The Protein Tyrosine Kinase $p56^{lck}$ Regulates Cell Adhesion Mediated by CD4 and Major Histocompatibility Complex Class II Proteins

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Summary

The CD4 protein is expressed on a subset of human T lymphocytes that recognize antigen in the context of major histocompatibility complex (MHC) class II molecules. Using Chinese hamster ovary (CHO) cells expressing human CD4, we have previously demonstrated that the CD4 protein can mediate cell adhesion by direct interaction with MHC class II molecules. In T lymphocytes, CD4 can also function as a signaling molecule, presumably through its intracellular association with $p56^{lck}$, a member of the *src* family of protein tyrosine kinases. In the present report, we show that $p56^{lck}$ can affect cell adhesion mediated by CD4 and MHC class II molecules. The expression of wild-type $p56^{lck}$ in CHO-CD4 cells augments the binding of MHC class II^+ B cells, whereas the expression of a mutant $p56^{lck}$ protein with elevated tyrosine kinase activity results in decreased binding of MHC class II^+ B cells. Using site-specific mutants of $p56^{lck}$, we demonstrate that the both the enzymatic activity of $p56^{lck}$ and its association with CD4 are required for this effect on CD4/MHC class II adhesion. Further, the binding of MHC class II^+ B cells induces CD4 at the cell surface to become organized into structures resembling adherens-type junctions. Both wild-type and mutant forms of $p56^{lck}$ influence CD4-mediated adhesion by regulating the formation of these structures. The wild-type *lck* protein enhances CD4/MHC class II adhesion by augmenting the formation of CD4-associated adherens junctions whereas the elevated tyrosine kinase activity of the mutant $p56^{lck}$ decreases CD4-mediated cell adhesion by preventing the formation of these structures.

Their cell–cell adhesion between T lymphocytes and their targets is an important prerequisite for efficient T cell stimulation. Initial interactions are mediated, in part, by members of the integrin family, including LFA-1, whose avidity for its counter-receptor on target cells can be upregulated through occupancy and signaling through the T cell receptor (reviewed in references 1, 2). The antigen-specific $\alpha\beta$ TCR recognizes foreign peptides displayed within the polymorphic antigen-binding cleft of MHC molecules (3). Both CD4, on helper T cells, and CD8, on cytotoxic T cells, also play an important role in efficient T cell activation. The role of CD4 and CD8 as co-receptors important for antigen recognition and signaling has now become established (4), however, their importance as adhesion molecules remains controversial. We have previously demonstrated an interaction between human CD4 and MHC class II molecules that can mediate cell adhesion even in the absence of the TCR (5, 6). Similar studies have demonstrated an interaction between CD8 and MHC class I molecules (7).

We have recently reported the characterization of CD4/class II adhesion using Chinese hamster ovary (CHO)[1] cells that express the human CD4 protein on the cell surface (CHO-CD4 cells) (6). Cell adhesion mediated by CD4 and MHC class II molecules is energy dependent and an intact cytoskeleton is required for the establishment and maintenance of stable cell conjugates. Analysis of the time course of cell binding revealed that the adhesion of radiolabeled MHC class II^+ Raji B cells was only detected after 2 h of incubation at 37°C, yet cell conjugates remained stably associated for at least 6–8 h thereafter. We therefore questioned whether the observed time course for CD4/class II adhesion was physiologically relevant and sought to determine whether other T cell molecules, not present in CHO cells, might be involved in regulating adhesion/de-adhesion events.

$p56^{lck}$, a lymphoid-specific tyrosine kinase, is associated intracellularly with CD4 (for review see references 8, 9), suggesting that the *lck* protein might somehow play a regulatory role in adhesion through CD4. To test this hypothesis, we expressed either the wild-type ($p56^{lck}$ Y505) or a mutant form ($p56^{lck}$ F505) of the *lck* protein in CHO-CD4 cells. In resting T lymphocytes, the $p56^{lck}$ protein is phosphorylated at its carboxy-terminal tyrosine residue (10–12) and replacement of Y505 by phenylalanine (F505) results in an activated form of the protein that has been shown to cause oncogenic

[1] *Abbreviation used in this paper:* CHO, Chinese hamster ovary.

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transformation when expressed in NIH 3T3 cells (13). Upon T cell activation, it is thought that the CD45 phosphatase acts to dephosphorylate Y505, thereby activating the $p56^{lck}$ protein (14, 15). Less well understood is the concomitant phosphorylation at Y394, that appears to be required for the kinase activity of the protein (16–18). In vivo phosphorylation at this site can be measured in cells expressing the F505 form of the kinase, and to a lesser extent, upon antibody-mediated cross-linking of surface CD4 on cells expressing the wild-type kinase (12, 18, 19). Antibody-mediated cross-linking of cell surface CD4 also results in the increased kinase activity of $p56^{lck}$ as measured by in vitro kinase assay (11). Mutation of tyrosine to phenylalanine at position 394 prevents the activation of $p56^{lck}$ by CD4 cross-linking, implying that phosphorylation at this site provides a crucial positive regulatory signal.

In this report, we demonstrate that the expression of $p56^{lck}$ can dramatically alter cell adhesion mediated by CD4 and MHC class II molecules. Both the magnitude and time course of B cell adhesion to CHO-CD4 cells are affected by the presence of the $p56^{lck}$ protein and mutations in the *lck* protein which affect its enzymatic activity alter its ability to enhance CD4/class II interactions. In the presence of the wild-type protein, cell surface CD4 protein becomes clustered at regions of cell–cell contact into structures resembling adherens-type junctions (20–22). Further, a large proportion of cell surface CD4 molecules are found to be associated with the actin cytoskeleton in the presence of the wild type protein. By contrast, adherens type junctions are not seen in the presence of the constitutively active F505 mutant of $p56^{lck}$ nor is the enhanced association of cell surface CD4 with the cytoskeleton. These observations suggest a mechanism by which the *lck* protein tethers CD4 molecules to the actin cytoskeleton thereby facilitating or stabilizing the clustering of CD4 molecules on the cell surface resulting in enhanced cell adhesion.

Materials and Methods

Cell Culture and Antibodies. B lymphoblastoid cell lines and hybridomas were cultured in RPMI (RPMI 1640; GIBCO BRL, Gaithersburg, MD) supplemented with 10% FCS, 15 mM Hepes, 2 mM glutamine, penicillin, and streptomycin. CHO-DUKX II ($CD4^-$) and CHO-CD4 cells were maintained in MEM-α (GIBCO BRL) as described (6, 23). Hybridomas secreting the mAbs TS1/18 (anti-LFA1β; CD18), TS1/22 (anti-LFA1α; CD11a; reference 24), OKT4 (mouse anti–human CD4), and GK1.5 (rat anti-L3T4) were obtained from the American Type Culture Collection (Rockville, MA) and used as culture supernatants or ascites fluid.

Transfection of $p56^{lck}$. CHO-DUKX II or CHO-CD4 cells were co-transfected with 20 μg of the pNUT recombinant expression vector containing the murine wild-type (Y505) or mutant (F505) $p56^{lck}$ cDNAs (generously provided by R. Perlmutter, University of Washington, Seattle, WA) and 2.0 μg of the pCDneo plasmid using the BES-(*N,N*-bis-[2-hydroxyethyl]-2-aminoethanesulfonic acid; Calbiochem-Novabiochem Corp., La Jolla, CA) calcium phosphate transfection technique (6, 25). After transfection, the cells were selected in media containing 150 μg/ml G418 (GIBCO BRL) and survivors were cloned by limiting dilution.

Northern Blot Analysis. Total cellular RNA was isolated by acid guanidium-phenol-chloroform extraction (26). 15 μg of RNA was size fractionated by formaldehyde-agarose gel electrophoresis (27) and blotted onto Hybond-N (Amersham Corp., Arlington Heights, IL) nylon membrane. Hybridization and washing was performed as described (28). The probe was the SmaI fragment (625–1,382 bp) of the $p56^{lck}$ cDNA that was radiolabeled with [^{32}P]dCTP using the random primed DNA labeling kit (United States Biochemical Corp., Cleveland, OH).

Immunoblotting. 5×10^6 cells were washed with PBS and lysed in a solution containing 50 mM Tris-HCl, pH 8.0, 1% NP-40, 2 mM EDTA, 100 μM Na_3VO_4, 18 mg/ml PMSF, and 20 μg/ml leupeptin. Human CD4 was immunoprecipitated from CHO-CD4 cells using the OKT4 mAb and murine CD4 was immunoprecipitated from LSTRA cells using the GK1.5 mAb. SDS-PAGE and immunoblot analysis with anti–$p56^{lck}$ rabbit polyclonal antisera (kindly provided by Dr. Roger Perlmutter, University of Washington) was also performed as described (29). Immunoreactive proteins were visualized using alkaline phosphatase–conjugated goat anti–rabbit IgG (GIBCO BRL) or ^{125}I-labeled protein A-Sepharose, as indicated.

Cell Adhesion Assay. The binding of radiolabeled Raji B cells to adherent CHO ($\pm$ $p56^{lck}$) cells was performed as previously described (6). All of the results presented in the text are averages of duplicate samples. For optimal binding, 0.25 ml of radiolabeled Raji B cells were added to each confluent CHO-containing well yielding a final volume of 0.5 ml containing 1×10^6 B cells/well and incubated at 37°C in 5% CO_2 for 1–4 h. Unbound labeled B cells were removed by repeated washes in PBS (+1 mM Ca^{2+}, Mg^{2+}) and the remaining bound cells were disrupted by hypotonic lysis. Radioactivity was measured in a β counter and the number of cells bound in each well was quantified by the following algorithm: cells bound = number of added cells × experimental value (cpm bound)/(total cpm).

Cytoskeletal Association Assay. The association between CD4 and the cytoskeleton was assessed as previously described (30). Briefly, CHO-CD4 cells, resuspended in trypsin-EDTA solution (GIBCO BRL), were stained with FITC-conjugated OKT4 mAbs (Ortho Diagnostic Systems Inc., Westwood, MA) for 1 h at 4°C. Molecules not associated with the cytoskeleton were solubilized by treatment with detergent (1.0% NP-40) for 20 min at room temperature. After two washes with PBS, samples were analyzed by flow cytometry (FACS®).

Adherens Junction Assay. CHO-CD4 cells, grown to confluence in eight-well chamber slides (Nunc Roskilde, Denmark), were incubated with 1×10^5 Raji B cells for 4 h at 37°C, washed at 4°C to remove bound Raji B cells, and fixed with 3.7% formaldehyde/PBS solution for 30 min at room temperature. After three washes with 50 mM NH_4Cl/PBS, the samples were permeabilized with 0.05% NP-40/PBS for 20 min at room temperature and washed with PBS. The samples were then incubated for 30 min each at room temperature with relevant primary and secondary antibodies. Samples were mounted with FluorSave (Calbiochem Novabiochem Corp., La Jolla, CA) to minimize photobleaching and observed by indirect immunofluorescence microscopy (×630, Zeiss). Adherens junction formation was scored in coded samples by assessing the fraction of CHO-CD4 cells displaying aggregated molecule(s). At least 100 CHO cells in at least 10 different fields of view were counted per sample and the data presented is representative of at least three separate experiments.

Site-directed Oligonucleotide Mutagenesis. Point mutants of the $p56^{lck}$ molecule were constructed by site-directed mutagenesis according to the method of Kunkel (31). The following mismatched oligonucleotides (mutation underlined): CA20 (cysteine to alanine at residue 20): $^{5'}$(ACATTGACGTG<u>G</u>CTGAA)$^{3'}$; YF394 (tyrosine to phenylalanine at residue 394): $^{5'}$(GGACAATGAGT<u>T</u>CA

CGG)3′, and KA273 (lysine to alanine at residue 273): 5′(TGG-CGGTGGCGAGTCTGA)3′, were annealed to M13-p56^{lck} single-stranded uracil-containing template DNA and second strand synthesis was carried out as recommended by the manufacturer (Bio-Rad Laboratories, Cambridge, MA). Mutants were screened by dideoxy-sequence analysis using the Sequenase enzyme (Version 2; United States Biochemical Corp.) and double-stranded mutant DNA was ligated into the pNUT expression vector (13) for amplification in *Escherichia coli* (DH-5α) and subsequent transfection into CHO cells.

Results

Expression of p56^{lck} in CHO-CD4 Cells. CHO-DUKX (CD4 negative) and CHO-CD4 (0.80 μm methotrexate [MTX]) cells were co-transfected with plasmids encoding resistance to neomycin and either wild-type p56^{lck} (Y505) or a previously described mutant p56^{lck} cDNA that encodes a phenylalanine in place of the tyrosine at residue 505 (p56^{lck} F505) (13). Subclones of transfected cells were isolated by limiting dilution. The presence of p56^{lck} was confirmed by Northern blot analysis and immunoblotting of CD4 immunoprecipitates with p56^{lck} antisera (Fig. 1). In addition, both wild-type and mutant forms of p56^{lck} were shown to promote in vivo tyrosine phosphorylation of cellular substrates in p56^{lck}-transfected CHO-CD4 cells as assessed by immunoprecipitation and Western blotting with phosphotyrosine-specific antibodies (data not shown).

p56^{lck} Regulates CD4/MHC Class II Adhesion. Cell adhesion assays were performed using transfected CHO-CD4 cells (±p56^{lck}) and MHC class II–positive Raji B lymphoblastoid cells. After incubation of the CHO-CD4 monolayers with radiolabeled Raji B cells for 1–4 h at 37°C, the plates were washed vigorously to remove unbound cells. The remaining bound cells were lysed and radioactivity was measured. In all cases, cells expressing wild-type p56^{lck} (CHO-CD4-Y505 cells) displayed enhanced binding of Raji cells as compared to CHO-CD4 cells lacking p56^{lck} (Fig. 2 *A*). Clones 5 and 7 are representative and were those used in the experiments presented in this report. Both the magnitude as well as the time course of CD4/MHC class II adhesion were profoundly altered (Fig. 2 *B*). Whereas adhesion of radiolabeled Raji B lymphocytes to CHO-CD4 monolayers was only detected after 2–4 h of incubation (1.5 × 10^5 radiolabeled B cells), similar levels of B cell adhesion to CHO-CD4-Y cells were readily observed within 1 h after the addition of radiolabeled B cells and continued to increase for 6 h until 5.3 × 10^5 B cells were bound. By contrast, the binding of Raji to CHO-CD4 cells transfected with the mutant F505 kinase (CHO-CD4-F505 clones 2 and 4) was comparable to or less than binding to CHO-CD4 cells (Fig. 2, *A* and *B*). Direct visualization of a typical binding assay, using tubulin-specific antibodies that stained both the CHO and Raji B cells, revealed that individual CHO-CD4-Y505 cells bound multiple B lymphocytes whereas CHO-CD4 and CHO-CD4-F505 clones bound single MHC class II$^+$ B cells (Fig. 3). All assays were performed in media containing antibodies directed against LFA-1 (mAb TS1/22) to prevent homotypic aggregation of human B lymphocytes (6). Therefore, we interpret this clustering to represent the simultaneous binding of multiple Raji B cells to individual CHO-CD4-Y505 cells. Increased binding was dependent upon the interaction of CD4 with MHC class II molecules, inasmuch as the binding of Raji

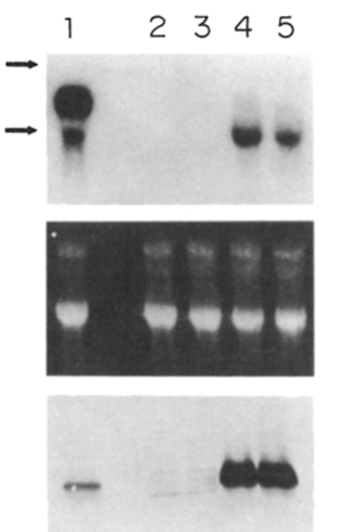

Figure 1. The expression of p56^{lck} in CHO-CD4 cells. Northern blot analysis of RNA (*top*) isolated from LSTRA, a murine thymoma that overexpresses p56^{lck} (lane *1*; reference 60) CHO-DUKX II (CD4-negative) (lane *2*), CHO-CD4 (lane *3*), CHO-CD4 cells transfected with p56^{lck} F505 (CHO-CD4-F505, clone 2) (lane *4*), and CHO-CD4 cells transfected with wild-type p56^{lck} Y505 (CHO-CD4-Y505, clone 5) (lane *5*) cells. The relative mobilities of 28S and 18S ribosomal RNA are indicated (*arrows*) and ethidium bromide staining of total RNA is also shown (*middle*). Immunoblot analysis of CD4 immunoprecipitates using p56^{lck} specific rabbit polyclonal antiserum (*bottom*).

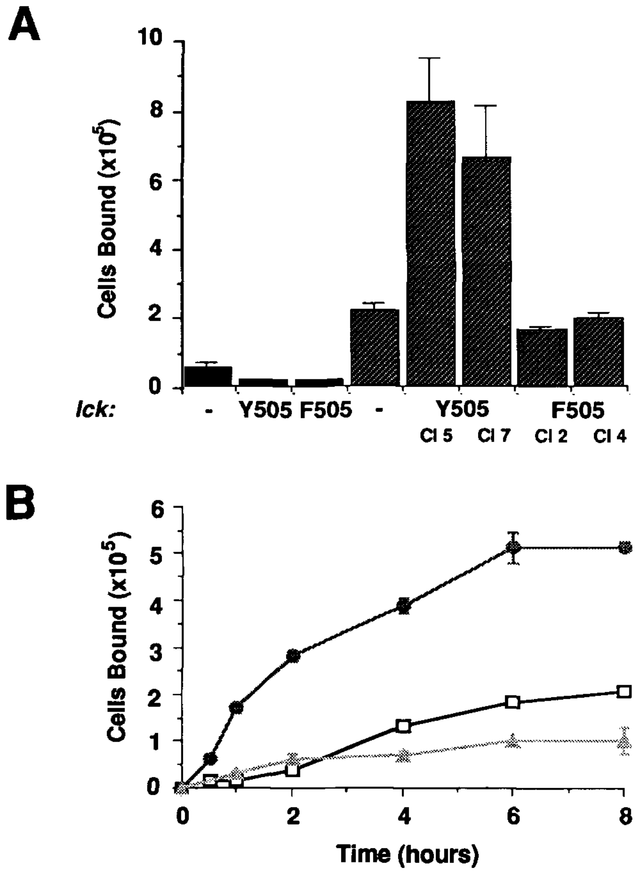

Figure 2. p56^{lck} regulates CD4/MHC class II adhesion. (*A*) The binding of radiolabeled Raji B cells to confluent monolayers of CHO-DUKX II (CD4-negative) (■) and CHO-CD4 (▨) clones expressing the wild-type (Y505) or mutant (F505) forms of p56^{lck}. Binding was assessed after 4 h of incubation at 37°C. (*B*) Time course of binding of radiolabeled Raji B cells to CHO-CD4 (□), CHO-CD4-Y505, clone 5 (●), and CHO-CD4-F505, clone 2 (▲).

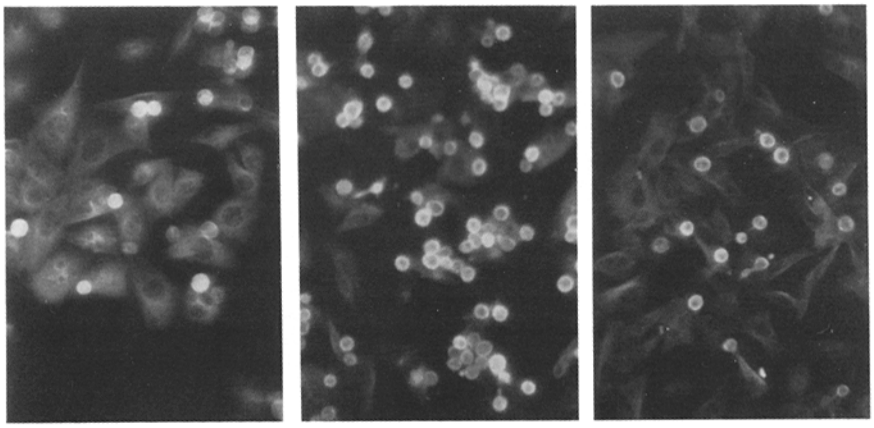

Figure 3. Multiple MHC class II$^+$ Raji B cells bind CHO-CD4-Y505 cells. The binding of Raji B cells to monolayers of CHO-CD4 (*left*), CHO-CD4-Y505, clone 5 (*middle*) and CHO-CD4-F505, clone 2 (*right*) cells after 4 h of incubation at 37°C was visualized by indirect immunofluorescence staining of both cell types with the tubulin-specific mAb YL1/2 (BioProducts for Science, Indianapolis, IN).

to CHO-DUKX II (CD4-negative) cells transfected with either wild-type or mutant kinase was equivalent to untransfected controls (Fig. 2 *A*, CHO-DUKX-Y505 and CHO-DUKX-F505). Moreover, the binding of Raji to CHO-CD4-Y505 and CHO-CD4-F505 cells was completely inhibited by CD4 and MHC class II–specific mAbs (data not shown).

A trivial explanation for the altered binding of MHC class II$^+$ B cells to CHO-CD4-Y505 and CHO-CD4-F505 cells is that expression of p56^{lck} resulted in increased levels of CD4 at the cell surface. Indeed, the expression of p56^{lck} in nonlymphoid cells inhibits CD4 endocytosis by excluding CD4 from coated pits (32, 33). However, despite a dramatic increase in adhesion, CHO-CD4-Y505, clone 5, displayed levels of CD4 at the cell surface that were comparable to CHO-CD4 cells, as determined by immunostaining and FACS® analysis (Fig. 4). Further, reduced adhesion of Raji to CHO-CD4-F505, clone 2, was observed even though this clone displayed more CD4 at the cell surface than CHO-CD4 cells or CHO-CD4-Y505, clone 5 (Fig. 4). Thus, the altered binding of Raji cells to CHO-CD4-Y505 cells or CHO-CD4-F505 cells was not the result of altered surface expression of CD4 in transfected cells.

Overexpression of *src* kinases has been demonstrated to cause changes in cell morphology and the expression of the activated form of p56^{lck} in NIH 3T3 cells has previously been shown to cause morphologic transformation (13). We therefore asked whether CHO-CD4–transfected cells were affected by the expression of either form of the *lck* protein. There were no obvious differences in growth rates, cell size, or shape. Cytoskeletal structure, including microtubule and microfilament organization, was comparable in transfectants (in the absence of Raji B cells) as determined by indirect immunofluorescence microscopy of permeabilized cells (data not shown). Finally, to determine whether expression of any member of the *src* family of tyrosine kinases in CHO-CD4 cells was sufficient to modify CD4–class II adhesion, we transfected a plasmid encoding the c-*src* protein into CHO-CD4 cells (Fig. 5 *A*). Over-expression of c-*src*, which does not specifically interact with CD4, did not significantly alter Raji binding ($P < 0.05$).

The Association of CD4 and p56^{lck} Is Required for the Regulation of MHC Class II Adhesion. In resting T lymphocytes, CD4 is stably associated with p56^{lck}. This intermolecular binding is facilitated by interactions between two pairs of cysteine residues in the amino terminal domain of p56^{lck} and in the cytoplasmic domain of CD4. Mutation of any of these cysteines completely abrogates the association of CD4 and p56^{lck} (29, 34, 35). To examine whether the association between CD4 and p56^{lck} is required for the regulation of CD4/MHC class II binding, an alanine was substituted for the cysteine at position 20 in the cDNA(s) encoding both the Y505 and F505 forms of p56^{lck}. The mutant constructs (Y505^{CA20} or F505^{CA20}) were then expressed in CHO-CD4 cells and control experiments were performed to demonstrate that the *lck* protein could not be immunoprecipitated in association with CD4 (data not shown). Adhesion assays were

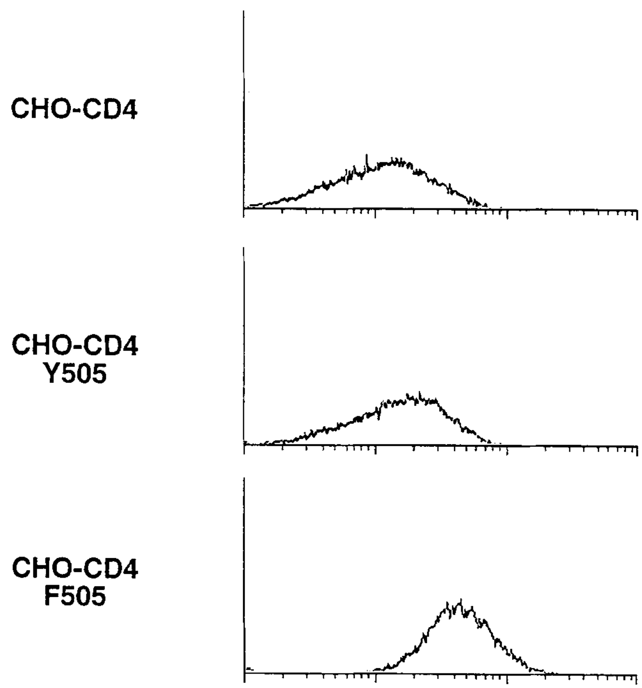

Figure 4. Cell surface expression of CD4 in CHO-CD4 cells (±p56^{lck}). CHO-CD4, CHO-CD4-Y505 (clone 5), and CHO-CD4-F505 (clone 2) cells were stained with the OKT4 mAb followed by FITC-conjugated goat anti–mouse IgG and analyzed by FACS®.

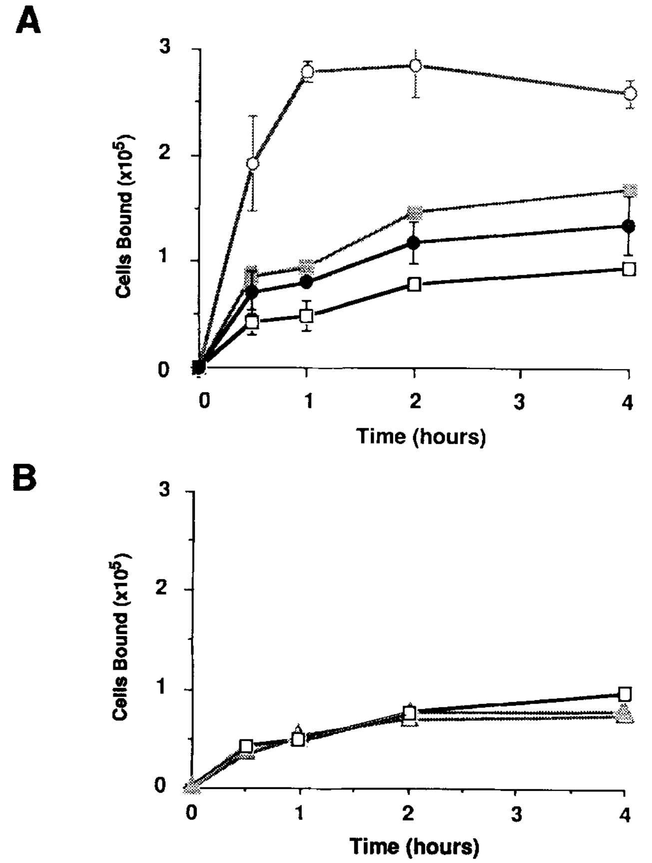

Figure 5. The association of CD4 and $p56^{lck}$ is necessary for enhanced MHC class II adhesion. Time course of binding of radiolabeled Raji B cells to monolayers of CHO-CD4 (□), CHO-CD4-Y505 (O), CHO-CD4-$Y505^{CA20}$ (●), and CHO-CD4^{src} (■) cells. (*B*) Time course of binding of radiolabeled Raji B cells to monolayers of CHO-CD4 (□), CHO-CD4-F505 (▲), and CHO-CD4-$F505^{CA20}$ (△) cells.

then performed as described above. The binding of Raji to CHO-CD4 cells expressing the single mutant ($Y505^{CA20}$) was decreased as compared with CHO-CD4-Y505 cells; displaying kinetics and magnitude of binding that were similar to CHO-CD4 cells (Fig. 5 *A*). By contrast, the binding of Raji to CHO-CD4-$F505^{CA20}$ cells was not significantly different from CHO-CD4-F505 cells (Fig. 5 *B*). These data demonstrate that the specific association of wild-type $p56^{lck}$ with CD4 is necessary for the rapid time course and augmented binding of MHC class II–expressing B cells.

The Enzymatic Activity of $p56^{lck}$ Is Required for Enhanced CD4/Class II Adhesion. To address whether the enzymatic activity of $p56^{lck}$ was important for its regulation of CD4/MHC class II adhesion, a lysine to alanine change was engineered at residue 273 (KA273) which has previously been shown to be essential for the phosphotransferase activity of the protein (36). CHO-CD4 cells expressing the kinase-deficient form of the protein (CHO-CD4-$Y505^{KA273}$) bound fewer Raji cells as compared with CHO-CD4-Y505 cells; both the time course and magnitude of adhesion were decreased (Fig. 6 *A*). To confirm further that the tyrosine kinase activity of wild-type $p56^{lck}$ was important for augmented CD4/MHC class II adhesion, the binding assay was performed in the presence of genistein; an ATP analogue that specifically binds the catalytic subunit of tyrosine kinases (Fig. 6 *B*) (37). The binding of Raji to genistein-treated CHO-CD4-Y505 cells was equivalent to parental CHO-CD4 cells; fewer Raji cells bound genistein-treated CHO-CD4-Y505 cells. Genistein had little or no effect in binding assays using CHO-CD4 cells or CHO-CD4-F505 cells presumably due to the small differences between the number of Raji cells bound to these transfectants at the 4 h time point when binding was measured. Thus, both the constitutively active ($p56^{lck}$ F505) as well as inactive forms of the wild-type kinase (p56lck$Y505^{KA273}$ or genestein-treated CHO-CD4-Y505 cells) fail to augment CD4/MHC class II adhesion. Rather, this appears to be a unique property of wild-type *lck* protein.

To further analyze the functional requirements of the transfected *lck* proteins expressed in CHO-CD4 cells, the tyrosine at position 394 was changed to phenylalanine (YF394) to abrogate the enzymatic activity of *lck*. Previous studies have demonstrated that the tyrosine kinase activity of $p56^{lck}$ is positively regulated by a critical tyrosine at residue 394 and mutation of this residue prevents the activation of *lck* (17). Again, mutations which rendered the protein kinase deficient resulted in a loss of phenotype ascribed to the kinase-active proteins. CHO-CD4-$Y505^{YF394}$ cells bound fewer Raji cells as compared with CHO-CD4-Y505 cells (Fig. 6 *C*), whereas the binding of Raji to CHO-CD4-$F505^{YF394}$ cells was slightly increased as compared to CHO-CD4-F505 cells (Fig. 6 *D*). These data are consistent with results obtained with KA273 mutants implying that the YF394 mutation is functionally equivalent to the KA273 mutation in this assay. Moreover, the observed differences in activity between constructs that are wild-type at position 394 and those in which the tyrosine has been changed to phenylalanine (YF394) suggest that $p56^{lck}$ can be regulated by phosphorylation at position 394 in CHO cells, consistent with reports that this site is autophosphorylated in vivo. Finally, the levels of the *lck* kinase in each of the transfectants were analysed by Western blotting using *lck*-specific antisera (Fig. 7). All of the transfectants appeared to express similar levels of wild-type or mutant forms of the protein suggesting that differences in adhesion observed with transfectants were not due to variability in the levels of $p56^{lck}$.

CD4-associated Adherens-type Junctions. A reorganization of CD4 and cytoskeletal elements within the cell membrane of T cells bound to APCs has been previously described by Kupfer and Singer (38–40). Thus we sought to determine whether the binding of MHC class II$^+$ B cells was associated with an altered distribution of CD4 within the cell membrane of CHO-CD4 cells. For these experiments, monolayers of CHO-CD4 cells were incubated with Raji B cells for 4 h at 37°C to promote optimal adhesion. Inasmuch as the maintenance of stable CD4/MHC class II adhesion is temperature dependent (6), the B cells were removed by incubation at 4°C. The monolayers were then fixed, permeabilized, immunostained with OKT4 mAbs, and visualized by indirect immunofluorescence microscopy. By contrast with the diffuse distribution of CD4 on control CHO-CD4 cells that had not encountered MHC class II$^+$ B lymphocytes,

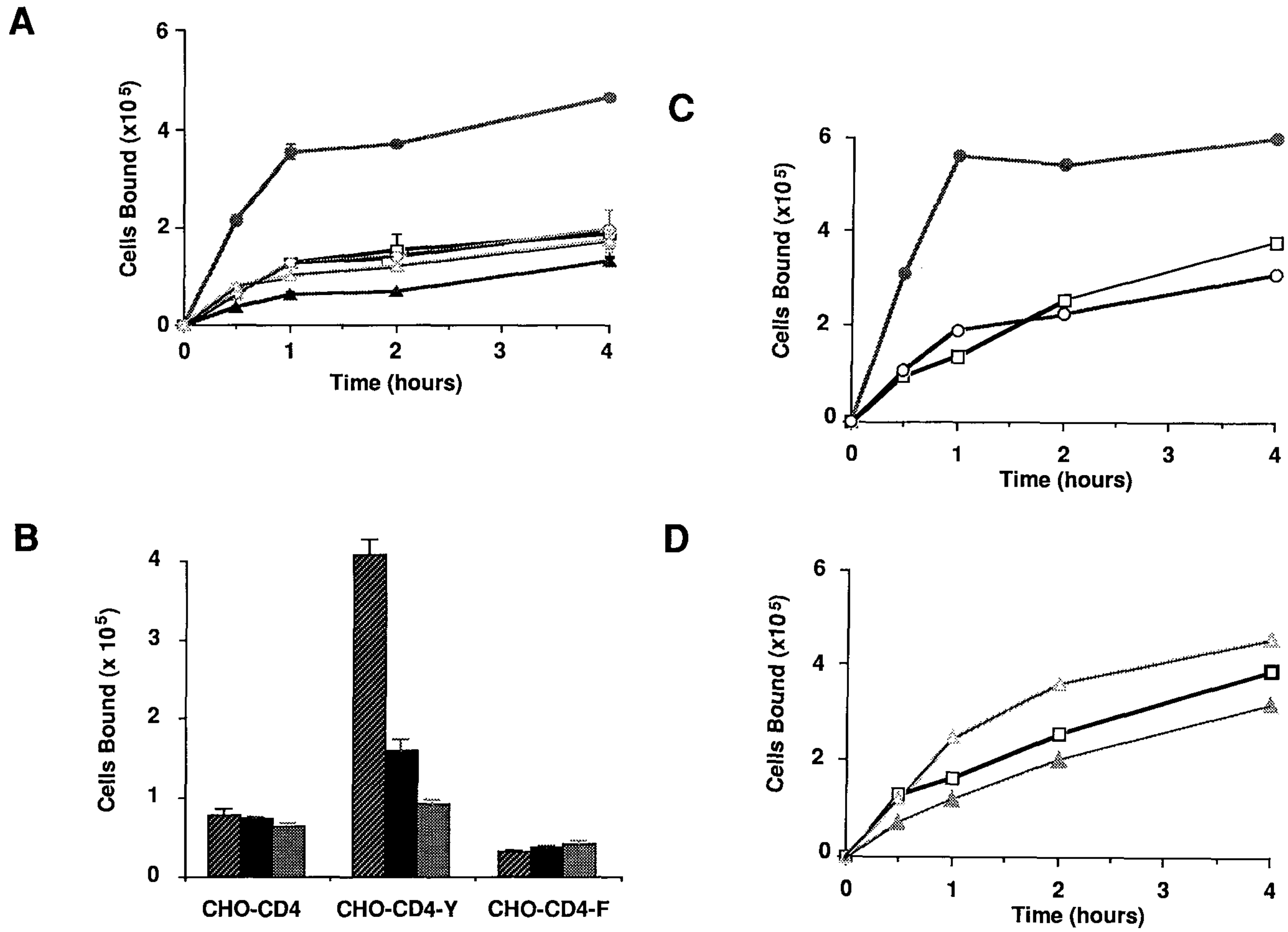

Figure 6. The protein tyrosine kinase activity of $p56^{lck}$ regulates CD4/MHC class II adhesion. (*A*) The binding of radiolabeled Raji B cells to monolayers of CHO-CD4 (□), CHO-CD4-Y505 (●), CHO-CD4-F505 (▲), CHO-CD4-Y505^{KA273} (○), and CHO-CD4-F505^{KA273} (△) cells. (*B*) The binding of radiolabeled Raji B cells to monolayers of CHO-CD4, CHO-CD4-Y505, or CHO-CD4-F505 cells in the absence (5 μl DMSO, ▨), or presence of genistein (20 μg/ml, ■; 100 μg/ml, ▩). Genistein was present throughout the entire four hour incubation. (*C*) The binding of radiolabeled Raji B cells to monolayers of CHO-CD4 (□), CHO-CD4-Y505 (●), CHO-CD4-Y505^{YF394} (○) cells. (*D*) The binding of radiolabeled Raji B cells to monolayers of CHO-CD4 (□), CHO-CD4-F505 (▲) and CHO-CD4-F505^{YF394} (△) cells.

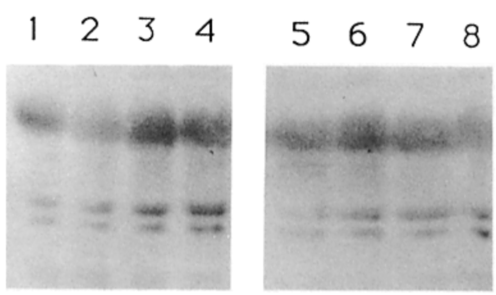

Figure 7. Expresion of wild-type and mutant $p56^{lck}$ proteins in transfected CHO-CD4 cells. Equivalent amounts of total cell protein were prepared from cell lysates of transfected cells as follows: (*1*) CHO-CD4-Y505, (*2*) CHO-CD4-Y505^{CA20}, (*3*) CHO-CD4-Y505^{KA273}, (*4*) CHO-CD4-Y505^{YF394}, (*5*) CHO-CD4-F505, (*6*) CHO-CD4-F505^{CA20}, (*7*) CHO-CD4-F505^{KA273}, (*8*) CHO-CD4-F505^{YF394} . Proteins were separated by SDS-PAGE, transferred to nitrocellulose and incubated with $p56^{lck}$-specific antiserum followed by ^{125}I-labeled protein A-Sepharose.

CD4 was found to be localized in patches at the cell surface after incubation with Raji B lymphocytes (Fig. 8). A corresponding redistribution of vinculin in CHO-CD4 cells was also detected after the binding of MHC class II–expressing B cells. Similar results were obtained with talin and α-actinin (data not shown). The selective enrichment and redistribution of cytoskeletal proteins with CD4 at junctional zones likely functions to stabilize CD4/MHC class II–mediated cell adhesion by increasing the local density of CD4 capable of interacting with MHC class II molecules.

$p56^{lck}$ Promotes the Association of CD4 with the Cytoskeleton. Interactions between cell surface molecules and the cytoskeleton can regulate the efficiency of ligand binding. For example, the binding of fibroblasts to extracellular matrix components is anchored by interactions between integrin molecules and components of the actin cytoskeleton (41). A similar accumulation of cadherins into adherens-type junctions stabilizes cell–cell interactions (42). Consequently, pharmacological agents that disrupt the cytoskeleton, such as cytochalasins, also inhibit integrin- and cadherin-mediated adhesion events.

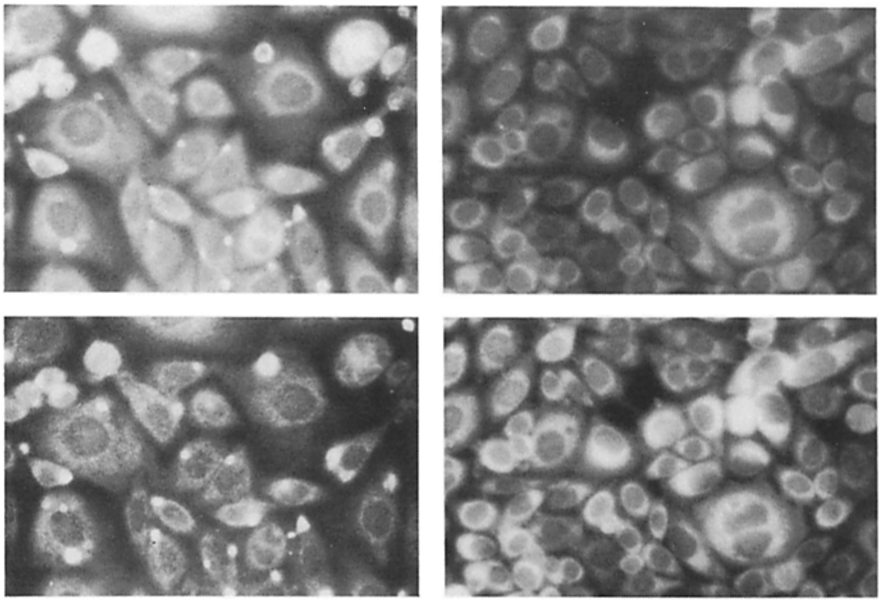

Figure 8. CD4 aggregation into adherens-type junctions. Monolayers of CHO-CD4 cells were incubated for 4 h in the presence (*left*) or absence (*right*) of Raji B cells. After the removal of bound MHC class II$^+$ B cells at 4°C, the monolayers were fixed, permeabilized, and stained for CD4 (OKT4 followed by rhodamine-conjugated goat anti-mouse IgG) (*top*) or vinculin (rabbit polyclonal sera followed by FITC-conjugated goat anti-rabbit IgG) (*bottom*). Staining was visualized by indirect immunofluorescence microscopy (×630).

Using similar strategies, we have observed that stable CD4/MHC class II adhesion requires an intact cytoskeleton. Pharmacological agents such as cytochalasin D, colchicine, and nocodazole, were shown to disrupt CHO-CD4-Raji B cell conjugates (6). To further examine how cytoskeletal interactions might regulate CD4/MHC class II binding and to determine the role of p56^{lck} in these interactions, we assessed the physical association of CD4 with the cytoskeleton by measuring whether cell surface CD4 (± p56^{lck}) was resistant to solubilization with non-ionic detergent. Using this technique, Geppert and Lipsky have previously demonstrated that a fraction (~20%) of CD4 on peripheral blood T cells is associated with the cytoskeleton (30). Cytoskeletal association can be evaluated experimentally by staining cell surface proteins with fluorescently labeled mAbs, treating the cells with detergent to remove molecules not associated with the cytoskeleton, and assessing the remaining bound label by flow cytometry. For these experiments, CHO-CD4, CHO-CD4-Y505, and CHO-CD4-F505 cells were labeled with OKT4-FITC mAb, treated with 1.0% NP-40, and analyzed by flow cytometry (Fig. 9). Virtually all of the CD4 at the cell surface of CHO-CD4-Y505 cells was resistant to NP-40 treatment thus implying a strong association with detergent-insoluble cytoskeletal components. By contrast, the majority of cell surface CD4 in CHO-CD4 and CHO-CD4-F505 cells was solubilized by detergent treatment and thus not associated with the cytoskeleton. These data indicate that the wild-type, but not the mutant (p56^{lck}F505), form of p56^{lck} promotes interactions between CD4 and the cytoskeleton thereby suggesting a mechanism by which p56^{lck} might regulate CD4/MHC class II adhesion via the formation of CD4-associated adherens junctions.

p56^{lck} Regulates the Formation of CD4-associated Adherens Junctions. Previous studies have shown that the level of protein tyrosine kinase activity regulates the formation and stability of focal adhesions. For example, the formation of these structures can be blocked with specific tyrosine kinase inhibitors (43). Elevated protein tyrosine kinase activity can also dissociate integrin-mediated junctional interactions (44, 45). Inasmuch as the level of tyrosine phosphorylation can differentially regulate the formation and stability of such cellular junctions, we sought to determine whether either form of the p56^{lck} tyrosine kinase influenced the formation of CD4-associated adherens junctions. After incubation with Raji, the fraction of CHO-CD4 cells displaying aggregated CD4 was measured by indirect immunofluorescence staining with the OKT4 mAb. Adherens junction formation is reported as the fraction of CHO-CD4 cells that display aggregated OKT4 staining (Fig. 10). In both CHO-CD4 and CHO-CD4-Y505 cells, the formation of adherens junctions preceded detectable binding (compare with Fig. 2 *B*). Moreover, CD4 adherens junctions were detected more rapidly in CHO-CD4-Y505 cells than in CHO-CD4 cells; consistent with the enhanced time course and magnitude of Raji adhesion to CHO-CD4-Y505 cells. Further, a greater fraction of CHO-CD4-Y505 cells displayed aggregated CD4 as compared with control CHO-CD4 cells after 4 h of incubation with Raji B cells (Fig. 10). Adherens junction formation was also analyzed on CHO-CD4 clones expressing the KA273 mutants of p56^{lck} (CHO-CD4-Y505^{KA273} and CHO-CD4-F505^{KA273} cells). Both the time course and magnitude of CD4 aggregation were decreased with CHO-CD4-Y505^{KA273} cells and more closely resembled the pattern of adherens junction formation seen in CHO-CD4 cells lacking p56^{lck} (Fig. 10). Thus, the protein tyrosine kinase activity of wild-type p56^{lck} likely increases CD4/MHC class II adhesion by augmenting the formation of CD4-associated adherens junctions. We propose that p56^{lck} mediates an increased association of CD4 with the cytoskeleton thereby facilitating the formation of adherens junctions, resulting in enhanced cell–cell adhesion.

Strikingly, CD4 aggregation could not be detected on CHO-CD4-F505 cells at any time after the introduction of MHC class II$^+$ B cells (Fig. 10). However, CD4 aggregation was detectable on CHO-CD4 clones expressing the

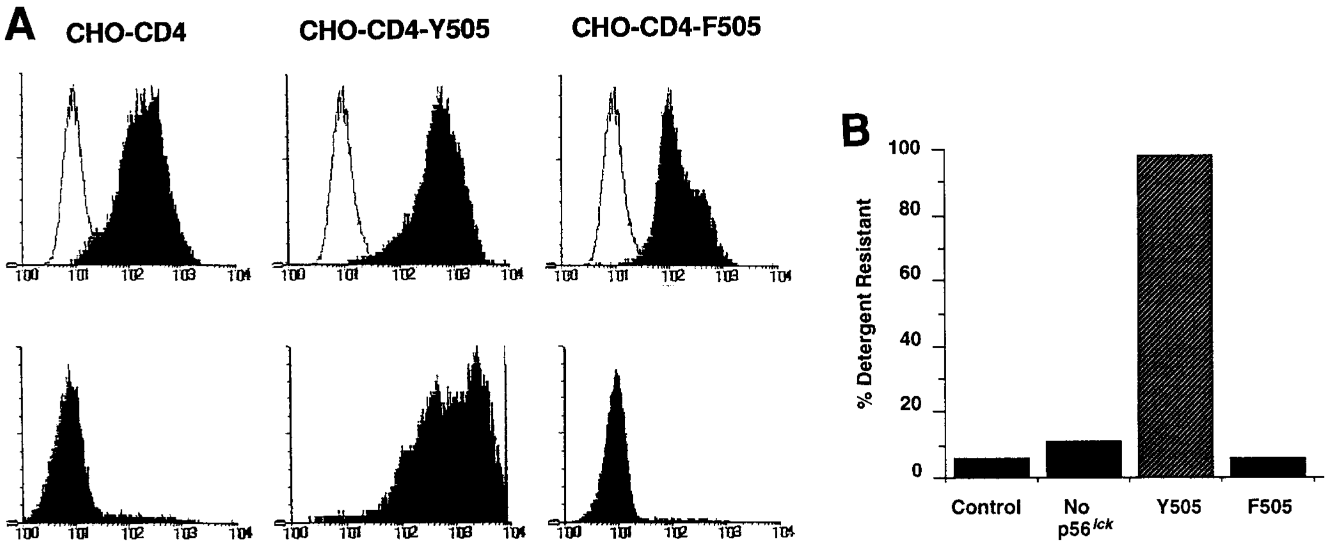

Figure 9. The association of CD4 with the cytoskeleton is enhanced by the presence of p56^{lck}. The association of CD4 with the cytoskeleton was assessed by immunostaining of CHO-CD4 cells (±p56^{lck}) with FITC-conjugated OKT4 mAbs followed by treatment with 1.0% NP-40. (*A*) FACS® profiles of CHO-CD4, CHO-CD4-Y505, and CHO-CD4-F505 cells before (*top*) or after (*bottom*) treatment with detergent. Staining with negative control P3X63 mAb is shown in open profiles, OKT4 staining in filled profiles. (*B*) The fraction of control (CHO-DUXK II, CD4-negative), CHO-CD4 (no p56^{lck}), CHO-CD4-Y505 (clone 5), and CHO-CD4-F505 (clone 2) cells expressing detergent-resistant CD4 molecules was assessed by flow cytometry. The results presented are representative of three different experiments.

kinase-deficient p56^{lck}F505^{KA273} double mutant (Fig. 10, △); suggesting that the enzymatic activity of the F505 mutant p56^{lck} prevents CD4 aggregation. Moreover, the inability of CD4 to aggregate on CHO-CD4-F505 cells correlates with the decreased binding of MHC class II$^+$ Raji B cells. In summary, these data strongly argue that elevated tyrosine phosphorylation by p56^{lck} decreases CD4/MHC class II adhesion by inhibiting the formation of CD4-associated adherens junctions that are required for stable cell–cell adhesion mediated by CD4 and MHC class II molecules.

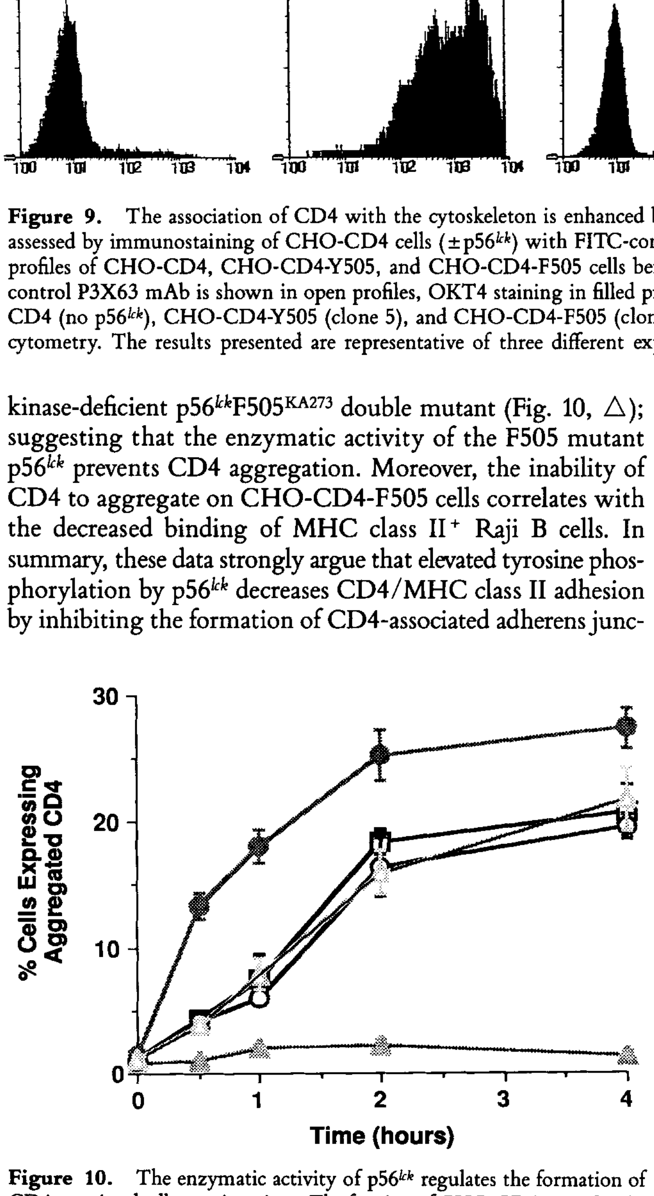

Figure 10. The enzymatic activity of p56^{lck} regulates the formation of CD4-associated adherens junctions. The fraction of CHO-CD4 (□), CHO-CD4-Y505 (●), CHO-CD4-Y505^{KA273} (O), CHO-CD4-F505 (▲), and CHO-CD4-F505^{KA273} (△) cells expressing aggregated CD4 after incubation with Raji B lymphocytes (4 h, 37°C). Adherens junction formation was assessed by indirect immunofluorescence staining with FITC-conjugated OKT4 mAb. At least 100 CHO cells representing at least 10 different fields of view were counted for each sample and the data is representative of at least three separate experiments.

Discussion

The data presented in this report demonstrate that the expression of a wild-type or constitutively activated form of the p56^{lck} protein in CHO-CD4 cells differentially regulates the adhesion of MHC class II$^+$ B cells. Both the magnitude and the time course of CD4/MHC class II adhesion are enhanced by the presence of wild-type p56^{lck} as compared to cells expressing mutant *lck* proteins. The *lck* protein likely influences CD4/MHC class II adhesion by effecting a redistribution of CD4, and other cytoskeletal proteins, at the cell surface into adherens-type junctions at the site of interaction with an MHC class II–expressing cell.

How might wild-type p56^{lck} augment CD4/class II adhesion and the formation of adherens junctions? p56^{lck} is found associated with the detergent-insoluble fraction in both T cells and transfected fibroblasts (8, 46), suggesting that p56^{lck} may interact directly with cytoskeletal elements. Similarly, we observed that the presence of wild-type p56^{lck} renders CD4 resistant to detergent solubilization. We propose that *lck* interacts with components of the actin cytoskeleton thus facilitating contacts with CD4 and providing a scaffold onto which adherens junctions can efficiently assemble. Potential targets for this interaction include vinculin, talin, α-actinin, and paxillin. Indirect immunofluorescence studies reveal that these proteins do, in fact, colocalize with CD4 within adherens junctions (our manuscript in preparation). While this scaffolding function may provide a mechanism to enhance CD4 adherens junction formation, the nature of the physical linkage is not known at present. One possibility is that the amino terminus of p56^{lck} interacts with CD4 while simultaneously binding one or more cytoskeletal components through its SH2 or SH3 domains (47, 48). Alternatively, p56^{lck} may phosphorylate specific cytosolic proteins which indirectly facilitate interactions between CD4 and the cytoskeleton. A newly discovered family of tyrosine-phosphorylated proteins including cortactin and p130, as well as tyrosine kinases such as p125FAK (focal adhesion kinase), may also be involved (49).

The interaction of a T helper cell with an antigen presenting cell results from a number of adhesive interactions including those of LFA-1 with its ICAM ligands, CD2 and LFA-3, and CD4 with MHC class II molecules. Whereas LFA-1/ICAM interactions are responsible for initial adhesion events, CD4–class II adhesive interactions are only detected after 2 h (this report and references 6 and 50), implying that CD4/class II binding is probably not involved in the initial formation of T cell/APC conjugates. The inability to detect CD4/MHC class II adhesion at these earlier times likely reflects the time required for the mobilization of CD4 into adherens-type junctions. The selective enrichment of CD4 at junctional zones likely stabilizes cell adhesion by increasing the overall avidity of its interaction with MHC class II molecules and may be important for the long-term maintenance of stable T cell adhesion. This is supported by our observation that cell adhesion mediated by CD4 and class II is a stable, long-lived interaction that may be essential to the function of the T helper cell, i.e., lymphokine secretion, and is in contrast with the rapid and transient interaction of cytolytic T cells with their targets (51).

The T cell receptor, LFA-1 and components of the microtubule organizing center colocalize with CD4 in the cell membrane of T helper cells, oriented towards the site of interaction with an MHC class II^{+} B lymphocyte (38–40, 51). The reorganization of cellular components correlates with antigen-specific recognition and is not detected in T cells bound to B cells expressing irrelevant antigen (39). LFA-/ICAM binding was recognized as the predominant adhesive interaction stabilizing T/B conjugates, and it was proposed that a redistribution of LFA-1 might facilitate TCR capping at the cell/cell junction (52). As CD4 did not aggregate in the absence of LFA-1–mediated adhesion, it was presumed that the transcellular bond between CD4 and MHC class II was too weak to cause CD4 aggregation. Rather, interactions between TCR and CD4 were thought to selectively enhance CD4 accumulation at the T cell/APC junction; thereby increasing the avidity of the weak binding of CD4 to MHC class II. Thus, CD4 could only interact with MHC class II ligands once sequestered within the cell contact region. Recent observations by our laboratory do not concur with this view of CD4 function. Substantial CD4 adhesion and redistribution within the membrane can be detected in the absence of TCR or LFA-1 molecules using this adhesion assay (this report and reference 6). Moreover, there is increasing evidence that the CD4–MHC class II interaction is of sufficient avidity to contribute to T cell adhesion inasmuch as conjugates with B lymphocytes can be disrupted by CD4-specific mAbs (50, 53; and our unpublished observations). Our data support a model in which the molecular interaction of CD4 with MHC class II proteins facilitates cell adhesion by inducing the reorganization of CD4 (and perhaps the TCR and cytosolic components) at sites of cell/cell contact.

How might this active reorganization of CD4 and cytoskeletal proteins into adherens type junctions influence T cell function in vivo? CD4 adherens junctions might stabilize cellular interactions at times when other adhesive interactions are no longer operative. This notion is supported by recent studies documenting the transient nature of LFA-1–mediated adhesions (54) and cascades of T cell adhesions (55). The formation of CD4 adherens junctions might also define the area of surface contact between a T lymphocyte and specific APC. Using alloantigen immobilized onto variably sized latex beads, Mescher determined that a minimum cell diameter of 4–5 μm is necessary for the productive recognition of presented antigen (56). The diameter of a CD4-associated adherens type junction encompasses a diameter of at least 6 μm on CHO-CD4 cells (our unpublished data), suggesting that the cell contact region defined by this structure is within the range defined for productive T cell interactions. The aggregation of CD4 might directly influence T cell signal transduction by aggregating p56^{lck} molecules at the intercellular junction. Clustering of p56^{lck} is known to induce its enzymatic activities (8), potentially via autophosphorylation and the assembly of CD4 adherens junctions might provide a mechanism for the induction of tyrosine phosphorylation. Finally, biophysical measurements of receptor/ligand interactions reveal that ligand binding can facilitate cell signaling by increasing the duration of receptor aggregation (57). Consequently, CD4 adherens junctions might enhance T cell signaling by increasing the length of time in which CD4 and TCR bind their common ligand, the MHC class II molecule.

Straus and Weiss have previously demonstrated that p56^{lck} is essential for T cell activation and IL-2 production using the JCAM-1 cell line which lacks the *lck* protein (58). Similarly, we demonstrate that mutants of the *lck* protein lacking kinase activity fail to support enhanced CD4/class II–mediated cell adhesion. These data appear to conflict with several recent reports which argue that the kinase function of p56*lck* is not essential for its ability to enhance the responsiveness of a class II–reactive hybridoma (36, 59). However, in both studies, kinase-deficient *lck* proteins were expressed in parent cells which contained wild-type protein. Moreover, those studies were designed to measure T cell responsiveness and the kinase activity of the *lck* protein may be dispensable for the enhanced responsiveness of a T cell hybridoma. By contrast, we are solely measuring CD4–class II adhesion and our data demonstrate that the kinase activity of p56^{lck} is essential for enhanced cell adhesion. Inasmuch as the importance of CD4–class II adhesion has remained somewhat controversial, a direct demonstration of the regulation of CD4-mediated cell adhesion by an associated protein tyrosine kinase provides new insight into the function(s) of both CD4 and p56^{lck}. Moreover, these results have general implications for the regulation of cell adhesion by tyrosine kinases and suggest that specific elements of the cytoskeleton may act as substrates for this family of protein tyrosine kinases.

Finally, the experiments presented in this report demonstrate that the constitutively elevated tyrosine kinase activity by p56^{lck} decreases the efficiency of CD4/MHC class II adhesion and prevents the clustering of CD4 at sites of cell–cell interaction. Enhanced levels of tyrosine phosphorylation of cellular substrates are observed in the presence of the F505 form of the kinase although we have not as yet identified these proteins. Elevated tyrosine kinase activity has previously been found to disrupt the aggregation of various integrin and cadherin adhesion molecules; thereby decreasing cell/substratum and cell/cell binding (44, 45). If a similar redistribution of the cytoskeleton is necessary for the maintenance of stable T cell/APC conjugates, elevated kinase activity could transduce a signal for de-adhesion. This is currently under investigation in our laboratory.

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