Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 3277–3281, May 1989
Immunology

The CD4 and CD8 antigens are coupled to a protein-tyrosine kinase ($p56^{lck}$) that phosphorylates the CD3 complex

ELIZABETH K. BARBER*, JAI DEV DASGUPTA†, STUART F. SCHLOSSMAN*‡, JAMES M. TREVILLYAN§, AND CHRISTOPHER E. RUDD*¶‖

Divisions of *Tumor Immunology and †Immunogenetics, Dana–Farber Cancer Institute, 44 Binney Street, and the Departments of ¶Pathology and ‡Medicine, Harvard Medical School, Boston, MA 02115; and §Department of Internal Medicine, Texas Tech University Health Sciences Center, Amarillo, TX 79106

Communicated by Baruj Benacerraf, December 28, 1988

ABSTRACT **Many mammalian receptors have been found to regulate cell growth by virtue of a protein-tyrosine kinase domain in their cytoplasmic tail. We recently described an association of the CD4 antigen with a T-cell-specific protein-tyrosine kinase ($p56^{lck}$; formerly termed $pp58^{lck}$; EC 2.7.1.112). This interaction represents a potential mechanism by which T-cell growth may be regulated and offers a model by which other members of the src family (products of c-*src*, c-*yes*, c-*fgr*, etc.) may interact with mammalian growth factor receptors. As in the case of the CD4 antigen, the CD8 antigen appears to serve as a receptor for nonpolymorphic regions of products of the major histocompatibility complex and has been implicated in the regulation of T-cell growth. In this study, we reveal that the human CD8 antigen is also associated with the T-cell-specific protein-tyrosine kinase ($p56^{lck}$). The associated $p56^{lck}$ kinase was detected by use of both *in vitro* and *in vivo* labeling regimes using an antiserum to the C terminus of $p56^{lck}$. Two-dimensional nonequilibrium pH-gradient gel electrophoresis and sodium dodecyl sulfate/polyacrylamide gel electrophoresis demonstrated the similarity of $p56^{lck}$ to the protein-tyrosine kinase associated with the CD4 antigen. The catalytic activity of $p56^{lck}$ was revealed by the autophosphorylation of the 55- to 60-kDa kinase and the occasional labeling of a 35-kDa protein. Last, we demonstrate directly that members of the CD3 complex, including the γ, δ, and ε chains, as well as a putative ζ subunit, can be phosphorylated at tyrosine residues by the CD4/CD8·$p56^{lck}$ complex.**

T-cell activation is initiated by the recognition of foreign and major histocompatibility complex (MHC) antigens by the T-cell receptor (Ti/T3, TcR) in conjunction with the CD4 and CD8 accessory structures (1, 2). CD4 and CD8 adhere to nonpolymorphic regions of MHC antigen, and they have been independently implicated in the regulation of T-cell growth (3–7). Current models of signal transduction involve the hydrolysis of phosphatidylinositol 4,5-bisphosphate to D-inositol 1,4,5-trisphosphate and diacylglycerol, which in turn leads to the activation of protein kinase C (8). By contrast, tyrosine phosphorylation is a relatively rare event (accounting for only 0.01–0.02% of phosphorylation in the cell) that has been causally linked to the activation and transformation of many mammalian cells (9, 10). However, a role for a tyrosine-related pathway in the activation process of T cells has been unclear. Recently, we described an association between human CD4 and a protein-tyrosine kinase (EC 2.7.1.112) termed $p56^{lck}$ in T lymphocytes (11, 12). Preliminary evidence also revealed that the CD8 antigen precipitates with kinase activity (12). The purified CD4·$p56^{lck}$ complex is catalytically active, as observed by its ability to undergo autophosphorylation. From this, we proposed that the association with $p56^{lck}$ might constitute the underlying molecular basis of CD4 function, either independently, or in conjunction with the Ti/CD3 complex on normal and malignant T cells. It may also play a role in regulating the ability of the human immunodeficiency virus (HIV) to enter and/or replicate in $CD4^+$ cells (11, 12).

Importantly, $p56^{lck}$ (formerly named lsk^T, tck, or $pp58^{lck}$) is a member of the src family of protein-tyrosine kinases, which includes the products of the c-*src*, c-*yes*, c-*fgr*, *fyn*, *hck*, *tkl*, and *lyn* protooncogenes (13–17). It is also structurally related to receptor kinases such as the receptors for the epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin-like growth factor (I-GF), and insulin (9, 10). Abnormally high levels of lck expression have been causally linked to the development of the murine thymic lymphoma LSTRA (18). $p56^{lck}$ is phosphorylated at serine residues by protein kinase C as a consequence of T-cell activation (19). In fact, a dramatic decrease in the presence of $p56^{lck}$ has been reported in response to mitogen and phorbol ester (20). $p56^{lck}$ itself lacks a transmembrane region, instead interacting with the inner face of the plasma membrane through an N-terminal myristic moiety (9). The CD4 antigen may thus act as the ligand-binding component of the CD4·$p56^{lck}$ complex, while $p56^{lck}$ may generate signals linked to the activation and/or down-regulation of T-cell growth. An association of murine $p56^{lck}$ with the L3T4/Lyt2 antigens has recently been confirmed in mice (21).

The association between CD4 and $p56^{lck}$ provides a model by which $p56^{lck}$ (and other members of the src family) may interact with other surface receptors in the regulation of cell growth. Previous associations have involved the binding of the middle-sized tumor antigen (MTAg) of the polyoma virus with the *src* and *yes* protooncogenes (9, 22). One such candidate is the CD8 antigen, which is found predominantly on the surface of cytotoxic and suppressor T cells and recognizes the nonpolymorphic regions of class I antigens of the MHC (1, 2). Structurally, the antigen exists as a polypeptide of 32–35 kDa that can form various disulfide-linked multimers up to 200 kDa (23, 24). CD8 also appears linked to the CD1 antigen on thymocytes and HLA class I antigens on peripheral blood lymphocytes (25–28). Monoclonal antibodies to the CD8 antigen have been reported to either down-regulate or potentiate the proliferation of T lymphocytes in a manner independent of MHC recognition (4, 5, 7). In particular, crosslinking is thought to increase T-cell proliferation by increasing the proximity of the Ti/CD3 complex to the CD8 antigens (4, 5). These observations have implied that the CD8 antigen may function to generate intracellular signals involved in the activation of the T lymphocyte.

In the present study, we demonstrate in more detail that the CD8 antigen is coupled to $p56^{lck}$ from human T lymphocytes.

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Abbreviations: Con A, concanavalin A; HLA, human leukocyte antigen; MHC, major histocompatibility complex; NEPHGE, nonequilibrium pH-gradient gel electrophoresis; PMA, phorbol 12-myristate 13-acetate.

‖To whom reprint requests should be addressed.

The associated $p56^{lck}$ kinase is catalytically active as demonstrated by the autophosphorylation of a 55- to 60-kDa protein. Two-dimensional nonequilibrium pH-gradient gel electrophoresis and sodium dodecyl sulfate/polyacrylamide gel electrophoresis (NEPHGE/SDS-PAGE) analysis revealed that the kinase associated with the CD8 antigen is similar to that associated with the CD4 antigen. *In vivo* labeling in the presence of phorbol ester allowed detection of the kinase in a manner independent of catalytic activity. In addition, a comparison of reduced and nonreduced gels revealed that the association of CD8 with $p56^{lck}$ is not a result of artifactual disulfide bonding in the detergent lysate. Last, we demonstrated directly that the CD4- and CD8-associated $p56^{lck}$ is capable of phosphorylating the γ, δ, and ε subunits and a putative ζ subunit of the human CD3 complex. The implications of these findings are discussed in reference to a role for the CD4/CD8·$p56^{lck}$ complex in the activation of human T cells.

MATERIALS AND METHODS

Monoclonal Antibodies, Antisera, and Cells. The production and characterization of the monoclonal antibodies to the CD4 antigen (19thy5D7; IgG2) and the CD8 antigen (21thy2D3; IgG1) have been described elsewhere (1). The antibody W6/32 against HLA-A,B,C antigens (29) and the anti-phosphotyrosine antibody have also been described (30). The anti-protein tyrosine kinase antiserum was generated against a synthetic peptide (16). The peptide was coupled to bovine serum albumin through the N-terminal cysteine by using 3-(2-pyridyldithio)propionic acid *N*-hydroxysuccinimide ester (31).

Human peripheral blood cells and the transformed human T-cell lines MOLT 4 and REX were cultured in RPMI 1640 medium with 10% (vol/vol) fetal calf serum and 1% (wt/vol) penicillin/streptomycin (GIBCO) at 37°C in an atmosphere containing 5% CO_2. The cell lines REX and MOLT 4 have the surface phenotypes $CD3^+$ $CD4^+$ $CD8^+$ and $CD3^-$ $CD4^+$ $CD8^-$, respectively (1).

Immunoprecipitation and Kinase Analysis. Cells at 35×10^6 per ml were solubilized in 1% (vol/vol) Nonidet P-40 or Triton X-100 lysis buffer in 20 mM Tris·HCl buffer, pH 8.0, containing 150 mM NaCl and 1 mM phenylmethanesulfonyl fluoride for 30 min at 4°C, as previously described (11, 12). Antibodies to the CD4 and CD8 antigens were coupled to Sepharose beads according to Schneider *et al.* (32). The immunoprecipitates were washed three times with Nonidet P-40 lysis buffer (see below), prior to incubation with 30 μl of 25 mM Hepes, pH 7.2, containing 0.1% Nonidet P-40 and 1–10 μCi (1 Ci = 37 GBq) of [γ-^{32}P]ATP (ICN). After an incubation of 15–30 min at 25°C, the reaction mixture was subjected to SDS-PAGE and autoradiography (33). For the analysis of reprecipitated antigen, the reaction mixture was supplemented to 1.0–2.0% (wt/vol) SDS, boiled for 5 min, and subsequently diluted 10- or 20-fold with lysis buffer to give 0.1% SDS. NEPHGE/SDS-PAGE analysis was conducted by using Ampholines (LKB) in the pH range 2–11 as described (34, 35).

For biosynthetic labeling, peripheral blood lymphocytes were stimulated with concanavalin A (Con A) at 5 μg/ml in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum and 1% penicillin-streptomycin for 48 hr prior to an incubation in phosphate-free RPMI medium (30×10^6 cells per ml) at 37°C for 30 min. Lymphocytes were then equilibrated for 2 hr in sodium [^{32}P]orthophosphate (NEN, 1 mCi per 10^8 cells) prior to the addition of phorbol 12-myristate 13-acetate (PMA) (Sigma) at 20 ng/ml for 20 min. Cells washed in RPMI 1640 medium were then solubilized in the Nonidet P-40 lysis buffer, which contains 1% (vol/vol) Nonidet P-40, 20 mM Tris base, 150 mM NaCl, 0.4 mM sodium vanadate, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 10 mM sodium fluoride, 10 mM sodium pyrophosphate, and 1 mM phenylmethanesulfonyl fluoride, for immunoprecipitation.

Phosphorylated amino acid analysis was conducted by eluting bands from fixed polyacrylamide gels and precipitating with trichloroacetic acid as described previously (36, 37). The precipitate was washed in acetone and hydrolyzed in 6 M HCl at 110°C for 2 hr. The individual phosphorylated amino acids were separated by electrophoresis (1000 V, 30 min) at pH 3.5 in pyridine/acetic acid/water (1:10:189, vol/vol). Nonradioactive standards were detected by ninhydrin, while radiolabeled phosphorylated amino acids were observed by autoradiography.

RESULTS

Recently, we reported that the CD4 receptor on T cells is associated in detergent lysates with a T-cell-specific protein-tyrosine kinase, $p56^{lck}$ (11, 12). The CD8 and CD4 antigens have a similar immunoglobulin-like structure and play similar roles in the associative recognition of foreign antigen by reciprocal subsets of T cells (1, 2, 38, 39). It was therefore of interest to determine whether the CD8 receptor was also associated with the kinase in T lymphocytes. In a phosphotransferase assay, the CD8 antigen and associated polypeptides were immunoprecipitated from unlabeled cells and tested for the ability to transfer the γ-phosphate of [γ-^{32}P]ATP to a substrate. Fig. 1*A* shows that a major band at 55–60 kDa was labeled in immunoprecipitates formed by an anti-CD8 antibody and extracts of the T-cell line REX (lane 3). A band at 35 kDa was also occasionally detected. Interestingly, the 55- to 60-kDa bands migrated with those precipitated by an anti-$p56^{lck}$ antiserum (lane 4). Other bands at 72 and 90 kDa were also occasionally precipitated by the antiserum. The 55- to 60-kDa band also migrated with immunoprecipitates formed by an anti-CD4 antibody (lane 2). In contrast, immunoprecipitates formed by an antibody against MHC class I antigens (W6/32) possessed no detectable phosphotransferase activity (lane 1). Specificity in the interaction was shown by the inability of the anti-CD8 antibody to precipitate kinase activity from the $CD3^-$ $CD4^+$ $CD8^-$ cell line MOLT 4 (lane 7), despite its presence in anti-CD4 (lane 6) and anti-$p56^{lck}$ precipitates (lane 8).

Many protein-tyrosine kinases such as $p56^{lck}$ can undergo autophosphorylation, an event which can regulate their catalytic activity (9, 10). Given the absence of a kinase domain in the cytoplasmic tail of CD8 (38, 39), the above data were consistent with the coprecipitation of a protein kinase with the CD8 antigen. To directly assess whether $p56^{lck}$ was associated with CD8, the labeled anti-CD8 precipitates were denatured by boiling in SDS and then reprecipitated by use of the anti-$p56^{lck}$ antiserum (Fig. 1*A*, lanes 9–11). From this assay, it was clear that the anti-$p56^{lck}$ serum recognized the 55- to 60-kDa band associated with the anti-CD8 precipitates (lane 10). In contrast, the control antibody against MHC class I antigens failed to precipitate material (lane 9). The band at 55–60 kDa was also precipitated by an anti-phosphotyrosine antibody (lane 11). The labeling at phosphotyrosine residue(s) was confirmed by phosphorylated amino acid analysis of the eluted 55- to 60-kDa band (Fig. 1*B*). Two-dimensional NEPHGE/SDS-PAGE further showed that the kinase associated with the CD8 antigen had the same molecular mass and charge (approximate pI of 4.8–5.8) as those associated with the CD4 antigen (Fig. 1*C Middle* and *Bottom*, respectively) and with the antigen in detergent lysates recognized directly by the anti-$p56^{lck}$ antiserum (Fig. 1*C Top*). Taken together, these data reveal that $p56^{lck}$, which is capable of undergoing autophosphorylation at tyrosine residue(s), exists in association with the human CD8 antigen.

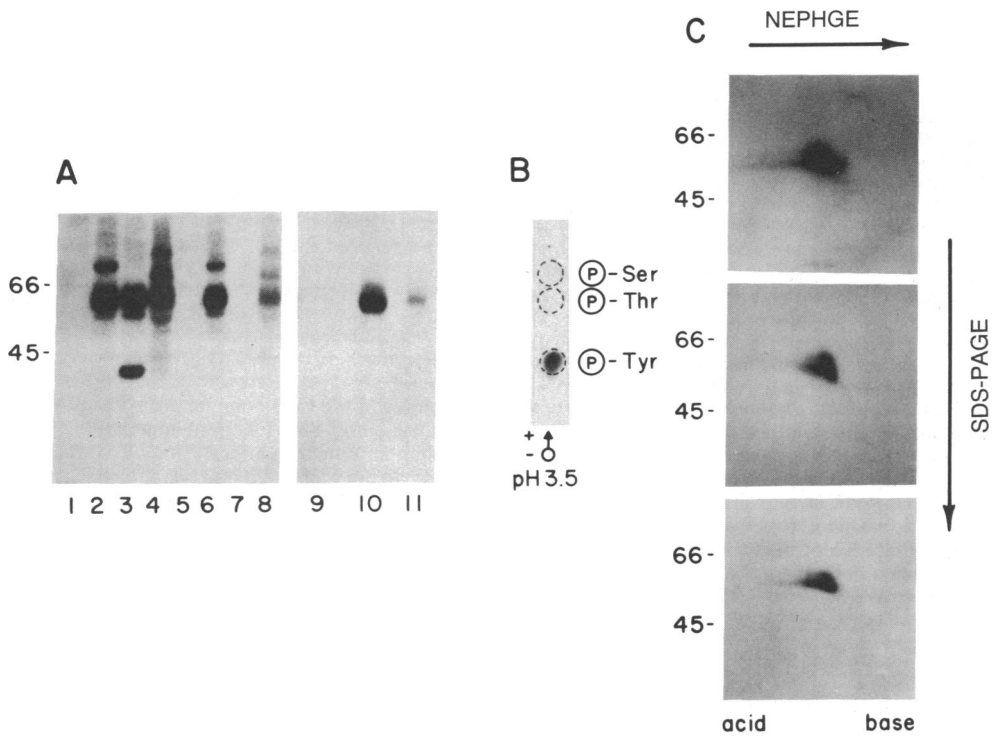

FIG. 1. $p56^{lck}$ is associated with the CD8 antigen. (*A*) Phosphotransferase activity of immunoprecipitates derived from REX (lanes 1–4) and MOLT 4 cells (lanes 5–8). Antibodies were as follows: lanes 1 and 5, rabbit anti-mouse (W6/32); lanes 2 and 6, anti-CD4; lanes 3 and 7, anti-CD8; and lanes 4 and 8, anti-$p56^{lck}$. In lanes 9–11, anti-CD8 precipitate was reprecipitated with the W6/32 antibody (lane 9), anti-$p56^{lck}$ serum (lane 10), or an anti-phosphotyrosine antiserum (lane 11). Positions of molecular mass markers (kDa) are indicated on the left. (*B*) Phosphorylated amino acid analysis of the 55- to 60-kDa band. (*C*) Two-dimensional NEPHGE/SDS-PAGE of $p56^{lck}$ labeled in the phosphotransferase assay after immunoprecipitation. (*Top*) Anti-$p56^{lck}$ antiserum; (*Middle*) anti-CD8 antibody; (*Bottom*) anti-CD4 antibody.

Another approach was to identify the physical association between $p56^{lck}$ and CD8 in a manner independent of the catalytic activity of the kinase. This was accomplished by the *in vivo* labeling of Con A-activated peripheral blood T cells with [^{32}P]orthophosphate in the presence of PMA. Fig. 2*A* shows the SDS-PAGE profile of immunoprecipitates formed by antibodies to MHC class I antigens (lane 2), the CD8 antigen (lane 3), and $p56^{lck}$ (lane 4). The autoradiograph was overexposed to reveal the presence of minor components in the immunoprecipitates. The anti-CD8 antibody precipitated a protein giving a CD8 band at about 32 kDa as well as two fainter bands at 55 and 60 kDa and two very faint bands at 72 and 90 kDa (lane 3). Each of the CD8-associated bands at 55, 60, 72, and 90 kDa appeared to migrate with those precipitated by the anti-$p56^{lck}$ antiserum (lane 4). In addition, the bands at 55 and 60 kDa migrated with the 55- to 60-kDA band derived from the phosphotransferase assay (lane 5). In contrast, the anti-class I control precipitated a single heavily labeled protein of 45 kDa (lane 2). Two-dimensional NEPHGE/SDS-PAGE analysis confirmed the identity of these polypeptides by showing that several of the CD8-associated spots focused in the same isoelectric position as the lower molecular mass polypeptides precipitated by the anti-$p56^{lck}$ serum (see arrows in Fig. 2*B*, *Bottom* and *Middle*, respectively). Several of the spots at 55–60 kDa in these patterns also migrated with the spots precipitated and labeled in the kinase assay (see arrow in Fig. 2*B*, *Top*). Peptide fingerprinting also showed the identity between the bands at 55–60 kDa (data not shown). The CD8 antigen itself focused as a series of six spots with a mass of 32 kDa, a spectrum most probably related to sialylation and phosphorylation (25–28).

To determine whether the CD8 antigen was covalently linked to $p56^{lck}$ by interchain disulfide bonds, the anti-CD8 precipitates that had undergone autophosphorylation were compared by SDS-PAGE under reducing and nonreducing conditions (Fig. 3*A*). A labeled doublet at 55–60 kDa was visualized under both reducing (lane 1) and nonreducing (lane 2) conditions, demonstrating the absence of disulfide bonds between these proteins.

Potentially important targets of the CD4- and CD8-associated $p56^{lck}$ are the CD3 subunits of the Ti/CD3 complex. An interaction mediated by $p56^{lck}$ could provide the molecular basis for the interaction between CD4/CD8 and the Ti/CD3 complex in the recognition of foreign antigen (1, 2). We therefore determined whether the CD3 complex could serve as an *in vitro* target for the CD8-associated kinase. In this assay, monoclonal antibodies to CD8 or CD3 were added

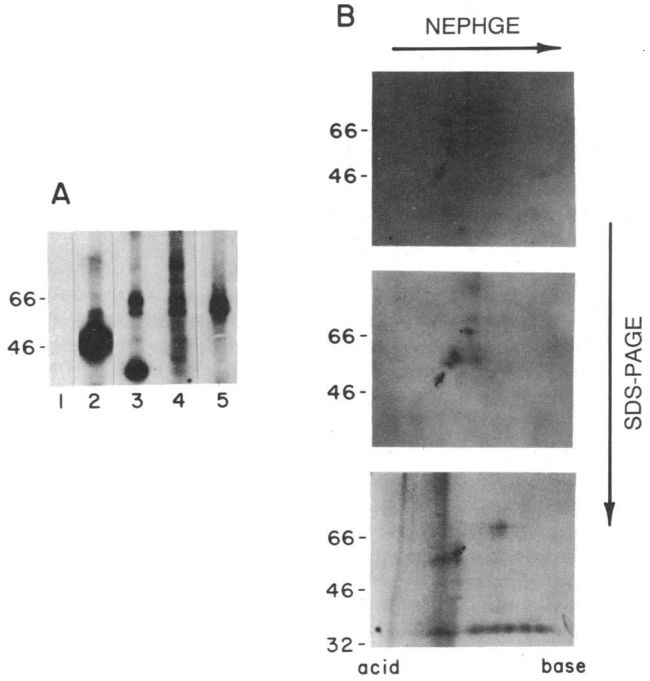

FIG. 2. *In vivo* detection of the $p56^{lck}$ association with the CD8 antigen. (*A*) SDS-PAGE profile of immunoprecipitates formed with rabbit anti-mouse antibody (lane 1), W6/32 antibody (lane 2), anti-CD8 antibody (lanes 3 and 5), and anti-$p56^{lck}$ antiserum (lane 4). The precipitate in lane 5 was derived from the kinase assay. (*B*) Two-dimensional NEPHGE/SDS-PAGE analysis of immunoprecipitates. (*Top*) Anti-$p56^{lck}$ labeled with [^{32}P]ATP in the kinase assay; (*Middle*) anti-$p56^{lck}$ labeled with [^{32}P]orthophosphate; (*Bottom*) anti-CD8 labeled with [^{32}P]orthophosphate.

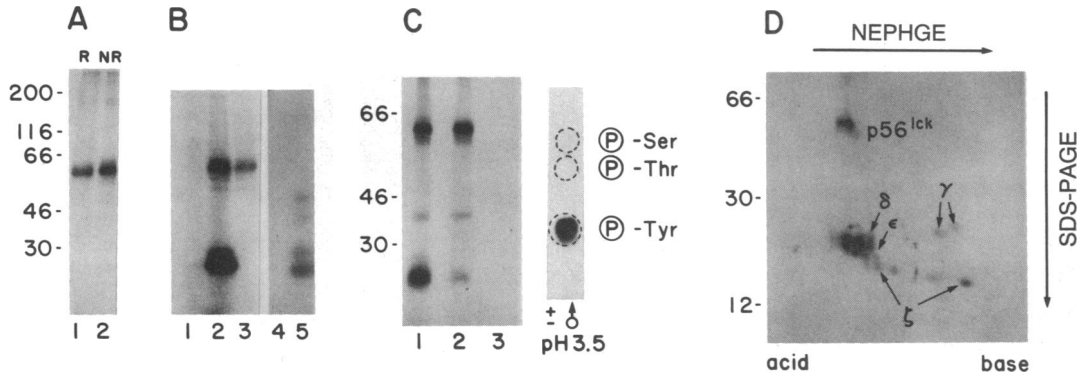

FIG. 3. The CD8·p56^{lck} complex is noncovalently linked and can phosphorylate various subunits of the CD3 complex. (*A*) SDS-PAGE of anti-CD8-associated p56^{lck} under reducing conditions (lane 1) and under nonreducing conditions (lane 2). (*B*) SDS-PAGE of immunoprecipitates formed with anti-CD3 (lane 1); combined anti-CD3 and anti-CD8 (lane 2); anti-CD8 (lane 3); rabbit anti-mouse antibody and a lysate derived from iodinated peripheral blood lymphocytes (lane 4); and anti-CD3 and a lysate derived from iodinated peripheral blood lymphocytes (lane 5). (*C*) Reprecipitation analysis of the [^{32}P]ATP-labeled CD3 subunits. Lane 1, anti-CD8/CD3 immunoprecipitate; lane 2, denatured anti-CD8/CD3 immunoprecipitate reprecipitated with the anti-phosphotyrosine antisera; and lane 3, denatured anti-CD8/CD3 immunoprecipitate reprecipitated with a W6/32 control. Right lane, phosphorylated amino acid analysis of the CD3 band. (*D*) Two-dimensional NEPHGE/SDS-PAGE analysis of the anti-CD8/CD3 immunoprecipitate labeled in the phosphotransferase assay.

to separate cell lysates, or added together in the same lysate prior to immunoprecipitation by staphylococcal protein A-Sepharose and analysis for phosphotransferase activity. Fig. 3*B* shows that while the anti-CD3 immunoprecipitate did not coprecipitate kinase activity (lane 1), the anti-CD8 antibody precipitated the labeled 55- to 60-kDa p56^{lck} (lane 3). However, the addition of [^{32}P]ATP to the CD8·p56^{lck} complex in the presence of CD3 resulted in the dramatic labeling of additional bands of 20–26 kDa (lane 2). These bands were clearly absent from the anti-CD8 precipitate alone (lane 3) and migrated with CD3 bands precipitated from iodinated T cells (lane 5). The anti-CD3 precipitate from iodinated cells included the α and β chains of the T-cell receptor at 42 and 49 kDa, a doublet generally not labeled in the phosphotransferase assay (lane 2). Reprecipitation analysis using an anti-phosphotyrosine antibody (Fig. 3*C*, lane 2) and phosphorylated amino acid analysis (Fig. 3*C*, right lane) confirmed that the CD3 band was labeled at a tyrosine residue.

Identical results were obtained when the CD4·p56^{lck} complex was analyzed by two-dimensional NEPHGE/SDS-PAGE to identify the phosphorylated CD3 subunits (Fig. 3*D*). These immunoprecipitates were derived from REX cells which had been solubilized in the detergent Triton X-100 to facilitate copurification of the ζ chain of the CD3 complex. Similar results were obtained with a combination of Triton X-100 and digitonin (data not shown). In both cases, the two-dimensional patterns revealed a spectrum of spots in the positions of the γ chain, 26 kDa, with an approximate pI of 6.5–7.2, as well as the δ and ε chains, 20 kDa, with a pI of 5.0–6.0 (40, 41). Digestion of these immunoprecipitates by endoglycosidases further showed the 26-kDa (γ) protein to be completely sensitive to endoglycosidase F, while the 20-kDa protein is composed of both endoglycosidase F-sensitive (δ) and -resistant ε polypeptides (data not shown). Interestingly, the two-dimensional pattern also showed a series of spots stretching from 16 to 20 kDa, whose mass and pI are consistent with the ζ chain of the human CD3 complex (42, 43). Collectively, these data indicated that the individual subunits of the CD3 complex can be phosphorylated *in vitro* by the CD4/CD8·p56^{lck} complex.

DISCUSSION

The demonstration that the CD8 antigen is coupled to the T-cell-specific protein-tyrosine kinase p56^{lck} could have important implications for our understanding of the molecular mechanisms that control T-cell growth. The catalytic domain of p56^{lck} shares some 85% amino acid identity with p60^{src} (13–17), and it is structurally related to domains within the cytoplasmic tail of receptors for the epidermal growth factor and insulin (9, 10). Two-dimensional NEPHGE/SDS-PAGE analysis showed that the p56^{lck} had the same mass and charge as the kinase previously found linked to the CD4 antigen (Fig. 1*C*) (11, 12). Both kinases were also recognized by the antiserum against the C terminus of p56^{lck} (Fig. 1*A*). A comparison of reduced and nonreduced gels showed that the association is not due to the formation of artifactual disulfide bonding (Fig. 3*A*), as has been observed between the heavy chains of the HLA class I antigens (44). In addition, the CD4 and CD8 antigens are the only T-cell antigens from a panel of some 30 surface antigens which have been found associated with p56^{lck} (45). Taken together, these observations support the argument that the CD8·p56^{lck} complex is likely to represent a specific interaction within the T cell. The selective association of p56^{lck} with CD4 and CD8 is also compatible with their similar roles as accessory structures in aiding the activation of T cells with low affinity for antigen (46).

Interestingly, p56^{lck} was found associated with both the CD4 and CD8 antigens when they are coexpressed on the surface of the same cell (Fig. 1, lanes 2 and 3, respectively). The T-cell thymoma REX has a CD3$^+$ CD4$^+$ CD8$^+$ phenotype (1). Therefore, the surface expression of CD4 antigen does not prevent the association of p56^{lck} with the CD8 antigen, and vice versa. It is presently unknown whether there is a competition for p56^{lck} and whether different events regulate its association with the two receptors. However, the linkage of p56^{lck} with both CD4 and CD8 is consistent with the notion that both receptors could utilize the kinase in the differentiation of CD3$^+$ CD4$^+$ CD8$^+$ thymocytes to a more mature CD3$^+$ CD4$^+$ CD8$^-$ or CD3$^+$ CD4$^-$ CD8$^+$ phenotype. The copurification of p56^{lck} from the T-cell leukemia line MOLT 4 suggests that the Ti/CD3 complex need not be expressed on the cell surface for p56^{lck} to associate with CD4 (Fig. 1, lane 6). MOLT 4 cells do not express detectable levels of CD3 on the cell surface (47, 48). Thus, although the Ti/CD3 complex may interact with the CD4·p56^{lck} complex, its presence on the cell surface does not appear to be required for the formation of the CD4·p56^{lck} complex.

Importantly, the CD8·p56^{lck} complex could be identified from peripheral blood T cells which had been labeled biosynthetically with [^{32}P]orthophosphate in the presence of PMA (Fig. 2). Several of the lower molecular mass (55–60 kDa) proteins migrated with those derived from the kinase assay. The occasional presence of higher molecular mass

spots from the kinase assay is probably related to differences in the degree of phosphorylation (Fig. 2*B*). Bands at 72 and 90 kDa were found in both the anti-CD8 and anti-p56^{lck} immunoprecipitates from biosynthetically labeled cells (Fig. 2*A*). The presence of these additional polypeptides suggests that other components within the cell may be involved in the CD8·p56^{lck} association.

The association of p56^{lck} with the CD8 and CD4 receptors may mediate the cooperative interaction between these accessory structures and the CD3/Ti complex. Copurification of the CD4/CD8·p56^{lck} complex with the CD3 complex clearly demonstrates that the various subunits of CD3 can be phosphorylated at a tyrosine residue(s) by p56^{lck} (Fig. 3 *B–D*). The labeled pattern includes the well-characterized γ, δ, and ε subunits, as well as a series of spots which focused in the position of the ζ subunit of the CD3 complex. Similar results have recently been obtained with the CD4·p56^{lck} complex from mutants of the T-cell line HPB-ALL which vary in their expression of the ζ chain (C.E.R., J. Sancho, and C. Terhorst, unpublished data). Each of the CD3 subunits possesses a potential tyrosine phosphorylation site within its cytoplasmic tail (48–50). It is noteworthy that the CD3 antigens were the only polypeptides from amongst 12 different T-cell antigens tested (CD1, CD2, CD5, CD6, CD9, CD25, CD29, CD45, etc.) which were found to act as a substrate for p56^{lck}. Thus, this type of analysis allows for a direct assessment of the potential targets of p56^{lck}. However, *in vivo* analysis will be required to demonstrate whether the substrate specificity of this interaction is more restricted inside the intact cell and whether different pathways of activation cause the phosphorylation of different CD3 chains. To date, only the murine and human ζ chains have been found to be phosphorylated at a tyrosine residue during T-cell activation (42, 43). By contrast, the γ and δ chains of the human CD3 complex have been found to be phosphorylated at a serine residue (51). Rapid dephosphorylation of substrates by the various tyrosine phosphatases such as the putative enzyme domain within the cytoplasmic tail of the CD45 antigen could interfere with the detection of the other substrates (52). It remains to be determined whether p56^{lck} can associate with the different forms of the CD8 antigen (i.e., CD1·CD8, HLA-A, B·CD8, and oligomers) found on the surface of thymocytes and peripheral blood cells and whether interactions with these surface molecules can regulate the CD8·p56^{lck} linkage and its ability to phosphorylate the CD3 complex.

Note Added in Proof. A comparison of CD4 and CD8 cytoplasmic sequences reveals a possible binding site for p56^{lck} at the CD4 sequence Lys-Lys-Thr-Cys-Gln-Cys-Pro-His-Arg-Phe-Gln-Lys-Thr and the CD8 sequence Arg-Arg-Val-Cys-Lys-Cys-Pro-Arg-Pro-Val-Val-Lys-Ser.

We thank Drs. Gene Levinson and Paul Kaplan for helpful suggestions in reading this manuscript, Drs. Jaime Sancho and Cox Terhorst for stimulating discussions, and Kristine Burgess for technical help. C.E.R. is a recipient of the Cancer Research Institute/Benjamin Jacobson Family Investigator Award. This work is also supported by National Institutes of Health Grants AI125505-01 and AI12069-16.

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