cells

All tissue culture reagents were purchased from Life Technologies (Paisley, United Kingdom). Other chemicals were from Sigma (Poole, United Kingdom) unless indicated otherwise, and radioactive reagents were from Amersham (Little Chalfont, United Kingdom). The murine anti-human CD4 mAbs Q4120, 4, and 19 were all immunoglobulin G1. Q4120 (Healey *et al.*, 1990) was obtained from Q. Sattentau through the Medical Research Council AIDS Reagents Program (National Institute for Biological Standards and Control, South Mimms, Potters Bar, United Kingdom); antibodies 4 and 19 (Endres *et al.*, 1996) were provided by J. Hoxie (University of Pennsylvania, Philadelphia, PA). Q4120 was radiolabeled using ¹²⁵I-3-(*p*-hydroxyphenyl)-propionic acid *N*-hydroxy-succinimide ester (Bolton and Hunter reagent) as described (Pelchen-Matthews *et al.*, 1998). Specific activities were typically 300–600 Ci/mmol.

HeLa cells were maintained in Dulbecco's modified Eagle's medium containing 4% FCS, 2 mM glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin.

Generation and Expression of CD4 Mutants

CD4 mutants made for this study are detailed in Figure 1. The truncation mutants, S to E and L to A substitutions, were made by oligonucleotide-directed, site-specific mutagenesis using the *dut/ung* phagemid system (Bio-Rad, Hemel Hempstead, United Kingdom). Other mutants were made using the QuikChange site-directed mutagenesis kit (Stratagene, Cambridge, United Kingdom). Mutagenesis reactions were performed using the oligonucleotides and templates described in Table 1.

All CD4 constructs were generated and expressed in the eukaryotic expression vector pSG5 (Stratagene). Sequences of constructs were checked using either Sequenase version 2.0 T7 DNA polymerase (Amersham) or the Applied Biosystems Prism dye terminator cycle sequencing ready reaction kit (Perkin Elmer-Cetus, Warrington, United Kingdom). Two oligonucleotides were used for

Table 1. Oligonucleotides used for site-directed mutagenesis

Construct	Oligonucleotide
K418	5'-AGGGCACTGGCATCACTTCTTCTCACT
R412	5'-CTTCTCACTGAGTCATCTTTGATCTG
H399	5'-CTTGGCGCCTTCACTGCGCGCAC
K418 S408A	5'-GAGCGGATGGCACAGATCAAG and 5'-CTTGTATCTGTGCCATCCGCTC
K418 S415A	5'-AGACTCCTCGCGAGAAGAAG and 5'-CTTCTTCTCTGCGAGGAGTCT
K418 S408A/ S415A	As for K418 S415A
K418 S408E	5'-TCTCTTGTATCTGTCCATCCGCTCTGC
K418 S415E	5'-CTTCTTCTCTCGAGGAGTCTCTT
K418 S408E/ S415E	As for K418 S415E
K418 L413A/ L414A	5'-CTTCTTCTCACTGGCGGCTCTTGTAT
K418 L413V	5'-CAGATCAAGCGAGTACTCAGTGAGAAGAAG and 5'- CTTCTTCTCACTGAGTACTCGCTTGTATCTG
K418 L413V/ L414V	5'-CAGATCAAGAGAGTGGTCACTGAGAAGAAG and 5'-CTTCTTCTCACTGACCACTCTTGTATCTG

sequencing, one directed to the 3' untranslated sequence of CD4 (5'-GTCCGCAGGCAGCAAACGCGG-3') and the other to the extracellular domain of the protein (5'-AACCTGAGGCGGGGAT-GTGG-3').

HeLa cells were transfected by electroporation. Plasmids encoding the CD4 constructs were transfected together with pSV2-Neo at a plasmid ratio of pSG5-CD4:pSV2-Neo of 10:1. Resistant colonies were selected in medium containing 1 mg/ml Geneticin sulfate (Life Technologies) and screened by immunofluorescence using Q4120 as described (Signoret *et al.*, 1997). DNA was prepared from the stable lines as described (Strauss, 1987), and the oligonucleotides 5'-CCA-CATCCCCGCTCAGGGT-3' and 5'-GTCCGCAGGCAGCAAA-CGCGG-3' were used to reisolate the transfected CD4 cDNAs by PCR. The PCR products were sequenced using the Applied Biosystems Prism system.

Measurement of CD4 Endocytosis

Endocytosis of CD4 was measured using ^{125}I -Q4120 essentially as described (Pelchen-Matthews *et al.*, 1989). Cells were seeded in 16-mm wells, in 24- or 4-well plates, and grown to confluence over 2 d. The cells were washed and labeled with 0.3 nM ^{125}I -Q4120 in BM (RPMI 1640 medium containing 0.2% BSA and buffered to pH 7.4 with 10 mM HEPES) for 2 h at 4°C. After labeling, unbound antibody was washed away, and the cells were either held on ice or warmed to 37°C for various times in the presence or absence of 100 ng/ml phorbol 12-myristate 13-acetate (PMA). Cells were warmed rapidly by immersion of plates in a large volume of prewarmed medium. After incubation at 37°C, the cells were cooled by immersion in 4°C BM and either harvested directly using 0.2 M NaOH (to give total cell-associated ^{125}I -Q4120 activity), or after treatment with pH 2.0 medium to remove the cell surface ^{125}I -Q4120 (to give the intracellular ^{125}I -Q4120 activity). Endocytosis was calculated as the proportion of acid-resistant to total cell-associated radioactivity for each time point. A background value for acid-resistant counts on cells maintained at 4°C (~200 cpm) was subtracted from each time point. Endocytosis rates were calculated by linear regression over the first 4 min after warmup or, for PMA-treated cells, after the 1-min lag phase (see below).

Fluid Phase Endocytosis

Fluid phase endocytosis was measured using HRP (type II) as described (Griffiths *et al.*, 1989). Cells, plated on 6-cm plates 2 d before the assay, were washed twice with 37°C BM and incubated in BM containing 5 mg/ml HRP at either 4 or 37°C. At the indicated times the cells were placed on ice and washed extensively with 4°C PBS. Cells were recovered in 2.5 mg/ml trypsin/EDTA, washed twice in 10 ml of 4°C PBS containing 0.5 mg/ml soya bean trypsin inhibitor, and lysed in ice-cold PBS containing 0.5% Triton X-100. HRP was quantitated using *o*-dianisidine in a microtiter plate assay (Pelchen-Matthews *et al.*, 1991). Cell protein was measured using bicinchoninic acid (Pierce and Warriner, Chester, United Kingdom) as described (Smith *et al.*, 1985).

Phosphate Labeling and CD4 Immunoprecipitation

Cells were cultured on 6-cm plates for 2 d before labeling. The cells were washed twice with phosphate-free medium and equilibrated with 0.5 mCi of [^{32}P]orthophosphoric acid for 4 h. The cells were incubated for 3 min in 37°C BM with or without 100 ng/ml PMA and then washed three times with 4°C PBS. Cell lysates were prepared by incubating the cells for 10 min in 4°C lysis buffer (20 mM Tris-HCl, pH 8.0, containing 0.5% NP40, 150 mM NaCl, 2 mM EDTA, protease inhibitors (1 mM PMSF and 10 $\mu\text{g}/\text{ml}$ chymostatin, leupeptin, antipain, and pepstatin), and phosphatase inhibitors (10 mM sodium pyrophosphate, 50 mM sodium fluoride, and 1 mM orthovanadate). The lysates were recovered by scraping, and the detergent-insoluble material was removed by centrifugation in a benchtop microfuge (13,000 rpm for 20 min at 4°C).

Before immunoprecipitation, the lysates were "precleared" by incubation for 30 min with 5 μl of a nonspecific rabbit serum and 20 μl of protein A-Sepharose CL-4B (Pharmacia, St. Albans, United Kingdom). The beads were removed by centrifugation (13,000 rpm for 1 min at 4°C), and the lysate was precleared twice more in the presence of 2.5 μl of nonspecific rabbit serum or beads alone. The final "preclears" were analyzed together with the immunoprecipitates. To immunoprecipitate CD4, lysates were mixed for 2 h, or overnight at 4°C, with protein A-Sepharose CL-4B covalently cross-linked to antibodies 4 and 19. The beads were collected by centrifugation and washed four times with lysis buffer and once in mixed micelle buffer (10 mM Tris-HCl, 0.3 M NaCl, 0.1% SDS, 0.05% Triton X-100, and 0.02% NaN_3). The precipitates were eluted with non-reducing sample buffer, separated on 10% SDS-polyacrylamide gels, and transferred to nitrocellulose. The blots were exposed to Kodak (Rochester, NY) X-OMAT AR film for autoradiography. Levels of ^{32}P incorporation were determined using a Bio-Rad GS250 Molecular Imager and/or by excision of the labeled bands and Cerenkov counting. To quantify the amount of CD4 precipitated in each lane, the transfers were immunoblotted with Q4120. Blots were blocked for 2 h at room temperature using 10% dried skimmed milk powder in PBS containing 0.1% Tween 20 and then incubated in Q4120 diluted 1:2000 in PBS and 0.1% Tween 20 for 1 h. After washing, the blots were probed with peroxidase-conjugated goat anti-mouse antibody (Pierce and Warriner), developed using ECL (Amersham) and quantified using the Bio-Rad Molecular Imager.

Preparation of Clathrin Adaptors

AP-1 and AP-2 clathrin adaptor complexes were purified from pig brain as described (Höning *et al.*, 1997). Briefly, adaptors were released from cell membranes by treatment with 0.5 M Tris, pH 7.8, and purified by gel filtration on Superose-6 by fast-performance liquid chromatography (Pharmacia). AP-1 was separated from AP-2 by hydroxyapatite chromatography. The purity of AP-1 and AP-2 was confirmed by Coomassie Blue staining fractions after SDS-PAGE and by Western blotting using AP-1-specific (100/3) and AP-2-specific (100/2) mAbs (Sigma). Before SPR analysis AP-1 and AP-2 preparations were centrifuged for 15 min at 275,000 $\times g$ to remove any aggregated material. The supernatants were analyzed

by gel filtration using a SMART system (Pharmacia) both before and after SPR analysis. No adaptor aggregation was detected under the experimental conditions used.

Peptide Synthesis

Peptides encoding the cytoplasmic domain of human CD4 from A398 to K418 (see Table 3) were synthesized by Chiron Technologies (Meltek Scientific, Feltham, United Kingdom). The peptides were synthesized with biotin at the N terminus to facilitate coupling for SPR analysis. Phosphorylated peptides were synthesized using phosphoserine instead of serine. The phosphorylation of these peptides was stable for >7 d in the buffers and conditions used for SPR analysis, as determined by mass spectroscopy.

SPR Analysis

The interaction of CD4 peptides with AP-1 and AP-2 adaptor complexes was analyzed by SPR (Jonsson *et al.*, 1991) using a BIAcore-2000 biosensor (BIAcore, Uppsala, Sweden). The peptides were coupled via their N-terminal biotin moiety to a streptavidin-coated SA5 sensor chip according to the manufacturer's instructions.

All interaction experiments were performed with buffer A (20 mM HEPES-NaOH, pH 7.0, 150 mM NaCl, 10 mM KCl, 2 mM MgCl₂, and 0.2 mM DTT) at a flow rate of 20 μl/min unless otherwise stated. Purified adaptors were used at 50–500 nM in buffer A. Association for 2 min was followed by dissociation for 2 min, during which buffer A alone was perfused. A short pulse injection (15 s) of 20 mM NaOH and 0.5% SDS was used to regenerate the sensor chip surface after each experimental cycle. The peptide-derivitized sensor chip remained stable and retained its specific binding capacity for >100 experimental cycles of association–dissociation and regeneration.

Determination of Kinetic Rate Constants

The rate constants (k_a for association and k_d for dissociation) and the equilibrium constant ($K_D = k_d/k_a$) for the interaction of the CD4 peptides and purified adaptors were calculated using the evaluation software of the BIAcore-2000 (version 3.0) assuming first-order kinetics $A + B = AB$. The mathematical models used are described in detail elsewhere (Schuck and Minton, 1996; Schuck, 1997). Association was determined 15–20 s after switching to the adaptor solution to avoid distortions caused by injection and mixing. The dissociation rate constants were determined 5–10 s after switching to buffer flow. After a dissociation phase of ~30 s, further dissociation of adaptors was slow. The model calculates the k_a and the steady-state response level R_{eq} by fitting data to the equation:

$$R = R_{eq}(1 - e^{-(k_a C_n + k_d)(t-t_0)})$$

where t is time in seconds; R_{eq} is the steady-state response level; and C is the molar concentration of adaptors in the injection solution. The steric interference factor N , which describes the valency of the interaction between the adaptors and the CD4 peptides, was set to 1. The k_d was determined by fitting data to the equation:

$$R = R_0 e^{-k_d(t-t_0)}$$

where R_0 is the response level at the beginning of the dissociation phase. This model, which has been applied to other adaptor–cytoplasmic domain interactions (Heilker *et al.*, 1996), is described in more detail elsewhere (Karlsson *et al.*, 1991; O'Shannessy *et al.*, 1993). It should be noted that these models allow the determination of rate constants without reaching equilibrium during the experimental cycle.

Mutant	Cytoplasmic domain sequence
wt CD4	RCRHHRRRQAERMSQIKRLLSEKKTCCPHRFQKTCSP1
K418	RCRHHRRRQAERMSQIKRLLSEKK
R412	RCRHHRRRQAERMSQIKR
H399	RCRH
K418 S408A	RCRHHRRRQAERMAQIKRLLSEKK
K418 S408E	RCRHHRRRQAERMEQIKRLLSEKK
K418 S415A	RCRHHRRRQAERMSQIKRLLAEKK
K418 S415E	RCRHHRRRQAERMSQIKRLLSEKK
K418 S408A/S415A	RCRHHRRRQAERMAQIKRLLAEKK
K418 S408E/S415E	RCRHHRRRQAERMEQIKRLLSEKK
K418 L413V	RCRHHRRRQAERMSQIKRVLSEKK
K418 L413V/L414V	RCRHHRRRQAERMSQIKRVVSEKK
K418 L413A/L414A	RCRHHRRRQAERMSQIKRAASEKK

Figure 1. CD4 and CD4 cytoplasmic domain mutants used in this study. The full-length cytoplasmic domain is illustrated for wt CD4 commencing at residue R396. Single amino acid substitutions at S408, L413, L414, and S415 are marked in bold.

RESULTS

The CD4 Endocytosis Signal Is Located between Residues 399 and 418

To examine the relationship between serine phosphorylation and CD4 recruitment into endocytic clathrin-coated vesicles, we expressed CD4 and CD4 cytoplasmic domain mutants in HeLa cells. HeLa cells do not express p56^{lck} and allow the endocytic properties of CD4 to be examined unfettered. To identify the location of the endocytosis signal(s) in CD4 we first used site-directed mutagenesis to replace amino acids R400, L413, and T419 with stop codons and to produce the truncation mutants H399, R412, and K418 (Figure 1). The constructs were transfected into HeLa cells by electroporation, and stable CD4-positive clones were selected. CD4 expression was monitored by immunofluorescence and SDS-PAGE. All three truncation mutants were expressed on the cell surface and, as expected, demonstrated increased mobility in SDS-PAGE compared with wild-type (wt) CD4 (Figure 2B).

An assay based on the internalization of prebound ¹²⁵I-Q4120 was used to measure CD4 uptake. In this assay, the amount of antibody internalized in the first 10 min after warming cells to 37°C gives a direct measure of the rate of CD4 endocytosis (Pelchen-Matthews *et al.*, 1989). To ensure that all cells were treated identically, plates of cells were warmed by immersion in 37°C medium. This allowed a more rapid rate of temperature change than previous procedures, and, as a consequence, we observed slightly faster CD4 uptake (cf. Pelchen-Matthews *et al.*, 1989). The rates of internalization were calculated for the first 4 min after warmup. Under these conditions full-length CD4 was internalized at ~5% of the cell surface pool per minute (Figure 2A and Table 2). K418 was internalized at a similar rate. By contrast, R412 and H399 were internalized at rates of 1–2%/min. The internalization of H399 is considered to occur through

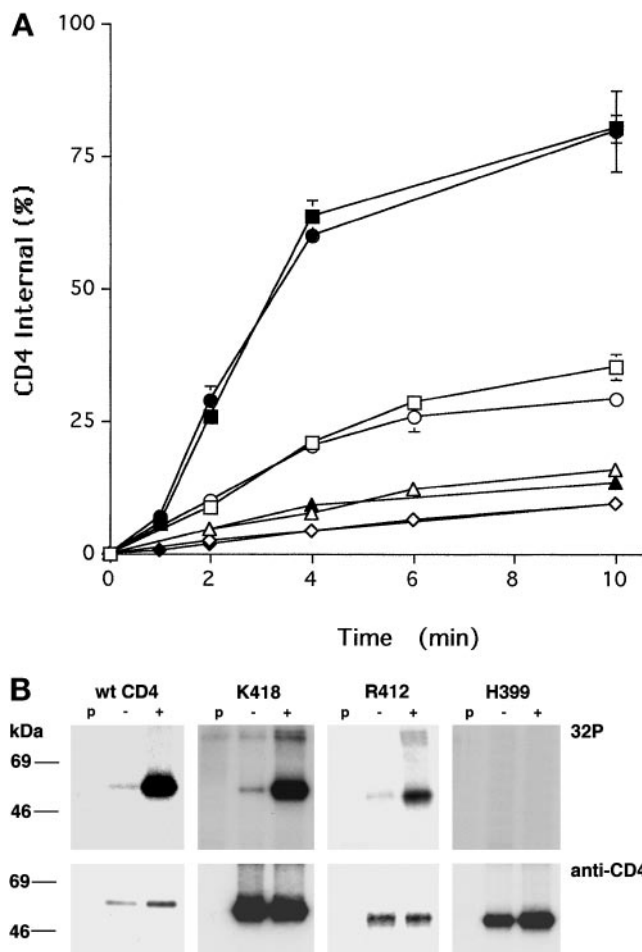


Figure 2. Endocytosis and phosphorylation of CD4 truncations mutants. (A) HeLa cells stably expressing CD4 or CD4 truncation mutants (Figure 1) were labeled with 0.3 nM ^{125}I -Q4120 at 4°C and then warmed to 37°C in binding medium (open symbols) or medium containing 100 ng/ml PMA (solid symbols) for the indicated times. The total and acid-resistant intracellular radioactivities were determined for each time point as described in MATERIALS AND METHODS. Data from one representative experiment are illustrated, and each point represents the mean \pm SD for duplicate samples. \square and \blacksquare , wt CD4; \circ and \bullet , K418; \triangle and \blacktriangle , R412; \diamond and \blacklozenge , H399. (B) Cells were labeled with ^{32}P orthophosphate (as described in MATERIALS AND METHODS) and incubated in medium with (+) or without (-) 100 ng/ml PMA for 3 min. The cells were rapidly cooled to 4°C and lysed, and the CD4 molecules were immunoprecipitated, separated by SDS-PAGE, and transferred to nitrocellulose paper. CD4 protein was visualized and quantitated by Western blot, and the ^{32}P -activity was determined on the same blots using a phosphorimager. The positions of the molecular weight standards are shown; p, preclear lane (see MATERIALS AND METHODS). Note that the K418 ^{32}P lanes illustrated here have been exposed for shorter times than the other panels.

basal endocytosis (Pelchen-Matthews *et al.*, 1991). The faster uptake of wt CD4 and K418 reflects constitutive signal-dependent endocytosis. R412 consistently showed slightly faster internalization rates than H399. Whether this indicates the presence of a weak endocytosis signal in R412 or reflects some nonspecific

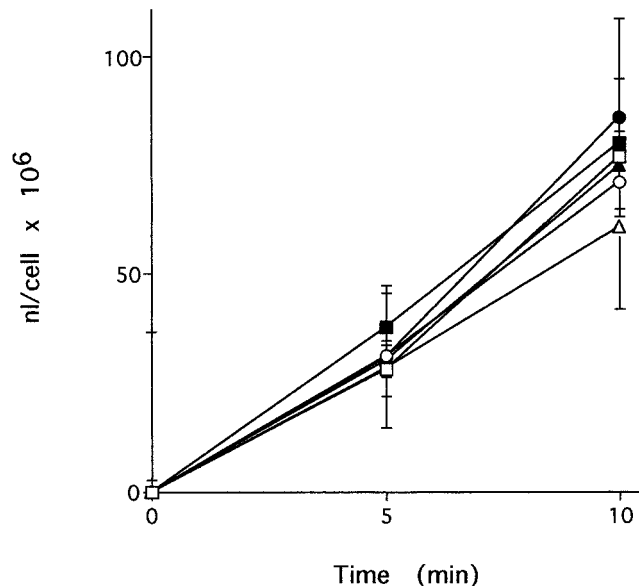


Figure 3. Effect of PMA on fluid phase endocytosis. Cells expressing CD4, K418, or H399 were incubated in BM containing HRP in the presence (filled symbols) or absence (open symbols) of 100 ng/ml PMA. At the end of the incubation period the cells were rapidly cooled to 4°C and washed extensively, and the cell-associated HRP was determined. \square and \blacksquare , wt CD4; \circ and \bullet , K418; \triangle and \blacktriangle , H399.

interaction of the truncated cytoplasmic domain with the endocytosis machinery is unclear.

As previously described (Pelchen-Matthews *et al.*, 1989, 1991), treatment of wt CD4 expressing cells with PMA rapidly induced a fourfold increase in the rate of CD4 endocytosis (Figure 2A and Table 2). This increase occurred after a lag of \sim 1 min (Figure 2A), which we attribute to the time required for the activation of PKC and phosphorylation of CD4. K418 showed a similar increase in endocytosis rate. However, PMA had no effect on the endocytosis of either H399 or R412 (Figure 2A). Together these data indicate that constitutive and PE-induced CD4 endocytosis require cytoplasmic domain elements residing between the transmembrane domain and K418, and that sequences downstream of K418 are not required for CD4 endocytosis.

The observation that H399 endocytosis was not modulated by PE treatment suggested that PMA had no effect on bulk flow endocytosis in these cells. To determine this directly, we measured the effect of PMA on fluid phase endocytosis using HRP. Similar levels of HRP were accumulated over 10 min for all cell lines in the presence and absence of PMA (Figure 3). A slight increase in HRP accumulation was observed after 30 min of endocytosis in the presence of PMA, as previously reported (Pelchen-Matthews *et al.*, 1993), and may reflect PE effects on later steps in the endocytosis pathway.

Table 2. Effects of phorbol ester on CD4 endocytosis and phosphate incorporation

Construct	Endocytosis rate ^a			Relative phosphate incorporation ^b		
	-PMA (%/min)	+PMA (%/min)	+PMA:-PMA	-PMA	+PMA	+PMA:-PMA
wt CD4	5 ± 0.9 n = 11	19.2 ± 3.3 n = 8	3.8	1 n = 7	6.1 n = 7	6.1
K418	4.2 ± 0.9 n = 3	18.2 ± 3.4 n = 3	4.3	1.2 n = 7	6.3 n = 7	5.2
R412	2.4 ± 0.2 n = 3	2.3 ± 0.4 n = 3	1	0.9 n = 1	4.4 n = 1	4.9
H399	1.2 ± 0.4 n = 10	1.0 ± 0.2 n = 8	1	ND ^c	ND	
Mutations in K418 background						
K418 S408A/S415A	1.7 ± 1.0 n = 3	4.2 ± 0.2 n = 4	2.4	ND	ND	
K418 S408E/S415E	1.9 ± 0.8 n = 3	4.1 ± 0.1 n = 3	2.2	ND	ND	
K418 S408A	1.7 ± 1.0 n = 3	7.1 ± 1.7 n = 3	4.1	0.7 n = 4	4.0 n = 4	5.7
K418 S408E	2.7 ± 0.5 n = 7	13.7 ± 4.5 n = 7	5	2.0 n = 3	8.3 n = 3	4.1
K418 S415A	2.1 ± 0.4 n = 3	6.6 ± 1.2 n = 3	3.1	0.1 n = 1	2.4 n = 1	24
K418 S415E	2.0 ± 0.3 n = 3	5.0 ± 0.6 n = 2	2.5	0.1 n = 2	2.5 n = 2	25
K418 L413A/L414A	2.2 ± 0.7 n = 4	2.5 ± 0.4 n = 3	1	1.1 n = 3	7.1 n = 3	6.5
K418 L413V	1.6 ± 0.4 n = 2	1.9 ± 0.2 n = 3	1	2.7 n = 2	5.9 n = 1	0.5
K418 L413V/L414V	1.9 ± 0.5 n = 2	2.3 ± 0.4 n = 3	1	1.7 n = 1	7.3 n = 1	4.4

^a Endocytosis rates were normally calculated over the first 4 min after warmup except in PE-treated cells. Because the effects of PE were observed after a lag of ~1 min (see Fig. 2A), the rates for these cells were calculated 1–4 min after warmup. The effects of PE on CD4 endocytosis are given as the ratio of the rates +PMA:-PMA.

^b Phosphate incorporation for each construct (- and +PMA) is compared with wt CD4 immunoprecipitated from untreated cells (see MATERIALS AND METHODS). Wild-type CD4 is set to 1. n, number of experimental data sets used in these calculations.

^c ND, not detected.

CD4 Phosphorylation

It has been demonstrated that PEs induce rapid phosphorylation of the three serine residues in the CD4 cytoplasmic domain (S408, S415, and S431) and that this phosphorylation is necessary, but not sufficient, for CD4 down-regulation (Shin *et al.*, 1991). Phosphorylation occurs mainly on S408, and substitution of this amino acid with alanine inhibits down-modulation (Shin *et al.*, 1990). To study the role of phosphorylation in CD4 endocytosis specifically, cells expressing wt CD4 and truncation mutants were labeled with [³²P]orthophosphate for 4 h and then incubated in BM with or without 100 ng/ml PMA for 3 min at 37°C. A 3-min time point was chosen, because we previously found that maximal clustering of CD4 in endocytic, clathrin-coated pits occurs 2–3 min after PMA addition (Pelchen-Matthews *et al.*, 1993). Subsequently, the cells were lysed and CD4 immunoprecipitated, and the immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose. CD4 was quantitated

by Western blotting, and CD4 ³²P incorporation was determined on the same blots. To compare phosphate incorporation for each construct, ³²P activity associated with each CD4 band was normalized for the amount of CD4 protein. The phosphate:protein ratios obtained were then compared with the phosphate:protein ratio for CD4 precipitated from cells that had not been treated with PE.

Wild-type CD4 was constitutively phosphorylated (Figure 2B). After 3 min of PE treatment, phosphate incorporation on this molecule was increased approximately sixfold. K418 and R412 were also constitutively phosphorylated and showed approximately fivefold increases in incorporation after PE treatment. As expected, H399 was not phosphorylated either constitutively or after PE treatment. Together, these data suggest that S415 phosphorylation does not require sequences downstream of K418, and S408 phosphorylation does not require sequences downstream of R412. The data also suggest that S431 is not signifi-

cantly phosphorylated after PE treatment. In addition, because R412 was phosphorylated to a similar level as wt CD4 and K418 but did not undergo efficient endocytosis, the data also indicated that phosphorylation of the truncated R412 molecule is not sufficient to induce its endocytosis. Thus elements between R412 and K418 are required for CD4 internalization.

Because the 23 membrane proximal amino acids in the CD4 cytoplasmic domain are sufficient for phosphorylation and endocytosis, and the C-terminal 15 amino acids make little contribution to the total level of phosphorylation or the endocytic activity of the molecule, the K418 construct was used for site-directed mutagenesis.

Two Leucine Residues Are Essential for CD4 Endocytosis

To investigate the role of leucines 413 and 414, we generated a mutant CD4 molecule in which these residues were substituted with alanine (K418 L413A/L414A; Figure 1). This molecule showed no constitutive or PMA-induced endocytosis (Figure 4). Strikingly, the molecule was constitutively phosphorylated to the same extent as K418 (Figure 4C), and the level of phosphorylation was increased approximately sevenfold after PE treatment. In addition, we made two mutants in which either one or both leucine residues were mutated to valine (K418 L413V and K418 L413V/L414V; Figure 1). Both mutants were internalized at basal endocytosis rates (Figure 4), despite the fact that both were phosphorylated with similar or higher efficiency than K418 (Figure 4C). Thus the loss of a single methyl group from the side chain of residue 413 is sufficient to disrupt the endocytosis signal.

The CD4 Endocytosis Signal Requires Serine Phosphorylation

Phosphorylation of S408 in R412, and of the leucine substitution constructs discussed above (Figure 4), was not sufficient to generate an active endocytosis signal. However, the basal phosphorylation of wt CD4 and K418, together with the increased phosphorylation that accompanied the PE-induced CD4 endocytosis, suggested that phosphorylation might be necessary for efficient CD4 endocytosis. We examined the role of phosphorylation by substituting S408 and S415 with alanine (K418 S408A/S415A; Figure 1). As expected, no basal or PE-induced phosphorylation of this double mutant was observed (Figure 5). Moreover, this construct was internalized at rates similar to the basal endocytosis of H399 (1.7%/min; Figure 5 and Table 2). Thus the constitutive internalization of wt CD4 and K418 may be due in part to the constitutive phosphorylation of these molecules.

After PE treatment, the rate of K418 S408A/S415A endocytosis increased from 1.7 to 4.2%/min (Figure 5

and Table 2). The uptake of the R412 and H399 constructs, which do not contain leucines 413 and 414, was not affected by PE (Figure 2 and Table 2). This suggests that PE treatment may induce some modification of the endocytic machinery so that it interacts more effectively with the nascent LL-containing endocytosis signal in CD4.

Both S408 and S415 Contribute to the CD4 Endocytosis Signal

The individual contributions of S408 and S415 to CD4 endocytosis were analyzed with constructs containing single alanine substitutions (K418 S408A and K418 S415A; Figure 1). K418 S408A was internalized at 1.7%/min (Figure 5), a rate comparable to H399 and R412 (Figure 2 and Table 2). On PMA treatment the rate of K418 S408A internalization increased ~4.5-fold to 7.1%/min. K418 S408A was constitutively phosphorylated (Figure 5), indicating that S415 could be phosphorylated in the absence of S408, although the level of constitutive phosphorylation was less than that seen for K418 (Table 2). PE treatment induced an approximately sixfold increase in phosphate incorporation.

The intermediate values for the rates of endocytosis seen with these constructs suggest that the interplay between phosphorylation and endocytosis is complex. The basal endocytosis of the S408A mutant indicates that the constitutive phosphorylation of S415 does not generate an efficient endocytosis signal. After PE treatment, however, the endocytosis and phosphorylation of S408A were increased by four- and sixfold, respectively. We suggested above that PE treatment may modify the interaction of adaptors with the nascent signal in CD4; some of the PE-induced increase in S408A uptake may be attributed to this effect. Nevertheless, in PE-treated cells the S408A construct was internalized faster than the double serine mutant (S408A/S415A), indicating that phosphorylation of S415 may activate the CD4 signal, although this activation is not as effective as phosphorylation on S408. The notion that phosphorylation of S415 may generate a weak signal was supported by *in vitro* SPR studies (see below).

K418 S415A was also internalized at a basal rate (2.1%/min; Figure 5). Very little constitutive phosphorylation was seen with this construct (Figure 5). PE treatment induced a threefold increase in the rate of internalization of this construct (Figure 5) and a significant increase in phosphorylation. The level of this increase was exaggerated by the low basal labeling, and the level observed was comparable to that of wt CD4 in unstimulated cells. Because the R412 construct was phosphorylated both constitutively and after PE (Figure 2), these results suggest that residues 413–418, and S415 in particular, may influence phosphorylation on S408.

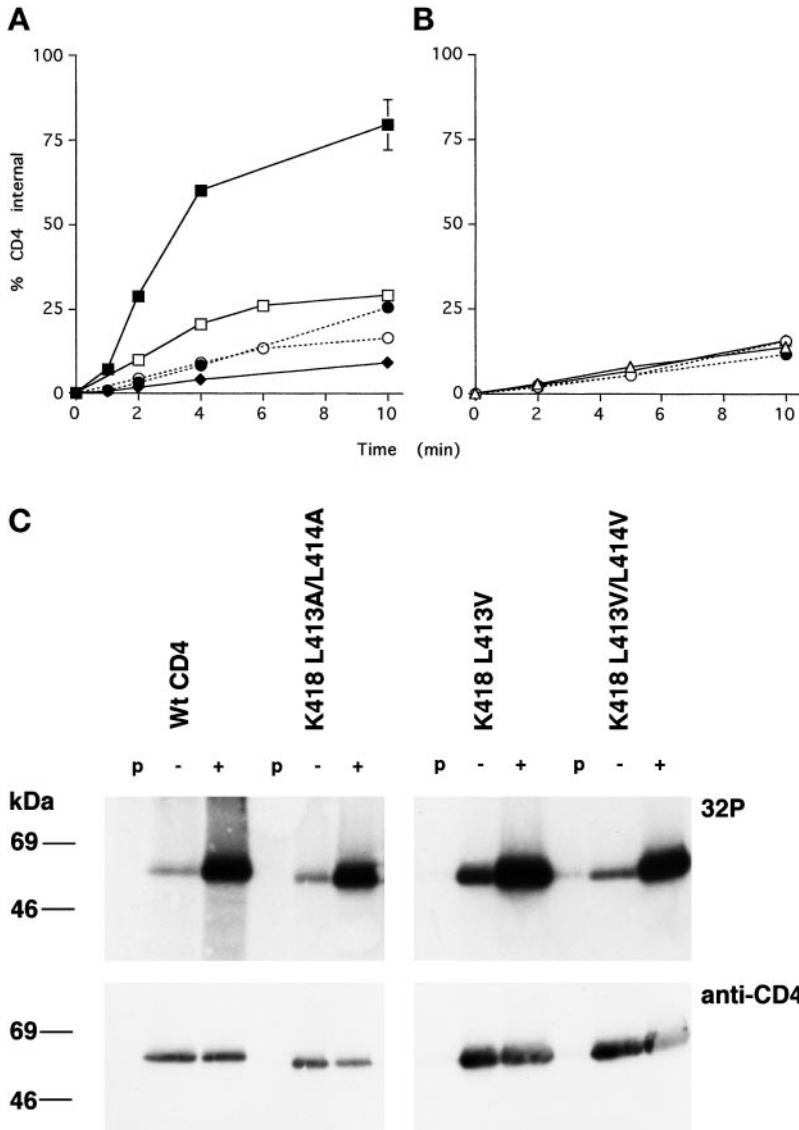


Figure 4. Effects of leucine substitutions on K418 CD4 endocytosis and phosphorylation. (A and B) Endocytosis of K418 CD4 L substitutions (Figure 1) was measured as described in Figure 2A. Open symbols indicate the constitutive endocytosis of constructs in the absence of PMA, and solid symbols indicate endocytosis in the presence of 100 ng/ml PMA. (A) □ and ■, K418; ○ and ●, K418 L413A/L414A; ◇ and ◆, H399. (B) △ and ▲, K418 L413V/L414V; ○ and ●, K418 L413V. (C) Cells expressing K418 CD4 L413/L414 substitutions were labeled with [³²P]orthophosphate, incubated in medium with (+) or without (-) 100 ng/ml PMA for 3 min, and CD4 immunoprecipitated as described in Figure 2B. The left six lanes of the top panel were exposed to film for twice as long as the right six lanes.

Glutamic Acid Does Not Substitute for Serine Phosphorylation in CD4 Endocytosis

A consequence of phosphorylation is the addition of negative charge to a protein. In some cases phosphoserine can be mimicked by glutamic or aspartic acid (for example, see Hicke *et al.*, 1998). To determine whether the introduction of negative charges into CD4 might generate an active endocytosis signal, S408E, S415E, and double S408E/S415E mutants were made in K418 (Figure 1). When these mutants were stably expressed in HeLa cells, cell surface expression equivalent to that of wt CD4 was observed by immunofluorescence and ¹²⁵I-Q4120 binding, indicating that the substitutions did not impede delivery of the protein to the plasma membrane. K418 S408E/S415E behaved identically to the K418 S408A/S415A mutant. Basal

internalization was seen (Figure 6), and PE induced an approximately twofold increase in the endocytosis rate, similar to the K418 S408A/S415A construct discussed above (Table 2).

Single glutamic acid substitutions exhibited some intermediate properties. S408E showed some constitutive endocytosis activity (2.7%/min; Table 2). This construct also showed relatively high constitutive phosphorylation (Figure 5). PE treatment increased the rate of S408E endocytosis approximately fivefold, and phosphate incorporation increased the rate fourfold (Figure 5). Together these data are consistent with the notion that phosphorylation on S415 can generate a weak endocytosis signal. Why this construct is phosphorylated more efficiently than S408A is unclear; possibly the presence of a negative

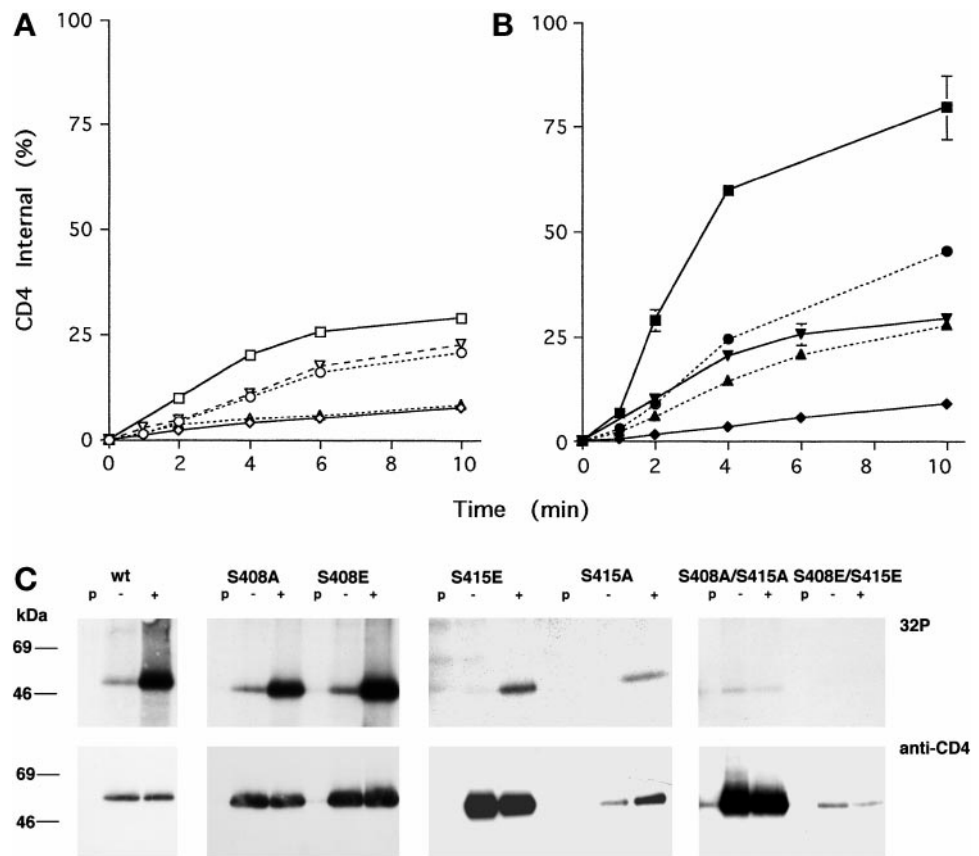


Figure 5. Effects of serine substitutions on K418 CD4 endocytosis and phosphorylation. (A and B) Endocytosis of K418 CD4 S to A substitutions (Figure 1) was measured as described in Figure 2A. (A) Constitutive endocytosis of the constructs in the absence of PMA. (B) Endocytosis in the presence of 100 ng/ml PMA. (C) Cells expressing K418 CD4 S to A or E (see Figure 5) substitutions were labeled with [³²P]orthophosphate, incubated in medium with (+) or without (-) PMA, and CD4 immunoprecipitated as described in Figure 2B.

charge at S408 may increase the efficiency of phosphorylation on S415.

S415E showed internalization kinetics similar to S415A (Table 2). In addition, as with S415A, there was little constitutive phosphorylation of S415E. S415E was phosphorylated and internalized after PE treatment (Figure 5), although the rates were slow compared with K418. Thus a negative charge at position 415 does not generate an active endocytosis signal, but, as suggested above, the residues in this domain of CD4 may influence S408 phosphorylation. Together the data indicate that glutamic acid residues do not substitute for phosphoserine in the CD4 tail and that negative charges alone are not sufficient to generate an active endocytosis or sorting signal.

SPR Analysis of CD4 Peptide-Adaptor Interactions

Previous reports have suggested that dileucine-containing endocytosis signals interact with clathrin adaptor complexes (Dietrich *et al.*, 1997; Bremnes *et al.*, 1998; Rapoport *et al.*, 1998), although the site of interaction may be different from that recognized by tyrosine-containing signals (Marks *et al.*, 1996; Rapoport *et al.*, 1998). Attempts to coprecipitate CD4 with adaptor protein complexes either under steady-state con-

ditions or after PE treatment were not successful. We therefore investigated the interaction of purified adaptors with CD4 cytoplasmic domain peptides using SPR. N-terminally biotinylated peptides equivalent to the cytoplasmic domain of K418 CD4 were synthesized. Peptides containing specific amino acid substitutions are indicated in Table 3. The N-terminal biotin modification facilitated coupling to a streptavidin-coated SPR chip such that the peptide was oriented with the C terminus free, as may occur on the internal face of the plasma membrane. Interactions with purified pig brain AP-2 and AP-1 adaptors were determined as described (Höning *et al.*, 1997).

Little interaction with AP-2 was seen using a wt K418 peptide in which neither serine was phosphorylated (Figure 7) or with a peptide containing L413A/L414A substitutions (Table 3). The observed signal was comparable to that seen when adaptor complexes were passed over a nonderivatized surface. However, a significant increase in AP-2 binding was seen when a wt K418 peptide with phosphoserine at position 408 was used (Figure 7). Analysis of the association and dissociation phases of the interaction suggested a K_D of 92 μ M for the wt peptide (Table 3), and that this value was increased >700-fold ($K_D \approx 120$ nM) for the

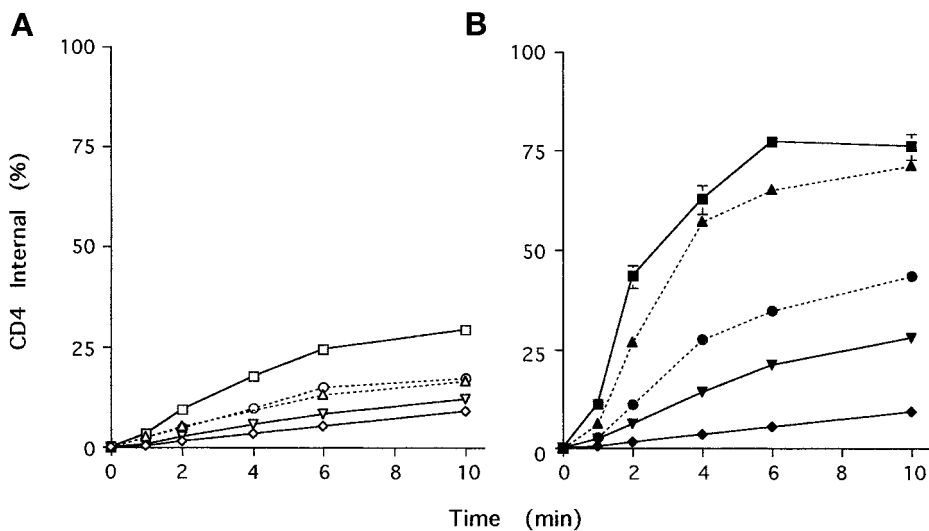


Figure 6. Effects of serine to glutamic acid substitutions on K418 CD4 endocytosis. Endocytosis of K418 CD4 S to E substitutions (Figure 1) was measured as described in Figure 2A. (A) Constitutive endocytosis of the constructs in the absence of PMA. (B) Endocytosis in the presence of 100 ng/ml PMA. □ and ■, wt CD4; △ and ▲, K418 S408E; ○ and ●, K418 S415E; ▽ and ▼, K418 S408E/S415E; ◇ and ◆, = H399.

phosphorylated peptide. AP-2 binding was also increased when a peptide with phosphoserine at 415 was used, supporting the notion that phosphorylation of S415 may generate a weak endocytosis signal. This later binding was subsequently reduced to background levels when the peptide was dephosphorylated using calf alkaline phosphatase (our unpublished results). Significantly, highest affinity binding was seen with a peptide in which both serine residues were phosphorylated. Binding was dependent on the presence of leucines 413 and 414, because peptides containing alanine at these positions failed to interact with the adaptors regardless of whether phosphoserine was present at either 408 or 415 or both. Moreover, substitution of S408 with glutamic acid also failed to elicit significant binding. We also observed binding of these peptides to the AP-1 complex. Binding was on the order of three- to fourfold lower affinity

than that seen for AP-2 but was also dependent on phosphorylation and the presence of the dileucine sequence. Together these data indicate that the endocytosis signal in CD4 binds the clathrin adaptor complexes AP-1 and AP-2 and that the sequence requirements for binding show a very close correlation to those required for endocytosis (see above).

DISCUSSION

Cell surface receptors, and other proteins, that traffic between the plasma membrane and endocytic compartments require signals that allow their efficient recruitment into endocytic vesicles. To date four classes of endocytosis signal have been identified that target surface proteins to clathrin-coated vesicles. First, tyrosine-based signals within FXNPXY or YXXØ motifs were initially identified in the low-density lipoprotein

Table 3. SPR analysis of CD4 peptide–adaptor complex binding

CD4 peptide	Sequence	K_D (μ M)	
		AP-1	AP-2
wt	Biotin-RHRRRQAERM <u>S</u> QIKRLLSEK	ND	92
L413A/L414A	Biotin-RHRRRQAERM <u>S</u> QIKRAASEK	ND	ND
S408	Biotin-RHRRRQAERM <u>S</u> QIKRLLSEK	0.60	0.12
S415	Biotin-RHRRRQAERM <u>S</u> QIKRLLSEK	0.76	0.35
S408/S415	Biotin-RHRRRQAERM <u>S</u> QIKRLLSEK	0.52	0.08
S408/L413A/L414A	Biotin-RHRRRQAERM <u>S</u> QIKRAASEK	ND	82
S408/S415/L413A/L414A	Biotin-RHRRRQAERM <u>S</u> QIKRAASEK	46	12
S408E/L413A/L414A	Biotin-RHRRRQAERME <u>S</u> QIKRAASEK	86	ND

CD4 cytoplasmic domain peptides were immobilized on streptavidin-coated sensor chips at similar densities (variation, <8%). Purified AP-1 and AP-2 complexes were used at concentrations ranging from 50 to 500 nM. The equilibrium constant (K_D = the association rate [k_a]/dissociation rate [k_d]) was calculated from the sensorgrams (see Fig. 7) as described in MATERIALS AND METHODS. Phosphoserine residues are indicated in underlined type; ND, reactions in which the interactions were too weak to determine the binding constants.

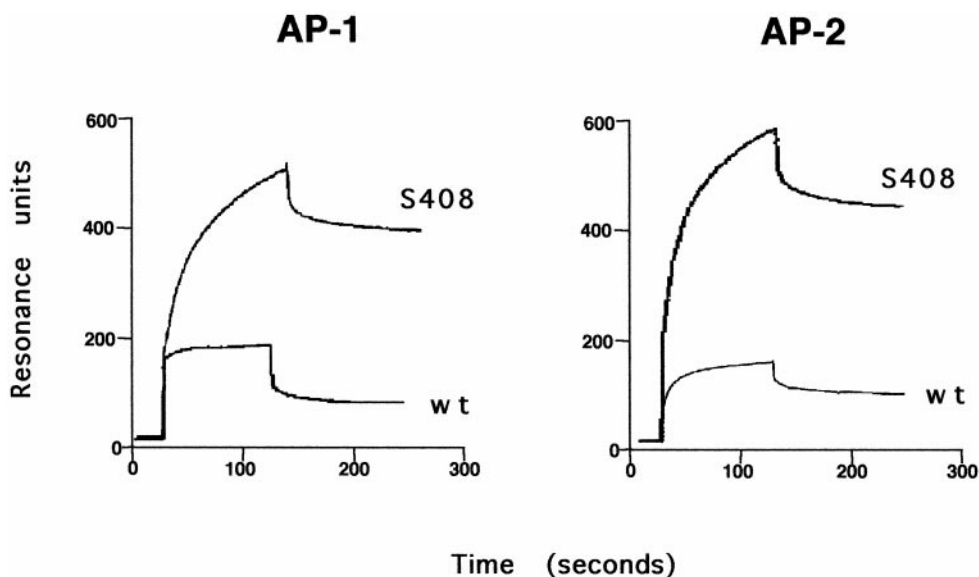


Figure 7. SPR analysis of CD4 peptide-adaptor complex binding. Biotinylated CD4 tail peptides were immobilized on an SA5 sensor surface and analyzed for binding of purified AP-2 and AP-1 adaptor complexes. The wt peptide bound both adaptor complexes with very low affinity (see Table 3); however, high-affinity adaptor binding was observed when peptides containing phosphorylated S408 were used.

and transferrin receptors, respectively (Trowbridge *et al.*, 1993). The low-density lipoprotein receptor FXN-PXY motif is reported to adopt a reverse turn configuration and to bind directly to clathrin heavy chains (Kibbey *et al.*, 1998), whereas at least some YXX \emptyset motifs interact with the μ chains of adaptor complexes (reviewed in Kirchhausen *et al.*, 1997; Owen and Evans, 1998). In many cases these signals are constitutively active; i.e., receptors containing the motifs undergo continuous rounds of endocytosis and recycling whether ligands are bound.

However, endocytosis signals can be regulated. For CTLA-4 at least, phosphorylation of a tyrosine can inactivate μ chain binding (Shiratori *et al.* 1997). By contrast, three other types of endocytosis signal can be activated by phosphorylation. The first type is signals involving pairs of hydrophobic amino acids, frequently leucines (Letourneur and Klausner, 1992), although isoleucine, methionine, or valine can substitute for one of the leucines in some circumstances (Bremnes *et al.*, 1994; Sandoval and Bakke, 1994; Pond *et al.*, 1995). These so-called "dileucine" signals also bind adaptor complexes through a site(s) different from that involved in YXX \emptyset motif recognition (Marks *et al.*, 1996; Dietrich *et al.*, 1997; Bremnes *et al.*, 1998; Rapoport *et al.*, 1998). Dileucine motifs have been implicated in the endocytic activities of a number of proteins and receptors, and for some at least, the endocytic activities are regulated by phosphorylation (Shin *et al.*, 1991; Corvera *et al.*, 1994; Dietrich *et al.*, 1994). A second type of regulated signal is contained in members of the family of seven-transmembrane-domain G protein-coupled receptors. For several of these receptors, in particular the β -adrenergic receptor, ligand-induced phosphorylation of serine residues

in the C-terminal domain of the molecule leads to recruitment of nonvisual arrestins that both uncouple associated heterotrimeric G proteins and act as adaptors to recruit the receptor into clathrin-coated pits (reviewed in Ferguson *et al.*, 1996a). Finally, in *Saccharomyces cerevisiae* ligand-induced phosphorylation of serines in the α factor receptor (Ste2p) C-terminal domain leads to ubiquitination of Ste2p and its subsequent internalization (Hicke *et al.*, 1998). A number of mammalian cell surface receptors are also ubiquitinated after ligand binding (Strous *et al.*, 1997), although a role in endocytosis is still to be clearly established. Nevertheless, it is apparent that in many instances endocytic signals are regulated, and this regulation occurs primarily through phosphorylation of serine residues.

Here we show that a dileucine-containing signal in CD4 is activated by phosphorylation and that this reaction allows the CD4 cytoplasmic domain sorting signal to bind clathrin adaptor complexes. Our study refines earlier work (Shin *et al.*, 1990, 1991) in which the complex phenomenon of down-modulation was analyzed. Moreover, we have combined direct endocytosis assays with determinations of serine phosphorylation and adaptor binding assays. The results indicate a mechanism through which CD4 endocytosis is controlled by a phosphorylation-regulated interaction with clathrin adaptors. We have shown that the endocytosis signal in CD4 requires at least two components: a pair of leucine residues that are essential for endocytosis and a neighboring phosphorylated serine residue.

A pair of leucine residues is essential for CD4 endocytosis. These amino acids are absolutely conserved in all CD4 sequences published to date. Modification of

either one or both of the leucines to alanine completely disrupts CD4 endocytosis regardless of whether the serines at 408 and 415 are phosphorylated. Moreover, substitution of just one of the leucines (L413) with valine, a change that removes a single methyl group from the side chain, is sufficient to disrupt the signal. The two leucine residues were also essential for *in vitro* adaptor binding: peptides containing leucine to alanine substitutions showed weak, or undetectable, binding to both AP-1 and AP-2 adaptors even when phosphorylated. We have not tested other hydrophobic substitutions; however, our studies with the CXCR4 chemokine receptor suggest that hydrophobic substitutions may be tolerated in some dileucine-containing signals (Signoret *et al.*, 1998). At present we know little about the architecture of the CD4 cytoplasmic domain that is recognized by the endocytic machinery. (A nuclear magnetic resonance structure for a CD4 cytoplasmic domain peptide has been published; however this peptide was not phosphorylated, and it is unclear whether the structure represents that of the native protein [Willbold and Roesch, 1996].) The fact that the endocytosis signal in CD4 is regulated may impose a tighter sequence requirement for two leucines than constitutively active dileucine-containing signals in which other hydrophobic amino acids can be tolerated.

The second component of the signal is a phosphorylated serine residue. The CD4 cytoplasmic domain contains three serine residues that are potential targets for PKC-mediated phosphorylation. Of these, S431 is phosphorylated least efficiently (Shin *et al.*, 1991) and has no apparent involvement in the endocytic properties of the molecule. However, both S408 and S415 influence endocytosis, and both residues undergo phosphorylation on treatment of cells with PEs. S408 appears to be phosphorylated more efficiently (Shin *et al.*, 1991), and the increase in the level of phosphate incorporation is similar to increases in the measured rates of CD4 endocytosis. Substitution of both S408 and S415 with either alanine or glutamic acid and single S408 substitutions completely abolish constitutive CD4 endocytosis, even though the dileucine sequence is intact. In addition, PE-induced endocytosis of these constructs is reduced (see below). Although serine phosphorylation is required for CD4 internalization, phosphorylation alone is not sufficient for endocytosis, because constructs with leucine mutations and the R412 protein fail to internalize, even though they are phosphorylated efficiently. In keeping with these results, *in vitro* binding of adaptor complexes was seen with peptides phosphorylated on either S408 or S415, although higher-affinity binding was seen when S408 was phosphorylated. Together, the data indicate that both constitutive and PE-induced CD4 endocytosis is dependent on serine phosphorylation, the normal CD4 molecule being phosphorylated to

some extent even in unstimulated cells. Only basal CD4 internalization, which occurs by bulk flow endocytosis, is independent of both phosphorylation and the dileucine sequence. The precise relationship between S408 and S415 is complex. Both residues appear to facilitate endocytosis when phosphorylated, because the single substitutions show PE-induced internalization. For this function, which involves interaction with the adaptor proteins, phosphoserine cannot be substituted with glutamic acid. However, the phosphorylation of S415 in particular may be influenced by the presence of a negative charge at position 408. In addition, residues 413–418 may modulate the phosphorylation of S408. Thus the efficacy of CD4 endocytosis is a balance between the interaction of kinases (and possibly phosphatases) that phosphorylate the cytoplasmic domain of the molecule and the subsequent recognition of the active endocytosis signal by clathrin adaptor complexes.

Why serine phosphorylation is required to activate the CD4 endocytosis signal is unclear. One notion is that phosphorylation of the CD4 cytoplasmic domain may increase the affinity of the motif for binding sites on AP-2. However, the substitution of the serine residues with glutamic acid did not generate a constitutively active endocytosis signal, indicating that the role of phosphorylation is not simply the introduction of a negative charge. This contrasts the situation in, for example, Ste2p, in which negatively charged amino acids can partially replace phosphoserine (Hicke *et al.*, 1998). Another possibility is that the incorporation of phosphate would induce a structural constraint or conformational change in CD4 that allows the display of the dileucine sequence in a form that can interact with the adaptor complexes. Such a conformational change may indeed occur as the interaction of CD4 with p56^{lck} is also regulated by serine phosphorylation. Under normal circumstances the cytoplasmic domain of CD4 interacts with the N-terminal domain of p56^{lck}. This interaction involves cysteines 421 and 423 in CD4, cysteines 21 and 23 in Lck, and the coordination of a zinc ion (Huse *et al.*, 1998). PE-induced phosphorylation of S408 triggers dissociation of this complex (Pelchen-Matthews *et al.*, 1993), whereas a complex formed between Lck and CD4 with an S408A mutation fails to dissociate in PE-treated cells. More detailed structural studies will be required to fully understand the nature of the CD4 endocytosis signal and the role of phosphoserine and the dileucine sequence in regulating endocytosis.

The clathrin adaptor complex associated with endocytic coated vesicles is AP-2 (Robinson, 1994). Peptides encoding a portion of the cytoplasmic domain of CD4 can bind purified AP-2 complexes *in vitro*. The features of the peptides required for this association closely parallel the endocytic properties of CD4. Thus we believe that the interactions observed by SPR are

likely to reflect events that occur within the cell. Binding is dependent on the presence of the two leucine residues and requires phosphorylation of the serine residues. The binding affinity measured by SPR is highest when S408 is phosphorylated. However, significant binding also occurs when S415 is phosphorylated, supporting the endocytosis data suggesting that a weak endocytosis signal may be generated after phosphorylation of S415. The highest affinity CD4 peptide-AP-2 interaction measured using SPR ($k_D = 100$ nM) was similar to that recorded for a synthetic dileucine-containing sequence (Heilker *et al.*, 1996) but is somewhat less than values determined for Y-based signals (Höning *et al.*, 1997). At present it is unclear how closely the affinities we have measured by SPR reflect signal-adaptor interactions in the cell. Nevertheless, it is clear that the properties we observe for binding in vitro correlate well with the endocytic properties of CD4.

Significantly, we also observed the CD4 peptides binding to AP-1 complexes. This interaction appeared to be weaker than that seen for AP-2 but required similar features in the peptides, i.e., the pair of leucines and phosphoserine. The relevance of this binding is at present unclear. AP-1 is believed to function in association with clathrin-coated vesicles derived from the trans-Golgi network (Robinson, 1994). At present we have no indication that CD4 is phosphorylated during transit through the biosynthetic pathway. Indeed our studies of p56^{lck} biosynthesis suggest that CD4 and p56^{lck} form a complex early in the exocytic pathway and then move together to the cell surface (Bijlmakers and Marsh, unpublished results). Because phosphorylation of the CD4 cytoplasmic domain induces dissociation of p56^{lck}, it seems unlikely that CD4 will be phosphorylated during transit through the Golgi apparatus. However, during down-modulation, internalized CD4 is delivered to early endosomes and from this site is sorted to late endosomes and lysosomes, where it is believed to be degraded (Pelchen-Matthews *et al.*, 1993). At present we know little of the machinery responsible for this endosomal sorting step. It is possible that AP-1 plays some role. Alternatively, the recently characterized AP-3 complex, which has been implicated in sorting to late endocytic organelles (Ooi *et al.*, 1997; Simpson *et al.*, 1997), might function in this step. We have yet to determine whether CD4 peptides are able to bind AP-3.

A number of dileucine-containing endocytosis and sorting sequences contain one or more negatively charged amino acids three or four positions upstream of the leucine pair. The role of these residues is unclear. An aspartic acid residue appears to be important for the binding of a dileucine motif from the T cell receptor-CD3 γ subunit to AP-1 and AP-2 (Dietrich *et al.*, 1997), although the endocytic activity of this signal

is regulated by phosphorylation (Dietrich *et al.*, 1994). By contrast, in the MHC II invariant chain, polar residues upstream of both dileucine-like signals can influence endocytosis but appear to be required for intracellular sorting events (Pond *et al.*, 1995). Significantly, upstream negatively charged amino acids have been shown to be important for the interaction of tyrosinase and the lysosomal integral membrane protein II, two proteins that are sorted to lysosomal-type organelles, with AP-3 complexes. Moreover, the dileucine signals in these proteins do not appear to interact with AP-1 and AP-2 (Höning *et al.*, 1998). A similar signal has been identified in the yeast t-SNARE Vam3p that mediates sorting of Vam3p to the vacuole via the AP-3-dependent pathway (Darsow *et al.*, 1998). Whether other amino acids adjacent to the leucines play critical roles in adaptor binding is also unclear. M407 and I410 in CD4 have been implicated in PE-induced CD4 down-modulation (Shin *et al.*, 1991), but it remains to be determined whether these residues modulate endocytosis or adaptor binding specifically. How dileucine motifs within specific sequence contexts interact with different adaptor complexes will require more detailed systematic analyses.

The binding experiments described here used purified adaptor complexes in which the two heavy chains (α/β_1 and γ/β_2), the medium chains (μ_1 and μ_2), and the small chains (σ_1 and σ_2) were present. The binding site(s) for dileucine-containing signals on adaptor complexes are unclear. Competition studies with overexpressed receptors indicate that dileucine-containing signals are not competed by YXX \emptyset based signals (Marks *et al.*, 1996). Experiments using phage display suggest that dileucine-type signals in the MHC II invariant chain interact with adaptor μ subunits (Bremnes *et al.*, 1998), whereas labeling experiments with dileucine-containing peptides suggest an interaction with β subunits (Rapoport *et al.*, 1998). Significantly, a phosphopeptide was used for these latter experiments, although the phosphoserine was not required for the binding activity (Rapoport *et al.*, 1998). Further experiments will be required to establish exactly how the endocytosis signal sequence in CD4 interacts with clathrin adaptor complexes.

The observation that PEs could increase the endocytosis of some CD4 constructs containing substitutions in both phosphorylation sites has interesting implications. The in vitro adaptor binding assays indicate that adaptors only bind to the phosphorylated CD4 peptides (Table 3). Moreover, PE treatment did not increase bulk flow or fluid phase endocytosis over the course of these experiments. Thus one explanation for the PE-induced uptake of some nonphosphorylated constructs is that PE treatment leads to modification of the adaptors, or other clathrin-associated proteins, to enhance recognition of the nascent endocytosis signal in CD4. Components of the adaptor

complex are known to be phosphorylated (Pauloin and Thurieau, 1994; Wilde and Brodsky, 1996; Bar-Zvi *et al.*, 1998). To date this phosphorylation has been linked to adaptor association with clathrin (Wilde and Brodsky, 1996). However, our preliminary results suggest that phosphorylation of AP-2 can also modulate the interaction of this adaptor with the cytoplasmic domains of some membrane proteins (Fingerhut and Höning, unpublished results). We are currently investigating the possibility that adaptors are phosphorylated in PE-treated cells and that this phosphorylation may modify the association of adaptor complexes with endocytic sorting signals.

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