A Raf-1-Related p110 Polypeptide Associates with the CD4-p56^{lck} Complex in T Cells

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The CD4 and CD8 antigens on T cells have been shown to associate with the Src family member p56^{lck} and a GTP-binding protein, p32. The identification of receptor interactions with intracellular mediators is essential in the elucidation of downstream signals mediated by engagement of these receptor complexes. In this study, we report the detection of an additional 110-kDa polypeptide (p110) associated with the CD4-p56^{lck} complex in human peripheral blood T lymphocytes and leukemic T-cell lines. p110 bound preferentially to CD4-p56^{lck} as an assembled complex and poorly, if at all, to the individual components. p110 was recognized directly by an antiserum to the C-terminal region of the serine/threonine kinase Raf-1 and is related to a p110 polypeptide detected in anti-Raf-1 immunoprecipitates. Despite its association with the CD4-p56^{lck} complex, p110 was found to be phosphorylated predominantly on serine residues. Furthermore, phorbal ester treatment of cells resulted in a transient increase in the detection of p110 associated with CD4-p56^{lck}, concomitant with the modulation of CD4-p56^{lck} from the cell surface. This Raf-1-related p110 is therefore likely to play a role in signals generated from the CD4-p56^{lck} complex. p110 may serve as a bridge between the CD4-p56^{lck} complex and the serine/threonine kinase pathways of T-cell activation.

The T-cell receptor-CD3 complex (TcR-CD3) interacts with the processed antigenic peptide bound to major histocompatibility complex (MHC) antigens on the antigen-presenting cell (46). This interaction is further strengthened by the binding of the T-cell coreceptors CD4 and CD8, which interact with the nonpolymorphic regions of MHC class II and I molecules, respectively (11, 46, 53). CD4 is a 55-kDa integral membrane protein with a short cytoplasmic tail. Apart from its interaction with the MHC class II molecule, it is also the receptor for human immunodeficiency virus type 1 (14, 28). CD4 and CD8 receptors also synergize with the T-cell receptor complex (TcR-CD3) in the stimulation of T-cell growth (4, 16). Growth signals are likely mediated by the association of CD4 and CD8 plus the TcR-CD3 complex with the protein-tyrosine kinases p56^{lck} and p59^{fyn}, respectively (6, 49, 50, 60). Antibody-induced cross-linking of CD4 and TcR-CD3 induces the Tyr and Ser/Thr phosphorylation of numerous substrates (1, 5, 15, 22). Rapid tyrosine phosphorylation of certain substrates precedes the activation of second messengers, such as phospholipase Cγ (26). Activation ultimately results in stimulation of the phosphatidylinositol pathway, leading to the activation of protein kinase C (PKC), increased free intracellular calcium levels, and the activation of various proto-oncogenes (3, 13).

Recent studies have indicated that protein-tyrosine kinases interact physically with the intracellular molecules which serve as substrates. This biochemical property has provided a strategy for uncovering enzyme-substrate interactions within the tyrosine kinase cascade. Much of the interaction is mediated by Src homology regions (SH2 and SH3) within these proteins and is dependent on tyrosine phosphorylation (30). pp60^{src} interacts with phosphatidylinositol 3-kinase, an interaction likely mediated by the SH2 region of pp60^{src}(9). Likewise, platelet-derived growth fac-

tor receptor (PDGF-R) and epidermal growth factor receptor interact with SH2 carrying intracellular molecules such as phospholipase Cγ, GTPase-activating protein, and phosphatidylinositol kinase (9). Tyrosine phosphorylation of phospholipase Cγ acts to regulate its enzymatic activity (20, 27).

In addition to tyrosine-mediated signaling, serine and threonine kinases such as PKC play important roles in growth regulation. Various isoforms of PKC are thought to phosphorylate substrates such as CD4 and p56^{lck} in T cells (2, 7, 24, 46, 47). Engagement of the TcR-CD3 complex on T cells (54, 61) and stimulation by interleukin-2 (59) have been reported to stimulate Raf-1 kinase activity. The 70- to 72-kDa Raf-1 kinase is the cellular homolog of v-Raf, the product of the transforming gene of murine sarcoma virus 3611 (43). The amino-terminal region of Raf-1 has homology with PKC (56). V-Raf transforms murine 3T3 fibroblasts, and transfection with c-Raf having 5' deletions results in the induction of DNA synthesis in serum-starved cells, indicating an important role for Raf in cell cycle progression (21, 31, 55–57). In fibroblasts, platelet-derived growth factor binding results in the rapid tyrosine/serine phosphorylation of Raf, increased activity, and a physical association with the PDGF-R (37, 38). This interaction is mediated by unknown sequences but provides a potential link between the tyrosine kinase and serine/threonine kinase pathways (34, 37). Unlike other receptor systems, Raf-1 does not appear to physically associate with the TcR-CD3 complex (54).

In our initial report, the CD4-p56^{lck} complex was found to copurify with small, reproducible levels of serine kinase activity (49). In this study, we extend this observation by demonstrating that a Raf-1-related polypeptide of 110 kDa (p110) can associate with the CD4-p56^{lck} complex in T cells. p110 bound preferentially to the assembled CD4-p56^{lck} complex, with negligible binding to the individual components. p110 is structurally related to the 110-kDa protein present in anti-Raf-1 precipitates and can be directly recognized by

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anti-Raf antiserum. Despite its association with the *lck* kinase, p110 was phosphorylated almost exclusively on serine residues. p110 may serve as a link between the CD4-p56^{lck} complex and the serine/threonine kinase pathways of T-cell activation.

MATERIALS AND METHODS

Chemicals. Nonidet P-40, digitonin, phorbol 12-myristate 13-acetate (PMA), phenylmethylsulfonyl fluoride (PMSF), and *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-trypsin were obtained from Sigma (St. Louis, Mo.), and [γ-^{32}P]ATP (specific activity, 3,000 Ci/mol) was from NEN (Billerica, Mass.). Sodium dodecyl sulfate (SDS)-polyacrylamide gels were prepared from premixed acrylamide and bisacrylamide from Protogel, National Diagnostics (Manville, N.J.). Protein A-Sepharose CL beads and Ficoll-Paque were obtained from Pharmacia (Piscataway, N.J.). *Staphylococcus aureus* (Pansorbin) was purchased from Calbiochem (San Diego, Calif.). Recombinant soluble CD4 (extracellular region) was obtained from Biogen (Boston, Mass.). All other reagents used were of analytical-grade quality.

Monoclonal antibodies and antisera. Rabbit anti-mouse immunoglobulin was obtained from Dako Corporation (Carpinteria, Calif.). Details regarding the monoclonal antibody against CD4, 19Thy5D7, have been published (44). The other anti-CD4 antibodies, T119, MT151, and Leu3A, were obtained from the Fourth International Workshop on Human Leucocyte Differentiation Antigens (Vienna, Austria, 1989). Antibodies 4B4 (anti-CD29) and 1F7 (anti-CD26) have been described before (35, 36, 48). Rabbit anti-Raf antiserum was generated against a synthetic peptide (CTLTTSPRLPVF) corresponding to amino acid residues 315 to 326 of v-Raf (generous gift of K. Wood and T. Roberts) and was affinity purified by the method of Schultz and coworkers (52). Anti-Lck antiserum was raised in rabbits against an amino-terminal peptide (residues 39 to 64) coupled to keyhole limpet hemocyanin.

Cells. The cells used included the T-lymphoblastoid cell lines HPB-ALL, REX, and MOLT-15, the monocytic cell line U937, and HeLa cells (obtained from the American Type Culture Collection). The cells were cultured in RPMI-1640 containing 10% (vol/vol) fetal bovine serum 2 mM L-glutamine, penicillin (50 U/ml), and streptomycin (50 mg/ml) at 37°C and 5% CO_2. The transfected cell lines were growth selected in RPMI medium supplemented with G418 at 1 mg/ml. Peripheral blood lymphocytes were isolated by Ficoll-Paque centrifugation of samples from normal human donors (Dana-Farber Cancer Institute blood bank).

Immunoprecipitation and kinase assay. Cells (50 × 10^6/ml) were lysed with a mixture of the nonionic detergents Nonidet P-40 (0.5%, wt/vol) and digitonin (0.5%, wt/vol) in 20 mM Tris-HCl (pH 8.3)–150 mM NaCl–1 mM PMSF for 30 min at 4°C (49). The lysate was then centrifuged at 12,000 × *g* for 10 min at 4°C. The supernatant was precleared with 100 μl of *S. aureus* (10%, vol/vol) suspension in lysis buffer. Immunoprecipitations were then carried out by incubation of the lysate with the antibody for 2 h at 4°C and then with 50 μl of protein A-Sepharose beads (10%, wt/vol) for 1 h. Immunoprecipitates were washed three times with ice-cold lysis buffer, suspended in 30 μl of kinase buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid, pH 7.0 to 7.2], 3.5 mM MgCl₂, 3.5 mM MnCl₂), containing 10 to 20 μCi of [γ-^{32}P]ATP, and incubated at room temperature for 10 min. The samples were then subjected to SDS-polyacrylamide gel electrophoresis (PAGE) analysis (32), and the

phosphorylated proteins were visualized by autoradiography. When necessary, the gels were treated with 1 M KOH for 2 h at 55°C to remove the alkali-labile phosphate groups from serine/threonine-phosphorylated proteins (12).

For reprecipitations, SDS (1%, wt/vol) and β-mercaptoethanol (0.1%, vol/vol) were added to the samples, which were then denatured by boiling for 5 min, diluted to 0.1% SDS with lysis buffer, and subjected to immunoprecipitation (49). In some experiments, a specific protein band was excised from the polyacrylamide gel, and the protein was extracted in 50 mM NH₄HCO₃–0.1% (wt/vol) SDS–0.1% (vol/vol) β-mercaptoethanol–0.1% bovine serum albumin. The extract was lyophilized, resuspended in a 10-fold dilution of lysis buffer, and subjected to immunoprecipitation.

Phosphoamino acid analysis and phosphotryptic peptide mapping. The extracted protein was subjected to acid hydrolysis (6 M HCl), and the phosphoamino acids were separated by two-dimensional thin-layer electrophoresis by the procedure of Cooper et al. (12). For two-dimensional tryptic peptide mapping, eluted proteins were exhaustively digested with TPCK-trypsin, and the peptides were separated on a 0.1-mm-thick cellulose plate (E. Merck, Darmstadt, Germany) by electrophoresis (pH 8.9, 1,000 V for 30 min) and then by ascending chromatography in butanol-pyridine-acetic acid-water (75:50:15:60) (23, 40).

RESULTS

To ascertain whether the CD4-p56^{lck} complex was associated with any intracellular proteins, immunoprecipitates formed by anti-CD4 antibody from peripheral blood lymphocytes and various T-lymphoblastoid and nonlymphoid cells were analyzed by phosphotransferase labeling. As shown in Fig. 1A, anti-CD4 coprecipitated a 110-kDa (p110) protein in addition to p56^{lck} from a variety of T cells including HPB-ALL (lane 2), peripheral T cells (lane 6), REX (lane 9), and MOLT-15 (lane 12) cells. Under the same labeling conditions, p110 was not detected in immunoprecipitates formed by rabbit anti-mouse immunoglobulin (lanes 1, 5, 8, 11, and 14) or anti-CD3 (lane 3). Other negative controls included anti-transferrin receptor, anti-GTPase-activating protein, anti-Src, anti-Fyn, anti-PKC, anti-retinoblastoma protein, antinucleolin, anti-CD26, and anti-CD29 antibodies, none of which coprecipitated detectable p110 (data not shown). In order to establish whether Lck was needed for the interaction, CD4 was purified from the CD4$^+$ Lck$^-$ monocytic cell U937 and HeLa cells which had been transfected with human CD4. Anti-CD4 failed to precipitate p110 from U937 cells (Fig. 1A, lane 15). Similarly, as shown in Fig. 1B, anti-CD4 failed to coprecipitate p110 from nonlymphoid HeLa cells, either wild type (lane 1) or transfected with CD4 (lane 3). These data indicate that the CD4 antigen associates with a p110 protein and that detection of the association requires the expression of p56^{lck}.

To further establish the specificity of the association, we next examined whether p110 could be precipitated by antibodies to different epitopes on the CD4 antigen. CD4 comprises four immunoglobulin domains, of which the N-terminal domains bind to class II antigens of the MHC (10). Each of the anti-CD4 antibodies examined, including T119, MT151, and Leu3A, coprecipitated p110 with the CD4-p56^{lck} complex (Fig. 2, lanes 4 to 7). These data indicate that coprecipitation of p110 is not dependent on the recognition of any particular epitope on the CD4 antigen. Antibodies 19Thy5D7 and Leu3A recognize distinct epitopes at the N-terminal domain, and MT151 reacts with the second
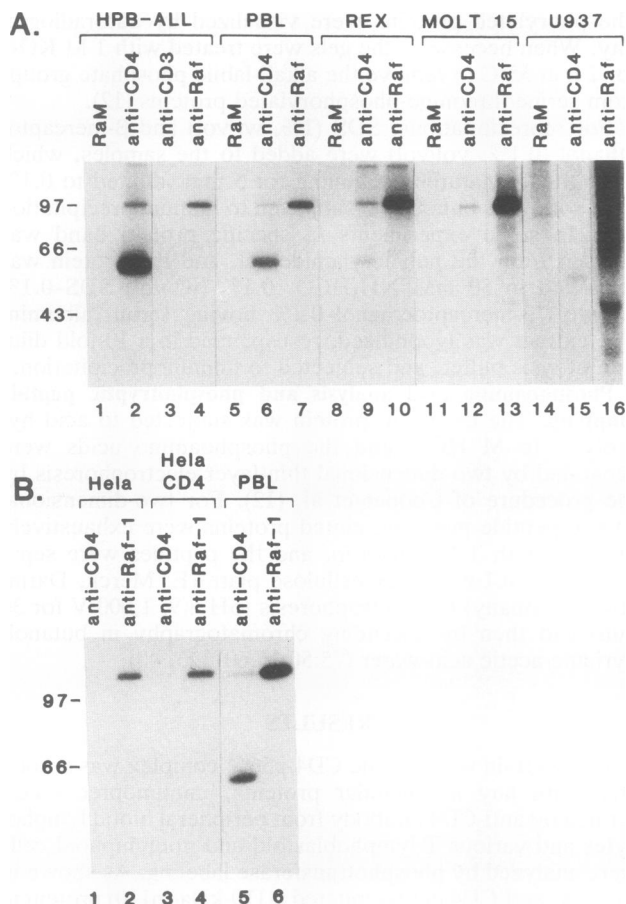
FIG. 1. p110 protein is associated with the CD4-p56^{lck} complex which comigrates with the Raf-1-associated p110. Immunoprecipitation and in vitro kinase analysis were conducted as described in Materials and Methods. (A) Human cell lines HPB-ALL (lanes 1 to 4), peripheral blood lymphocytes (PBL) (lanes 5 to 7), REX cells (lanes 8 to 10), MOLT-15 cells (lanes 11 to 13), and the monocytic cell line U937 (lanes 14 to 16). Antibodies and antisera include rabbit anti-mouse immunoglobulin (RaM) (lanes 1, 5, 8, 11, and 14), 19Thy5D7 (anti-human CD4) (lanes 2, 6, 9, 12, and 15), RW28C8 (anti-human CD3) (lane 3), and anti-Raf serum (lanes 4, 7, 10, 13, and 16). (B) HeLa (lanes 1 and 2), CD4-transfected HeLa (lanes 3 and 4), and peripheral blood (lanes 5 and 6) cells. Antibodies and antisera include 19Thy5D7 (anti-human CD4) (lanes 1, 3, and 5) and anti-Raf serum (lanes 2, 4, and 6). Sizes are shown to the left (in kilodaltons).
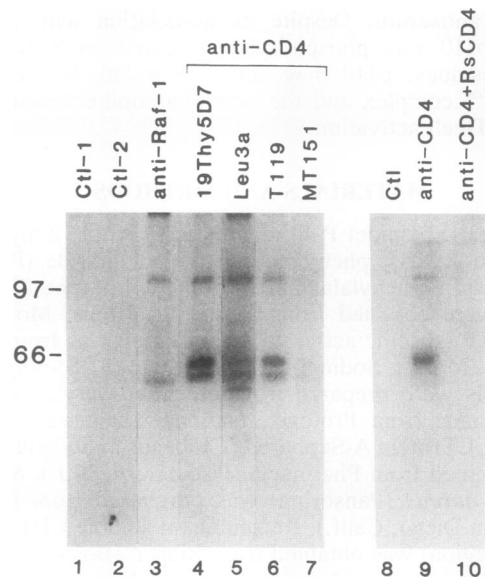

FIG. 2. Multiple antibodies to different epitopes of CD4 coprecipitate p110. HPB-ALL cells were treated with PMA (100 ng/ml, 30 min) and subjected to immunoprecipitation and in vitro kinase analyses. Anti-CD26 (Ctl 1) (lane 1), anti-CD27 (Ctl 2) (lane 2), and rabbit anti-mouse immunoglobulin (Ctl) (lane 8) served as negative controls. Other antibodies include anti-Raf (lane 3) and anti-CD4 antibodies 19Thy5D7 (lane 4), Leu3A (lane 5), T119 (lane 6), and MT151 (lane 7). An excess of recombinant soluble CD4 (RsCD4, 2 mg/ml) successfully prevented anti-CD4 (19Thy5D7) precipitation of p56^{lck} and p110 (lanes 9 versus 10). Sizes are shown at the left (in kilodaltons).

domain (10, 33, 41, 51). Furthermore, an excess of recombinant soluble CD4 (2.0 mg/ml) added to the lysate effectively blocked the detection of p56^{lck} and the vast majority of p110 in anti-CD4 immunoprecipitates, further supporting the specificity of the p110 association (Fig. 2, lanes 9 and 10).

In order to establish the identity of p110, anti-CD4 precipitates were compared with precipitates formed by an antiserum to the Raf-1 kinase. Previous studies had reported that a Raf-1-associated p110 subunit is the primary target of in vitro kinase labeling in anti-Raf immunoprecipitates (54, 61). Anti-Raf-1 precipitates revealed the presence of a 110-kDa band in all the cell lines examined (Fig. 1A, lanes 4, 7, 10, 13, and 16; Fig. 1B, lanes 2 and 4). The anti-Raf-precipitated p110 comigrated with CD4-associated p110. Little of the p72 Raf-1 kinase was labeled in this type of assay, as reported by others (54).

In order to establish the relationship between the anti-CD4- and anti-Raf-precipitable p110, bands were eluted from SDS-PAGE gels and subjected to two-dimensional peptide map analysis. The patterns of digested peptides from anti-Raf- and anti-CD4-precipitable p110 showed a single prominent peptide (spot 1) that migrated in an identical position (Fig. 3A versus B). This pattern demonstrated some structural similarity between the p110 molecules. However, despite the similarity, p110 also showed minor differences in the pattern of phospholabeled peptides. For example, peptide 2 was more prominent in anti-CD4 precipitates, while peptide 5 was more readily detected with anti-Raf. Peptide 3 was unique to anti-Raf-precipitable p110, and peptide 4 was unique to anti-CD4-precipitable p110. These data indicate that a structurally related p110 protein is found in both anti-CD4 and anti-Raf precipitates. The differences may reflect differences in the nature of covalent modifications, such as phosphorylation. Alternatively, different isoforms of the same protein may be present in the anti-CD4 and anti-Raf precipitates.

We next determined whether p110 could be directly recognized by the anti-Raf antiserum. Labeled anti-Raf or anti-CD4 immunoprecipitates were denatured by boiling in 1% SDS, diluted in excess lysis buffer, and reprecipitated. This approach has been used to demonstrate the association of p56^{lck} and p32 with CD4 (49, 58). As shown in Fig. 4, the anti-Raf antiserum specifically reprecipitated the p110 protein from anti-Raf immunoprecipitates (lane 5), a precipitation that was blocked by the presence of peptide to which the antiserum was raised (lane 6). Similarly, the anti-Raf serum was also capable of recognizing p110 from anti-CD4 immunoprecipitates (Fig. 4, lane 7). Lastly, eluted labeled p110
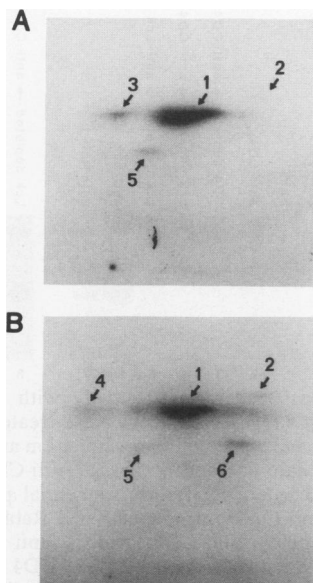
FIG. 3. Two-dimensional tryptic peptide map comparison of anti-CD4- and anti-Raf-precipitated p110. Phorbol ester-treated HPB-ALL cells were solubilized and immunoprecipitated with anti-CD4 (19Thy5D7) and anti-Raf antisera. After the kinase labeling, the samples were separated by SDS-PAGE, and radiolabeled p110 proteins were subjected to digestion with trypsin. Peptides were then separated by two-dimensional phosphopeptide mapping, as described in Materials and Methods. (A) Tryptic phosphopeptide map of the p110 present in anti-Raf-1 immunoprecipitate. (B) Tryptic phosphopeptide map of p110 associated with the CD4-p56*lck* complex.
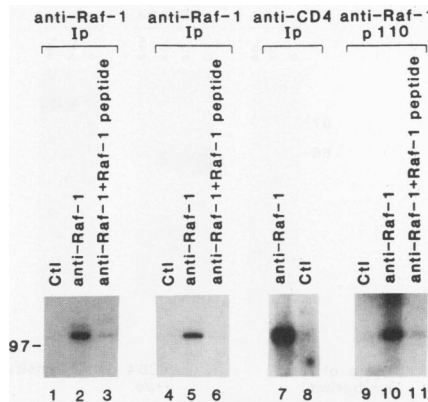

FIG. 4. Anti-Raf-1 antiserum directly recognizes CD4- and Raf-1-coprecipitated p110. Immunoprecipitates formed by anti-Raf (lanes 1 to 6) or anti-CD4 (lanes 7 and 8) were labeled by in vitro labeling with [γ-^{32}P]ATP, denatured in 1% (wt/vol) SDS, diluted 10-fold in lysis buffer, and subjected to reprecipitation with rabbit anti-mouse immunoglobulin (lanes 4 and 8) or affinity-purified anti-Raf (lanes 5, 6, and 7) serum. Reprecipitation was also conducted in the presence of the Raf peptide (1 mg/ml) to which the serum was raised (lanes 3 and 6). Alternatively, the labeled p110 band was also extracted from SDS-PAGE gels and subjected to reprecipitation with anti-Raf-1 antiserum in the absence (lane 10) or presence (lane 11) of Raf peptide. The position of the 97-kDa band is marked. Ctl, control. Note: lanes 1 to 3 represent primary immunoprecipitations only.

from gels was specifically reprecipitated by the anti-Raf serum (Fig. 4, lane 10), a precipitation which was effectively inhibited by the relevant peptide (lane 11). Neither preimmune serum nor anti-CD4 antibody was capable of reprecipitating p110 (data not shown). These results clearly demonstrated that the CD4- and Raf-associated p110 polypeptides have an epitope that can be recognized by an anti-Raf antiserum.

Two-dimensional phosphoamino acid analysis revealed predominantly serine phosphorylation on p110 associated with CD4-p56*lck* (Fig. 5B). Only prolonged exposures allowed the detection of small amounts of phosphorylation on tyrosine residues. Likewise, p110 derived from anti-Raf immunoprecipitates was labeled on serine residues (Fig. 5A), further demonstrating the similarity between the p110 polypeptides. By contrast, p56*lck* was phosphorylated predominantly on tyrosine residues (Fig. 5C).

Given the fact that p110 is phosphorylated on serine and threonine residues, we attempted to establish whether the detection of CD4-associated p110 could be altered by the activation of PKC. HPB-ALL cells were exposed for various times to phorbol ester (PMA, 100 ng/ml) prior to immunofluorescence and precipitation analyses. PMA activates the serine/threonine kinase PKC (39). Under this regimen, CD4-associated p110 underwent a dramatic increase in phosphorylation. As shown in Fig. 6B, PMA induced a time-dependent decrease in the density of CD4 on the surface of cells, as detected by flow cytometry. As previously noted by ourselves (46, 47) and others (24), the loss of surface CD4 expression was accompanied initially by a time-dependent shift in the mass of CD4-associated p56*lck* from 56 to 60 kDa,
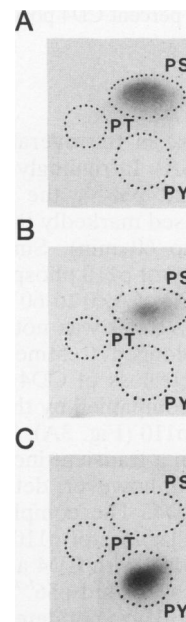

FIG. 5. p110 is phosphorylated predominantly on serine residues as a result of in vitro labeling. Two-dimensional phosphoamino acid analysis of the extracted p110 protein from gels of anti-CD4 (19Thy5D7) and anti-Raf precipitates was carried out as described in Materials and Methods. Samples were digested with 6 N HCl, and the phosphoamino acids were separated by two-dimensional thin-layer electrophoresis on cellulose plates. (A) p110 present in Raf-1 immunoprecipitates. (B) p110 associated with the CD4-p56*lck* complex. (C) p56*lck*. PY, PS, and PT represent phosphotyrosine, phosphoserine, and phosphothreonine, respectively.
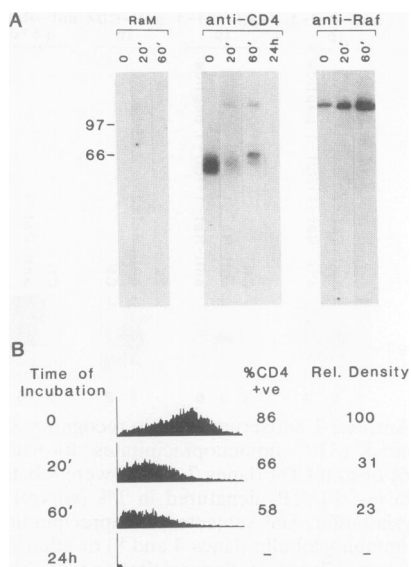
FIG. 6. Phorbol ester treatment increases the detection of p110 associated with CD4-p56^{lck}. (A) HPB-ALL cells were treated with PMA (100 ng/ml) for various times before solubilization, immunoprecipitation, and in vitro kinase analysis. The following antibodies were used: rabbit anti-mouse immunoglobulin (RaM, left panel), anti-CD4 (middle panel), and anti-Raf (right panel). Sizes are shown on the left (in kilodaltons). (B) Phorbol ester-induced downregulation of CD4, as assessed by flow cytometry. An aliquot of cells (5 × 10^5) was withdrawn at various times and reacted with the monoclonal antibody 19Thy5D7 and then with fluorescein-conjugated goat anti-mouse immunoglobulin antibody. Mean channel fluorescence intensity was used to calculate the relative density of CD4 receptors on the cell surface. Samples were drawn from the cell culture used for panel A. %CD4 +ve, percent CD4 positive. Times are shown in minutes or hours.
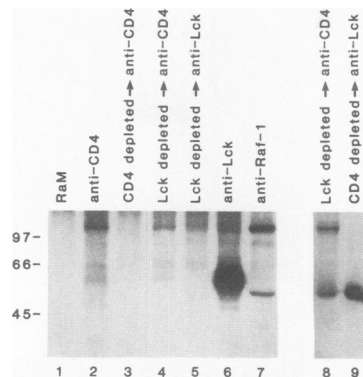

FIG. 7. p110 associates preferentially with CD4-p56^{lck} as an assembled complex. HPB-ALL cells were treated with PMA (100 ng/ml) for 30 min and subjected to precipitation and kinase analyses as described in Materials and Methods. Anti-CD4 and anti-p56^{lck} depletions were carried out by six sequential precipitations with 19Thy5D7 and anti-Lck sera, respectively. Rabbit anti-mouse immunoglobulin (lane 1), anti-CD4 (lane 2), anti-Lck (lane 6), and anti-Raf (lane 7). Samples were depleted of CD4 with anti-CD4 and then immunoprecipitated with either anti-CD4 (lane 3) or anti-Lck (lane 9). Alternatively, samples were partially depleted of Lck with anti-Lck and then precipitated with anti-Lck (lane 5) or anti-CD4 (lanes 4 and 8). Sizes are shown on the left (in kilodaltons).

followed by a decrease in the overall intensity of labeled p56^{lck} by 20 min (Fig. 6A). Intriguingly, despite the reduction in detectable CD4 and p56^{lck}, the appearance of CD4-associated p110 increased markedly in phorbol ester-treated T cells (Fig. 6A, 0 to 60 min). Similarly, there was an increase in the intensity of p110 phosphorylation in anti-Raf immunoprecipitates (Fig. 6A, 0 to 60 min). As observed in untreated samples, p72 Raf-1 was not visualized under this labeling regimen. Prolonged treatment with PMA (24 h) resulted in the complete loss of CD4 from the cell surface (Fig. 6B), an event accompanied by the concomitant loss of detectable p56^{lck} and p110 (Fig. 3A). Therefore, the activation of PKC resulted in a transient increase in the detection of CD4-associated p110; however, detection was dependent on the presence of CD4. The complete loss of CD4 was accompanied by a similar loss of p110.

The association of p110 with CD4 appeared to depend on the presence of CD4-associated p56^{lck}. This was shown by the observation that p110 was not detected in U937 or CD4$^+$ HeLa cells despite the expression of p110, as detected by anti-Raf-1 (Fig. 1A and B). We next addressed the question whether p110 binds to unbound p56^{lck} as well as to receptor-associated p56^{lck}. Initially, anti-CD4 precipitates were contrasted with anti-p56^{lck} precipitates from PMA-treated HPB-ALL cells for the presence of p110. PMA was used to enhance detection of the p110 polypeptide. As shown in Fig. 7, while anti-CD4 precipitated significant amounts of p110 (lane 2), antiserum to p56^{lck} precipitated only negligible

amounts of p110 despite the presence of well-labeled p56^{lck} (lane 6). The anti-p56^{lck} serum, which binds to the N-terminal region of Lck, appears to preferentially precipitate free, non-receptor-bound kinase (unpublished observations). In depletion experiments, anti-CD4 depleted even the faint p110 band coprecipitated by anti-Lck (Fig. 7, lane 9). Anti-CD4 also successfully depleted CD4-p56^{lck}-p110 complexes from the lysates (Fig. 7, lane 3). These data indicated that p110 associates with the assembled CD4-p56^{lck} complex and binds poorly, if at all, to free, unbound kinase. Attempts to deplete p56^{lck} from the lysates met with limited success. A series of seven anti-Lck immunoprecipitations incompletely reduced the levels of anti-CD4-precipitable Lck (Fig. 7, lanes 4 and 8). Nevertheless, even with a partial reduction in unbound Lck, anti-CD4 was still able to coprecipitate p110 (Fig. 7, lane 4). Taken together, these data demonstrate that p110 binds preferentially to the assembled CD4-p56^{lck} complex and poorly, if at all, to the individual CD4 and Lck proteins.

DISCUSSION

The CD4 and CD8 antigens have been shown to interact within the cytoplasm with the protein tyrosine kinase p56^{lck} (6, 49, 60). This interaction provides a molecular basis for CD4 and CD8 function in signalling events as mediated by protein-tyrosine phosphorylation (17, 19, 45). CD4 and CD8 receptor complexes have also been found to interact with a GTP-binding protein, p32 (58). These receptors are therefore oligomeric in nature, a characteristic that has been described for other receptor systems. Receptors such as the PDGF-R generate intracellular signals by means of the tyrosine kinase domains and associated intracellular proteins such as phospholipase C (30) and phosphotidylinositol 3-kinase (9). In the present study, we have identified a third intracellular protein, a Raf-1-related intracellular molecule of 110 kDa (p110), in the CD4-p56^{lck} complex. The p110 polypeptide was found associated with the complex in a variety of T cells, including peripheral blood T cells and the T-cell lines HPB-ALL,

MOLT-15, and REX (Fig. 1). Significantly, p110 was found to preferentially associate with CD4 and p56^{lck} as an assembled complex and not with the individual components of the complex. Detection of p110 depended on the coexpression of p56^{lck}, since it was not detected associated with CD4 from CD4$^+$ U937 or HeLa cells despite the fact that p110 was expressed, as detected by the anti-Raf antibody (Fig. 1A and B). This is unlikely to reflect a requirement for the kinase in the labeling of p110, since the protein was found to be phosphorylated almost exclusively on serine residues (Fig. 5). Furthermore, despite the requirement for p56^{lck}, anti-p56^{lck} serum coprecipitated little, if any, p110, and the small amount of material was readily depleted by anti-CD4 antibody (Fig. 7). These data are compatible with a model in which Lck binding to the CD4 receptor induces an altered conformation and/or phosphorylation that facilitates p110 association with the complex.

Although the exact identity and structure of CD4-Lck-associated p110 is unknown, the phosphopeptide map pattern of p110 showed similarities with the pattern of anti-Raf-precipitable p110 (Fig. 3). The patterns revealed a major common peptide as well as several minor peptides. The p110 proteins may represent the same protein with different posttranslational changes or different isoforms of the same protein. In either case, both were directly recognized by an anti-Raf serum, attesting to the similarity of these proteins (Fig. 4). The antiserum reacts with the C-terminal region of the kinase (52). A protein of 110 to 115 kDa has been noted as a prominent protein in anti-Raf precipitates labeled in in vitro kinase assays; however, its identity was unknown (54, 61). It had previously been assumed that p110 was associated with and therefore coprecipitated with p72 Raf. The antiserum reacts with a region outside the Raf catalytic domain and therefore does not provide any information on whether p110 is itself a kinase (52). Attempts to detect catalytic activity associated with p110 by ATP labeling on an insoluble matrix (polyvinylidene difluoride) or to label p110 with the nonhydrolyzable form of ATP (FSBA) failed (data not shown). cDNA cloning may be required to determine whether p110 is a serine kinase and/or a substrate.

T-cell activation induces a cascade of tyrosine and serine/threonine phosphorylation of substrates (29, 45). The identification of a Raf-1-related protein linked to the CD4-p56^{lck} complex provides a potential bridge between the tyrosine and serine/threonine pathways in T cells. Although a physical association between Raf-1 and the TcR-CD3 complex has not been shown, Siegel et al. (54) have provided compelling evidence of a link between TcR-CD3 ligation and the activation of p72 Raf-1 activity. Activation occurred in a PKC-dependent fashion. In this sense, it is interesting that the detection of CD4-associated p110 in T cells was greatly enhanced by exposure of the cells to phorbol ester. Over the time course, p56^{lck} shifted to a higher-mass form at 60 kDa and was reduced in intensity concomitant with the loss of CD4 (24, 46, 47). By contrast, the time-dependent loss of CD4 was accompanied by an initial dramatic increase in the detection of both CD4- and Raf-associated p110 (Fig. 2). Detection of p110 with CD4 may therefore be regulated by activation of PKC, or, if p110 is a kinase, its activity may be directly increased by phorbol ester. Such results are consistent with the predominance of serine phosphorylation of p110. Neither anti-CD4 nor anti-CD3 ligation induced in vivo phosphotyrosine labeling of CD4-associated p110 (data not shown), a result consistent with the absence of tyrosine phosphorylation in vitro (Fig. 5). The TcR-CD3 complex does not associate with detectable p110 (Fig. 1); however,

CD4 and the TcR-CD3 complex can physically interact (8), thereby introducing the possibility that CD4-associated p110 may play a role in the serine kinase pathway initiated by TcR-CD3 ligation.

It is presently unclear whether p72 Raf can also associate with the CD4-p56^{lck} complex. Raf has been reported to associate with the PDGF-R (37). In vitro labeling failed to label p72 Raf in either anti-Raf or anti-CD4 immunoprecipitates (Fig. 1). However, p72 Raf could be detected on immunoblots of whole-cell lysates or anti-Raf-1 immunoprecipitates when probed with anti-Raf-1 serum (data not shown). Other labeling techniques failed to consistently show either p72 or p110, presumably because of the low stoichiometry of the interaction (data not shown). We have therefore been unable to exclude the possible inclusion of p72 Raf in anti-CD4 precipitates. Nevertheless, it is possible that p110 may associate independently with CD4-p56^{lck}. In either case, the interaction of p110 with CD4-p56^{lck} provides further evidence that the CD4 antigen exists as an oligomeric complex in T cells. The association of p56^{lck} with CD4 is required for the optimal proliferation of certain T-cell clones to antigen (1, 19, 45). This requirement may also reflect a need for associated p110. p110 may play a role in the downstream signals derived from CD4 and the TcR-CD3 complex.

Raf-1 is known to transactivate the c-fos and β-actin promoters (25), to be required for v-src-induced activation of the mitogen-responsive transcription factor Egr-1 (42), and to be essential for the mitogenesis of fibroblasts (31) and may play a role in the cell cycle (61). Structural analysis and cDNA cloning of p110 should provide further insights into its role in T-cell function.

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