The Protein Tyrosine Kinase p56^{lck} Inhibits CD4 Endocytosis by Preventing Entry of CD4 into Coated Pits

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Abstract. The lymphocyte glycoprotein CD4 is constitutively internalized and recycled in nonlymphoid cells, but is excluded from the endocytic pathway in lymphocytic cells (Pelchen-Matthews, A., J. E. Armes, G. Griffiths, and M. Marsh. 1991. *J. Exp. Med.* 173: 575–587). Inhibition of CD4 endocytosis is dependent on CD4 expressing an intact cytoplasmic domain and is only observed in cells where CD4 can interact with the protein tyrosine kinase p56^{lck}, a member of the *src* gene family. We have expressed p56^{lck}, p60$^{c\text{-}src}$, or chimeras of the two proteins in CD4-transfected NIH-3T3 or HeLa cells. Immunoprecipitation of CD4 and in vitro kinase assays showed that p56^{lck} and the *lck/src* chimera, which contains the NH_2 terminus of p56^{lck}, can associate with CD4. In contrast, p60$^{c\text{-}src}$ and the *src/lck* chimera, which has the NH_2 terminus of p60$^{c\text{-}src}$, do not associate with CD4. Endocytosis assays using radioiodinated anti-CD4 monoclonal antibodies demonstrated that coexpression of CD4 with p56^{lck}, but not with p60$^{c\text{-}src}$, inhibited CD4 endocytosis, and that the extent of the inhibition depended directly on the relative levels of CD4 and p56^{lck} expressed. The uptake of mutant CD4 molecules which cannot interact with p56^{lck} was not affected. Measurement of the fluid-phase endocytosis of HRP or the internalization of transferrin indicated that the effect of p56^{lck} was specific for CD4, and did not extend to other receptor-mediated or fluid-phase endocytic processes. Immunogold labeling of CD4 at the cell surface and observation by electron microscopy demonstrated directly that p56^{lck} inhibits CD4 endocytosis by preventing its entry into coated pits.

THE cell surface glycoprotein CD4, which is expressed primarily on helper T lymphocytes, recognizes nonpolymorphic regions of the class II major histocompatibility complex ((MHC)[1], for review see references 34, 41). CD4 is believed to participate in T cell activation by antigen in two ways. First, it can act as an accessory molecule to the T cell receptor/CD3 complex, facilitating adhesion between T cells and antigen-presenting cells (9, 42). Second, it can be directly involved in signal transduction via the protein tyrosine kinase p56^{lck} (for review see reference 43). In humans, CD4 is also expressed on cells of the macrophage/monocyte lineage, dendritic cells, and eosinophils, where it may act as the receptor for lymphocyte chemoattractant factor (7). In addition, CD4 acts as a cellular receptor for the human immunodeficiency viruses (HIV-1 and -2; for reviews see references 41, 47). The structure of the CD4 molecule, a member of the immunoglobulin superfamily, is becoming increasingly well defined. The three-dimensional framework of the first two NH_2-terminal extracellular domains of the molecule has been solved by x-ray crystallography, and the epitopes involved in binding to both MHC class II and to the HIV surface glycoprotein gp120 have been mapped in detail (45, 56, and references therein). We have been investigating the endocytic trafficking of CD4 in order to gain a clearer understanding of the functions of CD4 internalization both in T cell activation and in the processes by which HIV infects CD4-bearing cells.

CD4 is a type I transmembrane protein containing a cytoplasmic domain of 38 amino acids (27). Our studies have shown that in CD4-transfected nonlymphoid HeLa or NIH-3T3 cells CD4 is constitutively internalized at rates of 2–3% of the cell surface pool per minute (28, 36, 37). CD4 molecules from which the cytoplasmic domain has been deleted (CD4^{cyt-}) are taken up two- to threefold less efficiently than intact CD4 (36, 37), being internalized at rates corresponding to the constitutive plasma membrane turnover (12, 37). Immunolabeling electron microscopy was used to demonstrate directly that CD4 is enriched in coated pits relative to its concentration in the plasma membrane, while CD4^{cyt-} molecules are neither concentrated into, nor excluded from,

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1. *Abbreviations used in this paper*: CD4^{cyt-}, CD4 molecules from which the cytoplasmic domain has been deleted; HIV, human immunodeficiency virus; MHC, major histocompatibility complex.

coated pits (37). These observations suggest that the cytoplasmic domain of CD4 may contain a signal that facilitates its endocytosis. We have also demonstrated that CD4 endocytosis is constitutive, is not induced by the presence of mono- or divalent antibody ligands, and is balanced by the recycling of the internalized molecules to the cell surface; at steady state ~40–50% of the CD4 pool is located in an intracellular compartment (28, 36).

As in the CD4-transfected HeLa and NIH-3T3 cells, CD4 molecules which are naturally expressed in cells of the monocyte/macrophage lineage (e.g., HL-60 cells) are internalized and recycled to the cell surface (37). In contrast, lymphocytic cell lines show very low levels of CD4 endocytosis (0.2–0.4% per minute) and steady-state levels of internal CD4 equivalent to only 5–7% of the cell surface pool (37, 38). These low levels of internalization are not due to a general defect in the endocytic properties of the lymphocytic cell lines, since the rates of uptake of fluid-phase markers, coated pit densities at the cell surface, and size of the endocytic compartment of these cells are comparable to those of nonlymphoid cells (37). Significantly, $CD4^{cyt-}$ molecules are internalized faster in lymphoid cells than the full-length CD4 molecule, at rates very similar to the $CD4^{cyt-}$ molecules in nonlymphoid cells (~1% per minute, with 20% of the $CD4^{cyt-}$ internal at steady state; references 37, 38). These observations suggest that in T cells, CD4 must be actively excluded from the endocytic pathway, presumably via an interaction of the cytoplasmic domain with another lymphoid-specific molecule(s).

In the present study, we demonstrate that a member of the *src* gene family, the lymphocyte-specific protein tyrosine kinase $p56^{lck}$, which has previously been shown to interact with the cytoplasmic domain of CD4 (44, 48, 51, 52), is responsible for these effects on CD4 endocytosis. By expressing the products of the *lck* or *c-src* genes, or chimeras of the two proteins, in CD4-transfected nonlymphocytic cell lines, we show that a direct physical interaction between CD4 and $p56^{lck}$ is required, and that $p56^{lck}$ prevents CD4 entering coated pits, thus inhibiting its endocytosis. This interaction between $p56^{lck}$ and CD4 represents a novel mechanism for regulating the endocytosis of a cell surface molecule.

Materials and Methods

Cells and Cell Culture

Cells were cultured as described (37). CD4-transfected NIH-3T3 or HeLa cells were used 3 d after subculture, when the cell surface expression of CD4 was maximal, while CEM (11) and A2.01/CD4-cyt399 cells (4) were used while growing exponentially.

Antibody Reagents

The anti-CD4 monoclonal antibodies used were Q4120 (reference 13; provided by Professor Peter Beverley, Imperial Cancer Research Fund, Human Tumour Immunology Group, University College, London, UK) and L120.3 (reference 13; provided by the Medical Research Council AIDS Directed Programme Reagents Programme). A rabbit polyclonal anti-CD4 serum, raised against recombinant soluble CD4 from CHO cells (37), was affinity purified using baculovirus-expressed soluble CD4 (reference 33; supplied through the AIDS Directed Programme Reagents Programme) immobilized on Reactigel (Pierce and Warriner, Chester, UK). Q4120 was radioiodinated as described (37).

A rabbit antiserum against a peptide covering residues 38–62 of $p56^{lck}$ (starting with the sequence IRNG; see Fig. 7) has been described (10); this is referred to as anti-$p56^{lck}$ [IRNG]. In addition, a peptide corresponding to amino acids 478–509 (KERP. . .) of $p56^{lck}$ (prepared by Dr. Torben Saermark, University of Copenhagen, Denmark, for the European Community Concerted Action program) was used to raise a second rabbit antiserum, anti-$p56^{lck}$ [KERP], which was affinity purified using the peptide immobilized on Reactigel (Pierce and Warriner). The anti-*src* mAb, 327, raised against *v-src* (24), was purchased from Oncogene Science, Inc., Manhasset, NY. Peroxidase-conjugated goat anti-rabbit and anti-mouse antibodies, as well as anti-mouse rhodamine and anti-rabbit FITC reagents, were purchased from Pierce and Warriner.

Transfections and Selection of Stable Cell Lines

Human CD4 cDNA in which the codon for cysteine 420 was mutated to a serine codon, provided by Dr. Andre Shaw (49), was cloned into the eukaryotic expression vector pSG5 (Stratagene Ltd., Cambridge, UK). pSV2 Neo was supplied by Dr. Alan Hall (Institute of Cancer Research, London). cDNAs encoding $p56^{lck}$, avian $p60^{c-src}$, and *src/lck* and *lck/src* chimeras in the expression vector pSM were as described (51). The pBabe/hygro vector (32) encoding the gene for hygromycin resistance was provided by Dr. Mary Collins (Institute of Cancer Research, London).

Transfections were carried out using calcium phosphate precipitates essentially as described (46). The plasmid encoding the mutant CD4 was transfected together with pSV2 Neo, while plasmids encoding kinase constructs were transfected together with pBabe/hygro at ratios of kinase plasmid to pBabe/hygro plasmid of 10–15 to 1. Resistant colonies were selected in the presence of 1 mg/ml Geneticin sulphate (G418, GIBCO Limited, Paisley, Scotland, UK) or 0.2 mg/ml hygromycin B (Sigma Chemical Company Ltd., Poole, Dorset, UK) as appropriate.

Cells expressing CD4 were identified by labeling with ^{125}I-Q4120. Expression of $p56^{lck}$ or other kinase constructs was detected either by measuring CD4 endocytosis, or by immunofluorescence staining. Cells were cultured on 13-mm glass coverslips, or on glass microscope slides using Flexiperm-Micro 12 silicone mould tissue culture chambers (Heraeus-Biotech, Brentwood, Essex, UK), fixed in 3% paraformaldehyde in PBS, quenched with 50 mM NH_4Cl, permeabilized with 0.1% Triton X-100, and preincubated in 0.2% gelatin in PBS. Cells were stained with either anti-$p56^{lck}$ [IRNG] serum at 1:2,000, anti-$p56^{lck}$ [KERP] at 1:500, or mAb 327 at 1:500 (0.2 μg/ml). The bound antibodies were detected using rhodamine-labeled goat anti-mouse or fluoresceinated goat anti-rabbit reagents.

Measurement of CD4 Endocytosis

Endocytosis of CD4 was measured using ^{125}I-Q4120 as described (36, 37). Briefly, cells were grown in 16-mm tissue culture wells and labeled with 0.5 nM ^{125}I-Q4120 for 2 h at 4°C. Cells were washed and held on ice, or warmed to 37°C for various times. Subsequently, the cells were cooled on ice and either harvested directly using 0.2 M NaOH (to give the total cell-associated level of ^{125}I-Q4120), or treated with medium adjusted to pH 2.0 to remove the cell surface ^{125}I-Q4120 before harvesting (to reveal the intracellular ^{125}I-Q4120 activity). All samples were counted using a γ-counter (Gamma 5500B; Beckman Instrs., Inc., Fullerton, CA). The proportion of acid-resistant to total cell counts was calculated for each time point (36), and the background value of acid-resistant material on cells held on ice throughout the experiment was subtracted. Endocytosis rates were usually calculated by linear regression of the percentages of ^{125}I-Q4120 internalized during the first 10 min at 37°C, while the steady-state levels of internalized CD4 were measured after 1 h of endocytosis and recycling (36, 37).

Preparation of Cell Lysates

Cells were washed once in Ca^{2+}/Mg^{2+}-free PBS, and adherent cells were harvested by scraping into PBS and centrifuging at 1,500 rpm for 5 min at 4°C. Cell pellets were suspended for 10 min 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). Detergent-insoluble material was removed by centrifugation at 4°C for 30 min at full speed in an Eppendorf microfuge. The supernatants were collected, aliquots were taken for protein determination using bicinchoninic acid (Pierce and Warriner), while the remaining samples were frozen in liquid nitrogen and stored at −70°C.

In Vitro Kinase Assays

CD4 was immunoprecipitated at 0–4°C from the NP-40 cell lysates prepared as above. Lysates were precleared by incubation for 30 min with 50 μl of packed prewashed protein A–Sepharose (Sigma Chemical Company Ltd.), and then immunoprecipitated by adding 4.5 μg of L120.3 for 1 h, and protein A–Sepharose (50 μl of a 50% slurry) for an additional 1.5 h. 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 (Sigma Chemical Company Ltd.), pH 7.2, containing 0.1% NP-40. For the kinase reaction, the beads were resuspended in 25 mM Hepes/0.1% NP-40, containing 10 mM $MnCl_2$ and 1 μCi of ^{32}P-γ-ATP, and incubated at 30°C for 25 min. The beads were washed once in 25 mM Hepes/0.1% NP-40, resuspended in 40 μl SDS-PAGE sample buffer containing 50 mM DTT, analyzed on 10% SDS-PAGE gels, and autoradiographed on Kodak X-Omat AR film for 10 min to 48 h. To quantitate the amount of kinase activity precipitated, the ^{32}P labeled bands were excised from some of the gels, and the levels of ^{32}P determined by Cerenkov counting.

Immunoblotting

For immunoblotting, aliquots of NP-40 cell lysates containing equal amounts of protein were diluted with SDS-PAGE sample buffer. Alternatively, cell pellets were washed in PBS and directly solubilized in SDS-PAGE sample buffer; and the released DNA was sheared using a microprobe sonicator. Samples containing equal amounts of protein were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose. Blots were blocked using 5% dried skimmed milk powder (Marvel Div. Dayton-Walther Corp., Richmond, IN) in PBS for 1 h at 45°C and 1 h at room temperature, and then incubated in primary antibody. The antisera anti-p56^{lck} [IRNG] and anti-p56^{lck} [KERP] were used at dilutions of 1:1,000 and 1:500, respectively, while the mouse mAb 327 was used at 0.1 μg/ml. For CD4 blotting, affinity-purified anti-CD4 antibodies were used at 200 ng/ml. After incubation with antibody and washing, the blots were probed with peroxidase-conjugated goat anti-rabbit antibodies (or goat anti-mouse for mAb 327), and developed using the ECL Western blotting detection system (Amersham International plc, Amersham, UK) according to the manufacturer's instructions.

Electron Microscopic Localization of CD4

For electron microscopy, cells were plated onto 22-mm^2 glass coverslips and grown to confluence for 3 d. Cells were cooled and incubated on ice with 8 nM Leu3a for 2 h. Excess antibody was washed away, and cells were labeled with 9-nm-diam protein A–gold particles (provided by Dr. Gareth Griffiths, EMBL, Heidelberg, Germany; see reference 37). After an additional 2 h, cells were washed extensively and fixed on ice with 2.5% glutaraldehyde in 50 mM sodium cacodylate buffer, pH 7.4, containing 50 mM KCl and 2.5 mM $MgCl_2$ (14). After postfixation in osmium tetroxide, cells were stained in Kellenberger's uranyl acetate, dehydrated, scraped off the coverslips, collected by centrifugation, and embedded in Epon. Thin sections were examined with a Philips CM12 electron microscope. For quantitative analysis, cell surfaces were examined systematically, noting the location of every gold particle encountered.

Other Methods

Fluid-phase endocytosis was measured on cells cultured on 60-mm-diam tissue culture plates using medium containing 3 mg/ml HRP (type II; Sigma Chemical Company Ltd.) essentially as described (12, 37). To measure endocytosis of transferrin (20), cells cultured on 60-mm tissue culture plates were preincubated in RPMI 1640 medium lacking bicarbonate, supplemented with 0.2% BSA, 10 mM Hepes, and 100 μg/ml desferrioxamine mesylate (Desferal; Ciba-Geigy Pharmaceuticals, Horsham, West Sussex, UK). Cells were labeled at 4°C in the same medium without Desferal, but containing 10 nM iron-loaded ^{125}I-labeled human transferrin (radioiodinated using iodobeads [Pierce and Warriner] to a specific activity of 50 Ci/mmol) for 2 h. Free ^{125}I-transferrin was washed away and the plates were held on ice or incubated at 37°C for various times. Subsequently, the cells were returned to ice and either harvested directly with 0.2 M NaOH, to measure total cell-associated activity, or surface stripped to remove cell surface ^{125}I-transferrin before harvesting. Surface stripping was performed by incubating cells for 5 min at 4°C in pH 2.2 medium containing 100 μg/ml Desferal, and then for an additional 5 min in neutral pH medium containing Desferal.

Results

Inhibition of CD4 Endocytosis in Lymphocytic Cells

To explain the differences in the rates of CD4 endocytosis observed in nonlymphoid and lymphoid cells (37), we have suggested that a protein or proteins, expressed specifically in lymphoid cells, must interact with the cytoplasmic domain of CD4 to prevent its internalization. In T cells, CD4 is known to associate with a protein tyrosine kinase of the *src* gene family, p56^{lck} (44, 52). To investigate whether p56^{lck} may modulate CD4 internalization, we analyzed the association of CD4 and p56^{lck} in a number of cell lines in which we have previously characterized CD4 endocytosis (37).

To detect CD4-associated p56^{lck}, we used an in vitro autophosphorylation assay (44, 48, 51). Cell lysates were prepared in NP-40 buffer, CD4 was immunoprecipitated using anti-CD4 antibodies and CD4-associated kinase activity was detected after incubation with ^{32}P-γ-ATP, analysis by SDS-PAGE, and autoradiography. In lysates of T cell lines such as CEM, anti-CD4 antibodies precipitated p56^{lck} as a ^{32}P-labeled band which migrated with a mobility of 56–58 kD (Fig. 1, *top*). This band was only detected in immunoprecipitates from lymphocytic cell lines, and could be precipitated with two different anti-CD4 antibodies (OKT4 and L120.3), but not with control antibodies to the transferrin receptor (not shown). Western blotting of CEM cell lysates with the anti-p56^{lck} [IRNG] serum again identified p56^{lck} as a band of 56–58 kD (Fig. 1, *middle*).

CD4-associated ^{32}P-labeled p56^{lck} was not observed in the nonlymphoid HeLa-CD4 or NIH-CD4 cells (Fig. 1). Direct immunoblotting of cell lysates with anti-p56^{lck} antibodies indicated that these nonlymphoid cells do not express detectable amounts of p56^{lck}. In the A2.01/CD4-cyt399 cell line, which expresses a CD4$^{cyt^-}$ molecule (4) and which shows CD4 internalization corresponding to bulk flow endocytosis (37), p56^{lck} was detected by immunoblotting (Fig. 1) but was not immunoprecipitated with anti-CD4 antibodies, indicating that the kinase did not interact with the truncated form of CD4 expressed in these cells (see also reference 51). Thus, CD4-associated p56^{lck} was only detected in cells which exhibited very low levels of CD4 uptake. Cells which show relatively efficient endocytosis of CD4 either do not express p56^{lck}, or express a form of CD4 which cannot interact with p56^{lck}.

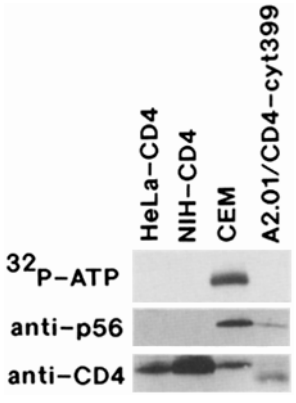

Figure 1. Association of CD4 and p56^{lck} in lymphoid and CD4-transfected nonlymphoid cells. p56^{lck} was detected after immunoprecipitation of CD4 and in vitro kinase assay (*^{32}P-ATP, top*) or by immunoblotting cell lysates with anti-p56^{lck}[IRNG] serum (*anti-p56, middle*). CD4 was detected by immunoblotting with rabbit anti-CD4 antibodies (*anti-CD4, bottom*).

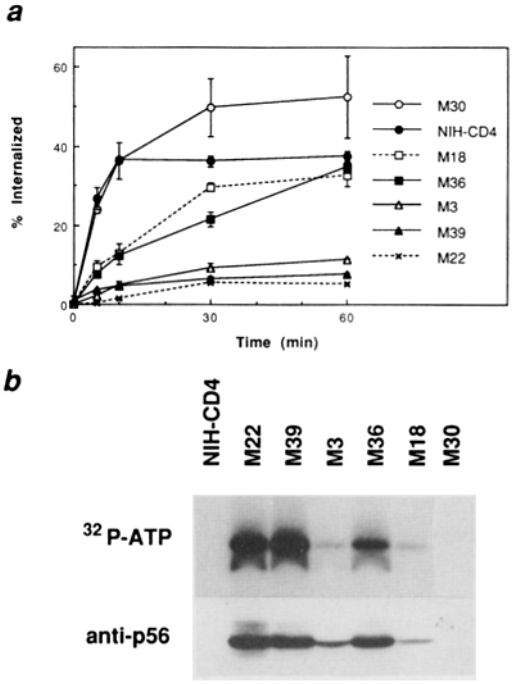

Figure 2. Analysis of p56^{lck}-transfected NIH-CD4 cells. (*a*) Time course of internalization of CD4 (traced with the ^{125}I-labeled anti-CD4 mAb Q4120) on NIH-CD4 cells, and on a series of hygromycin-resistant transfected clones (*M22, M39, M3, M36, M18,* and *M30*). The plot shows the ratio of acid-resistant (*% internalized*) ^{125}I-Q4120 to the total cell-associated label at various time points. (*b*) Analysis of the association of CD4 and p56^{lck} in NIH-CD4 cells and the hygromycin-resistant transfected clones. p56^{lck} was detected after immunoprecipitation of CD4 and in vitro kinase assays (*^{32}P-ATP, top*) or by immunoblotting of cell lysates with anti-p56^{lck}[IRNG] serum (*anti-p56, bottom*).

The Protein Tyrosine Kinase p56^{lck} Inhibits CD4 Endocytosis When Transfected into NIH-CD4 Cells

If p56^{lck} is indeed responsible for inhibiting CD4 endocytosis in T cells, then coexpression of p56^{lck} and CD4 in non-lymphoid cells may also inhibit CD4 endocytosis. To test this possibility, NIH-CD4 cells were transfected with the murine *lck* gene. The amino acid sequences of the murine and human p56^{lck} proteins are highly homologous (39), especially in the NH_2-terminal region which is known to be involved in the CD4/p56^{lck} association, and both proteins can interact with human CD4 (49, 51). NIH-CD4 cells were, therefore, cotransfected with two independent expression vectors, pSM/p56^{lck}, which contains the gene for murine p56^{lck} (51), and pBabe/hygro, a retroviral vector containing the gene encoding hygromycin resistance (32). 59 stable clones were selected with hygromycin B and screened by measuring CD4 internalization. Some clones exhibited CD4 endocytosis levels similar to that of the parent NIH-CD4 cell line; analysis of a number of these indicated that, although hygromycin resistant, they did not express p56^{lck}. However, CD4 endocytosis was significantly reduced in 26 of the transfected clones, and in some cases was almost completely inhibited.

Six of the hygromycin-resistant clones, representing groups of clones in which CD4 endocytosis was either greatly reduced, partially reduced, or unaffected compared with the parent NIH-CD4 cell line, were selected for detailed analysis. Fig. 2 *a* shows the results of a series of endocytosis assays in which cells were surface labeled at 0–4°C with ^{125}I-anti-CD4 mAb, washed, and then warmed to 37°C for 0–60 min. The parental NIH-CD4 cells internalized the labeled antibody at ~3.6% per minute; and at steady state, ~43% of the initial cell surface pool of CD4 was in an intracellular compartment (Table I). By contrast, two of the hygromycin-resistant clones, M39 and M22, showed 10- and 26-fold slower rates of CD4 endocytosis, respectively; and at steady state, only 11 and 6% of the label was intracellular (Table I). Other clones (M3, M36, and M18) had intermediate rates of CD4 endocytosis and intermediate steady-state levels of intracellular CD4, while CD4 uptake in clone M30 was similar to the untransfected NIH-CD4 cells.

The interaction of CD4 and p56^{lck} was analyzed in the six selected clones by assaying for CD4-associated kinase activity (Fig. 2 *b*). A ^{32}P-labeled band, comigrating with the p56^{lck} precipitated from CEM cells, was precipitated with anti-CD4 antibodies from lysates of all the clones except M30. In addition, the p56^{lck} protein was detectable on immunoblots in amounts which reflected the levels of CD4-

Table I. Analysis of NIH-CD4 Cell Lines Transfected with p56^{lck}

Cell line	Rate of CD4 endocytosis*	CD4 internal at steady state‡	CD4/p56^{lck} coprecipitation§	CD4 expression‖
	% per min	*%*	*cpm*	*fmol/mg protein*
NIH-CD4	3.6	43 ± 6 (8)	3,186	860 ± 150
M22	0.14	6 ± 1 (2)	323,046	1,960 ± 150
M39	0.37	11 ± 4 (4)	460,622	1,670 ± 270
M3	0.50	16 ± 6 (2)	27,299	700 ± 200
M36	1.3	31 ± 5 (2)	160,484	2,530 ± 1,120
M18	1.2	34 ± 2 (2)	19,057	1,570 ± 390
M30	3.6	53 ± 1 (2)	7,135	2,580 ± 790

* Endocytosis rates were measured over the first 10 min at 37°C.
‡ The level of acid-resistant ^{125}I-Q4120 tracer in cells kept 60 min at 37°C. Background acid-resistant counts at $t = 0$ have been subtracted (see Materials and Methods). Data were averaged from the number of experiments shown in brackets.
§ The autophosphorylated p56^{lck} bands (precipitated with anti-CD4 mAb from cell lysates containing equal amounts of protein) were cut from the gel corresponding to the autoradiograph in Fig. 2 *b* (*top*), and levels of ^{32}P present were estimated by counting the Cerenkov radiation.
‖ The CD4 levels expressed in the various cell lines were estimated from equilibrium binding measurements using ^{125}I-Q4120. Cells were labeled at 0–4°C with ^{125}I-Q4120 at 0.5 nM, the K_d concentration which would saturate approximately half of the CD4 molecules exposed at the cell surface. Levels of total cellular CD4 were estimated by doubling the results from these binding experiments and correcting for the intracellular CD4 pools.

associated kinase activity (Fig. 2 *b*). To obtain an approximate measure of the amount of p56^{lck} associated with CD4, we excised the ^{32}P-labeled bands from the gel of the autoradiograph shown in Fig. 2 *b* (*top*) and measured the incorporated radioactivity by Cerenkov counting (Table I). This analysis demonstrated an inverse correlation between the amount of p56^{lck} associated with CD4 and the efficiency of CD4 endocytosis (Fig. 3); i.e., the higher the level of p56^{lck} associated with CD4, the slower the rate of CD4 endocytosis (Fig. 3 *a*) and the smaller the pool of CD4 internalized at steady state (Fig. 3 *b*). Cells of the M3 clone, which did not fit optimally with this correlation, express very low levels of CD4 (Table I), and as a consequence, low levels of p56^{lck} are sufficient to inhibit CD4 endocytosis. By contrast, the M36 cells express comparatively high levels of CD4, and hence the amount of p56^{lck} in these cells only partially inhibited CD4 uptake.

The expression of p56^{lck} did not disrupt the overall endocytic properties of the transfected cells, since we observed identical rates of fluid-phase endocytosis on the NIH-CD4 cells and on clone M22, which expresses a large amount of p56^{lck} and in which CD4 endocytosis was almost completely inhibited (data not shown). Likewise, both the NIH-CD4 and M22 cells internalized similar levels of radioiodinated transferrin (Fig. 4), indicating that receptor-mediated endocytosis was not affected by p56^{lck}. Thus, the endocytic capacities of both sets of cells were equivalent. The effect of p56^{lck} was specific for CD4 endocytosis and did not extend to other endocytic processes.

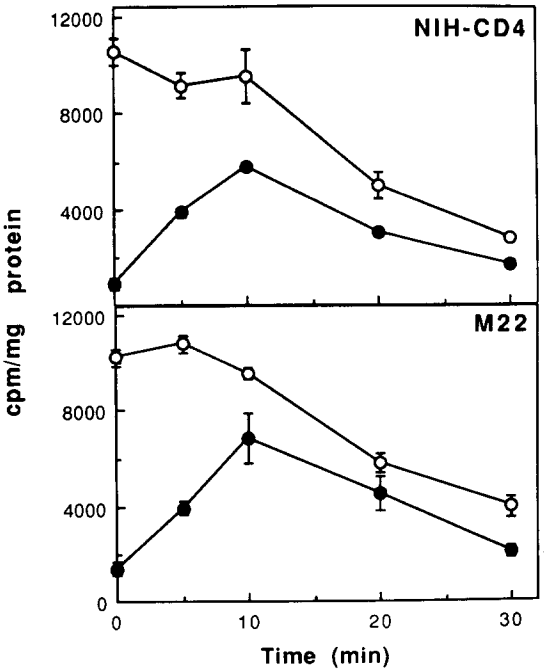

Figure 4. Receptor-mediated endocytosis of ^{125}I-transferrin on NIH-CD4 (*top*) and M22 cells (*bottom*). Open symbols show total levels of cell-associated ^{125}I-transferrin, while the closed symbols indicate intracellular ^{125}I-transferrin.

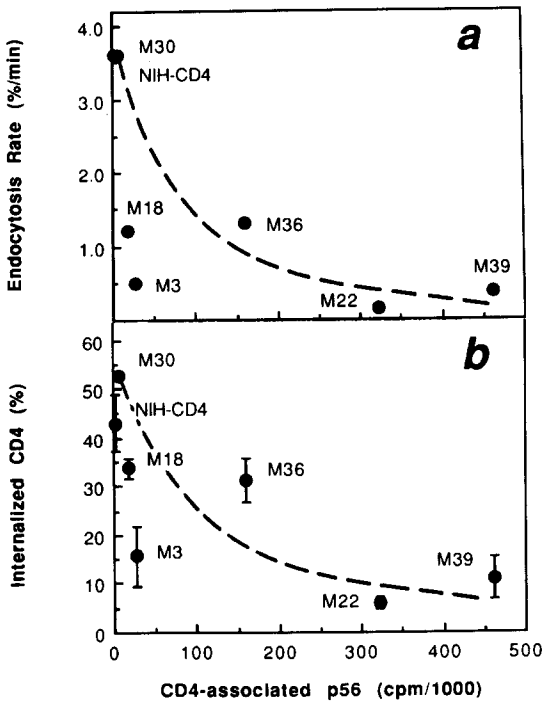

Figure 3. Correlation of the inhibition of CD4 endocytosis with the level of CD4-associated p56^{lck} in NIH-CD4 cells and the hygromycin-resistant p56^{lck}-transfected clones. The rate of CD4 endocytosis (*a*) and the level of CD4 internalized at steady state (*b*) were plotted against the level of ^{32}P incorporated in vitro into p56^{lck} precipitated with CD4 (measured by counting the Cerenkov radiation in excised bands; cf. Table I).

p56^{lck} Inhibits Endocytosis of CD4, but Not of Mutant CD4(C420→S)

We have also transfected the pSM/p56^{lck} vector into two human cell lines: HeLa-CD4 (36, 37) and a HeLa cell line transfected with a mutant CD4 molecule, CD4(C420→S), in which cysteine residue 420 was replaced by serine. This mutation disrupts the epitope in the cytoplasmic domain of CD4 (around amino acids 420–422) required for interaction with p56^{lck} (49, 51). In our hands it proved more difficult to obtain stable transfectants from the HeLa-CD4 than from NIH-CD4 cells, and when hygromycin B–resistant colonies were screened for p56^{lck} expression by immunofluorescent staining, we only obtained one positive cell line from each transfection. Immunoblotting with anti-p56^{lck} antiserum revealed that the level of expression of p56^{lck} in the HeLa-CD4/p56^{lck} cell line was very low compared to the M22 cells (above), while intermediate amounts of p56^{lck} were found in the HeLa-CD4(C420→S)/p56^{lck} cells (Fig. 5 *a*). Immunoprecipitation of CD4 and in vitro kinase assays demonstrated that the p56^{lck} in HeLa-CD4/p56^{lck} cells could associate with CD4. In contrast, no detectable p56^{lck} activity was precipitated with the CD4(C420→S) mutant (Fig. 5 *a*).

Endocytosis experiments with these cell lines showed that p56^{lck} significantly inhibited endocytosis of CD4 in the HeLa-CD4/p56^{lck} cells compared with the parent HeLa-CD4 cells (Fig. 5 *b*). The CD4(C420→S) molecule was internalized with similar kinetics to the wild-type CD4 and was not affected by coexpression of p56^{lck} (Fig. 5 *c*). Thus, the inhibition of CD4 endocytosis by p56^{lck} is not confined to NIH-CD4 cells and is dependent on the ability of p56^{lck} to interact directly with CD4.

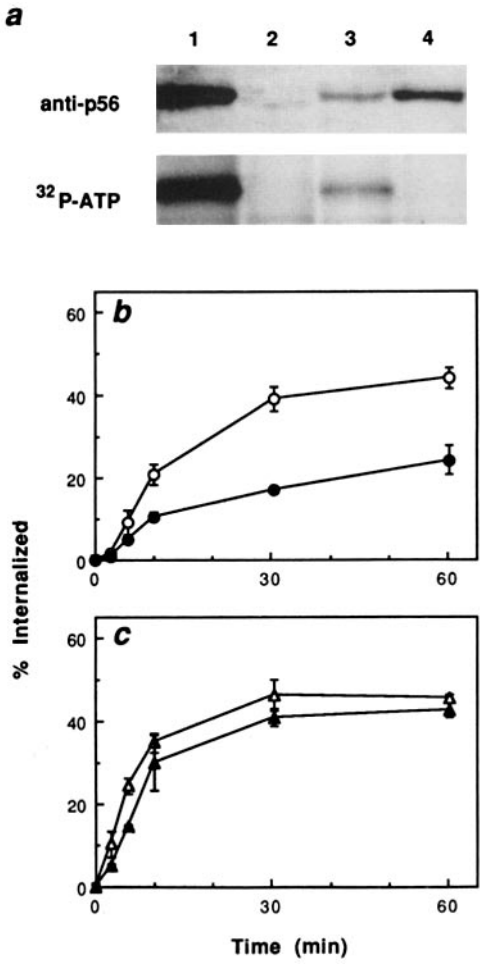

Figure 5. Transfection of p56^{lck} into HeLa-CD4 and HeLa-CD4-(C420→S) cells. (*a*) Analysis of the association of CD4 and p56^{lck} in M22 (lane *1*), NIH-CD4 (lane *2*), HeLa-CD4/p56^{lck} (lane *3*), and HeLa-CD4(C420→S)/p56^{lck} cells (lane *4*). p56^{lck} was detected by immunoblotting of cell lysates with anti-p56^{lck}[IRNG] serum (anti-p56, *top*) or after immunoprecipitation of CD4 and in vitro kinase assays (^{32}P-ATP, *bottom*). (*b* and *c*) Time courses of internalization of CD4 on HeLa-CD4 (○ in *b*), HeLa-CD4/p56^{lck} (● in *b*), HeLa-CD4(C420→S) (Δ in *c*), and HeLa-CD4(C420→S)/p56^{lck} (▲ in *c*) cells. The plots show ratios of acid-resistant (*% internalized*) ^{125}I-Q4120 to the total cell-associated label at various time points.

Transfection of NIH-CD4 Cells with p60^{c-src} Has No Effect on CD4 Endocytosis

To examine whether the inhibition of CD4 endocytosis was a specific property of p56^{lck}, or whether other kinases of the *src* gene family could similarly affect CD4 internalization, we transfected NIH-CD4 cells with the pSM vector containing the avian *c-src* gene, and again selected a number of hygromycin-resistant colonies. Clones expressing high levels of p60^{c-src} were identified by immunofluorescent staining with the anti-*src* mAb 327. NIH-3T3 cells express endogenous p60^{c-src} at levels below the limit of detection by immunofluorescence (8). Thus, only cell lines expressing p60^{c-src} from the transfected gene would be identified in this assay. Expression of p60^{c-src} in selected clones positive by immunofluorescence was confirmed by immunoblotting. CD4 endocytosis was measured in three clones: NIH-CD4/src-1, NIH-CD4/src-20, and NIH-CD4/src-23, which expressed the highest levels of p60^{c-src}. As shown in Fig. 6, the presence of the p60^{c-src} protein had no effect on the rate or extent of CD4 endocytosis.

Transfection of NIH-CD4 Cells with Chimeric Kinase Molecules

Kinases of the *src* gene family share three conserved regions: namely, the kinase domain, which comprises the COOH-terminal half of the molecule, and two *src* homology domains, SH2 and SH3 (23, 35). The NH_2 terminus consists of sequences unique to each member of the family, and in the case of p56^{lck} contains the residues required for interaction with CD4 (51). To examine in more detail which domains of the p56^{lck} molecule are required for inhibition of CD4 endocytosis, we generated NIH-CD4 cell lines stably transfected with the *src/lck* and *lck/src* chimeric molecules developed by Turner et al. (51). The *src/lck* construct contains the first 92 amino acids of the *c-src* gene, fused to the conserved domains (amino acids 72–509) of *lck*, and thus lacks the unique region of p56^{lck} required for CD4 binding. *lck/src* is the complementary chimera, consisting of the NH_2 terminus (amino acids 1–71) of *lck* fused to the conserved domains of *src* (Fig. 7 *a*). After transfection with vectors encoding these chimeras, hygromycin B–resistant colonies were screened for expression of the chimeric kinase molecules using two antisera raised against peptides of the p56^{lck} sequence. The anti-p56^{lck}[IRNG] serum (10) recognizes a peptide corresponding to amino acids 38–62 of p56^{lck}, and reacts with both p56^{lck} and the *lck/src* chimera, while the anti-p56^{lck}[KERP] serum, which was raised against amino acids 478–509 of p56^{lck}, binds the *src/lck* chimera as well as p56^{lck} (Fig. 7 *a*). In addition, the anti-*v-src* mAb 327, which binds to an epitope in the p60^{c-src} SH2 domain, recognized the *lck/src* chimera. All antibody reagents were active both by immunofluorescence and immunoblotting, allowing us to select cell lines by immunofluorescence screening, and then confirm expression of the chimeric kinases by immunoblotting. A number of cell lines were thus selected for measuring CD4 endocytosis.

As expected, the *src/lck* chimera, which lacks the NH_2-terminal sequence of p56^{lck} required for interaction with CD4 (51), did not affect CD4 endocytosis (Fig. 7 *b*). In contrast, four independent cell lines expressing the *lck/src* chimera, which is able to interact with CD4, showed reduced CD4 endocytosis (Fig. 7 *c*).

The levels of expression of the chimeras and p60^{c-src} were measured by immunoblotting of equal amounts of cell protein with the antisera to p56^{lck} or p60^{c-src}. Densitometric scanning of blots probed with the anti-p56^{lck}[KERP] antiserum indicated that the *src/lck*-14 cell line contained at least fivefold more of the COOH-terminal p56^{lck} epitope than the p56^{lck}-transfected M22 cell line (Fig. 8 *a*). When blots were probed with the anti-p56^{lck}[IRNG] antiserum, all four *lck/src*-expressing cell lines contained levels of the NH_2-terminal p56^{lck} epitope comparable to the level of p56^{lck} in the M22 cells. mAb 327 reacted with both p60^{c-src} and the *lck/src* chimera on immunoblots, and revealed that the *src*-transfected cell lines contained significantly higher levels of the *src* epitope than the *lck/src*-transfected cells (Fig. 8 *c*). Thus, all of the cell lines expressed the transfected kinases

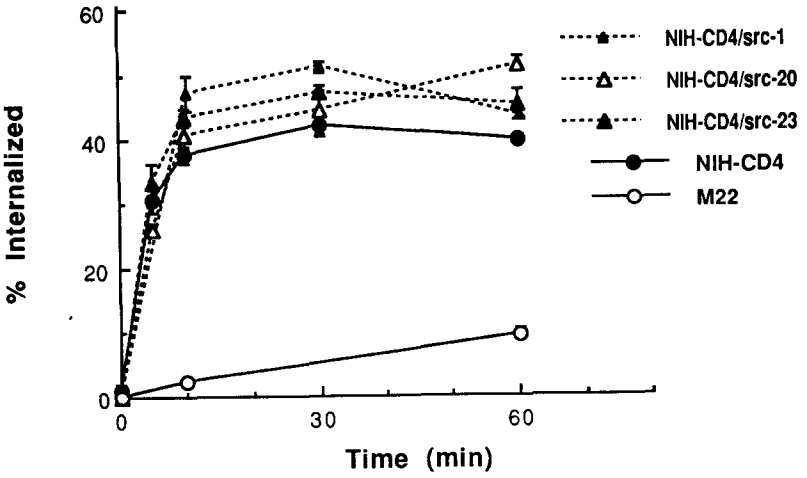

Figure 6. Endocytosis of CD4 in three $p60^{c\text{-}src}$-transfected NIH-CD4 cell lines. CD4 endocytosis was quantitated as described in Fig. 2 *a*. NIH-CD4 cells (●) and the $p56^{lck}$-transfected M22 cell line (○) are shown for comparison.

at levels comparable to, or higher than, $p56^{lck}$ in the M22 cells.

The association of the various transfected kinase constructs with CD4 was studied by immunoprecipitating CD4 from cell lysates and then measuring the CD4-associated kinase activity in vitro. Only the *lck/src* chimera and $p56^{lck}$ were able to associate with CD4 (Fig. 9). No kinase activity could be precipitated from the NIH-CD4 cell line, or from the cell lines transfected with $p60^{c\text{-}src}$ or the *src/lck* chimera. This agrees with a previous study (51) which demonstrated by immunoprecipitation followed by immunoblotting that $p60^{c\text{-}src}$ and the *src/lck* chimera cannot associate with CD4. Cerenkov counting of the labeled bands showed that the *lck/src* molecules incorporated >100-fold more ^{32}P than $p56^{lck}$, even though M22 and the *lck/src*-12 cell line contained similar levels of the respective proteins by immunoblotting (cf. Fig. 8 *b*), and suggested that the kinase activity of this chimeric molecule is elevated.

These studies demonstrate that the *lck/src* chimera can interact with CD4 and does decrease the rate of CD4 internalization, although it does not inhibit CD4 endocytosis to the extent of $p56^{lck}$. The *src/lck* chimera, which contains all of $p56^{lck}$ except the NH_2-terminal unique region, is unable to bind CD4 and has no effect on CD4 endocytosis.

$p56^{lck}$ Prevents CD4 Entry into Coated Pits

To investigate the mechanism by which $p56^{lck}$ inhibits CD4 endocytosis, we studied the distribution of CD4 at the cell surface by immunogold labeling electron microscopy. NIH-CD4 cells, the $p56^{lck}$-transfected M22 cells, as well as the NIH-CD4/*src*-23 and NIH-CD4/*lck/src*-12 cell lines were labeled on ice with Leu3a, and protein A–gold. Previous experiments using iodinated antibody together with protein A–gold indicated that the gold reagent does not affect the kinetics or extent of CD4 endocytosis (37). Thus, the distribution of CD4 at the cell surface is not influenced by the labeling method. Quantitative analysis of the distribution of CD4 showed that in the parent NIH-CD4 cells, 6.6% of all gold particles was located over coated areas of the plasma membrane (Table II). Similarly, 5.6% of the CD4 molecules on NIH-CD4/src-23 was found in coated pits. In contrast, only 3 gold particles out of 1,039 were located against coated membrane in the M22 cell line (Table II). As coated pits account for 1.5–2% of the cell surface area (3, 12, 37), these data confirm our previous observation that CD4 alone can be enriched three- to fourfold in coated pits. However, in the presence of $p56^{lck}$ CD4 is effectively prevented from entering coated pits. The NIH-CD4/*lck/src*-12 cells showed reduced levels (3.9%) of gold particles in coated pits (Table II), in agreement with the biochemical data which showed partial inhibition of CD4 endocytosis.

Discussion

The cell surface glycoprotein CD4 functions in T cell ontogeny and T cell activation, and as a receptor for both lymphocyte chemoattractant factor and HIV. The cell surface expression of CD4 is known to undergo modulation in both T lymphocytes and monocytic cells in response to a variety of stimuli (1, 6, 16, 40, 57). However, little is known of how these properties influence the diverse functions of the CD4 molecule. We have previously demonstrated that CD4 molecules expressed in nonlymphocytic cells, including cells of the macrophage/monocyte lineage, exhibit very different patterns of endocytosis than CD4 molecules expressed in T cells; i.e., CD4 in nonlymphoid cells is efficiently endocytosed, while that expressed in T cells is not (37, 38). Here we have shown that the protein tyrosine kinase $p56^{lck}$ is responsible for these differences in CD4 endocytosis. These results establish several important points. First, they indicate a novel mechanism through which the endocytosis of cell surface molecules may be regulated. As such they further refine current models of the molecular basis for coated pit–mediated endocytosis. Second, they indicate how the properties of CD4 may differ in the various cell types in which it is normally expressed, and show how the cell surface expression of CD4 may be rapidly and reversibly modulated in T cells.

Regulation of CD4 Endocytosis by $p56^{lck}$

In the present study we have demonstrated that the protein tyrosine kinase $p56^{lck}$ regulates CD4 endocytosis. In nonlymphoid cell lines which do not express $p56^{lck}$, such as cells of the myeloid lineage (e.g., HL-60), or in CD4-

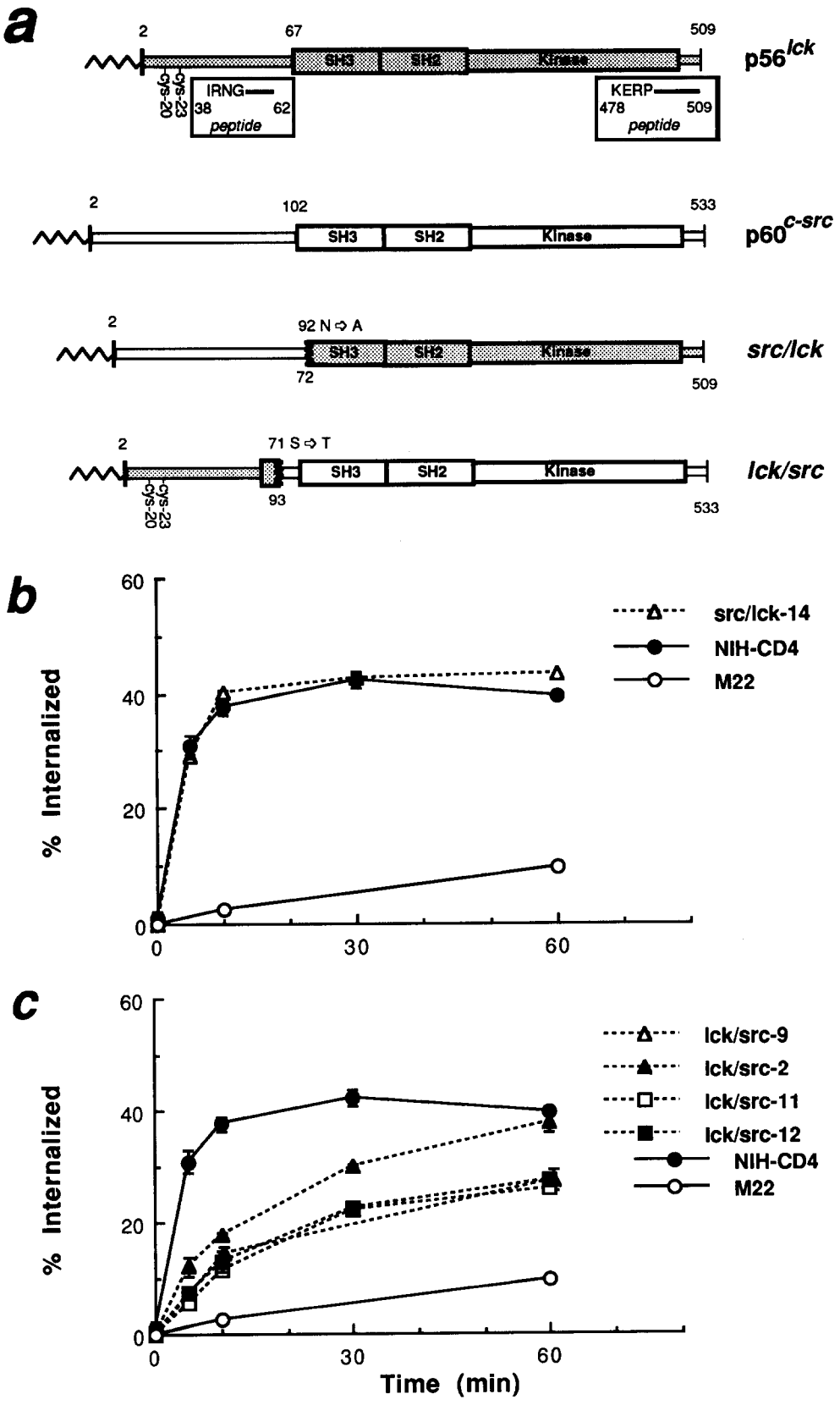

Figure 7. Effects of *src/lck* and *lck/src* chimeric kinases on CD4 endocytosis. (*a*) Structure of $p56^{lck}$ (*shaded*), $p60^{c\text{-}src}$, and two chimeras of these proteins, *src/lck* and *lck/src*. The position of the cysteine residues at position 20 and 23 of $p56^{lck}$, which are required for interaction with CD4, are indicated. The locations of the sequences of the peptides used to generate the anti-p56 antisera, anti-$p56^{lck}$[IRNG] and anti-$p56^{lck}$-[KERP], are also shown. SH2 and SH3 identify the *src*-homology regions. (*b* and *c*) Endocytosis of CD4 in a *src/lck*-transfected NIH-CD4 cell line (*b*) and in four *lck/src*-transfected NIH-CD4 cell lines (*c*). Endocytosis of CD4 was quantitated as described in Fig. 2 *a*. NIH-CD4 cells (●) and $p56^{lck}$-transfected M22 cells (○) are shown for comparison.

transfected HeLa or NIH-3T3 cells, CD4 is endocytosed through coated pits. The rates and extents of CD4 endocytosis vary in different cell lines, ranging from ~2–2.5% per minute in HeLa-CD4 cells to 3–4% per minute in NIH-CD4 or HL-60 cells (38). In agreement with these differences, the level of CD4 found in coated pits in the NIH-CD4 cell line (6.6%, Table II) is higher than that in HeLa-CD4 cells (4.5%, reference 37). While the rates of CD4 internalization

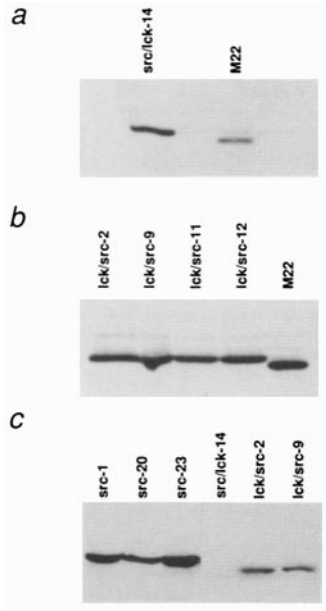

Figure 8. Detection of p56^{lck}, p60$^{c\text{-}src}$, and the *lck/src* and *src/lck* chimeras by immunoblotting. (*a*) Detection of kinases in lysates of the NIH-CD4/*src/lck*-14 and the p56^{lck}-transfected M22 cells by immunoblotting with the anti-p56^{lck}[KERP] antiserum (cf. Fig. 7 *a*). (*b*) Detection of kinases in lysates of the NIH-CD4/*lck/src* cell lines and the p56^{lck}-transfected M22 cells by immunoblotting with the anti-p56^{lck}[IRNG] antiserum. (*c*) Detection of kinases in lysates of the p60^{src}-transfected NIH-CD4 cells, and in *src/lck*- or *lck/src*-transfected NIH-CD4 cell lines by immunoblotting with the anti-*v-src* mAb 327. Note that the chimeras *lck/src* and *src/lck* migrate with an apparent molecular mass 2–3 kD higher than p56^{lck}, while p60$^{c\text{-}src}$ migrates with a molecular mass ~2 kD higher than the *lck/src* chimera, as predicted by the amino acid sequences of the proteins (see Fig. 7 *a*).

may not be as rapid as those reported for other receptors, such as the low density lipoprotein or transferrin receptors, it is nevertheless significant that the density of CD4 in coated pits in the NIH-CD4 cells is similar to the density at which

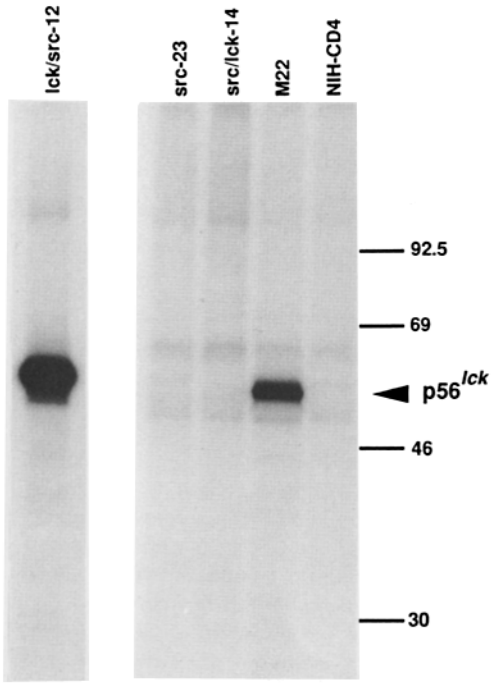

Figure 9. Association of various kinase constructs with CD4. CD4 was immunoprecipitated from lysates of NIH-CD4 or M22 cells, or from NIH-CD4 cells transfected with p60$^{c\text{-}src}$ or the *lck/src* or *src/lck* chimeras, and associated kinase activity was detected by autophosphorylation with ^{32}P-γ-ATP in vitro. After SDS-PAGE, labeled kinase molecules were detected by autoradiography for 10 min (*lck/src*-12) or 48 h (all other lanes). The molecular masses of marker proteins (in kD) are indicated.

the transferrin receptor is seen in coated pits on certain cell lines (31). Indeed, in the NIH-CD4 cells, CD4 was endocytosed at a similar rate to the transferrin receptor (cf. Fig. 4). This suggests that CD4 contains an endocytosis signal in its cytoplasmic domain, although the specific amino acid motifs involved have not yet been identified.

In contrast to the efficient endocytosis of CD4 in nonlymphoid cells, CD4 endocytosis is inhibited in cell lines expressing p56^{lck}, either naturally (e.g., lymphoid cell lines) or after transfection. We have observed p56^{lck} expression in all cell lines which showed an inhibition of CD4 endocytosis. Furthermore, all cell lines in which p56^{lck} was expressed, and could interact with CD4, had reduced CD4 internalization. The physical interaction of CD4 and p56^{lck} is a prerequisite for the regulation of CD4 endocytosis by p56^{lck}. Thus, endocytosis of tailless CD4 molecules (in the A2.01/CD4-cyt399 lymphocytic cell line) or of the CD4(C420→S) mutant, which cannot interact with p56^{lck}, is not affected by p56^{lck}. Similarly, kinase molecules that cannot interact with CD4 (p60$^{c\text{-}src}$ and the *src/lck* chimera) do not affect CD4 endocytosis. The inhibition of CD4 endocytosis appears to depend on the stoichiometric ratio of p56^{lck} to CD4 expressed. This was most clearly apparent with the series of clones derived by transfection of NIH-CD4 cells with p56^{lck} (the M clones). In these cell lines, there is a very good correlation between the amount of p56^{lck} expressed and associated with CD4, and the level of inhibition of CD4 endocytosis. The clones expressing the highest levels of p56^{lck}, M22 and M39, showed as little CD4 endocytosis as lymphocytic cell lines. Similarly, the HeLa-CD4/p56^{lck} cells, which expressed only low levels of p56^{lck}, showed only partial inhibition of CD4 endocytosis.

Electron microscopy of immunogold-labeled CD4 on the NIH-CD4 and M22 cells has demonstrated directly that p56^{lck} inhibits CD4 endocytosis by preventing entry of CD4 into coated pits. In the presence of p56^{lck}, the density of CD4 in coated pits was significantly lower than the density of CD4^{cyt-} molecules, which we have previously observed to be internalized as part of the bulk membrane flow (37). The mechanism by which p56^{lck} prevents CD4 entry into coated pits is not yet clear. Since p56^{lck} is a relatively large molecule, it may sterically prevent access of the cytoplasmic domain of CD4 to coated pits. It should be noted, however, that growth factor receptors such as the EGF, PDGF, and insulin receptors, which contain tyrosine kinase domains as part of their cytoplasmic sequences, are efficiently endocytosed through coated pits after binding their relevant growth factors (15). Thus, a large cytoplasmic kinase domain is not sufficient to inhibit endocytosis. Instead, p56^{lck} may prevent CD4 from entering coated pits through interaction of the CD4/p56^{lck} complex with other cytoplasmic components. Indeed, p56^{lck} has been reported to form oligomers with polypeptides of 110, 85, and 32 kD which can act as substrates for tyrosine phosphorylation (43). Alternatively, p56^{lck} appears to be able to interact with components of the cortical cytoskeleton (25), possibly via the SH2 or SH3 domains, which are known to be involved in protein–protein interactions and, at least in the case of SH3 domains, are found in a number of actin-binding proteins (23). Thus, p56^{lck} may serve to anchor CD4 to the submembraneous cytoskeleton. This idea would support the requirement for stoichiometric ratios of p56^{lck} and CD4 to effectively inhibit CD4 endocytosis. However, any cytoplasmic or cytoskeletal com-

Table II. Distribution of Gold-labeled CD4 on NIH-CD4 Cells

Cell line	Gold particles counted	Particles over noncoated plasma membrane		Particles over coated pits*		Unidentified‡
	n		%		%	
NIH-CD4	759	694	(91.4)	50	(6.6)	15
M22	1,057	1,039	(98.3)	3	(0.3)	15
NIH-CD4+*src*-23	571	517	(90.5)	32	(5.6)	22
NIH-CD4+*lck/src*-12	931	873	(93.8)	36	(3.9)	22

* Only particles directly juxtaposed to clathrin-coated membrane were counted.
‡ Gold particles over broken cell debris or too near grid bars to clearly classify were listed as unidentified.

ponents that are required for the p56^{lck} inhibition of CD4 uptake are presumably not T cell specific, as the lymphoid phenotype for CD4 endocytosis is observed when CD4 and p56^{lck} are expressed together in NIH-CD4 cells.

Like p56^{lck}, the *lck/src* chimera can associate with CD4 and inhibit CD4 endocytosis, although the inhibition observed with four different *lck/src*-transfected cell lines was only partial. Similarly, the *lck/src* chimera significantly reduced the amount of CD4 that could be observed in coated pits. Immunoblots with an antiserum directed to an NH_2-terminal epitope of p56^{lck} suggested that all four *lck/src*-transfected cell lines expressed levels of the protein comparable to the levels of p56^{lck} in the M22 cell line, in which CD4 endocytosis is completely inhibited. Thus, it appears that the *lck/src* chimera is less efficient than p56^{lck} in inhibiting CD4 endocytosis. Immunoprecipitation of CD4 and in vitro kinase assays indicated that the *lck/src* chimera could associate with CD4. In addition, the kinase activity of this chimera appeared significantly higher than that of p56^{lck}. We do not know whether the efficiency of association of *lck/src* with CD4 is impaired as a result of the increased kinase activity, thus liberating CD4 molecules which can be internalized. Alternatively, the interaction of the *lck/src* molecule with CD4 may mask the endocytosis signal on the cytoplasmic domain of CD4, but not prevent entry of the CD4–*lck/src* complex into coated pits. In the latter case, the CD4–*lck/src* complex would be expected to be internalized as a component of the bulk membrane flow, with kinetics similar to the CD4^{cyt-} molecules (37). The observed kinetics of CD4 internalization in the NIH-CD4/*lck/src* cell lines agree with this proposal. In the *lck/src* chimera, the anchoring interaction with the cytoskeleton could be disrupted, either because the relevant parts of p56^{lck} have been replaced by the *src* gene product or because of the increased activity of the *lck/src* kinase. Studies with constitutively activated *lck* mutants may resolve this issue.

Modulation of CD4 Endocytosis

The different rates of CD4 endocytosis observed in cells that express p56^{lck} (such as lymphocytes) compared with cells that do not (e.g., macrophages and monocytes) may be important in understanding the mechanisms underlying the different functions of CD4 as a receptor for lymphokine or coreceptor for MHC-II. Furthermore, the dissimilar patterns of CD4 endocytosis may be responsible for the different susceptibilities of T cells or cells of the macrophage/monocyte lineage to HIV infection (30).

For T cells, the interaction of CD4 and p56^{lck} provides a mechanism to rapidly and reversibly modulate CD4 cell surface expression during activation. Previous studies have demonstrated that the cell surface expression of CD4 can be down-regulated during antigen stimulation (5, 40, 57), that this down-regulation can be mimicked by treatment with phorbol esters (1, 16), and that down-regulation occurs by processes involving endocytosis (16, 17). Our studies suggest, at least in part, how this down-regulation may occur. Phorbol ester treatment activates protein kinase C, and triggers the phosphorylation of serine residues in both the cytoplasmic domain of CD4 (1, 50) and in p56^{lck} (29, 53). This phosphorylation induces the dissociation of the CD4/p56^{lck} complex (19), and the liberated CD4 is then free to enter coated pits and internalize. Whether or not these are the only steps required in down-regulation is at present unclear. Endocytosis of CD4 from the cell surface to the endosome compartment would bring about a redistribution of CD4. We have found that phorbol ester–induced phosphorylation of CD4 also increases the rate of CD4 endocytosis and may, thereby, enhance the extent of the down-regulation observed (Pelchen-Matthews, A., and M. Marsh, unpublished data). In addition to the effects of protein kinase C activation, incubation of lymphocytic cells with cross-linking anti-CD4 antibodies (52, 54) or with the HIV surface glycoprotein gp120 (21) have been reported to trigger the dissociation of CD4 from p56^{lck} and may, therefore, be expected to influence the endocytosis and cellular distribution of CD4. Whether similar reactions occur during the interaction of CD4 with MHC class II molecules remains to be determined.

p56^{lck} itself has tyrosine kinase activity and is part of a network of interacting kinases and phosphatases involved in T cell activation (2, 18, 22). Cross-linking of CD4 with certain anti-CD4 antibodies can increase the kinase activity of p56^{lck} (26, 54, 55), and lead to phosphorylation of cellular proteins such as the T cell receptor ζ chain (54). Although CD4 lacks a tyrosine residue in its cytoplasmic domain (27) and is not itself a substrate for p56^{lck}, it remains unclear whether tyrosine phosphorylation of p56^{lck} in vivo or its kinase activity influence the interaction of p56^{lck} and CD4 or affect CD4 endocytosis. However, the observation that the CD4/p56^{lck} interaction is reversible and subject to modulation indicates a mechanism through which the endocytosis of CD4 and the cellular distributions of both CD4 and p56^{lck} may be precisely regulated according to the physiological requirements of the cells.

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References

1. Acres, B. R., P. J. Conlon, D. Y. Mochizuki, and B. Gallis. 1986. Rapid phosphorylation and modulation of the T4 antigen on cloned helper T cells induced by phorbol myristate acetate or antigen. *J. Biol. Chem.* 34:16210–16214.
2. Alexander, D. R., and D. A. Cantrell. 1989. Kinases and phosphatases in T-cell activation. *Immunol. Today.* 10:200–205.
3. Anderson, R. G. W., M. S. Brown, and J. L. Goldstein. 1977. Role of the coated endocytic vesicle in the uptake of receptor-bound low density lipoprotein in human fibroblasts. *Cell.* 10:351–364.
4. Bedinger, P., A. Moriarty, R. C. von Borstel II, N. J. Donovan, K. S. Steimer, and D. R. Littman. 1988. Internalization of the human immunodeficiency virus does not require the cytoplasmic domain of CD4. *Nature (Lond.).* 334:162–165.
5. Blue, M., D. A. Hafler, K. A. Craig, H. Levine, and S. F. Schlossman. 1987. Phosphorylation of CD4 and CD8 molecules following T cell triggering. *J. Immunol.* 139:3949–3954.
6. Clapham, P. R., R. A. Weiss, A. G. Dalgleish, M. Exley, D. Whitby, and N. Hogg. 1987. Human immunodeficiency virus infection of monocytic and T-lymphocytic cells: receptor modulation and differentiation induced by phorbol ester. *Virology.* 158:44–51.
7. Cruikshank, W. W., J. L. Greenstein, A. C. Theodore, and D. M. Center. 1991. Lymphocyte chemoattractant factor induces CD4-dependent intracytoplasmic signaling in lymphocytes. *J. Immunol.* 146:2928–2934.
8. David-Pfeuty, T., and Y. Nouvian-Dooghe. 1990. Immunolocalization of the cellular *src* protein in interphase and mitotic *c-src* overexpresser cells. *J. Cell Biol.* 111:3097–3116.
9. Doyle, C., and J. L. Strominger. 1987. Interaction between CD4 and class II MHC molecules mediates cell adhesion. *Nature (Lond.).* 330:256–259.
10. Fischer, S., R. Fagard, I. Boulet, and J. C. Gesquière. 1987. The amino terminal region of the p56^{lck} from LSTRA exerts negative modulation on the tyrosine kinase activity. *Biochem. Biophys. Res. Commun.* 143:819–826.
11. Foley, G. E., H. Lazarus, S. Farber, B. G. Uzman, B. A. Boone, and R. E. McCarthy. 1965. Continuous culture of human lymphoblasts from peripheral blood of a child with acute leukemia. *Cancer (Phila.).* 18: 522–529.
12. Griffiths, G., R. Back, and M. Marsh. 1989. A quantitative analysis of the endocytic pathway in baby hamster kidney cells. *J. Cell Biol.* 109:2703–2720.
13. Healey, D., L. Dianda, J. P. Moore, J. S. McDougal, M. J. Moore, P. Estess, D. Buck, P. D. Kwong, P. C. L. Beverley, and Q. J. Sattentau. 1990. Novel anti-CD4 monoclonal antibodies separate human immunodeficiency virus infection and fusion of CD4$^+$ cells from virus binding. *J. Exp. Med.* 172:1233–1242.
14. Helenius, A., J. Kartenbeck, K. Simons, and E. Fries. 1980. On the entry of Semliki Forest virus into BHK-21 cells. *J. Cell Biol.* 84:404–420.
15. Hopkins, C. 1985. Coated pits and their role in membrane receptor internalization. *In* Molecular mechanisms of transmembrane signalling. P. Cohen and M. D. Houslay, editors. Elsevier/North Holland, Amsterdam. 337–357.
16. Hoxie, J. A., D. M. Matthews, K. J. Callahan, D. L. Cassel, and R. A. Cooper. 1986. Transient modulation and internalization of T4 antigen induced by phorbol esters. *J. Immunol.* 137:1194–1201.
17. Hoxie, J. A., J. L. Rackowski, B. S. Haggarty, and G. N. Gaulton. 1988. T4 endocytosis and phosphorylation induced by phorbol esters but not by mitogen or HIV infection. *J. Immunol.* 140:786–795.
18. Hunter, T. 1989. Protein-tyrosine phosphatases: the other side of the coin. *Cell.* 58:1013–1016.
19. Hurley, T. R., K. Luo, and B. M. Sefton. 1989. Activators of protein kinase C induce dissociation of CD4, but not CD8, from p56^{lck}. *Science (Wash. DC).* 245:407–409.
20. Jing, S., T. Spencer, K. Miller, C. Hopkins, and I. S. Trowbridge. 1990. Role of the human transferrin receptor cytoplasmic domain in endocytosis: localization of a specific signal sequence for internalization. *J. Cell Biol.* 110:283–294.
21. Juszczak, R. J., H. Turchin, A. Truneh, J. Culp, and S. Kassis. 1991. Effect of human immunodeficiency virus gp120 glycoprotein on the association of the protein tyrosine kinase p56^{lck} with CD4 in human T lymphocytes. *J. Biol. Chem.* 266:11176–11183.
22. Klausner, R. D., and L. E. Samelson. 1991. T cell antigen receptor activation pathways: the tyrosine kinase connection. *Cell.* 64:875–878.
23. Koch, C. A., D. Anderson, M. F. Moran, C. Ellis, and T. Pawson. 1991. SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins. *Science (Wash. DC).* 252:668–674.
24. Lipsich, L. A., A. J. Lewis, and J. S. Brugge. 1983. Isolation of monoclonal antibodies that recognize the transforming proteins of avian sarcoma viruses. *J. Virol.* 48:352–360.
25. Louie, R. R., C. S. King, A. Macauley, J. D. Marth, R. M. Perlmutter, W. Eckhart, and J. A. Cooper. 1988. p56^{lck} protein-tyrosine kinase is cytoskeletal and does not bind to polyomavirus middle T antigen. *J. Virol.* 62:4673–4679.
26. Luo, K., and B. M. Sefton. 1990. Cross-linking of T-cell surface molecules CD4 and CD8 stimulates phosphorylation of the *lck* tyrosine protein kinase at the autophosphorylation site. *Mol. Cell Biol.* 10:5305–5313.
27. Maddon, P. J., D. R. Littman, M. Godfrey, D. E. Maddon, L. Chess, and R. Axel. 1985. The isolation and nucleotide sequence of a cDNA encoding the T cell surface protein T4: a new member of the immunoglobulin gene family. *Cell.* 42:93–104.
28. Marsh, M., J. E. Armes, and A. Pelchen-Matthews. 1990. Endocytosis and recycling of CD4. *Biochem. Soc. Trans.* 18:139–144.
29. Marth, J. D., D. B. Lewis, M. P. Cooke, E. D. Mellins, M. E. Gearn, L. E. Samelson, C. B. Wilson, A. D. Miller, and R. M. Perlmutter. 1989. Lymphocyte activation provokes modification of a lymphocyte-specific protein tyrosine kinase (p56^{lck}). *J. Immunol.* 142:2430–2437.
30. Meltzer, M. S., D. R. Skillman, D. L. Hoover, B. D. Hanson, J. A. Turpin, D. C. Kalter, and H. E. Gendelman. 1990. Macrophages and the human immunodeficiency virus. *Immunol. Today.* 11:217–223.
31. Miller, K., M. Shipman, I. S. Trowbridge, and C. R. Hopkins. 1991. Transferrin receptors promote the formation of clathrin lattices. *Cell.* 65:621–632.
32. Morgenstern, J. P., and H. Land. 1990. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res.* 18:3587–3596.
33. Morikawa, Y., H. A. Overton, J. P. Moore, A. J. Wilkinson, R. L. Brady, S. J. Lewis, and I. M. Jones. 1990. Expression of HIV-1 gp120 and human soluble CD4 by recombinant baculoviruses and their interaction *in vitro*. *AIDS Res. Hum. Retroviruses.* 6:765–773.
34. Parnes, J. R. 1989. Molecular biology and function of CD4 and CD8. *Adv. Immunol.* 44:265–311.
35. Pawson, T. 1988. Noncatalytic domains of cytoplasmic protein-tyrosine kinases: regulatory elements in signal transduction. *Oncogene.* 3:491–495.
36. Pelchen-Matthews, A., J. E. Armes, and M. Marsh. 1989. Internalization and recycling of CD4 transfected into HeLa and NIH3T3 cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:3641–3649.
37. Pelchen-Matthews, A., J. E. Armes, G. Griffiths, and M. Marsh. 1991. Differential endocytosis of CD4 in lymphocytic and nonlymphocytic cells. *J. Exp. Med.* 173:575–587.
38. Pelchen-Matthews, A., J. E. Armes, and M. Marsh. 1992. Endocytosis of CD4 in lymphoid and nonlymphoid cells. *In* Current Topics in Endocytosis, NATO ASI Series, Vol. H62. P. J. Courtoy, A. Dautry-Varsat, J. Gruenberg, L. Mata, M. Marsh, A. Ryter, and B. van Deurs, editors. Springer Verlag, Berlin, Germany. 355–361.
39. Perlmutter, R. M., J. D. Marth, S. F. Ziegler, A. M. Garvin, S. Pawar, M. P. Cooke, and K. M. Abraham. 1988. Specialized protein tyrosine kinase proto-oncogenes in hematopoietic cells. *Biochim. Biophys. Acta.* 948:245–262.
40. Rivas, A., S. Takada, J. Koide, G. Sonderstrup-McDevitt, and E. G. Engleman. 1988. CD4 molecules are associated with the antigen receptor complex on activated but not resting T cells. *J. Immunol.* 140:2912–2918.
41. Robey, E., and R. Axel. 1990. CD4: collaborator in immune recognition and HIV infection. *Cell.* 60:697–700.
42. Rosenstein, Y., S. J. Burakoff, and S. H. Herrmann. 1990. HIV-gp120 can block CD4-class II MHC-mediated adhesion. *J. Immunol.* 144:526–531.
43. Rudd, C. E. 1990. CD4, CD8 and the TCR-CD3 complex: a novel class of protein-tyrosine kinase receptor. *Immunol. Today.* 11:400–406.
44. Rudd, C. E., J. M. Trevillyan, J. D. Dasgupta, L. L. Wong, and S. F. Schlossman. 1988. The CD4 receptor is complexed in detergent lysates to a protein-tyrosine kinase (pp58) from human T lymphocytes. *Proc. Natl. Acad. Sci. USA.* 85:5190–5194.
45. Ryu, S.-E., P. D. Kwong, A. Truneh, T. G. Porter, J. Arthos, M. Rosenberg, X. Dai, N. Xuong, R. Axel, R. W. Sweet, and W. A. Hendrickson. 1990. Crystal structure of an HIV-binding recombinant fragment of human CD4. *Nature (Lond.).* 348:419–426.
46. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Second Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 16.30.
47. Sattentau, Q. J., and R. A. Weiss. 1988. The CD4 antigen: physiological ligand and HIV receptor. *Cell.* 52:631–633.

48. Shaw, A. S., K. E. Amrein, C. Hammond, D. F. Stern, B. M. Sefton, and J. K. Rose. 1989. The *lck* tyrosine protein kinase interacts with the cytoplasmic tail of the CD4 glycoprotein through its unique amino-terminal domain. *Cell.* 59:627–636.
49. Shaw, A. S., J. Chalupny, J. A. Whitney, C. Hammond, K. E. Amrein, P. Kavathas, B. M. Sefton, and J. K. Rose. 1990. Short related sequences in the cytoplasmic domains of CD4 and CD8 mediate binding to the amino-terminal domain of the $p56^{lck}$ tyrosine protein kinase. *Mol. Cell. Biol.* 10:1853–1862.
50. Shin, J., C. Doyle, Z. Yang, D. Kappes, and J. L. Strominger. 1990. Structural features of the cytoplasmic region of CD4 required for internalization. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:425–434.
51. Turner, J. M., M. H. Brodsky, B. A. Irving, S. D. Levin, R. M. Perlmutter, and D. R. Littman. 1990. Interaction of the unique N-terminal region of tyrosine kinase $p56^{lck}$ with cytoplasmic domains of CD4 and CD8 is mediated by cysteine motifs. *Cell.* 60:755–765.
52. Veillette, A., M. A. Bookman, E. M. Horak, and J. B. Bolen. 1988. The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase $p56^{lck}$. *Cell.* 55:301–308.
53. Veillette, A., I. D. Horak, and J. B. Bolen. 1988. Post-translational alterations of the tyrosine kinase $p56^{lck}$ in response to activators of protein kinase C. *Oncogene Res.* 2:385–401.
54. Veillette, A., M. A. Bookman, E. M. Horak, L. E. Samelson, and J. B. Bolen. 1989. Signal transduction through the CD4 receptor involves the activation of the internal membrane tyrosine-protein kinase p56. *Nature (Lond.).* 338:257–259.
55. Veillette, A., J. C. Zúñiga-Pflücker, J. B. Bolen, and A. M. Kruisbeek. 1989. Engagement of CD4 and CD8 expressed on immature thymocytes induces activation of intracellular tyrosine phosphorylation pathways. *J. Exp. Med.* 170:1671–1681.
56. Wang, J., Y. Yan, T. P. J. Garrett, J. Liu, D. W. Rodgers, R. L. Garlick, G. E. Tarr, Y. Husain, E. L. Reinherz, and S. C. Harrison. 1990. Atomic structure of a fragment of human CD4 containing two immunoglobulin-like domains. *Nature (Lond.).* 348:411–418.
57. Weyand, C. M., J. Goronzy, and C. G. Fathman. 1987. Modulation of CD4 by antigenic activation. *J. Immunol.* 138:1351–1354.